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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 715–721
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Identification of unknown impurities in simvastatin substance and tablets by liquid chromatography/tandem mass spectrometry

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Received 19 February 2004; received in revised form 19 November 2004; accepted 19 November 2004
Available online 25 December 2004

Abstract

Unknown impurities were detected in simvastatin substance and tablets at a 0.2% level using the liquid chromatography technique with UV (DAD) detection. The impurity structures were elucidated by a direct hyphenation of liquid chromatograph to high-resolution mass spectrometer with electrospray ionisation interface using solutions of formic acid in water and in acetonitrile as the mobile phase. Peak tracking was performed using the column-switching technique. Accurate mass measurements by quadrupole time-of-flight mass spectrometer equipped with lock-spray provided information about elemental composition of intact molecules and fragments of impurities. Measurement accuracy for precursor ions was around 3 ppm and for fragment ions between 4 and 13 ppm. Mass resolving power was around 6500. Deduced molecular formulae for A1, A2 and A3 impurities were $C_{27}H_{44}O_6$, $C_{26}H_{43}O_6$ and $C_{26}H_{41}O_5$, respectively. The structures proposed for all three impurities revealed modifications of simvastatin molecule on the lactone ring. Impurity A1, detected in simvastatin tablets, was identified as ethyl ester, while the impurities A2 and A3, detected in simvastatin substance, were identified as methyl ester and methyl ether of simvastatin. The impurity from tablets was synthesized and its structure confirmed by LC–UV, LC–MS/MS, and NMR techniques.

Keywords: Simvastatin; Impurities; Identification; LC–MS/MS; Pharmaceuticals

1. Introduction

Simvastatin or [1*S*-[1α,3α,7β,8β(2*S**,4*S**),8αβ]]-1,2,3,7,8,8a-hexahydro-3, 7-dimethyl- 8- [2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-naphtalenyl-2,2-dimethylbutanoate is an antilipemic agent similar to lovastatin, mevastatin and pravastatin [1,2]. It is a prodrug activated in organism after enzymatic hydrolysis. Simvastatin in β-hydroxy acid form acts as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [3], that is, as a regulator of cholesterol synthesis [4,5]. It is mainly used for the treatment of primary hypercholesterolemia, as it effectively reduces the levels of total and low-density level cholesterol (LDL), triglycerides, and apolipoprotein B in plasma. Simvastatin is obtained by synthesis from lovastatin, by replacement of 2-methylbutyryl side chain with 2,2-dimethylbutyryl group [5]. Lovastatin is produced biosynthetically from the fungus *Aspergillus terreus* [6,7].

Strict regulatory guidelines of the International Conference on Harmonization (ICH), have led to an increasing need for identification and quantification of trace impurities in drugs. All impurities, defined by ICH as any component of a pharmaceutical product which is not the chemical entity of active substance or excipient, present at levels higher than 0.1% or in some cases higher than 0.2%, depending on daily recommended dosage, need to be identified and qualified with appropriate toxicological studies. If impurities were expected to be very toxic, then identification and qualification would be required even at lower concentrations [8].

Isolation and purification of sufficiently large quantities of impurity required for its unambiguous identification and characterisation by different instrumental techniques,

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doi:10.1016/j.jpba.2004.11.047

including nuclear magnetic resonance is a very complex and time-consuming process. The problem is particularly complex when dealing with formulations like tablets with low quantities of active substance, e.g. 1–10 mg per tablet. The use of hyphenated systems of a highly efficient separation technique like high performance liquid chromatography and specific and sensitive detection by mass spectrometry is a promising approach to overcome these difficulties. This analytical task might be accomplished using different types of MS instruments, such as those with single or triple quadrupole analysers, ion traps, time-of-flight or quadrupole time-of-flight analysers. Each provides different kind and quality of information, and requires different time for identification. The main advantage of a high resolution quadrupole time-of-flight instrument is the ability to perform accurate mass measurement, not only of the product ion, but also of fragment ions gained through MS/MS experiment, thus giving an additional dimension to MS/MS data. Recently, the determination of elemental composition has become one of the main and very important tools in characterisation of substances by mass spectrometry, especially in pharmaceutical industry [9].

