Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 729–736

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Isolation and structure determination of oxidative degradation products of atorvastatin

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ARTICLE INFO

Article history:
Received 9 March 2009
Received in revised form 24 May 2009
Accepted 3 June 2009
Available online 12 June 2009

Keywords:
Atorvastatin
Oxidative degradation products
Preparative HPLC
HRMS
NMR

ABSTRACT

Methods were developed for the preparation and isolation of four oxidative degradation products of atorvastatin. ATV-FX1 was prepared in the alkaline acetonitrile solution of atorvastatin with the addition of hydrogen peroxide. The exposition of aqueous acetonitrile solution of atorvastatin to sunlight for several hours followed by the alkalization of the solution with potassium hydroxide to pH 8–9 gave ATV-FXA. By the acidification of the solution with phosphoric acid to pH 3 ATV-FXA1 and FXA2 were prepared. The isolation of oxidative degradation products was carried out on a reversed-phase chromatographic column Luna prep C18(2) 10 μm applying several separation steps. The liquid chromatography coupled with a mass spectrometer (LC-MS), high resolution MS (HR-MS), 1D and 2D NMR spectroscopy methods were applied for the structure elucidation. All degradants are due to the oxidation of the pyrrole ring. The most probable reaction mechanism is intermediate endoperoxide formation with subsequent rearrangement and nucleophilic attack by the 5-hydroxy group of the heptanoic fragment. ATV-FX1 is 4-[1b-(4-Fluoro-phenyl)-6-hydroxy-6-isopropyl-1a-phenyl-6a-phenylcarbamoyl-hexahydro-1,2-dioxa-5a-aza-cyclopropa[a]inden-3-yl]-3-(R)-hydroxy-butyric acid and has a molecular mass increased by two oxygen atoms with regard to atorvastatin. ATV-FXA is the regioisomeric compound, 4-[6-(4-Fluoro-phenyl)-6-hydroxy-1b-isopropyl-6a-phenyl-1a-phenylcarbamoyl-hexahydro-1,2-dioxa-5a-aza-cyclopropa[a]inden-3-yl]-3-(R)-hydroxy-butyric acid. Its descendants ATV-FXA1 and FXA2 appeared without the atorvastatin heptanoic fragment and are 3-(4-Fluoro-benzoyl)-2-isobutyryl-3-phenyl-oxirane-2-carboxylic acid phenylamide and 4-(4-Fluoro-phenyl)-2,4-dihydroxy-2-isopropyl-5-phenyl-3,6-dioxa-bicyclo[3.1.0]hexane-1-carboxylic acid phenylamide, respectively. Quantitative NMR spectroscopy was employed for the assay determination of isolated oxidative degradation products. The results obtained were used for the determination of the UV response factors relative to atorvastatin.

1. Introduction

Atorvastatin (βR,δR)-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid as the calcium salt belongs to the group of statins. All statins, including atorvastatin, reduce the production of cholesterol in the liver by the competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in the biosynthesis of cholesterol. Their common characteristic is a fragment of β,δ-dihydroxy-heptanoic acid, an analogue of mevalonate [1].

Atorvastatin is a synthetic substance discovered through the systematic testing of pyrrole derivatives and comparison of their inhibitory potency [2–4]. The solubility and equilibrium between the dihydroxycarboxylic acid and lactone forms are strongly dependent on pH [5]. The study of metabolic processes indicated several active and inactive metabolites [6,7]. Several impurities and degradation products of atorvastatin have been identified [8–10]. The most frequently encountered impurity, as well as degradation product, is atorvastatin lactone [11].

An important and characteristic sensitivity to photooxidation under intensive sunlight was observed [12–14]. The oxidation is localized to the pyrrole ring. The suggested pathways predict the formation of endoperoxide and hydroperoxide intermediates followed by formation of epoxides and lactam rings, rearrangements, opening of the pyrrole ring and cleavage of the statin side chain. Detailed studies of photooxygenations of pyrrole and its derivatives have confirmed the existence of unstable intermediates and have yielded several reaction mechanisms which depend on the pyrrole substituents and reaction conditions [15,16]. Reviews on

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doi:10.1016/j.jpba.2009.06.008

the photooxygenation of heterocycles including pyrrole give a broad account of reaction mechanisms and transformations [17,18]. These studies corroborate the reaction mechanisms suggested above for atorvastatin (photo)oxygenations.

