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Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 323–340

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Direct-injection LC–MS–MS method for high-throughput simultaneous quantitation of simvastatin and simvastatin acid in human plasma

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Accepted 11 January 2000

Abstract

A direct-injection liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) method was developed and validated for the simultaneous quantitation in human plasma of the widely used cholesterol-lowering prodrug simvastatin and its in vivo generated active drug, simvastatin acid. The plasma samples were injected into the LC–MS–MS system after simply adding the internal standard solution in an aqueous buffer and centrifuging. The analytes in the buffered plasma samples were found to be stable for at least 24 h at 4°C. The method was successfully validated under the challenging condition of using a large number of quality control (QC) samples including those in which the ratio of the simvastatin concentration to the simvastatin acid concentration was different from the concentration ratio in the calibration curve standards. Under the dual stabilizing conditions of lower temperature (4°C) and lower plasma pH of 4.9, the in-process hydrolysis of simvastatin to simvastatin acid or the lactonization of simvastatin acid to simvastatin was minimized to $\leq 1.0\%$. Although the entire run time for on-line cleanup and analysis was only 2.5 min, chromatographic base-line separation of simvastatin from simvastatin acid, which was required to avoid the interference by simvastatin acid with the simvastatin selected reaction monitoring channel, was achieved. The desired lower limit of quantitation of 0.5 ng/ml was achieved by injecting only an equivalent of 8.0 μl of the plasma sample. The extraction column lasted for at least 500 injections.

Keywords: Liquid chromatography–mass spectrometry–mass spectrometry method; Simvastatin; Simvastatin acid; Human plasma

1. Introduction

Simvastatin (Fig. 1) is a cholesterol-lowering agent widely used to treat hypercholestrolemia [1–4]. As a lactone prodrug administered orally, it hydrolyzes in vivo to simvastatin acid (Fig. 1), which is a potent inhibitor of 3-hydroxy-3-methyl-

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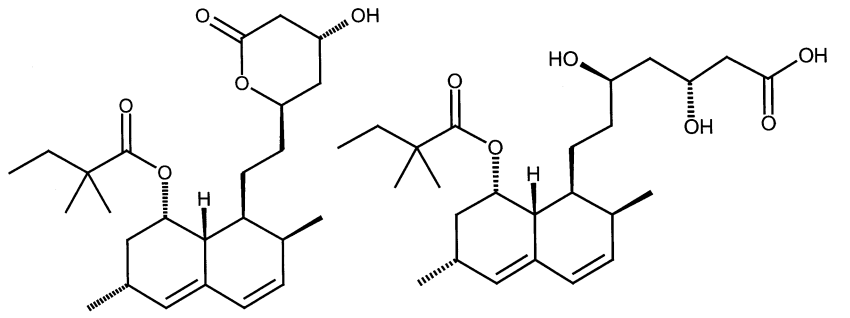
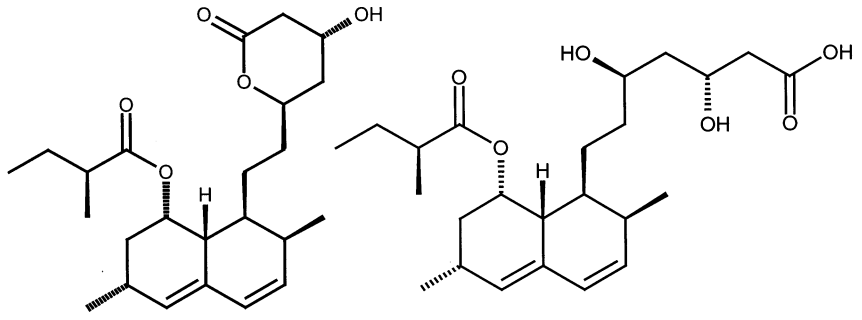
0731-7085/00/$ - see front matter
PII: S0731-7085(00)00309-5

glutaryl-coenzyme A (HMG-CoA) reductase. The quantitative determination of simvastatin and simvastatin acid has been reported using high performance liquid chromatography (HPLC) with ultra violet (UV) detection [5,6], HPLC with fluorescence detection [7], gas chromatography/mass spectrometry (GC/MS) [8,9], and atmospheric pressure chemical ionization (APCI) liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) [10]. The published methods suffer from long, arduous sample preparation and/or long chromatographic run times. In the GC/MS, LC–MS–MS and HPLC-fluorescence methods, simvastatin was separated from simvastatin acid by solid-phase extraction and then the two compounds were converted into the appropriate esters for the mass spectrometric or fluorescence analysis. Thus, two separate chromatographic runs were required in order to quantitate the prodrug and the active drug. In the HPLC–UV method, the sample preparation was comparatively less arduous but still required multiple manual steps of mixing, protein precipitation, centrifugation, ultrafiltration, evaporation, and reconstitution. By today's high-throughput industrial standard, the chromatographic run time was long (10 min).



Simvastatin
$C_{25}H_{38}O_5$
Molecular weight: 418.58
Monoisotopic exact mass: 418.27

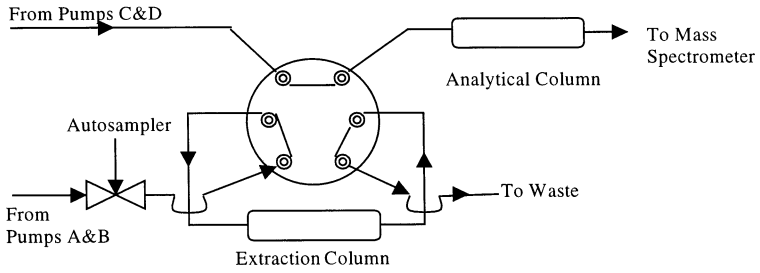
Simvastatin acid
$C_{25}H_{40}O_6$
Molecular weight: 436.59
Monoisotopic exact mass: 436.28



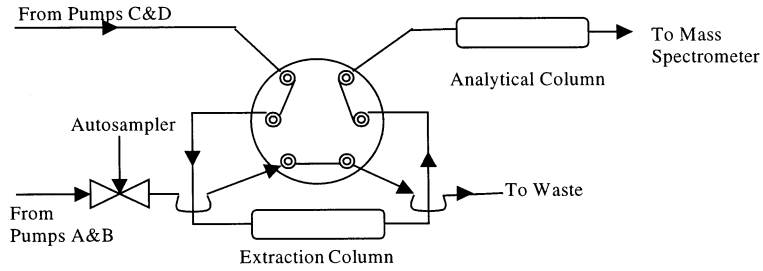
Lovastatin
$C_{24}H_{36}O_5$
Molecular weight: 404.55
Monoisotopic exact mass: 404.26

Lovastatin acid
$C_{24}H_{38}O_6$
Molecular weight: 422.56
Monoisotopic exact mass: 422.27

Fig. 1. Chemical structures of simvastatin, simvastatin acid and the two internal standards, lovastatin and lovastatin acid.



Configuration (a): Loading sample, extraction and equilibration



Configuration (b): Elution

Time	Flow rate (mL/min)				Configuration	On-line extraction stage
	Pump A	Pump B	Pump C	Pump D		
0.00	3.6	0.4	0.25	0.75	(a)	Extraction and cleanup
0.30	3.6	0.4	0.25	0.75	(a)	
0.40	0.005	0.095	0.25	0.75	(b)	Elution
1.00	0.0	4.0	0.25	0.75	(b)	
1.50	3.6	0.4	0.25	0.75	(a)	Equilibration to recondition the extraction column
2.00	3.6	0.4	0.25	0.75	(a)	
2.01	0.09	0.01	0.25	0.75	(a)	
2.50	0.09	0.01	0.25	0.75	(a)	

Pumps A and C: 3.0 mM formic acid in water (mobile phase A). Pumps B and D: 3.0 mM formic acid in acetonitrile (mobile phase B). The valve was switched at 0.3 and 1.0 min to obtain the two configurations. The mobile phase flows were decreased at 0.40 and 2.01 min to reduce waste.

Fig. 2. A schematic representation of the on-line extraction LC–MS–MS system.

