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Simultaneous Determination of Simvastatin, Simvastatin Acid, Ramipril and Ramiprilate in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

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A rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the determination of simvastatin and its *in vivo* generated drug simvastatin acid along with ramipril and its active metabolite ramiprilate in human plasma. After solid phase extraction (SPE) the analytes and IS were chromatographed on a hypurity C18 (150 mm × 4.6 mm i.d, 5 µm particle size) column using 50 µL injection volume with a run time of 6 min. An isocratic mobile phase consisting of 4 mmol/L ammonium acetate (pH 3.00): acetonitrile (20:80, v/v) was used to separate all these drugs. The precursor and product ions of these drugs were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring mode (MRM) with polarity switch. The proposed method was validated over the range of 0.25 ng/mL to 50 ng/mL for simvastatin and simvastatin acid, 0.25 ng/mL to 40 ng/mL for ramipril and 1.00 to 40 ng/mL for ramiprilate. Inter-batch and intra-batch precision (% CV) across 5 validation runs (LLOQ, LQC, MQC, HQC and ULOQ QC) was less than 14 for all the analytes. The accuracy determined for all the analytes at these levels was within ± 14 % in terms of relative error.

Key Words: Simvastatin, Simvastatin acid, Ramipril, Ramiprilate, Lovastatin acid, Quinapril, Quinaprilat LC-MS/MS, Multiple reaction monitoring.

INTRODUCTION

Simvastatin[1,2], [1S-[1α,3α,7β,8β(2S*,4S*)8aβ]]-2,2-dimethyl-butanoic acid 1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl)ethyl]-1-napthalenyl ester is a hypolipidemic drug belonging to the class of pharmaceuticals called 'statins'. Simvastatin is used to control hypercholesterolemia (elevated cholesterol levels) and to prevent cardiovascular disease. Simvastatin as a lactone prodrug administered orally, it hydrolyzes *in vivo* to simvastatin acid, which is a potent inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase, an enzyme that catalyzes an early rate-limiting step in biosynthesis of

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cholesterol. Ramipril[1,2], ([2s-{1(R*(R*)} 2α3a,β, 6aβ]}-1-[2-[[1-(Ethoxycarbonyl)-3-phenyl propyl]aminol]-1-oxopropyl]-octo hydrocyclo penta (b) pyrole-2-carboxylic acid) is an antihypertensive drug which is an angiotension converting enzyme inhibitor. Ramipril is hydrolyzed by esterases in the liver to its active metabolite ramiprilate. Ramiprilate inhibits the enzyme dipeptidylcarboxypeptidase I, also called angiotensin-converting enzyme (ACE). There are methods reported for estimation of simvastatin and simvastatin acid by mass spectrometry[3-5] and by HPLC[6]. Ramipril and its metabolite ramiprilate are also reported to be analyzed by LC-MS[7] and by HPLC[6]. The literature revealed no method was available for simultaneous determination of all the drugs in such pharmaceutical preparations by LC-MS-MS. Therefore an LC-MS-MS method was developed for simultaneous determination of simvastatin, simvastatin acid, ramipril and ramiprilate.

The aim of this study was to develop a sensitive, selective and high throughput method for simultaneous determination of simvastatin, simvastatin acid, ramipril and ramiprilate (Fig. 1) in human plasma for therapeutic drug monitoring and pharmacokinetic studies. As a part of our ongoing research in this area, we have developed and validated a LC-MS/MS assay for these drugs in human plasma. Special emphasis was given to optimize the extraction step in order to get quantitative and reproducible recovery for the analytes. The method presents a simple and clean SPE procedure with drying and reconstitution steps. The analytes and internal standards were well separated with minimum matrix interference in a run time of 6.0 min under isocratic conditions. The LLOQ for simvastatin, simvastatin acid and ramipril was 0.250 and 1.00 ng/mL for ramiprilate.

EXPERIMENTAL

Working standards of simvastatin, simvastatin acid and lovastatin acid (IS) were provided by Lupin Laboratories Ltd. (Mumbai, India) and ramipril and ramiprilate were provided by Aventis Pharma Ltd. (Goa, India) having purity more than 99 %. Quinapril (IS) and quinaprilat (IS) were supplied by Shasun Chemicals and Drugs Ltd. (Pondicherry, India). HPLC grade methanol and acetonitrile were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade ammonium acetate was procured from Qualigens Ltd. (Mumbai, India). Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Banglore, India). Blank human plasma was collected in house with heparin as an anticoagulant and stored at -70 °C. Orochem, 30 mg; 1 mL DVB HL solid phase extraction (SPE) cartridges were procured from orochem (India).