Several simvastatin-related impurities have been studied by LC–MS in literature so far [10]. All of these impurities were, nevertheless, standard substances, which are commercially available or already described in literature. Most of the published LC–MS methods are quantitative methods for determination of simvastatin and simvastatin acid in biological fluids or biomatrices [11–13].

Stability testing of simvastatin tablets containing 10 mg of active substance revealed about 20 μg of an unknown impurity per tablet. This result was obtained by high performance liquid chromatography with ultraviolet diode array detection. The impurity exceeded the 0.1% identification threshold, as did the quantity of two unknown impurities detected during the synthesis of simvastatin substance, which called for structural identification. HPLC retention times of the impurities did not coincide with any officially available standard substances of impurities. The purpose of this study was to identify these unknown impurities using on-line analytical techniques. The hyphenated system used was a liquid chromatograph/quadrupole time-of-flight (Q-ToF) mass spectrometer.

The structure of the unknown impurity in simvastatin tablets was additionally confirmed by chemical synthesis of this compound and its characterisation by NMR, IR, MS and LC–UV (DAD) techniques.

2. Experimental

2.1. Apparatus

The LC–UV (DAD) analyses were performed on a Waters LC system with a Waters 2795 autosampler and pump, and Waters 2996 photodiode array detector (Waters, Milford, USA). The UV spectra of all peaks were recorded from 190 to 400 nm, and the working wavelength was 238 nm.

The LC–MS/MS analyses were performed by coupling the LC system with a quadrupole time-of-flight mass spectrometer Q-ToF Micro equipped with the lock-spray (Micromass, Manchester, UK).

The LC column was Zorbax C8, 150 mm × 4.6 mm i.d. of 3.5 μm particle size (pore size 30 nm), from Agilent (Newport, USA). The column and autosampler temperatures were 30 and 5 °C, respectively. In LC–UV (DAD) analysis, a solution of 1 ml of 85% phosphoric acid in 1000 ml of water (14 mmol l^{-1}) was used as mobile phase A, and acetonitrile was used as mobile phase B. In LC–MS/MS analysis, the mobile phase A was a solution of 1 ml of 98% formic acid in 1000 ml of water (26 mmol l^{-1}) and mobile phase B was 1 ml of 98% formic acid in 1000 ml of acetonitrile (26 mmol l^{-1}). Gradient conditions for the analysis of simvastatin tablet samples for both types of experiments were: from 0 to 15 min, 50% of mobile phase A; from 15 to 25 min, linear gradient up to 25% of mobile phase A; from 25 to 30 min, linear gradient up to 15% of mobile phase A; from 30 to 45 min, 15% of mobile phase A; and finally, reconditioning column for 5 min on starting conditions. Gradient conditions for the analysis of simvastatin substance samples for both types of experiments were: from 0 to 10 min, 45% of mobile phase A; from 10 to 12 min, linear gradient up to 30% of mobile phase A; from 12 to 16 min, 30% of mobile phase A; from 16 to 20 min, linear gradient up to 15% of mobile phase A; from 20 to 30 min, 15% of mobile phase A; and finally, reconditioning column for 5 min on starting conditions. Mobile phase flow rate was 1.5 ml min^{-1}, injection volume around 50 μl and split ratio 1:20 was used for the analysis on the MS system.

The MS and MS/MS spectra were obtained under following conditions: ionisation, ESI positive; capillary voltage, 3000 V; sample cone voltage, 35 V; extraction voltage, 3 V; low mass resolution, 10 V; high mass resolution, 10 V; ion energy, 2 V; MCP detector, 2700 V; desolvation temperature, 150 °C; source temperature, 80 °C; cone gas, 0 l h^{-1}; desolvation gas, 450 l h^{-1}; collision energy from 10 to 15 V.

Lock-spray: scan frequency was 5 s and cone voltage was 35 V. Leucine enkephalin was used as a reference mass.