In this paper, we report a direct-injection electrospray LC–MS–MS method for the simultaneous quantitation of simvastatin and simvastatin acid in human plasma. The method does not involve sample preparation except for adding the internal standard solution to the plasma samples prior to analysis by direct-injection LC–MS–MS. The total run time per sample was only 2.5 min. The validity of this method was tested under a rigorous condition using a large number of quality control (QC) samples that realistically represent post-dose

samples obtained from a study of simvastatin in humans.

2. Experimental

2.1. Chemicals and reagents

Simvastatin and lovastatin (internal standard for simvastatin, Fig. 1) were obtained from U.S.P. Simvastatin acid and lovastatin acid were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. Acetonitrile (HPLC grade) and formic acid (88%) were purchased from VWR Scientific (Piscataway, NJ, USA). Glacial acetic acid (ACS grade) and sodium acetate trihydrate (ACS grade) were from J.T. Baker (Phillipsburg, NJ, USA). House deionized water, further purified with a Milli-Q water purifying system (Millipore Corp., Bedford, MA, USA), was used. Drug-free human plasma was purchased from Bioreclamation (E. Meadow, NY, USA).

Sodium acetate buffer (0.1 M, pH 4.2) was prepared by dissolving 13.6 g of sodium acetate trihydrate in 1000 ml of water. The pH was adjusted to 4.2 with glacial acetic acid. A 3.0 mM formic acid solution in water was prepared by dissolving 128 μl of formic acid in 1000 ml of water (mobile phase A).

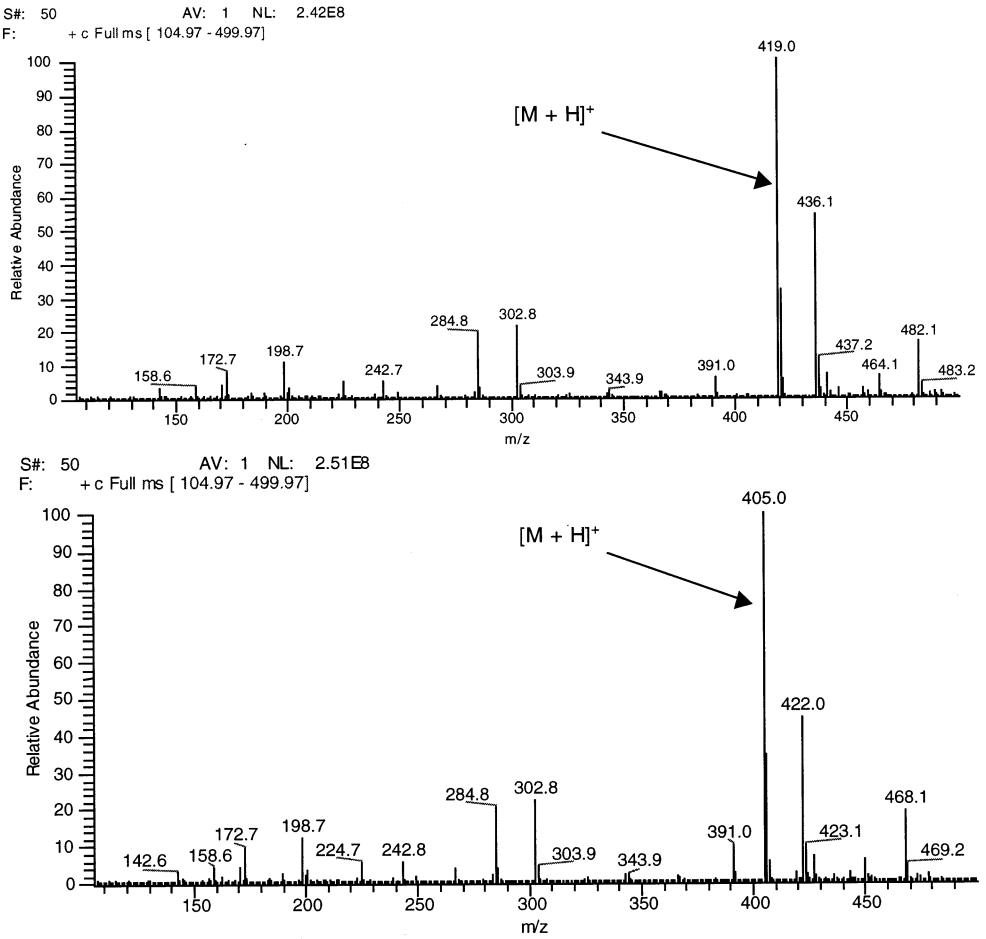


Fig. 3. Electrospray positive ion Q1 mass spectra for simvastatin (top) and its internal standard (bottom) in a mobile phase of 75% acetonitrile and 25% of aqueous 3.0-mM formic acid.

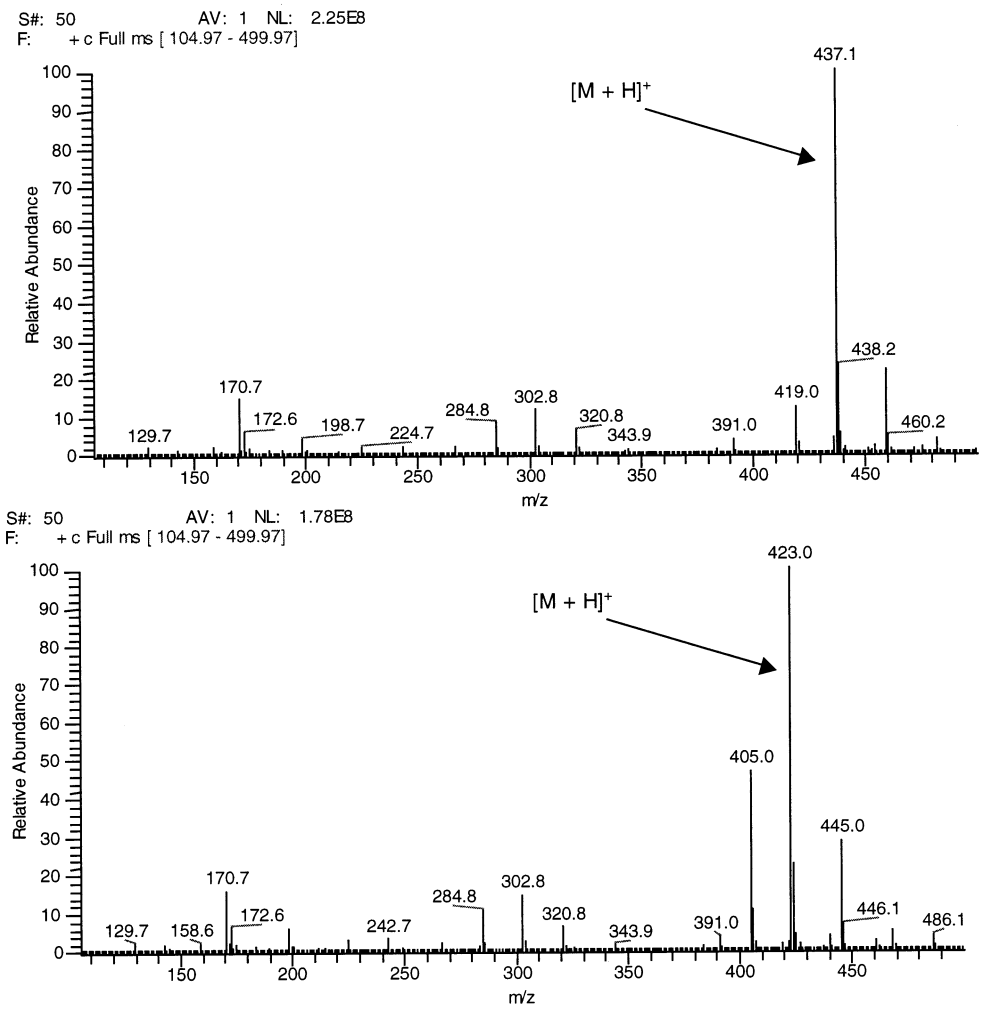


Fig. 4. Electrospray positive ion Q1 mass spectra for simvastatin acid (top) and its internal standard (bottom) in a mobile phase of 75% acetonitrile and 25% of aqueous 3.0-mM formic acid.