Liquid chromatography and mass spectrometric condition: An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC-10AD prominence pump, autosampler (SIL-HTc) and solvent degasser (DGU-14) were used for all the analysis. For separation, the samples were applied without any guard column to Hypersil Hypurity C18 (150 mm × 4.6 mm i.d., 5 μ particle size) analytical column from Thermo (I) Pvt Ltd (India). The flow rate of the mobile phase under isocratic condition

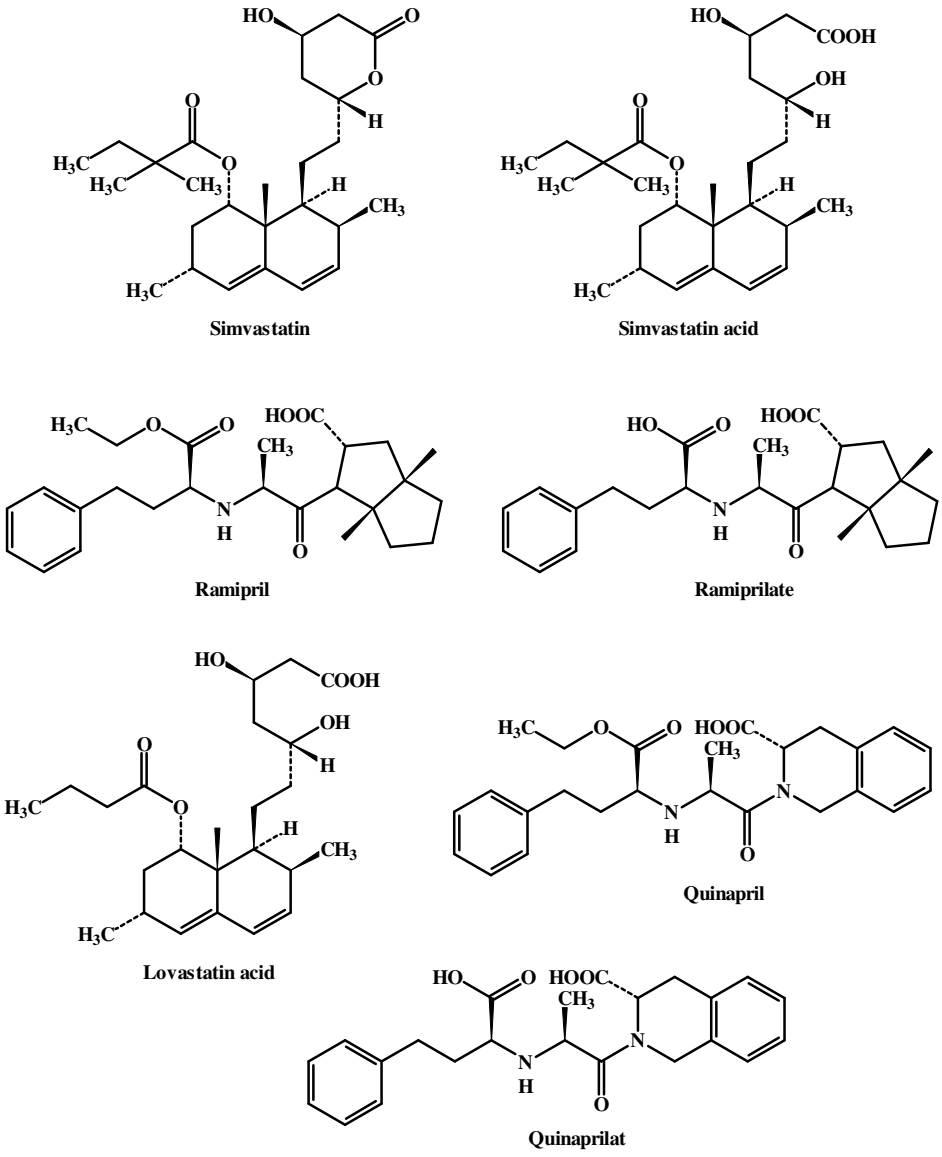


Fig. 1. Chemical structure of diffeernt compounds

was kept at 0.8 mL/min with split ratio 1:1. The auto sampler temperature was set at 4 °C and the injection volume was 50 mL. The mobile phase consisted of 4 mmol/L ammonium acetate (pH 3.0): acetonitrile (20:80 v/v). The total LC run time was 6.0 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-3000, (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray ionization source and operating in both positive and negative ion modes

simultaneously. Simvastatin, simvastatin acid and lovastatin acid (IS) were analyzed in positive mode and ramipril, ramiprilate, quinapril (IS for ramipril) and quinaprilat (IS for ramiprilate) were analyzed in negative mode. Analyst software version 1.4 was used to control all parameters of LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion (m/z) transitions for simvastatin (419 → 199.2), simvastatin acid (437.1 → 303.2), ramipril (415.2 → 154.0), ramiprilate (387.1 → 154.0) and internal standards lovastatin acid (423.3 → 303.8), quinapril (437.2 → 187.8) and quinaprilat (409.1 → 176.0), respectively.