Analytical methods for atorvastatin usually combine reversed-phase chromatographic methods and UV detection at characteristic absorption maxima or different modes of MS detection. Several methods have been reported for the quantitative determination of atorvastatin in biological samples [19–21], aqueous samples [22,23] and tablets [24–27]. A combination of mass spectrometry and NMR spectroscopy methods was recognized as crucial for the identification of new degradation products of atorvastatin [12,14].

Several degradation products are formed during the exposure of atorvastatin to the oxidative environment which is usually present during the production process, the storage of the substance and the pharmaceutical formulation. An HPLC method with fluorescence detection was developed for atorvastatin in bulk form and tablets subjected to various stress conditions including oxidation [28]. One study reported on the systematic forced degradation behaviour of atorvastatin under stress conditions prescribed by the International Conference on Harmonization (ICH) and World Health Organization (WHO), which provided many degradation products and a comprehensive fragmentation pattern [29].

The process of isolation and identification of four main oxidative degradation products of atorvastatin (OXDP) gave us an impulse to improve our knowledge of the pharmaceutical product and its safety [30]. We need to emphasize that according to our experience the oxidation of atorvastatin is not such a critical issue for the crystalline form as it is for the amorphous form. Different ways of preparing OXDP were investigated. We found that the oxidation of atorvastatin calcium solution in acetonitrile/water mixture by either H_2O_2 or photooxidation by the sunlight was successful and led to the same products as the oxidation of the same substance in the solid form or in pharmaceutical formulations by atmosphere oxygen during the production or storage. After the OXDP preparation procedure we isolated four products by preparative HPLC and, subsequently, determined the molecular structures by MS and NMR spectroscopy. OXDP substances can be hazardous with regard to toxicity issues. For that reason improving analytics of atorvastatin is very important.

The characterization and quantitation of each impurity-related substance or degradation product present in the active substance or pharmaceutical formulation is regularly performed by HPLC-UV analytical techniques. It is particularly important to determine the UV response factor of the impurity since it can be different from that of the active substance. In such cases it could happen that the active substance is declared to be pharmaceutically confirmed according to the requirements, although the levels of its impurities may be outside of the permitted values.

2. Experimental

2.1. Chemicals

Atorvastatin calcium was obtained from Lek d.d., solvents, salts, and bases were HPLC grade supplied by Merck, phosphoric acid by Riedel de Haen and deionised water was prepared by Elga UHQ. Deuterated solvents for NMR experiments were also from Merck.

Sodium salt of atorvastatin was prepared by a desalting procedure on a reverse phase HPLC column, with the addition of a stoichiometric amount of sodium hydroxide solution, followed by the lyophilization.

2.2. Compound preparation methods

2.2.1. Preparation of ATV-FX1 compound

800 ml of the solution of atorvastatin was prepared in acetonitrile containing 10 mg of atorvastatin per 1 ml. 4 ml of 12 M sodium hydroxide and 40 ml of 30% hydrogen peroxide were added. The solution was stirred at 55 °C for 5 h. The reaction mixture was allowed to cool and decanted. The supernatant was evaporated under reduced pressure to ca. 50 ml. The remaining water was discarded and the solid residue was washed with water. The solid residue was then dissolved in acetonitrile.

2.2.2. Preparation of ATV-FXA, ATV-FXA1 and ATV-FXA2 compounds

The solution of atorvastatin was prepared in a mixture of 80% acetonitrile and 20% (v/v) water, containing 1 mg of atorvastatin per 1 ml. The solution was put in a shallow crystallizer dish that was exposed to the sun radiation for 5 h. Immediately after that, the solution was alkalized with 1 M solution of potassium hydroxide to pH 8–9 to prepare ATV-FXA compound or acidified with 0.5 M phosphoric acid to pH 3.0 to prepare ATV-FXA1 and ATV-FXA2 compounds.

We also monitored by HPLC the consecutive transformation of ATV-FXA to ATV-FXA1 and ATV-FXA2.

2.3. Preparative HPLC

The isolation of oxidative degradation products was carried out using several steps of preparative HPLC using a reversed-phase chromatographic column Luna prep C18(2) 10 μm (200 mm × 50 mm).

Two chromatographic separation steps were necessary to obtain pure compounds. The first separation was carried out using a 10 mM ammonium acetate buffer at pH 4.5 adjusted with acetic acid as mobile phase A and a mixture of 5% tetrahydrofuran and 95% acetonitrile as mobile phase B. Four fractions were collected and purified again in the second step. The stability of substances in particular preparative fractions were strongly dependent on the pH of the solutions. After the elution of substances from the chromatographic column, pH was fixed using suitable sodium phosphate buffers or 1 M potassium hydroxide and 1 M hydrochloric acid. The buffer pH was adjusted using a phosphoric acid or sodium hydroxide solution according to the stability preferred pH presented in Table 1.