2.2. Materials and equipment

Shimadzu LC-10AD VP pumps (Shimadzu, Columbia, MD, USA) and Perkin Elmer 200 autosampler with a temperature controlled tray (Perkin Elmer, Norwalk, CT, USA) were used. The on-line extraction HPLC column used was Oasis® HLB column (1 × 50 mm, 30 μm) from Waters (Milford, MA, USA). The analytical HPLC column used was Symmetry C18 column (3.9 × 50 mm, 5 μm) from Waters. A Finnigan (San Jose, CA, USA) TSQ-7000 triple quadrupole mass spectrometer, equipped with an atmospheric pressure ionization (API) electrospray interface, six-port switching valve and interactive chemical information system (ICIS), was used.

2.3. Preparation of standard and quality control samples

Simvastatin stock solution (0.300 mg/ml) was prepared in acetonitrile. Simvastatin acid stock solution (0.300 mg/ml) was prepared in water: acetonitrile (90:10). Lovastatin and lovastatin acid stock solutions (0.300 mg/ml) were prepared as above. Separate or combined working solutions

of simvastatin and simvastatin acid were prepared by diluting portions of the appropriate stock solutions with acetonitrile. QC samples were prepared using working solutions prepared from stock solutions other than those used for the preparation of the standard curve. Internal standard working solution was prepared by diluting portions of lovastatin and lovastatin acid stock solutions in sodium acetate buffer (0.1 M, pH 4.2). The concentration of each internal standard was 200 ng/ml.

The calibration curve consisted of eight plasma standards, 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, 100 and 200 ng/ml of plasma. All the standards contained both simvastatin and simvastatin acid in the 1:1 concentration ratio. The 200 ng/ml standard was prepared by adding the appropriate volume of a 5.0 μg/ml combined working solution of simvastatin and simvastatin acid in acetonitrile into human plasma. The other standards were prepared by serial dilution starting with the 200 ng/ml standard. Three groups of QC samples were prepared in human plasma, depending on the ratio of the simvastatin concentration to the simvastatin acid concentration. The first group contained both simvastatin and simvastatin acid in the 1:1 concentration ratio. This group consisted of four QC

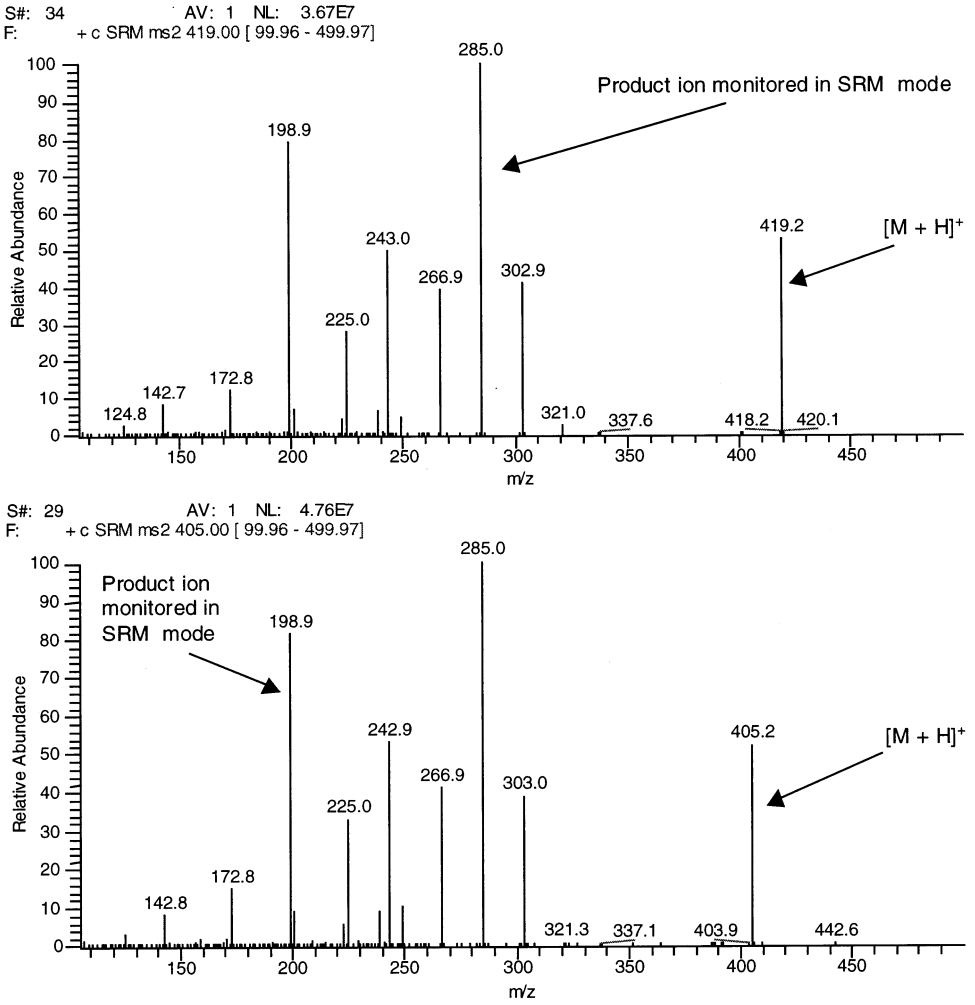


Fig. 5. Electrospray positive ion MS/MS product ion mass spectra of the $[M+H]^+$ precursor ion for simvastatin (top) and its internal standard (bottom) in a mobile phase of 75% acetonitrile and 25% of aqueous 3.0-mM formic acid.

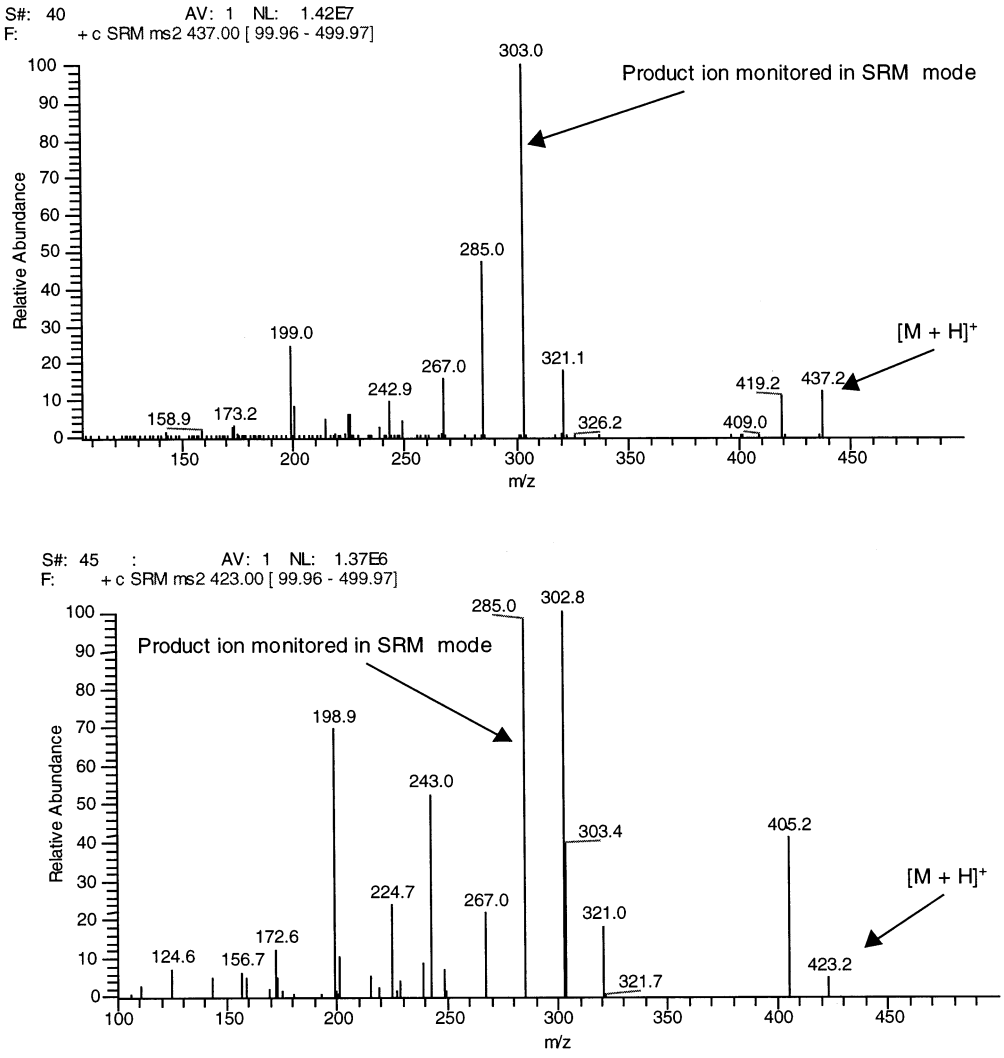


Fig. 6. Electrospray positive ion MS/MS product ion mass spectra of the $[M + H]^+$ precursor ion for simvastatin acid (top) and its internal standard (bottom) in a mobile phase of 75% acetonitrile and 25% of aqueous 3.0-mM formic acid.

