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Development and validation of a highly sensitive and robust LC-ESI-MS/MS method for simultaneous quantitation of simvastatin acid, amlodipine and valsartan in human plasma: application to a clinical pharmacokinetic study†

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ABSTRACT: A high-throughput, simple, highly sensitive and specific LC-MS/MS method has been developed for simultaneous estimation of simvastatin acid (SA), amlodipine (AD) and valsartan (VS) with 500 μL of human plasma using deuterated simvastatin acid as an internal standard (IS). The API-4000 LC-MS/MS was operated under the multiple reaction-monitoring mode (MRM) using electrospray ionization. The assay procedure involved precipitation of SA, AD, VS and IS from plasma with acetonitrile. The total run time was 2.8 min and the elution of SA, AD, VS and IS occurred at 1.81, 1.12, 1.14 and 1.81 min, respectively; this was achieved with a mobile phase consisting of 0.02 M ammonium formate (pH 4.5):acetonitrile (20:80, v/v) at a flow rate of 0.50 mL/min on an X-Terra C_{18} column. A linear response function was established for the range of concentrations 0.5–50 ng/mL ($r > 0.994$) for VS and 0.2–50 ng/mL ($r > 0.996$) for SA and AD. The method validation parameters for all three analytes met the acceptance as per FDA guidelines. This novel method has been applied to human pharmacokinetic study. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: simvastatin acid; amlodipine; valsartan; human plasma; method validation; LC-MS/MS; pharmacokinetics; humans

Introduction

Control of hypercholesterolemia is important for the prevention of coronary artery disease (CAD). Currently, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor is the first-choice therapeutic agent for patients with hypercholesterolemia. Simvastatin is widely used to reduce morbidity and mortality from CAD (Shepherd, 1998; Matsubara *et al*., 2003; Williams and Feely, 2002; Nishio *et al*., 2005). Simvastatin is an inactive lactone prodrug administrated orally; it hydrolyzes *in vivo* to simvastatin acid (SA, Fig. 1), which is a potent inhibitor of HMG-CoA (Vickers *et al*., 1990a,b; Prueksaritanont *et al*., 1990). Hypercholesterolemia is often accompanied by hypertension, an associated risk factor for CAD (Kannel, 2000; Sander and Giles, 2002; Nishio *et al*., 2005). Calcium channel blockers have been widely used in the treatment of hypertension and/or angina pectoris, and are often prescribed in association with a lipid-lowering agent such as simvastatin (Abernethy, 1992; Jukema *et al*., 1996, 1998). More than one antihypertensive agent is indicated for the patients with multiple cardiovascular risk factors. Combination therapy with calcium channel blocker and an angiotensin II receptor blocker would be expected to provide enhanced antihypertensive activity (Philipp *et al*., 2007). One such preparation available in the market is Exforge®, which is a combination of amlodipine (AD, Fig. 1) and valsartan (VS, Fig. 1) in a single pill (Norvasc; www.norvasc.com/high-blood-pressure-medicine/about-norvasc.asp). Valsartan is a nonpeptide, orally active and specific competitive angiotensin II antagonist acting on the AT1 receptor subtype, which mediates all known effects of angiotensin II on the cardiovasular system. Angiotensin receptor blockers have been hypothesized to have synergistic effects with statins (Ledingham and Laverty, 2005; Rajagopalan *et al*., 2007).

In this present study, a simple, robust and reproducible method has been developed and validated to estimate simvastatin acid, amlodipine and valsartan concentrations simultaneously in human plasma. This method meets the requirements and provides high degree of accuracy, sensitvity and specificity by simple precipitation using high-performance liquid chromatography and detection by electrospray tandem mass spectrometry. This method will be particularly useful to estimate the plasma samples of patients receiving simvastatin and Exforge® simultaneously. The application of this assay in a clinical pharmacokinetic study following oral administration of VS is described.