External calibration was performed in mass range from *m*/*z* 80 to 1000 using a calibration mixture of 10% formic acid–0.1 mol l^{-1} sodium hydroxide–acetonitrile (1:1:8, v/v/v).

2.2. Chemicals

Formic acid minimum 98% p.a., acetonitrile gradient grade for chromatography, sodium hydroxide extra pure, silica gel 60 for column chromatography (particle size, 0.063–0.200 mm) were purchased from Merck (Darmstadt, Germany). Phosphoric acid minimum 85% p.a., ethanol absolute p.a., chloroform p.a., ethyl acetate p.a., acetone p.a., sodium chloride p.a. were products of Kemika (Zagreb, Croatia). Methanesulfonic acid 99.5% and leucine enkephalin synthetic 98% were purchased from Sigma–Aldrich (St. Louis, USA). LC-grade water (resistivity less than 18.2 MΩ cm at

25 °C and total organic carbon less than 5 μg l^{-1}) was prepared by purifying distilled water with a Milli-Q water purification system from Millipore (Bedford, USA).

2.3. Preparation of simvastatin tablets and simvastatin substance solutions

Simvastatin tablets were dissolved in a mixture of 0.1% formic acid–acetonitrile (1:1, v/v) and sonicated for 45 min. Simvastatin substance was dissolved in a mixture of 0.1% formic acid–acetonitrile (1:1, v/v). The final concentration of simvastatin in a sample solution was about 0.5 mg ml^{-1}.

2.4. Synthesis of 7-[8-(2,2-dimethyl-butyryloxy)-2,6-dimethyl-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl]-3,5-dihydroxy-heptanoic acid ethyl ester (A1)

A solution of ammonium-7-[8-(2,2-dimethyl-butyryloxy)-2, 6-dimethyl-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl]-3,5-dihydroxy-heptanoate (10.00 g, 0.022 mol) and methanesulfonic acid (1.5 ml, 0.023 mol) in absolute ethanol (150 ml) was stirred under nitrogen for 6.5 h at ambient temperature. Ethanol was removed under reduced pressure, ethyl acetate (200 ml) was added and the solution was washed with water and brine. After evaporation of the solvent, the crude product was purified by column chromatography using a mixture of chloroform–acetone–ethyl acetate (6:1:3, v/v/v) as eluting solvent. 7-[8-(2,2-Dimethyl-butyryloxy)-2,6-dimethyl-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl]-3, 5-dihydroxy-heptanoic acid ethyl ester was obtained (3.49 g; 34.2% yield) as colourless oil.

3. Results and discussion

The main purpose of stability-indicating method is to determine the amount of active ingredient and all impurities produced as a result of aging process of a drug [14]. Of the impurities detected during stability testing of a certain drug only those exceeding 0.1 or 0.2%, depending on maximum daily