Source dependent parameters optimized were gas 1(Nebuliser gas): 12 psi; gas 2(heater gas flow): 8000 cc/min; temperature (TEM): 450 °C for all analytes in both positive and negative modes. Ion spray voltage (ISV) was 5500 V and -4500 v in positive and negative mode, respectively. Compound dependent parameters like declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were 30, 20 V; 8, 10 V; 102, 70 V; 16.50, 15.00 eV; 14, 10 V for simvastatin and simvastatin acid, respectively in positive mode. For negative mode declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) were -46, -36 V; -10 -10 V; -250, -160 V; -36, -28 eV; -11, -11 V for ramipril and ramiprilate, respectively. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 12 psi. Quadrupole 1 and Quadrupole 3 both were maintained at unit resolution and dwell time was set at 200 each for simvastatin, simvastatin acid, ramipril and ramiprilate, respectively.

Analytical data processing: Chromatographic data were collected and integrated using Analyst software version 1.4. Peak area ratio of the analyte to IS was utilized for the construction of calibration curve. A weighing of 1/x (least-squares linear regression analysis, where x is the analyte concentration) was used for curve fitting. Concentration in unknown samples were calculated from the best-fit equation ($y = mx + c$), where y is the peak area ratio. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level.

Standard and quality control preparation: Standard stock solution of all analytes (100 µg/mL) and internal standards (100 µg/mL) were separately prepared in methanol. Working solutions in the required concentration range were prepared by appropriate dilution of their stock solutions in methanol:water (50:50 v/v). All the solutions were stored at 2-8 °C and were brought to room temperature before use.

The calibration standards and quality control (QC) samples were prepared by spiking working solutions (5 %) with blank plasma. Calibration samples were made at concentrations 0.25, 0.50, 1.00, 5.00, 10.00, 20.00, 40.00 and 50.00 ng/mL for simvastatin and simvastatin acid, 0.25, 0.50, 1.00, 5.00, 10.00, 16.00, 32.00 and 40.00 ng/mL for ramipril, 1.00, 2.00, 5.00, 7.50, 10.00, 16.00, 32.00 and 40.00 ng/mL for ramiprilate. Quality control samples were prepared at 0.75 ng/mL (LQC), 12.00

ng/mL (MQC) and 36.00 ng/mL (HQC) for simvastatin and simvastatin acid, 0.75 ng/mL (LQC), 18.00 ng/mL (MQC) and 36.00 ng/mL (HQC) for ramipril and 3.00 ng/mL (LQC), 18.00 ng/mL (MQC) and 36.00 ng/mL (HQC) for ramiprilate. Spiked plasma samples were stored at -70 °C.

Sample preparation: All frozen calibration standards and quality control samples were thawed at room temperature. The samples were adequately vortexed to mix. 0.5 mL of plasma sample was dispensed into eppendorf tubes and 50 mL of internal standard (0.500 µg/mL of quinapril, 0.500 µg/mL of quinaprilat and 0.250 µg/mL of lovastatin acid) was added, followed by vortexing for 10 s. Then 0.5 mL of 50 mmol/L ammonium acetate (pH = 4.3 ± 0.05) was added and vortex again each tube for about 30 s. The samples were centrifuged at 15000 rpm at 10 °C for 5 min and then loaded on Orochem DVB-HL cartridges preconditioned with 1 mL of methanol followed by 1 mL of 50 mmol/L ammonium acetate (pH = 4.3 ± 0.05). Further, plasma was drained out under nitrogen pressure and cartridges were washed with 1 mL water followed by 1 mL of 2 % v/v acetic acid in methanol:water (10:90 v/v) twice to clean up the samples. After proper drying of the cartridge, elution was carried out using 1 mL methanol. The eluate was evaporated to dryness under nitrogen at 50 °C. The residue was reconstituted by 300 mL mobile phase and transferred into vials, capped and placed in an autosampler rack for injection.

Method validation: A thorough and complete method validation of simvastatin, simvastatin acid, ramipril and ramiprilate in human plasma was done following the USFDA guidelines[8]. The method was validated for selectivity, sensitivity, linearity, accuracy and precision, recovery, stability, matrix effect and dilution integrity.