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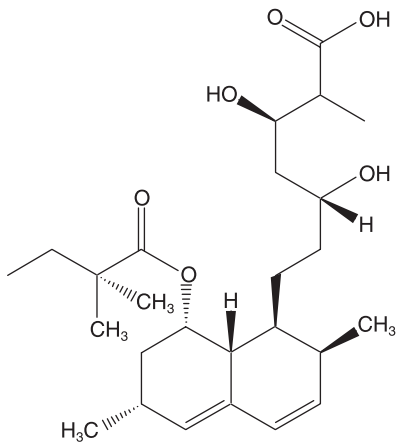
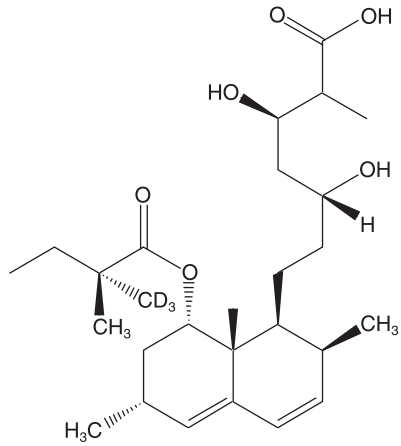
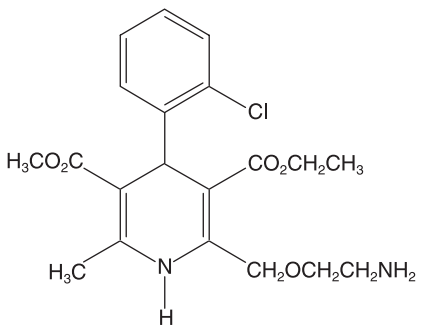
Abbreviations used: AD, amlodipine; CAD, coronary artery disease; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; SA, simvastatin acid; VS, valsartan.



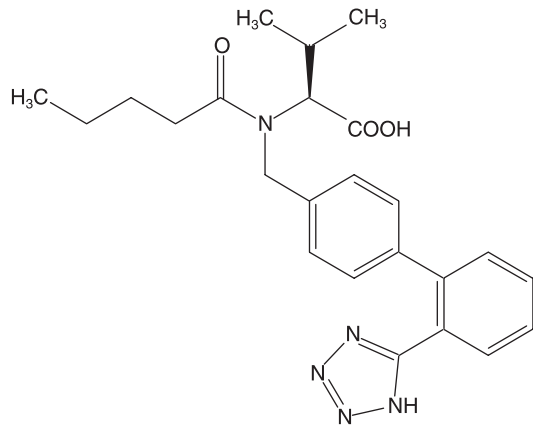
Simvastatin acid



Deuterated simvastatin acid (IS)



Amlodipine



Valsartan

Figure 1. Structural representation of simvastatin acid (SA), deuterated simvastatin acid (IS), amlodipine (AD) and valsartan (VS).

Experimental

Chemicals and Reagents

SA (>98.7%) was obtained from Biocon Ltd, Bangalore, India. AD (>98%) and VS (>98%) were obtained from Unit-II, Dr Reddy's Laboratories Ltd (DRL), Hyderabad. Deuterated SA (>96.8%, Fig. 1) was obtained from Syncom, Groningen, The Netherlands. HPLC-grade acetonitrile was purchased from RFCL Limited, New Delhi, India. Analytical-grade ammonium formate was purchased from HiMedia Laboratories, Pvt Ltd, Mumbai, India. Formic acid was purchased from Sisco Research Laboratories, Pvt Ltd, Mumbai, India and acetic acid was purchased from Qualigens Fine Chemicals, Mumbai, India. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control K_2EDTA human plasma was purchased from Cauvery Diagnostics and Blood Bank, Secunderabad, India.

Instrumentation and Chromatographic Conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1200 series LC system equipped with degasser (G1322A) and isopump (G1311A Quat pump) along with an auto-sampler (G1367B) was used to inject 20 μL aliquots of the processed samples on an X-Terra MS C_{18} column (4.6 × 50 mm, 2.5 μm, Water Corporation, Ireland, UK), which was kept at room temperature. The isocratic mobile phase, a mixture of 0.02 M ammonium formate (pH 4.5) and acetonitrile mixture (20:80, v/v) was delivered at 0.50 mL/min into the mass spectrometer's electrospray ionization chamber.

Quantitation was achieved with MS-MS detection in positive ion mode for all the analyte and IS, using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 500°C. The ion spray voltage was set at 5500 V. The common parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 35, 11, 40 and 4 psi, respectively. The compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 50, 16, 10, 10 V for SA, 46, 14, 10, 16 V for AD, 48, 25, 10, 21 V for VS and 52, 14, 10, 10 V for IS. Detection of the ions was performed in the multiple-reaction monitoring (MRM) mode, by monitoring the transition pairs of *m/z* 437.30 precursor ion to the *m/z* 303.20 for SA, *m/z* 409.20 precursor ion to the *m/z* 238.0 for AD, *m/z* 436.20 precursor ion to the *m/z* 291.20 for VS and *m/z* 440.40 precursor ion to the *m/z* 303.20 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.4.2).