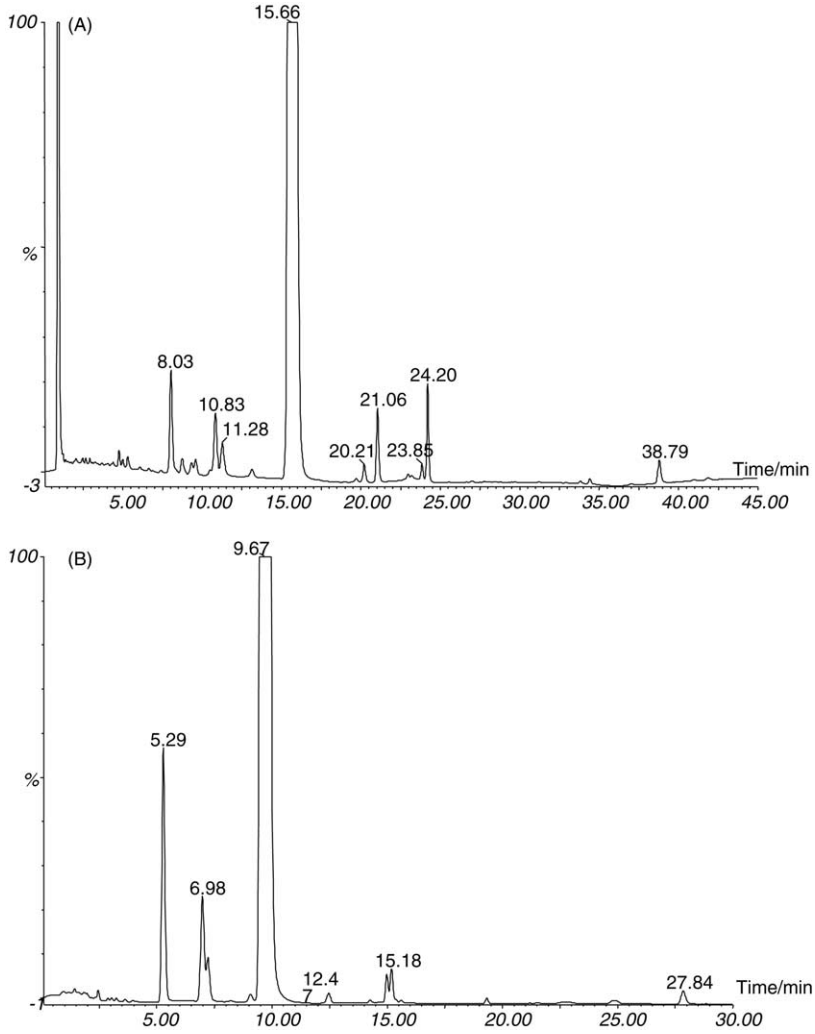

Fig. 1. LC–UV (DAD) chromatogram of simvastatin tablets 10 mg at 238 nm with A1 impurity peak at retention time (RT) = 21.06 min (A) and LC–UV (DAD) chromatogram of simvastatin substance sample with A2 and A3 impurity peaks at RT = 12.47 and 15.18 min, respectively (B).

drug dose, need to be identified [8,15]. A stability-indicating LC–UV (DAD) method for the determination of the active compound and impurities in simvastatin tablets (10 mg) was developed. The method was validated and proven to be suitable for this particular analytical task. During stability testing, simvastatin tablets were stored in appropriate stability chambers in conditions of controlled temperature and relative humidity (RH). Stability was tested at 25 °C/60% RH, 30 °C/60% RH and 40 °C/75% RH. Under all of these conditions, along with known related compounds of simvastatin, an unknown impurity (A1) was detected at relative retention time of 1.23 (Fig. 1). The amount of A1 significantly increased after 1 month and remained on the 0.2% level throughout the stability-testing period. In preliminary experiments, stability samples of simvastatin tablets were heated at 100 °C for different times in order to get larger quantities of the target impurity. However, the analysis of heated samples showed a gradual decrease in the quantity of A1 and its total disappearance in samples heated for 1 h. No new peaks in LC–UV (DAD) chromatogram were detected as a consequence of heating. By the end of the identification process, this information turned out to be very useful. Stress testing of tablet samples performed with 1 mol l^{-1} hydrochloric acid, 1 mol l^{-1} sodium hydroxide and 3% hydrogen peroxide did not increase the amount of A1. According to the UV spectra of simvastatin and A1 (data not shown), there was a great probability of structural similarity between the two compounds. Retention times in LC–UV (DAD) chromatogram indicated lower polarity of A1 compared to the active compound.

The two unknown impurities detected by LC–UV (DAD) analysis in simvastatin substance—A2 at the relative retention time of 1.29 and A3 at the relative retention time of 1.57—were produced during the synthesis (Fig. 1). They were also less polar than simvastatin, and the comparison of their UV spectra with that of simvastatin (data not shown) indicated a structural similarity of all three compounds. Although providing some structural information on A1, A2 and A3 impurities, the LC–UV (DAD) data were not sufficient to elucidate the exact structures of these compounds. Therefore, the solutions of simvastatin tablets and substance were