In the second step, selectivity was changed by the use of the following buffer solutions. For fraction one 10 mM ammonium hydrogen carbonate, for fraction two 10 mM phosphate buffer at pH 7.0 adjusted with phosphoric acid or sodium hydroxide solution, for fractions three and four 5 mM hydrochloric acid were employed. The pure fractions were evaporated under reduced pressure, non-volatile buffer salts were removed by desalting procedure on a reverse phase column and, finally, fractions were lyophilized.

2.4. Analytical HPLC

HPLC analyses were carried out on an Agilent Technologies 1100 System (Waldbronn, Germany), with the XBridge Shield RP 18

Table 1
Preparation and pH stability data for oxidative degradation products.

Fractions	OXDP	Method of synthesis	Stable at pH
1	ATV-FXA	O_2/photooxidation	$\gg$7
2	ATV-FX1	O_2/H_2O_2	$\geq$7
3	ATV-FXA2	O_2/photooxidation	$\leq$7
4	ATV-FXA1	O_2/photooxidation	$\ll$7

3.5 μm (150 mm × 4.6 mm) column (Waters, Massachusetts, USA) at a flow rate of 2 ml/min. The tray temperature was 10 °C and the column oven temperature was 25 °C. The gradient elution was carried out using 5% acetonitrile (mobile phase A) and 75% acetonitrile (mobile phase B) in 20 mM ammonium acetate pH 4.0 adjusted with acetic acid. The mobile phase gradient was started at 45% of B, and after 14 min increased to 66% B within 8 min then increased to 100% B within 5 min and held for 3 min. The injection volume was 10 μl. The UV detector was set to 248 nm. The split ratio of flow to the mass spectrometer was 1:7.

2.5. LC-MS and HRMS

HPLC instrument was coupled to Micromass Q TOF Ultima Global mass spectrometer (Manchester, UK) equipped with electrospray ionisation (ESI) in positive mode. ESI source parameters were: source temperature set to 120 °C, desolvation temperature set to 350 °C, capillary voltage 3 kV, cone gas (nitrogen) 200 l/h, desolvation gas (nitrogen) 650 l/h. Detector MCP was set to 2300 V. TOF geometry was in V mode.

The high resolution mass spectra of isolated OXDP were obtained using the same instrument Micromass Q TOF Ultima Global employing the positive ESI mode. Samples were introduced into the mass spectrometer by direct infusion. The source temperature was set to 100 °C, desolvation temperature to 200 °C, cone gas 0 l/h and desolvation gas 200 l/h. W geometry of TOF analyzer was employed. The sample was dissolved in 50% solution of 5 mM ammonium acetate and acetonitrile and infused to the mass spectrometer with constant flow of 10 μl/min. The concentration of sample solution was 0.05 mg/ml.

Atorvastatin was used as an internal standard for exact mass measurements. A concentration of 0.01 mg/ml was employed.

2.6. NMR spectroscopy

^{1}H NMR spectra were recorded on Varian spectrometers Inova 300 and 600 operating at 300 and 600 MHz. Instruments were equipped with inverse detection probes. ^{13}C NMR spectra were recorded on the Varian spectrometer Unity+ 300 at 75 MHz. The instrument had a direct detection dual broad band probe. Homo- and heteronuclear chemical shift correlations were determined by COSY, HSQC and HMBC 2D NMR methods. Standard Varian pulse sequences were applied by running Varian Vnmr software. Typical samples prepared for ^{13}C NMR measurements were 20 mg per 0.70 ml of the solvent and 5–10 mg per 0.70 ml for ^{1}H measurements. ^{1}H and ^{13}C chemical shifts were referenced to the residual signals of the deuterated solvents (CD_3OD, 3.31, 49.0 ppm; DMSO-d6, 2.50, 39.5 ppm; $CDCl_3$, 7.27, 77.0 ppm).

Quantitative ^{1}H NMR, employed for the assay determination, was based on the comparison of the integrals of the measured substance and the internal standard in the NMR spectrum. The sample and the internal standard, 4-dimethylaminopyridine (DMAP), with the certified assay were weighed in a stoichiometric ratio (minimum 3 mg of DMAP and corresponding mass of measured substance) into the same vial and dissolved in 1 ml of deuterated methanol (deuteration degree min 99.9%). The solution was transferred into the NMR tube. NMR measurements was carried out using 16 transients and a recycle delay of 30 s. Integrals of the aromatic protons were used for the assay calculation. The relative standard deviation of the quantitative NMR determination was previously estimated to be 2%.