Standard Solutions

Primary stock solutions of SA, AD, VS and IS for preparation of standard and quality control (QC) samples were prepared by weighing separately. The primary stock solutions (1.0 mg/mL) of the analytes and IS were prepared in acetonitrile:water (90:10, v/v) and stored at −20°C; they were found to be stable for one month (data not shown). Appropriate dilutions were made in acetonitrile:water for SA, AD and VS to produce working stock solutions of 500, 200, 100, 80, 60, 20, 10, 5 and 2 ng/mL on the day of analysis and these stocks were used to prepare the calibration curve (CC). The stock solution of 2 ng/mL was not used for CC of VS. Another set of working stock solutions was made in acetonitrile:water (90:10, v/v from primary stock) at 400, 250, 15, 6, 5 and 2 ng/mL, of which 400 and 250 ng/mL were used for high QC (HQC) and medium QC (MQC). Working stock solutions at 6 and 2 ng/mL were used for low QC (LQC) and lower limit of quantitation (LLOQ) of SA and AD; 15 and 5 ng/mL were used for QC low and LLOQ for VS. A working IS solution (1.00 μg/mL) was also prepared in acetonitrile:water (90:10, v/v). Calibration samples were prepared by spiking 450 μL of control human plasma with the appropriate amount of analytes (50 μL) and IS (10 μL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations (0.20, 0.60, 25.0 and 40.0 ng/mL as LLOQ, LQC, MQC and HQC, respectively, for SA and AD; and 0.50, 1.50, 25.0 and 40.0 ng/mL as LLOQ, LQC, MQC and HQC, respectively for VS) and 500 μL volumes were aliquoted into different tubes and, depending on the nature of experiment, samples were stored at −80 ± 10°C until analysis.

Sample Preparation

Samples were prepared by taking 500 μL of human plasma sample, IS solution (10 μL) equivalent to 20 ng was added, buffered with 50 μL of 0.02 M ammonium formate (pH 4.5) and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India), followed by precipitation with 3.00 mL of acetonitrile. The mixture was vortexed for 2 min, followed by centrifugation for 4 min at 3200 rpm on Multifuge 3 S-R (Heraeus, Germany). The organic layer (2.7 mL) was separated and evaporated to dryness at 50°C using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 200 μL of the reconstitution solvent (0.1% formic acid:acetonitrile: 20:80, v/v) and 20 μL was injected onto LC-MS/MS system.

Method Validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation (US DHHS, FDA, CDER, 2001).

Specificity and selectivity. The specificity of the method was determined by analyzing six different batches of human plasma to demonstrate the lack of chromatographic interference from endogenous plasma components.

Recovery. The recovery of SA, AD, VS and IS was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 4$) at LQC, MQC and HQC with the response of analytes from post-extracted plasma standard sample at equivalent concentrations (Dams *et al*., 2003). Recovery of the IS was determined at a single concentration of 20.0 ng/mL.

Matrix effect. The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the responses of the post extracted plasma standard QC samples ($n = 4$) with the response of analytes from neat samples at equivalent concentrations (Hubert *et al*., 1999; Dams *et al*., 2003). Matrix effect was determined at same concentrations for each analyte and IS as in recovery experiment.

Calibration curve. Calibration curves were acquired by plotting the peak area ratio of the transition pair of analytes to that of IS against the nominal concentration of calibration standards. The concentrations used were 0.20, 0.50, 1.00, 2.00, 6.00, 8.00, 10.0, 20.0 and 50.0 ng/mL for SA and AD. For VS the concentrations used were 0.50, 1.00, 2.00, 6.00, 8.00, 10.0, 20.0 and 50.0 ng/mL. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS, FDA, CDER, 2001).

Precision and accuracy. Inter- and intra-assay precision and accuracy were determined by analyzing six replicates at four different QC levels as described above on four different days. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of SD (US DHHS, FDA, CDER, 2001).