The selectivity towards endogenous and exogenous plasma matrix components was assessed in 12 different batches (6 normal, 2 hemolyzed and 2 lipemic) of human plasma samples by analyzing blank and spiked samples at LLOQ level. It was performed in two sets, in the first set, plasmas were extracted and directly injected for LC-MS/MS detection and in the second set, blank plasmas spiked with LLOQ working solution of mixture of simvastatin, simvastatin acid, ramipril and ramiprilate were extracted and analyzed. The second set was also used for sensitivity determination.

The linearity of the method was determined by analysis of standard plots associated with an 8-point standard calibration curve. Five linearity curves containing eight non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio *versus* concentration were drawn. The concentration of the analytes was calculated from the simple linear equation using regression analysis of spiked plasma calibration standard with reciprocate of the drug concentration as a weighting factor (1/concentration, i.e. 1/x). The peak area ratio values of calibration standards were proportional to the concentration of the drugs in plasma over the range tested. Inter-batch and intra-batch accuracy and precision was evaluated at 5 different concentrations (lower limit of quantification-LLOQ, lower quality control-LQC, medium quality control-MQC, higher quality control-HQC and upper limit of quantification-

ULOQ). Mean and standard deviation (SD) were obtained for calculated drug concentration at each level. Accuracy and precision were evaluated in terms of relative error (RE) and % CV respectively.

Recovery presents the extraction efficiency of a method. It was performed at LQC, MQC and HQC levels. The relative recoveries were evaluated by comparing peak area of extracted samples to that of unextracted samples.

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions. Stock solution stability was performed by comparing area response of stability sample of analytes and internal standards with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze thaw stability, long-term stability were performed at LQC and HQC level using six replicates at each level.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different lots of heparinized plasma. Two replicates each of LQC and HQC were prepared from different lots of plasma (total 24 QC samples).

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations above upper limit of quantification (ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.6 times the ULOQ concentration for all the analytes. Six replicates each of 0.5th and 0.25th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 against the freshly prepared calibration curve.

RESULTS AND DISCUSSION

For simultaneous detection up to nanogram level of analytes with internal standards in human plasma, it was necessary to adjust not only the chromatographic conditions and mass parameters but also to develop an efficient extraction method that gives consistent and reproducible recovery of analytes from plasma. Parent ions and product ions were optimized by infusing 500 ng/mL solutions of into mass spectrometer in 5-500 m/z range, in both positive and negative polarity mode using electro spray ionization technique. Best intensity for $[M+H]^+$ ions was found in positive mode for the simvastatin and simvastatin acid. Ramipril and ramiprilate was found more intense in negative polarity mode.

Chromatographic analysis of the analytes and internal standards was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Separation was tried using various combinations of acetonitrile and buffer with varying contents of each component on variety of columns like C8 and C18 Hypersil, hypurity; C18 advance high purity and symmetry shield RP 18 to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape.

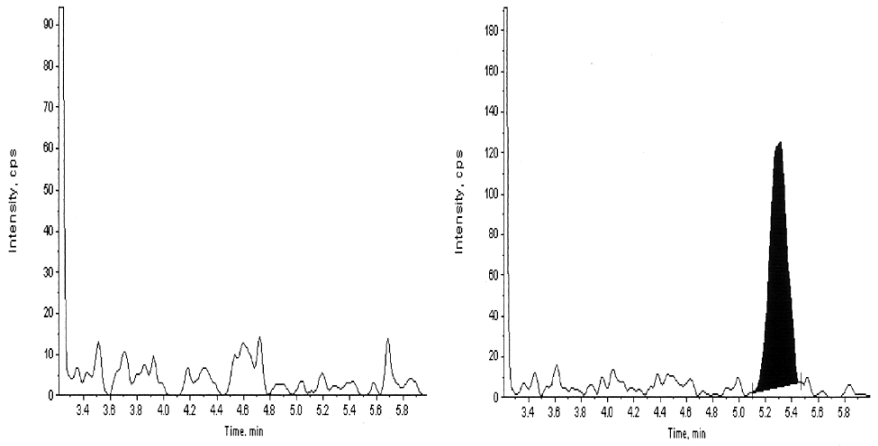
Use of buffer helped in achieving good response for MS detection operating in both the positive and negative mode. To get a good chromatographic separation with desired response it was observed that mobile phase as well as selection of column is an important criterion. Thus, a mobile phase consisting of 4 mmol/L ammonium acetate with pH adjusted to 3.0 with acetic acid: acetonitrile (20:80 v/v) was found suitable as all analytes and internal standards were well separated by this phase. High content of acetonitrile (80 %) in the mobile phase helped in eluting the analytes and internal standards within 6 min at a flow rate of 0.8 mL/min with LC split ratio 1:1. Hypersil hypurity C18 (150 x 3.9 mm, 5 μ particle size) column gave good peak shape and response even at LLOQ level for all the analytes including internal standards. Low injection volume of 50 μL reduced overloading of column with analytes, thereby ensuring more number of analyses on the same column.