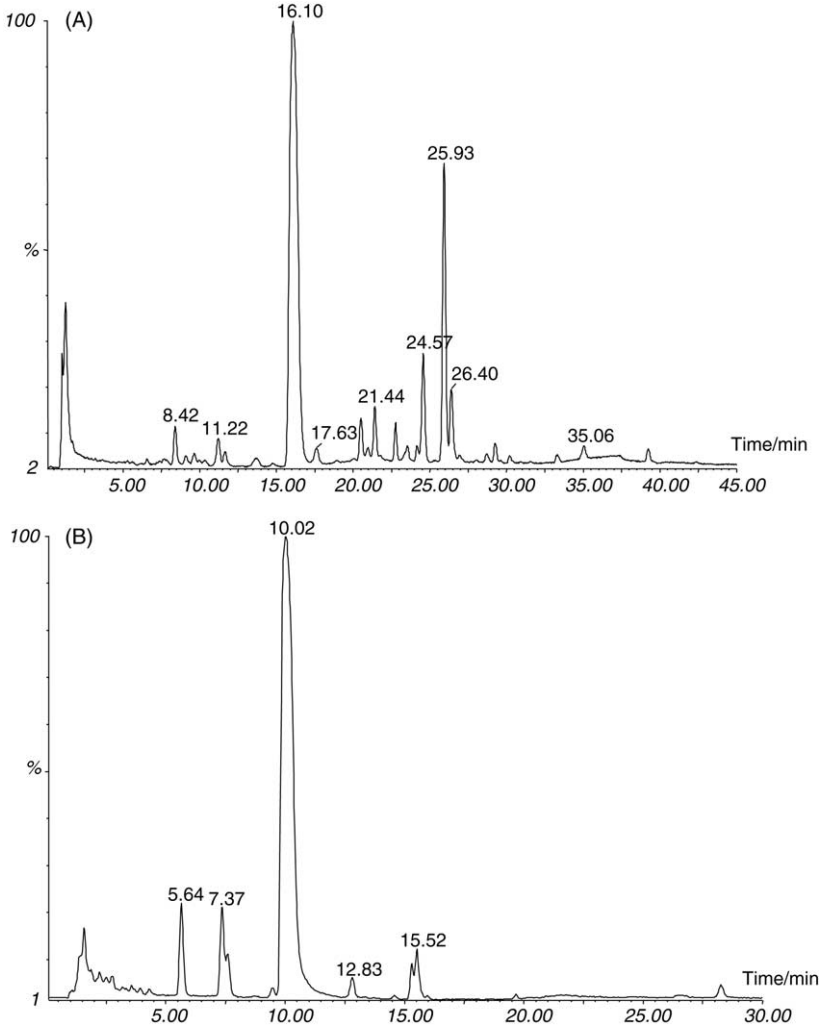

Fig. 2. LC–MS chromatogram of simvastatin tablets 10 mg in total ion current (TIC) mode with A1 impurity peak at RT = 21.44 min (A) and LC–MS chromatogram in TIC mode of simvastatin substance sample with A2 and A3 impurity peaks at RT = 12.83 and 15.52 min, respectively (B).

further analysed by LC coupled with a quadrupole time-of-flight mass spectrometer.

As mobile phases used for LC–UV (DAD) analysis of simvastatin tablets and substance contained non-volatile inorganic phosphoric acid, it was necessary to modify them in order to meet the conditions required for LC–MS. Formic acid, acetic acid and trifluoroacetic acid at low concentration could be used as substitutes [16]. We obtained the best chromatographic results with formic acid. LC–MS chromatograms were comparable to LC–UV (DAD) chromatograms obtained in stability testing of simvastatin tablets and impurity determination in simvastatin substance (Fig. 2). Peaks were tracked by comparing the peak areas, retention times and UV spectra of separated components, but the final confirmation of compound identity was achieved by the column-switching technique. This technique is basically described as a collection of mobile phase zone containing the unknown impurity peak, after passing through a UV (DAD) detector cell, in a holding loop, which will act as an injection loop on another LC system with MS compatible mobile phase [8,17]. After adjustment of all chromatographic parameters and optimisation of ESI-MS conditions to achieve the satisfactory sensitivity of the method (the obtained detection limit was around 400 pg of simvastatin injected on-column), the focus was on the evaluation and interpretation of the results obtained from various MS and MS/MS experiments, designed according to a specific analytical goal.