3. Results and discussion

During the pharmaceutical formulation stability testing in the presence of oxidative atmosphere, we were able to establish experimental conditions that stimulate atorvastatin degradation. HPLC methods with UV and MS detection were developed to monitor the degradation and to support the peak identification procedure. In LC-MS chromatogram of partially degraded substance atorvastatin calcium four main oxidative degradation products were detected (Fig. 1).

The four compounds designated in the chromatogram in Fig. 1 were isolated according to the experimental procedure described above. We succeeded in preparing a sufficient quantity of each OXDP which then enabled us to determine the structures in a relatively straightforward way. 1D and 2D NMR experiments and High Resolution MS techniques were a successful combination that led to the final identification of four molecules.

Different deuterated solvents were tested for NMR analysis of atorvastatin and isolated OXDP. We used both methanol and chloroform or their mixture in order to increase the solubility. In the first step, the assignment of signals in the ^{1}H and ^{13}C spectra of atorvastatin was carried out. The sodium salt of atorvastatin was chosen as we expected that isolated OXDP were sodium salts. Buffers with sodium salts were used during the preparative isolations.

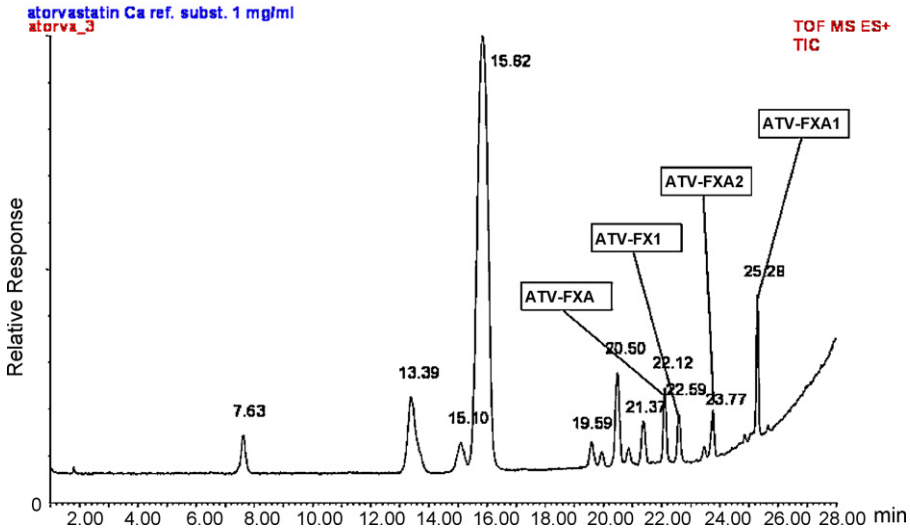


Fig. 1. HPLC-MS chromatogram of the crude substance atorvastatin calcium exposed to the oxidative environment with marked peaks of the four main oxidative degradation products.

Table 2
A comparison of ^{1}H chemical shifts (in ppm) between atorvastatin and two isolated oxidative degradation products in CD_3OD.

Proton	ATV	ATV-FX1	ATV-FXA
7	4.08 and 3.93	3.36 and 2.97	3.25 and 2.87
3	3.90	4.13	4.30
5	3.67	3.74	4.40
$(CH_3)_2\underline{CH}$	3.38	2.50	3.17
2	2.26	2.37 and 2.27	2.54
6	1.71	2.07 and 1.25	1.40 and 1.80–2.10
4	1.54 and 1.42	1.85 and 1.64	1.80–2.10
$(\underline{CH_3})_2CH$	1.47	1.26 and 1.28	1.24 and 1.31

For the assignment of the side chain protons COSY was applied. Dimethylsulfoxide was also used to observe exchangeable protons. The signals of exchangeable protons were detected in DMSO-d6 at 4.8 and 6.0 ppm for hydroxy groups and at 9.8 ppm for the amide NH proton. The ^{13}C NMR spectrum was assigned by DEPT, HSQC and HMBC experiments. The assignment procedure was the starting point for the identification of the four unknown structures. The structure determinations were based on the comparison of spectra and analysis of differences in the chemical shifts and multiplicities of signals between atorvastatin and isolated OXDP.