samples, 1.50, 80.0, 160 and 500 ng/ml for each analyte. The second group contained simvastatin and simvastatin acid in 9:1 or 1:9 concentration ratios. This group consisted of two QC samples: 72.0/8.00 (with 72.0 ng/ml for simvastatin and 8.00 ng/ml for simvastatin acid) and 8.00/72.0 (with 8.00 ng/ml for simvastatin and 72.0 ng/ml for simvastatin acid). The third group contained only simvastatin or only simvastatin acid. This group consisted of two QC samples, 80.0/0.00 (with 80.0 ng/ml for simvastatin and 0.00 ng/ml for simvastatin acid); and 0.00/80.0 (with 0.00 ng/ml for simvastatin and 80.0 ng/ml for simvastatin acid).

2.4. Sample preparation

A portion, 25 μl, of the working internal standard solution (in 0.1 M sodium acetate, pH 4.2) was added to 0.1 ml of each calibration standard and QC sample in a 0.25 ml polyethylene microvial. The pH of the resulting sample was 4.9.

The samples were then capped, vortexed for 30 s, and centrifuged for 5 min to remove any particulates. The concentration of the internal standard in the human plasma samples was 50 ng/ml. The processed samples were then injected (10 μl) into the LC–MS–MS system described below.

2.5. Chromatography

Chromatography was carried out using a Shimadzu SLC-10A VP system controller with four LC-10AD VP pumps and a Perkin Elmer 200 autosampler. Two HPLC columns, an Oasis® HLB column (1 × 50 mm, 30 μm) and a C18 column (3.9 × 50 mm, 5 μm), were used. The first column served as the sample extraction column and the second column served as the analytical column. The connection of Oasis® extraction column to the six-port switching valve, the analytical column and the mass spectrometer is shown in Fig. 2. A 10 μl portion of the processed human plasma standard or QC sample in vials placed in a chilled (4°C) autosampler tray was transferred by the autosampler onto the Oasis® column, using a mobile phase of 10% acetonitrile and 90% aqueous 3.0 mM formic acid at a flow rate of 4.0 ml/min, with the effluent directed to waste (Fig. 2, configuration a). This was the sample extraction

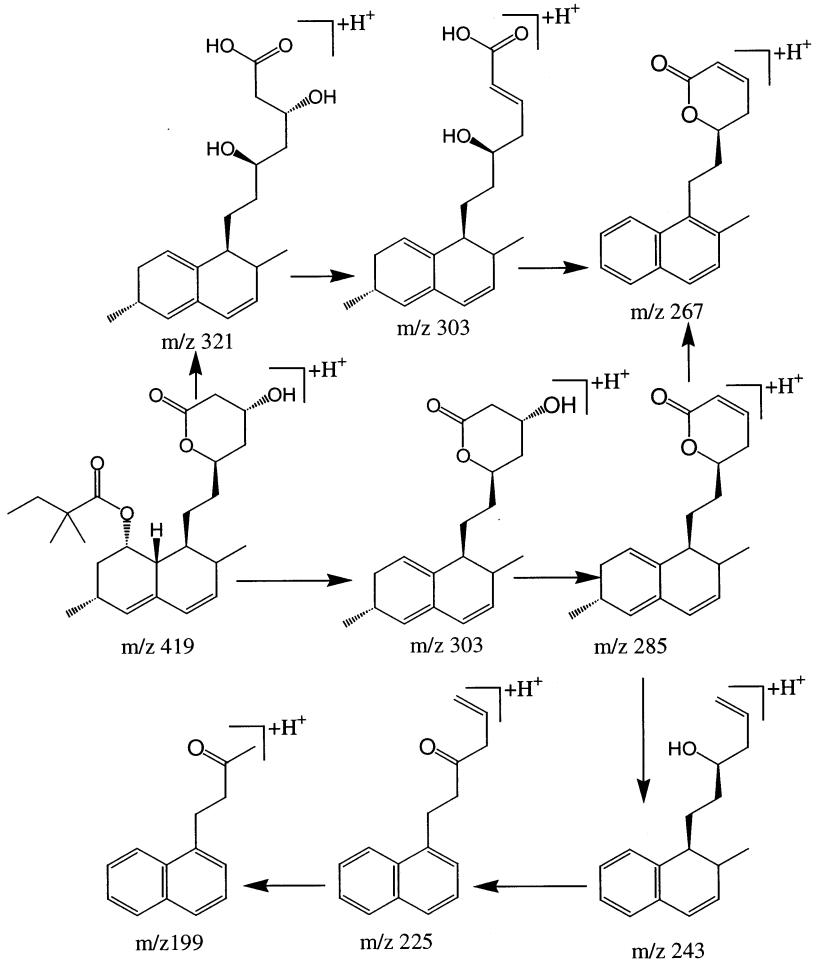


Fig. 7. Proposed CID fragmentation pathways for the $[M + H]^+$ ion of simvastatin (m/z 419) to produce the prominent product ions.

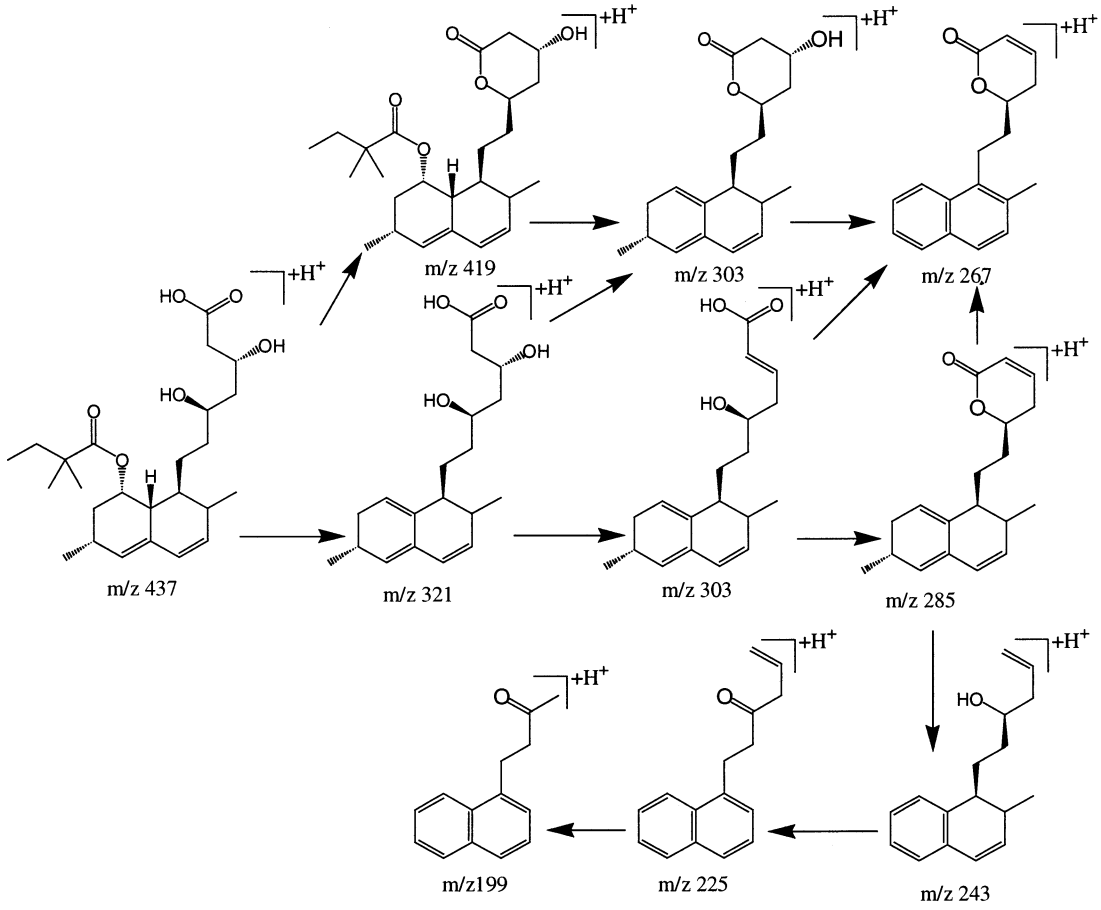


Fig. 8. Proposed CID fragmentation pathways for the $[M + H]^+$ ion of simvastatin acid (m/z 437) to produce the prominent product ions.