Stability experiments. The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 24 h (in auto-sampler) after the initial injection. The peak-areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the biomatrix after 8 h exposure in an ice bath (bench top) was determined at two concentrations in six replicates. Freezer stability of the analytes in biomatrix was assessed by analyzing the QC samples stored at −80 ± 10°C for at least 30 days. The stability of analytes in biomatrix following repeated three freeze–thaw cycles (stored at −80 ± 10°C between cycles) was assessed using QC samples spiked with analytes. Samples were processed as described above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD) (US DHHS, FDA, CDER, 2001).

Dilution effect. Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. SA, AD and VS separately spiked human plasma samples prepared at two concentrations (15 and 4000 ng/mL) of SA, AD and VS were diluted with pooled human plasma at dilution factors of 10 and 100 in six replicates and analyzed. The six replicates should have precision of ≤15% and accuracy of 100 ± 15%.

Human Pharmacokinetic Study

A pharmacokinetic study was performed in healthy ($n = 3$) male subjects. The ethics committee approved the protocol and the volunteers provided written informed consent. Blood samples were obtained following oral administration of 160 mg VS into polypropylene tubes containing K_2 EDTA solution as an anti-coagulant at pre-dose, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, 12, 24 and

36 h. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760***g*** for 5 min and stored frozen at −80 ± 10°C until analysis. Plasma (500 μL) samples were spiked with IS and processed as described above. Along with clinical samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among calibrators and unknown samples in the analytical run; not more than 33% of the QC samples were greater than ±15% of the nominal concentration. Plasma concentration–time data of each analyte was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

Results

Specificity and Selectivity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with analytes at LLOQ and IS is shown in Fig. 2(a–c). No interfering peaks from endogenous compounds were observed at the retention times of analyte and IS. The retention time of SA, AD, VS and IS were 1.81, 1.12, 1.14 and 1.81 min, respectively. The total chromatographic run time was 2.8 min.

Recovery

The extraction recoveries of the analytes from plasma were calculated by comparing the response of plasma samples spiked with the analytes prior to extraction to those spiked following extraction. Recovery was assessed for each analyte at low, mid and high QC concentrations and peak area ratios (analyte/IS) were used for the calculations. Recovery was not concentration-dependent and the mean percentage recoveries of SA, AD and VS were 104.9 ± 7.4, 104.1 ± 9.1 and 91.7 ± 6.4, respectively. The mean percentage recovery of IS at 20.0 ng/mL was 95.66 ± 6.7.