For extraction of all analytes and internal standards with quantitative recovery and negligible matrix effect from plasma samples, an efficient extraction method was mandatory. A simple and rapid solid phase extraction method was developed using orochem, 30 mg; 1 mL DVB HL solid phase extraction (SPE) cartridges. Extraction of analytes was carried out with 1 mL of methanol. The eluate was subjected for drying and reconstitution to increase the sensitivity (lower LLOQ) with improved response upto nanogram level. No interference was observed from any endogenous or exogenous plasma matrix.

It was difficult to find a compound which could ideally mirror the analytes to serve as a good IS. Several compounds were investigated to find a suitable IS and finally lovastatin acid belonging to same class of simvastatin in positive mode and quinapril, quinaprilat belonging to same class of ramipril and ramiprilate in negative mode was found most appropriate for the present purpose. There was no significant effect of IS on analytes recovery, sensitivity or ion suppression. The results of method validation using lovastatin acid, quinapril and quinaprilat as the IS were acceptable in this study based on FDA guidelines.

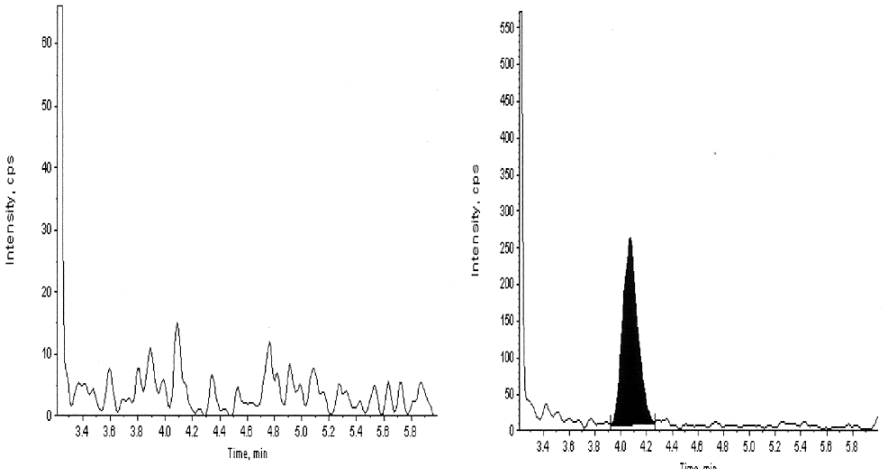
Selectivity and sensitivity (LLOQ): The selectivity of the method towards endogenous plasma matrix was evaluated in ten different batches of human plasma by analyzing blanks and spiked samples at LLOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma batches. Figs. 2-5 demonstrates the selectivity results with the chromatograms of blank plasma and the peak response of analytes at LLOQ level. The response was calculated in terms of signal to noise (S/N) ratio for spiked and unspiked plasmas. The mean S/N ratio for 10 plasma samples found was 97.61, 60.66, 175.96 and 837.86 for simvastatin, simvastatin acid, ramipril and ramiprilate, respectively. The mean accuracy (%) for back calculated concentration for normal, heamolyzed and lipemic was within 91-105 % with % CV between 4.68 and 9.69 %.

Linearity, accuracy, precision and recovery: The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 0.25-50.00 ng/mL for simvastatin and simvastatin



(A) (B)