and cleanup stage and lasted for 0.3 min. The valve was then switched so that the Oasis® column was in line with the analytical column and the mass spectrometer, and the mobile phase was changed to 75% acetonitrile and 25% aqueous 3.0 mM formic acid at a flow rate of 1.0 ml/min (Fig. 2, configuration b). This was the elution stage. The analytes were eluted from the Oasis® column to the analytical column for detection by the mass spectrometer. The effluent from the analytical column was split, allowing only 50% of the effluent to enter the mass spectrometer (0.5 ml/min). The elution stage was accompanied by the equilibration stage to first wash the extraction column, autosampler and other parts of the system with a reverse gradient mobile phase (from 100 to 10% acetonitrile) and then recondition the extraction column with the mobile phase composition used in the extraction stage (Fig. 2, configuration a). The total run time was 2.5 min. The retention times for simvastatin, simvastatin acid, lovastatin and lovastatin acid were 2.06, 1.49, 1.68, and 1.30 min, respectively.

2.6. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion electrospray mode. The heated capillary temperature was set to 350°C and its potential to 4.5 kV. Nitrogen was used as the sheath

and auxiliary gas and set to 80 psi and 40 (arbitrary) units, respectively. The argon collision gas pressure was set to 2.5 mTorr. The collision energy was set to 15 eV for all the compounds. The selected reaction monitoring (SRM) scheme employed involved transitions of the $[M+H]^+$ precursor ions to selected product ions, m/z 419 → m/z 285 for simvastatin; m/z 437 → m/z 303 for simvastatin acid; m/z 405 → m/z 199 for lovastatin; and m/z 423 → m/z 285 for lovastatin acid. The half-height mass-peak width was set to 0.7 Da for both Q1 and Q3. The dwell time was 0.3 s for each SRM channel.

2.7. Method validation

The accuracy at the lowest level of quantitation (LLQ) was assessed by spiking six different

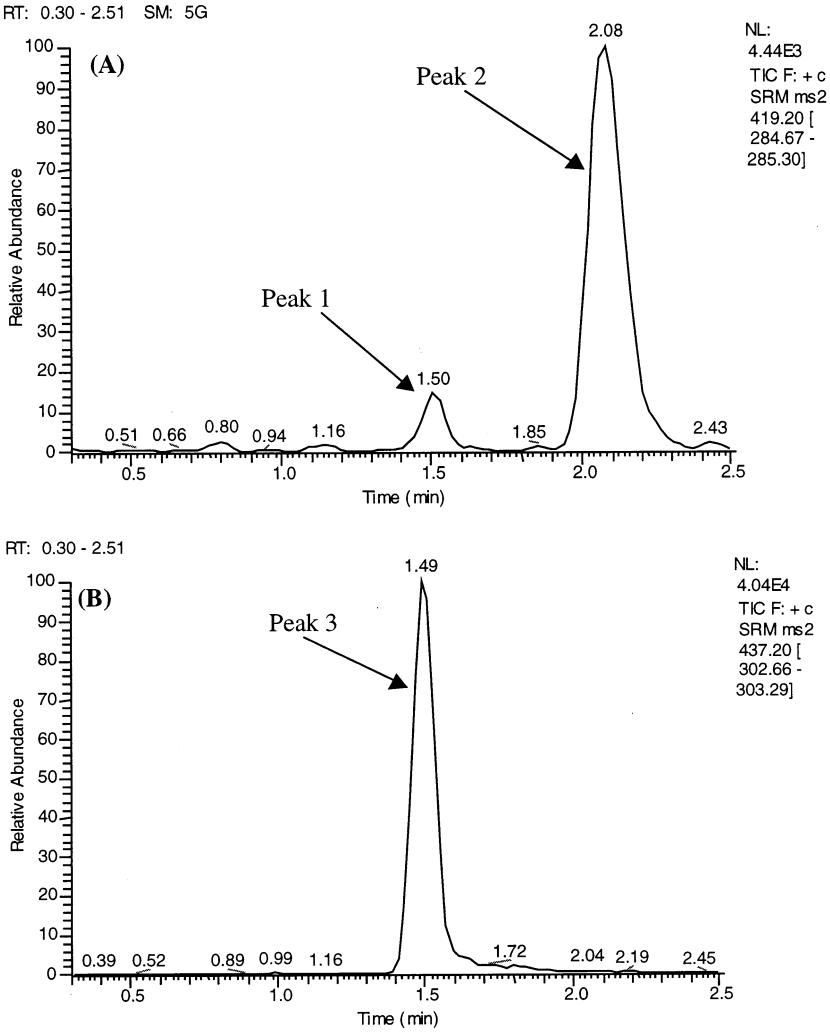


Fig. 9. (A) Selected reaction monitoring chromatogram for simvastatin (m/z 419 → m/z 285 channel) obtained from a human plasma sample containing simvastatin at 8.00 ng/ml and simvastatin acid at 72.0 ng/ml. Peak 1 is due to in-source lactonization of the simvastatin acid in the sample; peak 2 is due to the simvastatin in the sample; (B) selected reaction monitoring chromatogram for simvastatin acid (m/z 437 → m/z 303 channel) obtained from the same human plasma sample. Peak 3 is due to the simvastatin acid in the sample.