Subsequent to determining the molecular mass of impurities A1, A2 and A3, accurate mass measurements of precursor and fragment ions were performed in separate experiments (Fig. 3). Results showing deduced elemental compositions are presented in Table 1 for precursor ions and in Table 2 for four indicative fragment ions. Mass measurement accuracy, as experimentally determined, was within the specified limit for this instrument (better than 5 ppm), in experiments performed on intact compounds, while the results obtained by MS/MS experiments were at or above the 5 ppm level. The lowest accuracy was obtained for fragment ion at *m/z* 173, which was expected due to its very low intensity. Resolving power of the instrument was around 6500 (full width at half maximum [FWHM] of the peak definition) in both types of experiment. Still, these results were adequate for the deduction of elemental formulas of fragments. The discrepancy between MS and MS/MS accurate measurement results is probably due to technical limitations of a Q-ToF Micro as a bench-top instrument, e.g. it has no W-optics (a W reflectron

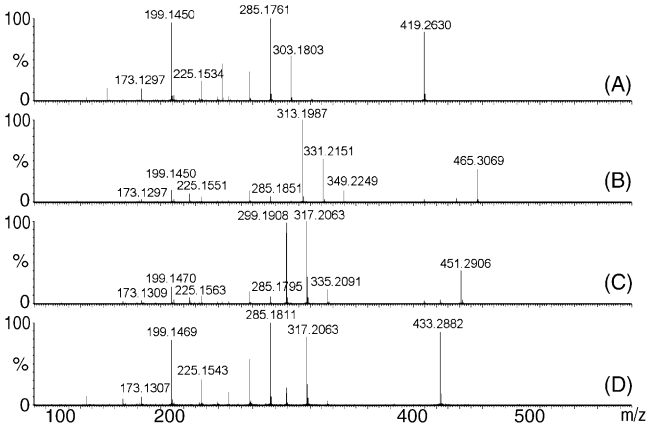

Fig. 3. MS/MS spectra of simvastatin (A), A1 (B), A2 (C) and A3 (D) in range from *m/z* 80 to 600.

or ion mirror) which could provide improved resolution and better accuracy of accurate TOF MS/MS measurements. The intensity of fragment ions must be properly adjusted during accurate mass measurement and should be neither to high (not more then 200 ion counts per second) because of the limitations caused by dead time of detector, nor too low because the peak shape would not be stable enough for acceptable measurement. When performing accurate MS/MS measurement, it might be difficult to achieve the same ion intensity level for all fragments. To a certain extent this could be improved by optimising the collision energy, collision gas pressure or cone voltage. Another very important factor in accurate mass measurement is a proper calibration of instrument in the mass range of interest. Calibration should be performed after completing the tuning procedure. Mass errors also significantly depend on temperature changes during the experiment, although this has lately been resolved to a certain extent by such technical devices as temperature correction sensors.

The fragmentation pattern for simvastatin was proposed (Fig. 4A**)** and confirmed through results obtained by MS/MS analysis of simvastatin molecule (Fig. 3). It was adopted as a template for the evaluation of A1, A2 and A3 fragmentation results.

The information about elemental composition of impurities has set molecular boundaries in which structural modifications of simvastatin molecule had to be determined. By comparing the results obtained from accurate MS/MS analysis of simvastatin with those obtained from accurate MS/MS

Table 1
Measured accurate mass results obtained for simvastatin, A1, A2 and A3

Compound	Averaged measured accurate mass of precursor ion	Calculated monoisotopic mass of precursor ion	Averaged measurement error (ppm)	Deduced elemental formula of protonated molecule
Simvastatin	419.2788	419.2797	−2.3	$C_{25}H_{39}O_5$
A1	465.3222	465.3216	1.3	$C_{27}H_{45}O_6$
A2	451.3045	451.3060	−3.2	$C_{26}H_{43}O_6$
A3	433.2950	433.2954	−1.0	$C_{26}H_{41}O_5$

The results were averaged from five consecutive MS experiments.