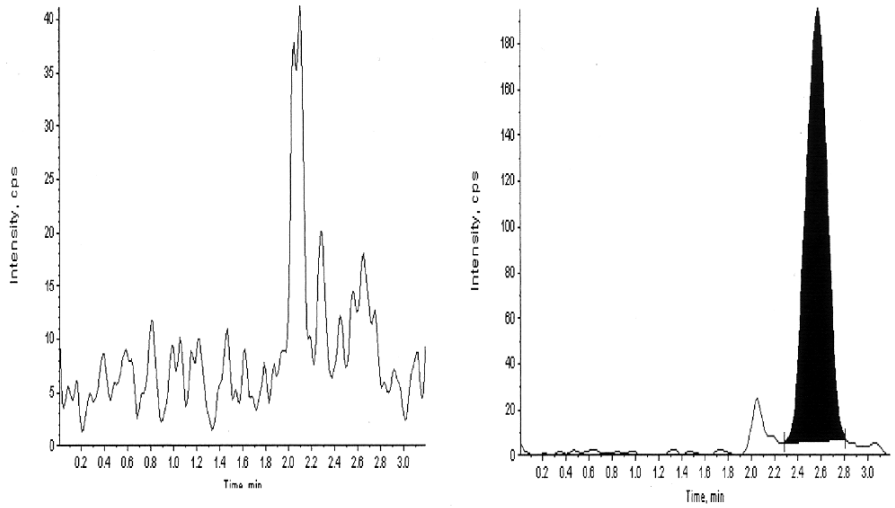
Fig. 2. Representative chromatogram of plasma blank (A) and LLOQ (B) for simvastatin



(A) (B)

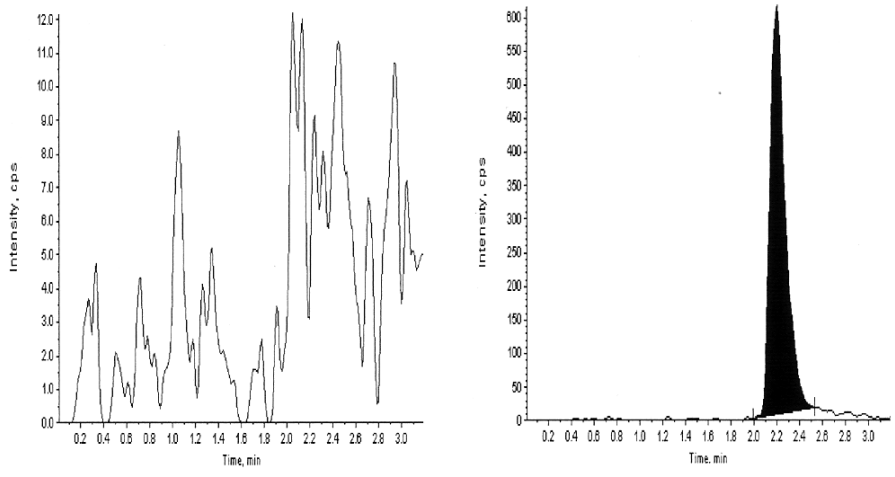
Fig. 3. Representative chromatogram of plasma blank (A) and LLOQ (B) for simvastatin acid

acid, 0.25-40.00 ng/mL for ramipril and 1.00-40.00 ng/mL for ramiprilate. The calibration curves appeared linear and were well described by least squares lines with correlation coefficient ≥ 0.9990, 0.9992, 0.9987 and 0.9971 for simvastatin, simvastatin acid, ramipril and ramiprilate. A weighing factor of 1/concentration (1/x) was chosen to achieve homogeneity of variance. The observed mean back calculated concentration with accuracy (%) and precision (% CV) of five linearties are given in Table-1.



(A) (B)

Fig. 4. Representative chromatogram of plasma blank (A) and LLOQ (B) for ramipril



(A) (B)

Fig. 5. Representative chromatogram of plasma blank (A) and LLOQ (B) for ramiprilate

The precision and accuracy of the developed method was determined by analysis 5 quality control samples. Intra-day variation of the assay was assessed by injecting 2 batches containing these samples on the same day. Inter-day variation was assessed by injecting 1 batch on 3 different days. Each run consisted of 6 replicates at 5 concentration levels (LLOQ QC, LQC, MQC, HQC and ULOQ QC). Intra-batch precision was less than 14 % for each analytes. Precision observed for inter-batch

TABLE-1
SUMMARY OF CALIBRATION CURVE STANDARDS (n=5)

Analyte	Nominal conc. (ng/mL)	Mean back calculated conc. (ng/mL)	Coefficient of variation (%)	Relative error (%)
Simvastatin	0.250	0.263	5.80	5.12
	0.500	0.459	12.93	-8.16
	1.000	1.029	5.74	2.94
	5.000	4.835	1.62	-3.29
	10.000	10.091	5.32	0.91
	20.000	19.038	3.85	-4.81
	40.000	39.173	2.21	-2.07
	50.000	51.830	2.62	3.67
Simvastatin acid	0.250	0.232	6.90	-7.36
	0.500	0.506	1.67	1.12
	1.000	1.069	2.38	6.88
	5.000	4.951	3.43	-0.99
	10.000	10.508	1.80	5.08
	20.000	18.721	4.32	-6.40
	40.000	40.254	1.82	0.64
	50.000	50.511	2.35	1.02
Ramipril	0.250	0.265	9.24	6.00
	0.500	0.505	3.42	0.96
	1.000	1.088	7.08	8.78
	5.000	4.661	6.69	-6.78
	10.000	9.810	6.82	-1.90
	16.000	14.727	5.04	-7.95
	32.000	32.242	2.37	0.76
	40.000	41.488	0.67	3.72
Ramiprilat	1.000	1.034	5.93	3.36
	2.000	1.835	2.89	-8.26
	5.000	5.259	2.96	5.18
	7.500	7.758	4.88	3.43
	10.000	10.676	2.98	6.76
	16.000	14.031	2.84	-12.31
	32.000	31.273	2.31	-2.27
	40.000	41.634	2.15	4.09