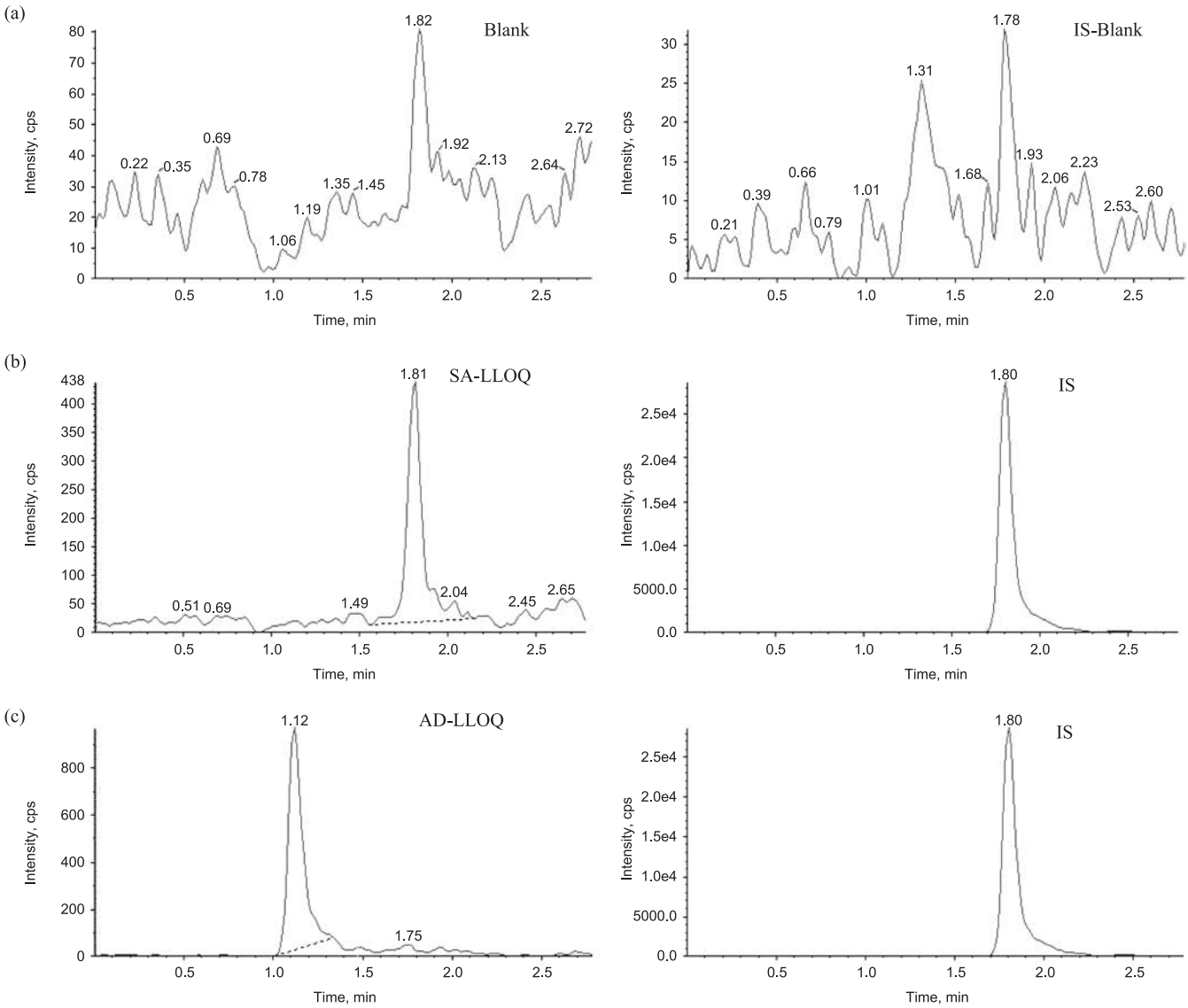


Figure 2. Typical MRM chromatograms of analyte (left panel) and IS (right panel) in (a) human blank plasma, (b) human plasma spiked with SA at LLOQ (0.2 ng/mL) and IS, (c) human plasma spiked with AD (0.2 ng/mL) at LLOQ and IS, (d) human plasma spiked with VS (0.5 ng/mL) at LLOQ and IS and (e) an *in vivo* plasma sample showing VS peak obtained following an oral dose of VS tablet to a healthy volunteer along with IS.