Table 2
Measured accurate mass results obtained for simvastatin, A1, A2 and A3

Compound	Averaged measured accurate mass of fragment ion	Calculated monoisotopic mass of fragment ion	Averaged measurement error (ppm)	Deduced elemental formula of fragment ion
Simvastatin	303.1982	303.1960	7.2	$C_{19}H_{27}O_3$
	285.1835	285.1855	−6.9	$C_{19}H_{25}O_2$
	225.1653	225.1643	4.3	$C_{17}H_{21}$
	173.1340	173.1330	5.6	$C_{13}H_{17}$
A1	349.2361	349.2379	−5.1	$C_{21}H_{33}O_4$
	285.1839	285.1855	−5.5	$C_{19}H_{25}O_2$
	225.1653	225.1643	4.3	$C_{17}H_{21}$
	173.1345	173.1330	8.5	$C_{13}H_{17}$
A2	335.2250	335.2222	6.2	$C_{20}H_{31}O_4$
	285.1872	285.1855	6.1	$C_{19}H_{25}O_2$
	225.1621	225.1643	−9.9	$C_{17}H_{21}$
	173.1346	173.1330	9.1	$C_{13}H_{17}$
A3	317.2094	317.2117	−7.2	$C_{20}H_{29}O_3$
	285.1840	285.1855	−5.1	$C_{19}H_{25}O_2$
	225.1660	225.1643	7.4	$C_{17}H_{21}$
	173.1353	173.1330	13.1	$C_{13}H_{17}$

The results were averaged from five consecutive MS/MS experiments.

analysis of unknown impurity compounds, it was enabled to define parts of a molecule that remained the same and parts that changed during stability testing of the tablets or substance synthesis.

Deduced elemental composition $C_{27}H_{44}O_6$ for A1 showed that the difference from elemental composition of simvastatin molecule $C_{25}H_{38}O_5$ was in two carbon atoms, six hydrogen atoms and one oxygen atom. Deduced elemental compositions of fragment ions (Table 2) indicated that A1 molecule fragmentation most likely began by the elimination of the ester side chain (*m*/*z* 465 → *m*/*z* 349), as in simvastatin molecule (*m*/*z* 419 → *m*/*z* 303), or by the neutral loss of ethanol group on opened lactone ring (*m*/*z* 465 → *m*/*z* 419). Fragments at *m*/*z* 285, 267, 225 and 173 were observed for both A1 (all of them cannot be represented by simplified fragmentation pattern shown in Fig. 4B due to molecular rearrangements which took place during fragmentation process) and for simvastatin. This and the evaluation of observed ions for A1 at *m*/*z* 349 → *m*/*z* 331 → *m*/*z* 313 representing neutral losses of water suggested that the modification took place on the lactone ring. Even more, knowing details on procedure of simvastatin tablets formulation (during the process of wet granulation ethanol was used) and on simvastatin chemistry enabled proposal of the structure shown in Fig. 4B for A1. It is already known from literature that hydroxy acids, hydroxy esters and hydroxylated acid derivatives can be converted to lactones by intramolecular acid-catalysed condensation at