was also almost 15 % at lower level (LLOQ QC) and less than 14 % for remaining levels. Accuracy was within 86 to 106 % of their nominal concentration respectively as given in Table-2.

Six replicates at LQC, MQC and HQC level were prepared for recovery determination. Mean relative recovery found was 70.73, 91.72, 78.56 and 72.62 % with a precision (% CV) of 4.44, 6.88, 5.79 and 6.91 for simvastatin, simvastatin acid, ramipril and ramiprilate, respectively. Recovery of IS was 78.06, 72.91and 83.25 % with % CV of 5.40, 9.77 and 6.75 for lovastatin acid, quinapril and quinaprilat,

TABLE-2
INTRA-BATCH AND INTER-BATCH PRECISION AND ACCURACY

Analytes	Level	Conc. added (pg/mL)	Intra-batch			Inter-batch		
			Mean conc. found (ng/mL)*	CV (%)	RE (%)	Mean conc. found (ng/mL)**	CV (%)	RE (%)
Simvastatin	LLOQ	0.250	0.251	13.28	0.30	0.255	13.60	2.13
	LQC	0.750	0.757	4.33	0.89	0.787	8.37	4.99
	MQC	12.000	11.241	5.59	-6.33	12.067	4.79	0.55
	HQC	36.000	34.199	4.39	-5.00	36.441	7.58	1.22
	ULOQ	50.000	48.912	5.81	-2.18	52.357	3.66	4.71
Simvastatin acid	LLOQ	0.250	0.215	9.06	-13.93	0.232	11.85	-7.09
	LQC	0.750	0.733	3.61	-2.30	0.740	8.71	-1.34
	MQC	12.000	11.584	4.75	-3.47	11.907	6.97	-0.78
	HQC	36.000	34.406	2.53	-4.43	33.352	7.67	-7.35
	ULOQ	50.000	49.313	1.44	-1.37	49.639	7.73	-0.72
Ramipril	LLOQ	0.250	0.263	6.77	5.20	0.258	15.49	3.36
	LQC	0.750	0.753	8.20	0.44	0.737	4.75	-1.77
	MQC	18.000	16.316	6.58	-9.35	16.956	8.63	-5.80
	HQC	36.000	36.912	6.11	2.53	36.625	11.62	1.74
	ULOQ	40.000	41.932	9.18	4.83	41.057	5.39	2.64
Ramiprilate	LLOQ	1.000	0.959	10.99	-4.12	1.037	13.64	3.69
	LQC	3.000	2.768	2.66	-7.72	2.785	3.32	-7.18
	MQC	18.000	18.893	5.04	4.96	19.105	4.83	6.14
	HQC	36.000	35.648	2.49	-0.98	35.347	8.35	-1.81
	ULOQ	40.000	39.961	2.18	-0.10	40.100	3.59	0.25

RE = Relative error, CV = Co efficient of variance; *Mean of six replicates observations at each concentration; **Mean of 24 replicates observations over four different analytical runs.

respectively. This indicates that the extraction efficiency for the analytes as well as internal standards was consistent and reproducible.

Stability, matrix effect and dilution integrity: Stock solution of analytes and internal standards were stable at room temperature for 24 h and at 2-8 °C for 21 d. Simvastatin, simvastatin acid, ramipril and ramiprilate in control human plasma at room temperature was stable at least for 24 h and for minimum of 5 freeze and thaw cycles. Process stability was of 24 h at 4 °C. Spiked plasma samples stored at -70 °C for long term stability experiment were stable for minimum 105 d. Different stability experiments in plasma and the values for the precision and percent change are shown in Table-3. There was no significant degradation observed since the deviations in concentration was within 15 % of their nominal values.