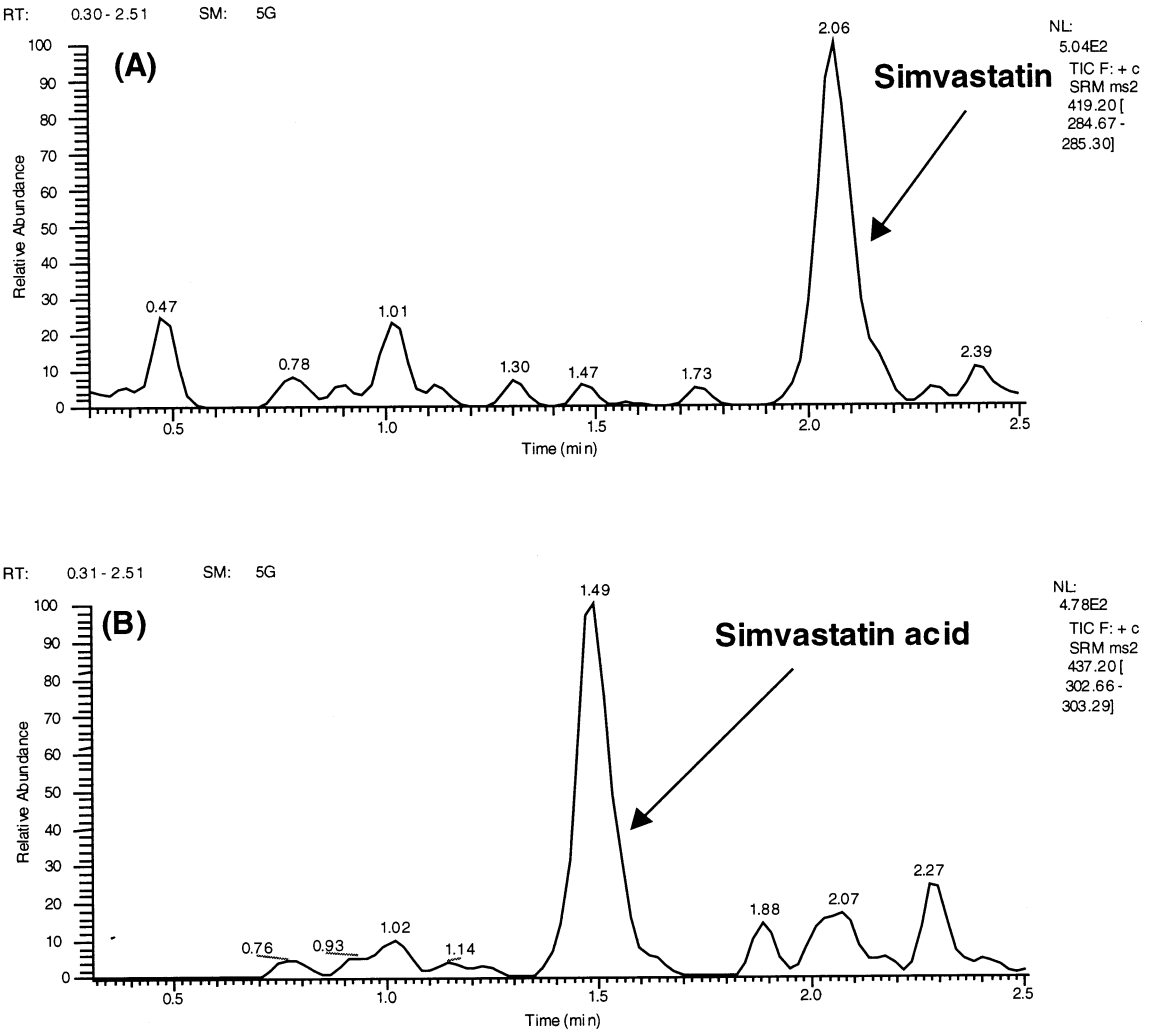


Fig. 10. Selected reaction monitoring chromatograms for simvastatin and simvastatin acid from a human plasma containing simvastatin and simvastatin acid at lower limit of quantitation (0.500 ng/ml) and their internal standards at 50.0 ng/ml: (A) simvastatin channel (m/z 419 → m/z 285); (B) simvastatin acid channel (m/z 437 → m/z 303).

batches of human plasma with simvastatin and simvastatin acid at the LLQ level and analyzing them as unknowns against a standard curve. The specificity of the method was determined by analyzing six different batches of human plasma as is (i.e. with no spiking) and after spiking with only the internal standards. For the determination of intra- and inter-assay precision and accuracy, the eight QC samples described in Section 2.3 were analyzed in replicates of five on three different days. The room temperature and 4°C stability of simvastatin and simvastatin acid in human plasma as is or buffered to pH 4.9 was studied using three concentrations of the 1:1 combination QC samples, one concentration each of simvastatin-only and simvastatin acid-only QC samples. The same set of five QC samples were used to study storage stability at −70°C and freeze–thaw stability of simvastatin and simvastatin acid in human plasma. The stability of processed samples (autosampler stability) was studied using the same set of five QC samples. For this, immediately follow-

ing the addition of the internal standard working solution, the processed samples were analyzed against a standard curve processed together with the QC samples. The same processed QC samples were reanalyzed after 48 h at 4°C against a freshly prepared standard curve. The recovery of simvastatin and simvastatin acid from human plasma during on-line extraction was determined at 1.5,

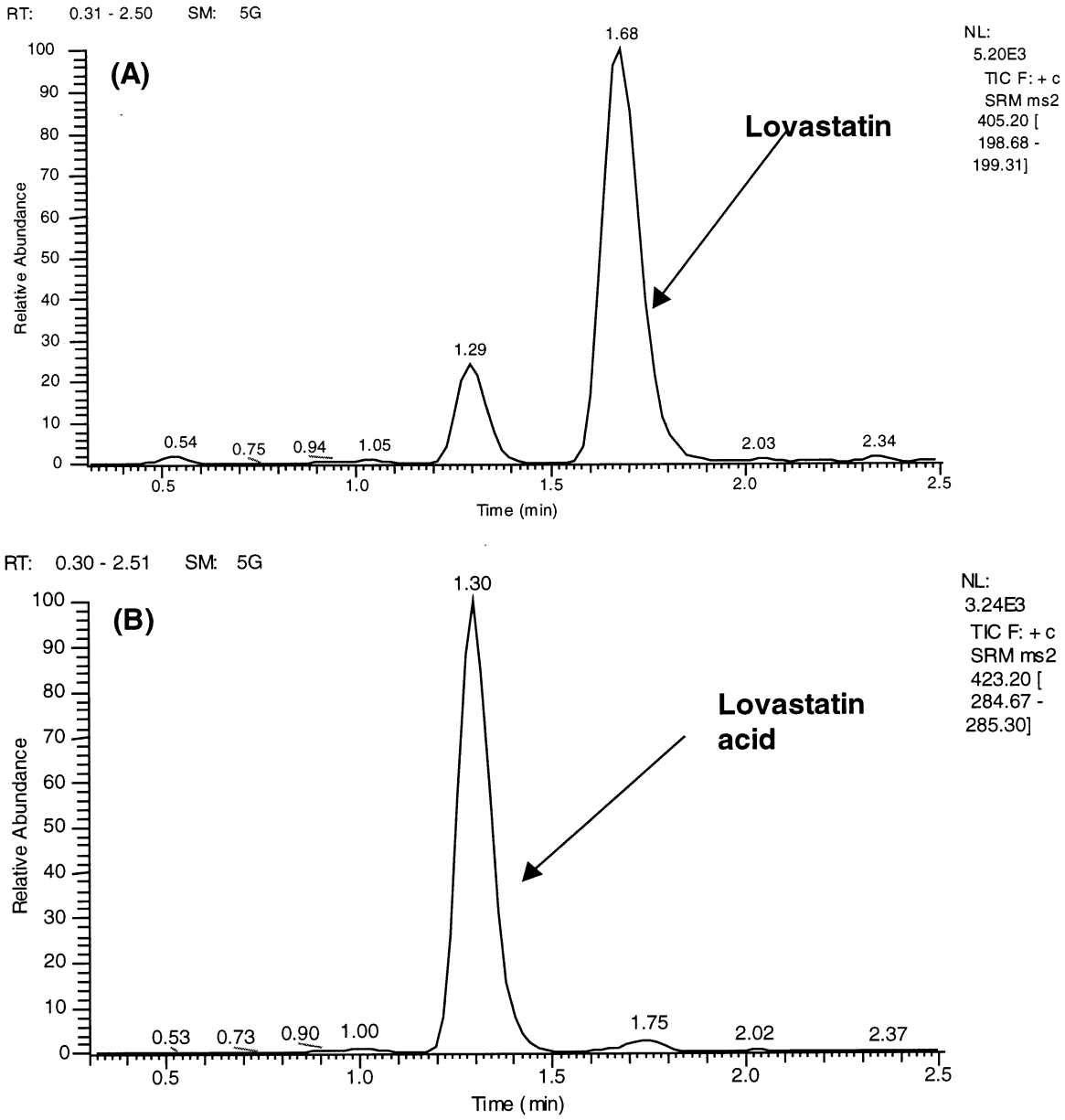


Fig. 11. Selected reaction monitoring chromatograms for lovastatin (internal standard of simvastatin) and lovastatin acid (internal standard of simvastatin acid) from a human plasma containing simvastatin and simvastatin acid at lower limit of quantitation (0.500 ng/ml) and the internal standards at 50.0 ng/ml: (A) lovastatin channel (m/z 405 → m/z 199); (B) lovastatin acid channel (m/z 423 → m/z 285).

Table 1
Lowest level of quantitation for simvastatin and simvastatin acid in human plasma[a]

Sample	Simvastatin		Simvastatin acid	
	Measured conc (ng/ml)	% Dev	Measured conc (ng/ml)	% Dev
1	0.539	7.7	0.560	12
2	0.503	0.5	0.540	8.0
3	0.560	12	0.464	−7.2
4	0.523	4.6	0.539	7.9
5	0.456	−8.8	0.767	54
6	0.546	9.2	0.560	12
Mean	0.521	4.2	0.572	14
% C.V.	7.2		18	

[a] % Dev, percent deviation from nominal concentration; conc, concentration.