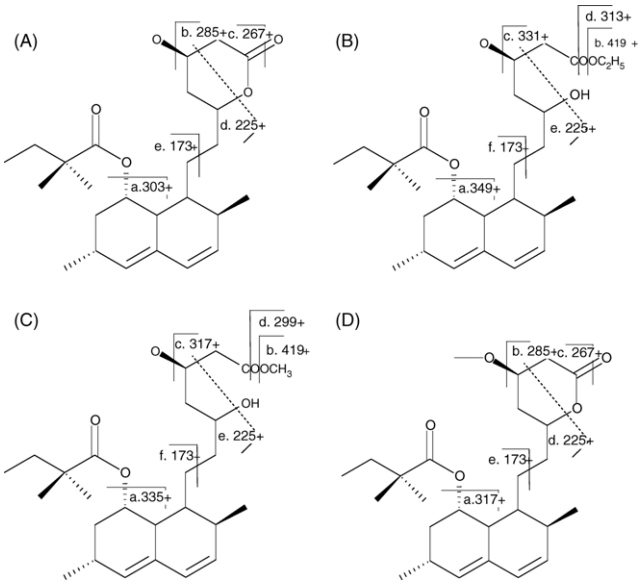

Fig. 4. Proposed structures and fragmentation patterns for simvastatin at *m*/*z* 419 (A), A1 at *m*/*z* 465 (B), A2 at *m*/*z* 451 (C) and A3 at *m*/*z* 433 (D).

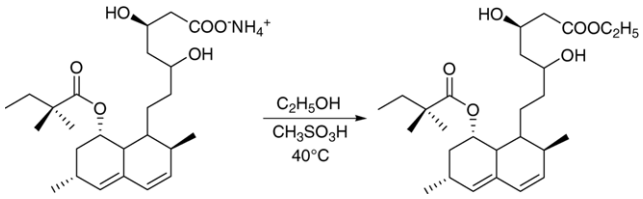

Fig. 5. Chemical reaction scheme for the synthesis of A1.

elevated temperatures [18,19], thus explaining the results of the experiment performed in the beginning of this study in which simvastatin tablets were heated to 100 °C. Based on the proposed structure, the unknown impurity A1 detected during stability studies of simvastatin tablet samples was synthesized by esterification of ammonium-7-[8-(2,2-dimethyl-butyryloxy)-2,6-dimethyl-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl]-3,5-dihydroxy-heptanoate in absolute ethanol in the presence of methanesulfonic acid (Fig. 5) and analysed by LC–UV (DAD), LC–MS/MS, NMR and IR techniques (data not shown) as a final confirmation of proposed molecular structure at the end of the identification process. Simvastatin was also formed during the reaction in 2:1 ratio to A1.

Deduced elemental compositions for A2 and A3 were $C_{26}H_{43}O_6$ and $C_{26}H_{41}O_5$ (Table 1). The evaluation of data obtained for A2 and A3 showed the same set of fragment ions with the elemental composition corresponding to that of simvastatin molecule fragment ions (Table 2**)**, at *m*/*z* 173, 225, 267 and 285 in the lower mass range. Fragmentation probably also began by the elimination of the ester side chain for both A2, confirmed by observed ions at *m*/*z* 451 and 335, and for A3 confirmed by observed ions at *m*/*z* 433 and 317. The conclusion for A2 was quite similar as for A1. They differed only in one carbon, two hydrogen and one oxygen atom. The abundance of fragment ions in the mass spectra of A2 and A3 was also quite similar suggesting the structure for A2 proposed and shown in Fig. 4C. A1 was identified as ethyl ester of simvastatin while the proposed structure for A2 belonged to methyl ester of simvastatin. Process of formation of the two impurities could be best described as nucleophilic substitution on carboxy atom in lactone ring by ethanol and methanol molecules.

Experimental results obtained for A3 also point to the modification on lactone ring, while the difference from simvastatin molecule in one carbon and two hydrogen atoms led us to a conclusion that the structure of A3 corresponded to the one shown in Fig. 4D. Additional confirmation for the proposed structure is neutral loss of methanol molecule represented with ions at *m*/*z* 317 and 285 in the mass spectrum. Since this part of simvastatin synthesis was performed by drying the substance from methanol solution, it was expected that the hydroxyl group on lactone ring would be replaced with the methoxy group through nucleophilic substitution. Most probably, the first step in reaction was elimination of hydroxyl group followed by addition of methanol. In the case of A2, a lactone ring also reacted in the methanol solution and converted into a methyl ester.

Acknowledgments

The authors would like to thank Dr. Vlasta Drevenkar, M.Sc. Katica Lazaric and Dr. Ante Krstulovic for their comprehensive help and support.

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