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. It was performed with the aim to see the matrix effect by processing six lots of different plasma samples in quadruplet

TABLE-3
STABILITY RESULTS

Analytes	Stability	Level	A	CV (%)	B	CV (%)	Change (%)
Simvastatin	Bench top (24 h at room temp.)	LQC (n = 6)	0.807	2.48	0.787	2.56	-2.54
		HQC (n =6)	36.743	1.53	36.370	1.11	-1.02
	Autosampler (24 h, 4 °C)	LQC (n = 6)	0.807	2.48	0.777	3.08	-3.80
		HQC (n =6)	36.743	1.53	35.794	2.32	-2.58
	5^{th} Freeze Thaw Cycle	LQC (n = 6)	0.770	4.01	0.747	5.60	-2.88
		HQC (n =6)	38.871	2.17	38.626	2.87	-0.63
	Long term (105 days, -70 °C)	LQC (n = 6)	0.738	4.25	0.721	3.59	-2.28
		HQC (n =6)	34.812	2.58	34.808	1.99	-0.01
Simvastatin acid	Bench top (24 h at room temp.)	LQC (n = 6)	0.786	4.18	0.772	5.19	-1.76
		HQC (n =6)	36.718	2.12	37.711	1.28	2.70
	Autosampler (24 h, 4 °C)	LQC (n = 6)	0.786	4.18	0.792	3.13	0.81
		HQC (n =6)	36.718	2.12	37.754	1.89	2.82
	5^{th} Freeze Thaw Cycle	LQC (n = 6)	0.748	3.09	0.753	2.61	0.65
		HQC (n =6)	36.993	2.21	36.864	2.97	-0.35
	Long term (105 days, -70 °C)	LQC (n = 6)	0.793	5.46	0.766	6.62	-3.38
		HQC (n =6)	34.615	2.57	33.908	1.48	-2.04
Ramipril	Bench top (24 h at room temp.)	LQC (n = 6)	0.741	4.60	0.747	2.21	0.74
		HQC (n =6)	40.009	8.22	37.362	5.47	-6.62
	Autosampler (24 h, 4 °C)	LQC (n = 6)	0.741	4.60	0.705	7.26	-4.92
		HQC (n =6)	40.009	8.22	37.800	6.29	-5.52
	5^{th} Freeze Thaw cycle	LQC (n = 6)	0.749	7.58	0.774	10.51	3.29
		HQC (n =6)	43.780	3.08	44.422	4.73	1.47
	Long term (105 days, -70 °C)	LQC (n = 6)	0.668	5.84	0.723	6.51	8.18
		HQC (n =6)	35.616	4.18	36.501	6.59	2.48
Ramiprilate	Bench top (24 h at room temp.)	LQC (n = 6)	3.050	3.96	3.125	1.22	2.48
		HQC (n =6)	37.229	5.62	38.151	2.90	2.48
	Autosampler (24 h, 4 °C)	LQC (n = 6)	3.050	3.96	3.038	8.63	-0.38
		HQC (n =6)	37.229	5.62	39.387	4.37	5.79
	5^{th} Freeze Thaw Cycle	LQC (n = 6)	2.786	8.02	2.905	4.18	4.26
		HQC (n =6)	38.828	2.30	39.070	3.98	0.62
	Long term (105 days, -70 °C)	LQC (n = 6)	3.263	3.36	3.100	7.24	-4.99
		HQC (n =6)	36.840	3.92	36.441	4.21	-1.08

A = Mean comparison sample conc. (ng/mL); B = Mean stability sample conc. (ng/mL)
CV = Coefficient of variation

(n = 4). LQC and HQC stock solutions were spiked post extraction in duplicate. Aqueous recovery solutions of LQC and HQC along with internal standard were also prepared. The results found were well within the acceptable limits. Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantification of analytes and IS peak. Thus, the extraction

method was rugged enough and gave accurate and consistent results when applied to subject sample analysis.

The mean back calculated concentrations for 0.50 and 0.25 dilution samples were within 85-115 % of their nominal. The coefficient of variation (% CV) for 0.50 and 0.25 dilution samples of simvastatin, simvastatin acid, ramipril and ramiprilate were less than 6.0 %.

Concusion

The developed simultaneous LC-MS/MS assay for simvastatin, simvastatin acid, ramipril and ramiprilate is selective, rugged and suitable for routine measurement of subject samples. This method has significant advantages in terms of clean and reproducible SPE extraction procedure and a short chromatographic run time of 6.0 min. The extraction method gave consistent and reproducible recoveries for analytes and internal standards from plasma, with minimum matrix interference and ion suppression. The reconstituted residue after evaporation (50 µL) is directly submitted for LC-MS analysis to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of simvastatin, simvastatin acid, ramipril and ramiprilate.

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