Table 2
Accuracy and precision for simvastatin in human plasma[b]

Nominal conc (ng/ml)	Mean measured conc (ng/ml)	% Dev	Intra-assay precision (% C.V.)	Inter-assay precision (% C.V.)
0.00	<LLQ	N/A	N/A	N/A
1.50	1.48	−1.4	6.6	6.8
8.00	7.47	−6.6	5.7	0.99
72.0	68.2	−5.2	5.6	0.00[a]
80.0	75.4	−5.7	6.3	2.1
80.1	76.6	−4.4	4.2	4.5
160	145	−9.3	4.0	0.00[a]
500	502	0.40	5.0	3.9

[a] No significant additional difference variation was observed as a result of performing the assay in different runs. % Dev, percent deviation from nominal concentration; conc, concentration; <LLQ, lower than the lower limit of quantitation of 0.5 ng/ml; N/A, not applicable since the nominal concentration is 0.00 ng/ml.

[b] The 1.50, 80.0, 160 and 500 ng/ml QC samples, as listed in the table above, contained simvastatin and simvastatin acid at equal concentrations. The 8.00 ng/ml QC samples contained 8.00 ng/ml simvastatin and 72.0 ng/ml simvastatin acid. The 72.0 ng/ml QC samples contained 72.0 ng/ml simvastatin and 8.00 ng/ml simvastatin acid. The 0.00 QC sample contained only simvastatin acid (80 ng/ml). The 80.1 ng/ml QC sample contained only simvastatin (80.1 ng/ml).

80 and 160 ng/ml. The responses of samples prepared by spiking the analytes into human plasma were compared with the responses of samples prepared by spiking the compounds into 50% acetonitrile in 0.1 M sodium acetate solution (pH 4.2). The recoveries of the two internal standards were determined similarly at 50 ng/ml.

3. Results and discussion

The full-scan electrospray positive ion mass spectra for simvastatin and its internal standard are presented in Fig. 3. The corresponding spectra for simvastatin acid and its internal standard are shown in Fig. 4. For all four compounds, the predominant signal was from the $[M+H]^+$ ion. The MS/MS product ion mass spectra of the $[M+H]^+$ precursor ions for simvastatin and its internal standard, and for simvastatin acid and its internal standard are presented in Figs. 5 and 6, respectively, with the product ions of the SRM channels used for quantitation shown. The proposed collision-induced dissociation (CID) pathways for simvastatin and simvastatin acid to the prominent product ions are presented in Figs. 7

and 8, respectively. Identical pathways (not shown) can be proposed to explain the SRM product ions monitored for the corresponding internal standards. By comparing the Q1 mass spectrum of simvastatin (Fig. 3) with that of simvastatin acid (Fig. 4), it can be seen that the m/z 419 ion, which is due to the protonated simvastatin, is present not only in the spectrum of simvastatin but also in the spectrum of simvastatin acid. The m/z 419 ion seen in the simvastatin acid spectrum arises from the post-column in-source lactonization of simvastatin acid. Thus, simvastatin acid would contribute to the m/z 419 $\rightarrow$ m/z 285 SRM transition used for the quantitation of simvastatin, unless the two compounds are chromatographically separated. This is illustrated in Fig. 9, which shows the separate chromatograms for the simvastatin SRM channel and simvastatin acid SRM channel obtained from a human plasma sample containing 8.00 ng/ml of simvastatin and 72.0 ng/ml of simvastatin acid. Peak 1 in the simvastatin channel arises from the post-column in-source ionization of the simvastatin acid in the sample. There is the same kind of common-ion problem between lovastatin and lovastatin acid and hence chromatographic separation between the two is needed. This type of in-source common ion formation, and hence the need for chromatographic separation, has been reported previously for different of classes of compounds vis-à-vis their potential biotransformation products [11]. It should be noted that the m/z 419 ion is present not only in the Q1 spectrum of simvastatin acid (Fig. 4) but also in the product ion spectrum of simvastatin acid (Fig. 6). However, the m/z 419 product ion signal will not contribute to the m/z 419 $\rightarrow$ m/z 285 SRM transition used for the quantitation of simvastatin even in the absence of chromatographic separation between the two compounds provided that there is no carry-over cross-talk between the SRM channels.

The Q1 spectra of the two analytes and the two internal standards showed $[M + NH_4]^+$ and $[M + Na]^+$ ions (in addition to the $[M + H]^+$ ion) although the mobile phase contained no known sources of the ammonium or sodium ion. The overall and relative responses of the $[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$ ions were affected not only by the presence of mobile phase additives (such as ammonium acetate) but also by the heated capillary temperature. The conditions finally selected favored the formation of the $[M + H]^+$ ion. It is important to point out the implication of the presence of the $[M + NH_4]^+$ ion in addition to the $[M + H]^+$ ion in the Q1 spectra of

Table 3
Accuracy and precision for simvastatin acid in human plasma[b]

Nominal conc (ng/ml)	Mean measured conc (ng/ml)	% Dev	Intra-assay precision (% C.V.)	Inter-assay precision (% C.V.)
0.00	<LLQ	N/A	N/A	N/A
1.50	1.53	2.0	9.7	1.5
8.00	7.33	−8.4	7.0	0.00[a]
72.0	66.4	−7.8	5.3	1.1
80.0	77.1	−3.6	7.0	0.00[a]
80.1	74.5	−7.0	4.5	3.8
160	152	−4.7	5.3	0.00[a]
500	499	−0.13	7.2	3.8

[a] No significant additional difference variation was observed as a result of performing the assay in different runs. % Dev, percent deviation from nominal concentration; conc, concentration; <LLQ, lower than the lower limit of quantitation of 0.5 ng/ml; N/A, not applicable since the nominal concentration is 0.00 ng/ml.

[b] The 1.50, 80.0, 160 and 500 ng/ml QC samples, as listed in the table aobe, contained simvastatin and simvastatin acid at equal concentrations. The 8.00 ng/ml QC samples contained 72.0 ng/ml simvastatin and 8.00 ng/ml simvastatin acid. The 72.0 ng/ml QC samples contained 8.00 ng/ml simvastatin and 72.0 ng/ml simvastatin acid. The 0.00 QC sample contained only simvastatin (80 ng/ml). The 80.1 ng/ml QC sample contained only simvastatin acid (80.1 ng/ml).

analytes such as simvastatin and simvastatin acid, whose molecular ions are separated by only 18 Da (due to H_2O). The $[M+H]^+$ ions of simvastatin and simvastatin acid are m/z 419 and m/z 437, respectively. The $[M+NH_4]^+$ ions of simvastatin and simvastatin acid are m/z 436 and m/z 454, respectively. Thus, the $[M+NH_4]^+$ ion from simvastatin at m/z 436 would contribute to (and hence interfere with) the m/z 437 $\rightarrow$ m/z 303 SRM transition used for the quantitation of simvastatin acid, unless there is a unit mass resolution at Q1 (to resolve m/z 437 from m/z 436) and/or a baseline chromatographic separation. The potential for this type of interference should be kept in mind when the Q1 spectra of lactone/acid analytes contain both the $[M+H]^+$ and $[M+NH_4]^+$ ions and when the $[M+H]^+$ ions are used as precursor ions for the SRM transitions of both compounds.

Sample preparation involved simply aliquotting the plasma samples into autosampler vials, adding the internal standard working solution, vortex-mixing and centrifuging. The samples were then injected into the direct injection system for on-line purification. The basis of this type of high-speed direct injection LC–MS–MS system has been described previously [12,13].