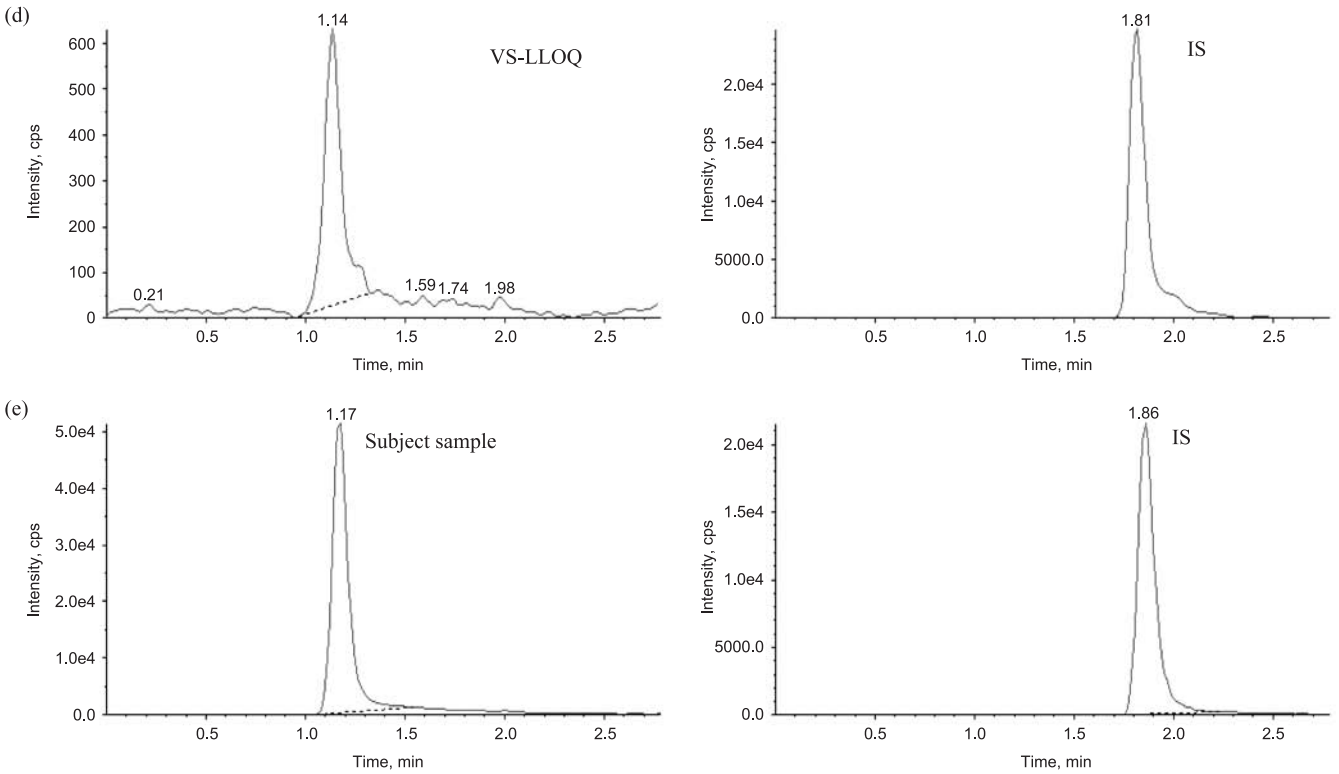


Figure 2. (*Continued*).

Matrix Effect

In our study, the matrix effect was evaluated by analyzing three batches of QC samples (LQC, MQC and HQC) for all the three analytes. Average matrix effect values obtained were 4.80–8.43% for SA, 7.60–12.8% for AD and 9.48–10.9% for VS. No significant peak area differences were observed. The matrix effect on IS was found to be 4.34% at the tested concentration of 20.0 ng/mL.

Calibration Curve

The plasma calibration curve was constructed using calibration standards of 0.20–50.0 ng/mL for SA and AD but 0.50–50.0 ng/mL for VS. The standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to the $y = mx + c$ using a weighing factor ($1/X^2$). The average regression ($n = 4$) for SA, AD and VS was found to be ≥0.996, 0.996 and 0.994, respectively. The lowest concentration with the RSD <20% was taken as LLOQ and was found to be 0.2 ng/mL for SA and AD, and 0.5 ng/mL for VS. The percentage accuracy observed for the mean of back-calculated concentration for four linearities was within 93.98–102.73, 93.00–106.50 and 92.84–104.63% for SA, AD and VS, respectively. The precision (% CV) values were 1.36–11.85, 2.16–13.57 and 5.47–13.35% for SA, AD and VS, respectively.

Precision and Accuracy

The accuracy, intra- and inter-assay precisions which were determined by analyzing six replicates of QC samples at four concentrations on four different days are shown in Table 1.

Stability

The predicted concentrations for each analyte at LQC and HQC samples deviated within ±15% of the nominal concentrations in a batter of stability tests viz. autosampler (24 h), bench-top (8 h), repeated three freeze–thaw cycles and at −80 ± 10°C for at least 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Dilution Effect

The standard curve can be extended up to 4000 ng/mL without affecting the final concentrations. The results have shown that the precision and accuracy for six replicates of diluted samples were within the acceptance range (data not shown).

Pharmacokinetic Study

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of VS in humans. Profiles of the mean plasma concentration vs time were shown in Fig. 2(d). Maximum concentrations in plasma (C_{max} 3290 ± 150 ng/mL) were achieved at 3.5 ± 1.4 h (T_{max}). The half-life ($t_{1/2}$) was found to be 5.52 ± 1.45 h, while the $AUC_{(0-\infty)}$ was 20.80 ± 2.51 μg h/mL.

Discussion

So far there have been no published methods available for the simultaneous determination of SA, AD and VS. Validated methods are essential for the determination of SA, AD and VS concentrations in human plasma for bioequivalence studies. The validated method is very robust, rugged and it utilizes a short

Table 1. Intra- and inter-day precision data of the analytes in human plasma

Quality control	Run	Measured concentration of SA (ng/mL)				Measured concentration of AD (ng/mL)				Measured concentration of VS (ng/mL)			
		Mean	SD	RSD	Accuracy (%)[a]	Mean	SD	RSD	Accuracy (%)[a]	Mean	SD	RSD	Accuracy (%)[a]
Intra day variation (six replicates at each concentration)													
LLOQ	1	0.20	0.02	10.6	100	0.21	0.01	6.38	104	0.47	0.06	12.8	94.3
	2	0.18	0.01	5.75	89.9	0.20	0.02	11.0	100	0.55	0.05	8.75	108
	3	0.20	0.03	12.5	100	0.17	0.01	5.56	86.4	0.56	0.07	13.0	111
	4	0.22	0.03	12.8	108	0.18	0.02	11.5	92.0	0.51	0.05	10.4	101
LQC	1	0.56	0.02	2.68	94.0	0.62	0.07	10.6	102	1.43	0.14	9.65	95.0
	2	0.56	0.03	5.18	93.1	0.54	0.04	7.24	89.8	1.34	0.03	2.36	89.0
	3	0.59	0.06	10.3	98.1	0.62	0.06	9.35	103	1.51	0.12	8.05	100
	4	0.53	0.05	8.77	88.7	0.52	0.06	10.9	86.7	1.50	0.20	12.9	100
MQC	1	21.3	0.19	0.88	85.4	24.4	2.43	9.96	97.6	23.1	2.47	10.6	92.4
	2	21.8	0.22	0.99	87.4	21.6	0.67	3.07	86.6	22.3	1.43	6.38	89.2
	3	25.0	1.09	4.35	100	25.0	0.93	3.72	100	24.7	1.70	6.88	99.0
	4	23.2	0.39	1.69	93.0	21.7	0.62	2.84	87.0	24.9	0.72	2.89	99.7
HQC	1	36.3	0.49	1.33	90.8	40.3	2.67	6.62	100	34.2	2.55	7.45	85.5
	2	38.1	0.86	2.25	95.2	38.8	1.13	2.92	97.0	34.9	1.61	4.61	87.2
	3	42.7	1.93	4.52	106	42.8	2.19	5.11	107	40.9	2.92	7.14	102
	4	38.3	0.52	1.36	95.9	36.4	0.96	2.63	91.0	41.9	4.66	11.1	104
Inter-day variation (24 replicates at each concentration)													
LLOQ		0.20	0.03	12.6	99.8	0.19	0.02	11.3	95.9	0.52	0.06	12.3	104
LQC		0.56	0.05	8.00	93.5	0.57	0.07	11.9	95.2	1.44	0.15	10.0	96.2
MQC		22.8	1.58	6.88	91.5	23.2	2.03	8.75	92.9	23.7	1.94	8.17	95.1
HQC		38.8	2.60	6.69	97.2	39.6	2.97	7.49	99.0	38.0	4.60	12.1	95.0

RSD, relative standard deviation (SD × 100/mean). LLOQ and LQC for SA and AD were 0.2 and 0.6 ng/mL, respectively, whereas for VS it was 0.5 and 1.5 ng/mL, respectively. MQC and HQC for all the three analytes were 25 and 40 ng/mL.
[a]Accuracy: (mean assayed concentration – nominal concentration)/(nominal concentration) × 100.

Table 2. Stability data of the analytes in human plasma

Quality control	Stability	Measured concentration of SA (ng/mL)				Measured concentration of AD (ng/mL)				Measure concentration of AD (ng/mL)			
		Mean	SD	Accuracy (%)*	% CV	Mean	SD	Accuracy (%)[a]	% CV	Mean	SD	Accuracy (%)[a]	% CV
LQC	0 h	0.59	0.06	100	10.3	0.62	0.06	100	9.35	1.51	0.12	100	8.05
	In injector (24 h)	0.60	0.04	102	6.18	0.63	0.06	101	8.98	1.53	0.19	100	12.3
	Bench top (8 h)	0.60	0.02	101	4.05	0.65	0.06	105	3.00	1.52	0.18	100	11.7
	Third freeze–thaw	0.57	0.05	96.3	8.11	0.59	0.02	96.1	3.05	1.40	0.15	92.5	10.5
	30 days at −80°C	0.58	0.03	99.1	5.39	0.59	0.03	95.7	4.57	1.49	0.09	98.2	6.03
HQC	0 h	42.7	1.93	100	4.52	42.8	2.19	100	5.11	40.9	2.92	100	7.14
	In injector (24 h)	44.3	1.52	103	3.41	42.3	1.78	98.7	4.21	41.9	3.63	102	8.64
	Bench top (8 h)	39.3	3.42	92.1	8.69	45.0	4.10	105	9.10	44.5	5.71	108	12.8
	Third freeze–thaw	38.6	0.57	90.5	1.48	39.0	1.10	91.1	2.83	41.1	1.73	100	4.20
	30 days at −80°C	41.8	0.83	98.0	1.99	41.6	1.51	97.1	3.64	42.8	0.60	104	1.39

LLOQ and LQC for SA and AD are 0.2 and 0.6 ng/mL whereas for VS it is 0.5 and 1.5 ng/mL, respectively. MQC and HQC for all the three analytes are 25 and 40 ng/mL
% CV (precision), coefficient of variation.
[a]Accuracy: (mean assayed concentration – nominal concentration)/(nominal concentration) × 100.