Typical SRM chromatograms of LLQ samples are presented in Figs. 10 and 11. The method was highly specific and the responses at the retention times and SRM channels of the analytes were significantly smaller in the blank samples compared with those in the LLQ samples. Note that the peak at 1.29 min in the lovastatin SRM chromatogram (Fig. 11) arises from the lovastatin acid post-column lactonization in the source. The LLQ experiment demonstrated that LLQ of 0.5 ng/ml has been achieved for each analyte as the deviations of the measured concentrations from the nominal concentration were within 20% for at least five of the six LLQ samples (Table 1). The summary results of the analysis of three types of QC samples (Section 2.3) from the accuracy and precision experiments are presented in Tables 2 and 3. The accuracy, as measured by mean deviation, was within 10% for both analytes. The intra-assay precision was within 10% (% C.V.) and the inter-assay precision was within 7% (% C.V.) for both analytes. For the assessment of accuracy and precision, it was important to include QC samples in which the ratio of the simvastatin concentration to the simvastatin acid concentration was different from the corresponding concentration ratio in the calibration curve standards, since such QC samples more realistically represent post-dose study samples in which the concentration ratio is expected to vary from sample-to-sample. It is important to stress that it is inadequate to validate a method for the quantitation of two analytes (such as simvastatin and simvastatin acid), that can potentially interconvert during the multiple steps of bioanalysis, solely using QC samples that contain the two analytes in the same concentration ratio as in the calibration curve standards.

The results of the study of the effect of temperature (room temperature vs. 4°C) and pH (as is plasma pH vs. pH 4.9) on the stability of human plasma QC samples are summarized in Tables 4 and 5. The samples were either treated by adding 25 μl of sodium buffer solution (0.1 M, pH 4.2) to a 100-μl portion of each plasma sample (buffered sample) or used as is (unbuffered sample) and then kept at room temperature or at 4°C for 2, 4 and 24 h. Simvastatin acid was stable under all the conditions studied. However, simvastatin in unbuffered plasma was significantly unstable after 24 h at room temperature as evidenced by the decrease in simvastatin concentration (Table 4) and the concomitant increase in the simvastatin acid concentration (Table 5). The instability of simvastatin was due to hydrolysis to simvastatin acid. The hydrolysis of simvastatin to simvastatin acid in plasma after 24 h was insignificant when either the temperature or the pH of the plasma sample was lowered. It was for this reason that, during method validation, the plasma samples were buffered to pH 4.9 and kept at 4°C at all stages of analysis. As shown in Tables 2–5, the hydrolysis of simvastatin or the lactonization of simvastatin acid was insignificant ($\leq 1.0\%$) under the conditions of the assay. It is interesting to note that the rate of hydrolysis of simvastatin to simvastatin acid in unbuffered plasma at room temperature or at 4°C appears to be significantly slower than that of other HMG-CoA reductase inhibitors, atorvastatin lactone to atorvastatin [14], cerivastatin lactone to cerivastatin [15], and pravastatin lactone to pravastatin (unpublished data from our laboratory).

Table 4
Stability of human plasma QC samples: monitoring simvastatin[a]

Sample condition	Nominal conc (ng/ml)								
	1.50		80.0		80.1		0.00	160	
	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	Meas'd conc (ng/ml)	% Dev
0 h	1.58	5.3	86.2	7.8	80.2	0.25	<LLQ	155	−3.1
2 h (Room temperature)	1.52	1.3	81.9	2.4	88.1	10	<LLQ	154	−0.38
4 h (Room temperature)	1.50	0.00	88.3	10.4	84.9	6.1	<LLQ	156	−2.5
24 h (Room temperature)	0.906	−39	61.7	−23	60.9	−24	<LLQ	124	−23
2 h (4°C)	1.49	−0.66	83.5	4.4	85.1	6.4	<LLQ	170	6.2
4 h (4°C)	1.52	1.3	86.4	8.0	84.4	5.5	<LLQ	162	1.2
24 h (4°C)	1.20	−20	82.1	2.6	79.5	−0.63	<LLQ	153	−4.4
2 h (Room temperature, buffered)	1.56	4.0	87.2	9.0	71.1	−9.3	<LLQ	156	−2.5
4 h (Room temperature, buffered)	1.55	3.2	85.6	7.0	87.5	9.4	<LLQ	166	3.8
24 h (Room temperature, buffered)	1.50	0.00	87.3	9.1	88.8	11	0.770	141	−11.8
2 h (4°C, buffered)	1.70	13	78.5	−1.9	81.8	2.2	0.518	166	3.8
4 h (4°C, buffered)	1.50	0.00	88.3	10.3	90.6	13.3	<LLQ	154	−3.8
24 h (4°C, buffered)	1.64	9.3	86.6	8.3	88.3	10.4	0.630	159	−0.62

[a] Meas'd, measured; conc, concentration; % Dev, percent deviation from nominal concentration. The 1.50, 80.0 and 160 ng/ml QC samples contained simvastatin and simvastatin acid at equal concentrations. The 0.00 QC sample contained only simvastatin acid (80 ng/ml). The 80.1 ng/ml QC sample contained only simvastatin (80.1 ng/ml).

Table 5
Stability of human plasma QC samples: monitoring simvastatin acid[a]

Sample condition	Nominal conc (ng/ml)								
	1.50		80.0		80.1		0.00	160	
	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	% Dev	Meas'd conc (ng/ml)	Meas'd conc (ng/ml)	% Dev
2 h (Room temperature)	1.56	4.0	82.8	3.5	81.0	1.3	1.55	147	−8.1
4 h (Room temperature)	1.42	5.3	83.1	3.9	86.9	8.6	4.14	151	5.6
24 h (Room temperature)	1.93	29	98.1	23	79.8	−0.30	25.4	211	32
2 h (4°C)	1.53	2.0	77.2	−3.5	80.5	0.63	<LLQ	148	−7.5
4 h (4°C)	1.44	−4.0	75.0	−6.3	70.8	−11	0.554	141	−12
24 h (4°C)	1.35	−10	79.2	−1.0	73.1	−8.6	2.54	153	−4.4
2 h (Room temperature, buffered)	1.42	−5.3	75.7	−5.4	75.0	−6.3	<LLQ	147	−8.1
4 h (Room temperature, buffered)	1.56	4.0	73.4	−8.3	76.9	−3.9	<LLQ	149	−6.9
24 h (Room temperature, buffered)	1.16	−23	73.9	−7.6	77.1	−3.6	1.53	149	−6.9
2 h (4°C, buffered)	1.51	0.67	71.9	−10	78.5	−1.9	<LLQ	149	−6.9
4 h (4°C, buffered)	1.44	−4.0	73.6	−8.0	72.8	−9.0	<LLQ	148	−7.5
24 h (4°C, buffered)	1.49	−0.67	77.8	−2.8	73.2	−8.5	<LLQ	148	−7.5

[a] Meas'd, measured; conc, concentration; % Dev, percent deviation from nominal concentration. The 1.50, 80.0 and 160 ng/ml QC samples contained simvastatin and simvastatin acid at equal concentrations. The 0.00 QC sample contained only simvastatin (80 ng/ml). The 80.1 ng/ml QC sample contained only simvastatin acid (80.1 ng/ml).

Both simvastatin and simvastatin acid in human plasma were stable for at least 2 months at −70°C. The two compounds were also found to be stable for at least three freeze–thaw cycles. Processed samples were stable for at least 48 h at 4°C. The recoveries were ≥75 and ≥38% at all levels tested for the analytes simvastatin and simvastatin acid, respectively. The recoveries were 52, and 57% for the internal standards lovastatin and lovastatin acid, respectively.

4. Conclusions

The direct-injection LC–MS–MS method developed for the simultaneous quantitation of simvastatin and simvastatin acid in human plasma was shown to be accurate, precise, sensitive and specific. The method was successfully validated under the rigorous condition of using a large number of QC samples including those in which the ratio of the simvastatin concentration to the simvastatin acid concentration was different from the concentration ratio in the calibration curve standards. With the dual stabilization conditions of lower temperature (4°C) and acidic plasma sample pH (4.9), the ex vivo hydrolysis of simvastatin to simvastatin acid was minimized to ≤1.0%. The run time of only 2.5 min was adequate to achieve the required chromatographic separation of simvastatin from simvastatin acid and lovastatin from lovastatin acid. The sample preparation involved simply pipetting the plasma samples into autosampler vials, adding the internal standard working solution, vortex-mixing and centrifuging. The sample preparation can further be simplified by automating the sample aliquotting and internal standard addition into 96-well plates for direct injection from the plates. We plan to accomplish this with the acquisition of an autosampler with temperature-controlled 96-well injection capability.

Acknowledgements

The authors are grateful to Iffaaz Salahudeen for his contribution to the early phase of method development, and to Sanaullah Khan for his contribution to the elucidation of the CID fragmentation pathways for simvastatin and simvastatin acid.

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