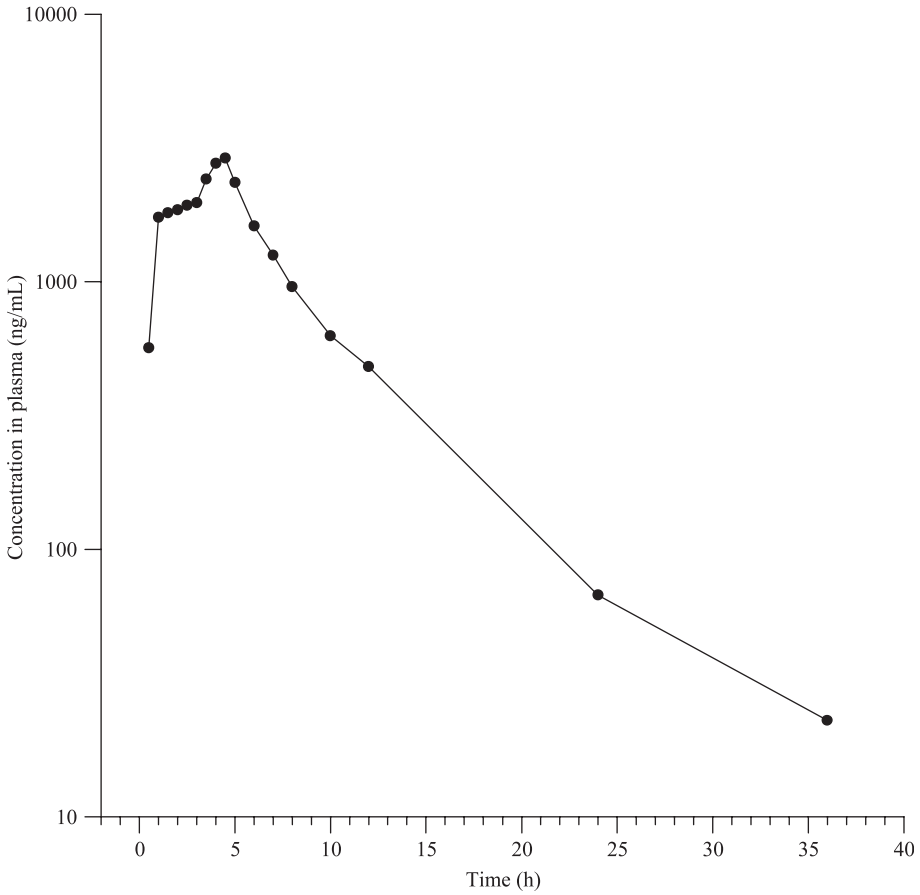


Figure 3. Plasma concentration–time profile of VS in human plasma following oral dosing of VS tablet to a healthy volunteer.

runtime of 2.8 min for each sample analysis. Here we have developed a method for the determination of SA, AD and VS in human plasma with good sensitivity (LLOQ 0.2 ng/mL for SA and AD; 0.5 ng/mL for VS). The method requires a single IS for determination of all three analytes. Sample preparation is very simple, involving simple protein precipitation of plasma with acetonitrile followed by separation of supernatant, evaporation and reconstitution of the dry extract with reconstitution solvent before injecting on to the LC-MS/MS. We have also confirmed that the presence of either parent (simvastatin) or putative metabolite (4-hydroxyvalsartan) did not interfere the either detection or separation of all three analytes investigated in this method (data not shown).

Conclusions

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS assay to quantify SA, AD and VS simultaneously using single IS. To the best of our knowledge, this is the first time all these three analytes were estimated simultaneously. In addition, the present method utilizes a single-step protein precipitation method and offers high throughput because of shorter run time. From the results of all the validation parameters, we can conclude that the present method can be useful for BA/BE studies and routine therapeutic drug monitoring (TDM) with desired precision and accuracy along with high-throughput.

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