The mechanism of oxidation of the pyrrole ring in atorvastatin most probably includes the formation of unstable endoperoxides [12–14] (see Scheme 1). Two pathways become viable. They both include nucleophilic attack of 5-OH group to the activated pyrrole ring and cyclization forming a 6-membered ring and a new hydroxy group, one forms an O–C bond at the 2′ position (ATV-FX1) and the other at the 5′ position (ATV-FXA). It is extremely interesting that the latter compound can undergo further rearrangement by opening of the pyrrole ring and releasing the heptanoic fragment, thus forming an open structure with two carbonyl groups (ATV-FXA1). This latter compound is capable of reacting with a water molecule to create a furane ring (ATV-FXA2). The compound designated as ATV-FXA1 was previously discovered by Hurley et al. [12].

3.1. ATV-FX1 and ATV-FXA

ATV-FX1 and ATV-FXA show notable changes in the ^{1}H and ^{13}C chemical shifts with regard to atorvastatin (ATV), especially for those arising from the side chain and the pyrrole ring (Tables 2 and 3). Differences were also observed between the signals of the isopropyl group. ^{1}H and ^{13}C NMR spectra confirmed that the phenyl rings deriving from atorvastatin are intact. Therefore, structural modifications were expected in the part of the side chain close to the pyrrole ring.

Signals of the exchangeable protons were observed in the ^{1}H NMR spectrum of ATV-FX1 and ATV-FXA in DMSO-d6. However, only two of the three expected signals were detected, at 6.2 and 10.0 ppm for ATV-FX1 and at 5.8 and 9.7 ppm for ATV-FXA. Low field signals correspond to the amide NH, while higher field signals could be assigned to 5′ and 2′, respectively. We believed that the signal 3-OH is missing, most probably due to an intramolecular H-bond with the carboxylic function, a well-known phenomenon in β-hydroxycarboxylic acids [31].

Table 3
A comparison of ^{13}C chemical shifts (in ppm) between atorvastatin and two isolated oxidative degradation products in CD_3OD.

Carbon	ATV	ATV-FX1	ATV-FXA
1	180.5	180.2	180.5
Pyrrole $\underline{C}$-5′	139.2	96.9	94.9
Pyrrole $\underline{C}$-2′	129.7	95.1	97.2
Pyrrole $\underline{C}$-3′	123.4	74.5	70.6
Pyrrole $\underline{C}$-4′	118.2	70.0	74.4
3, 5	69.0 and 68.9	67.5 and 70.4	69.3 and 70.4
2	45.5	45.2	46.4
4	44.6	44.7	45.0
7	42.4	38.3	37.4
6	40.3	31.0	30.5
$(CH_3)_2\underline{CH}$	27.7	36.6	29.5
$(\underline{CH_3})_2CH$	22.9	19.7 and 19.0	19.5 and 18.3

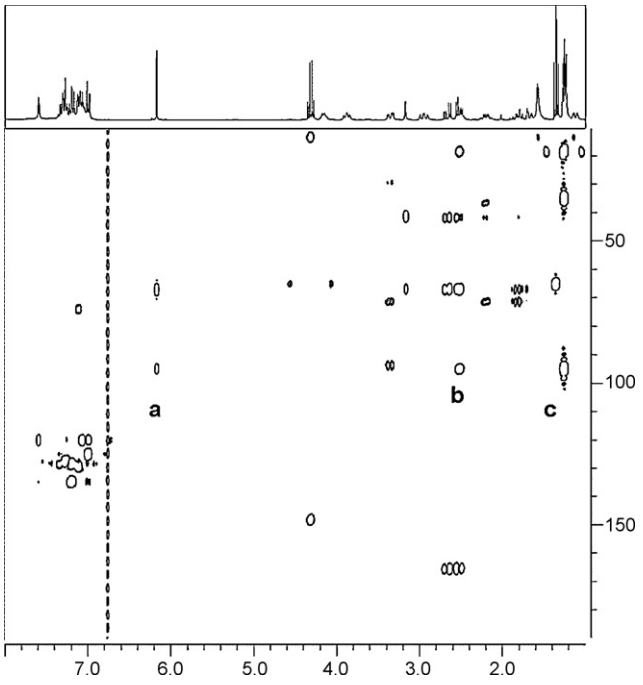


Fig. 2. 2D HMBC NMR spectrum of the ethylchloroformate derivative of ATV-FX1 in $CDCl_3$, with assigned key correlations: (a) C5′, 5′-OH; (b) C5′, $(CH_3)_2\underline{CH}$; (c) C5′, $(\underline{CH_3})_2CH$.

In a further step, we synthesized a derivative of ATV-FX1 with ethylchloroformate [32] and isolated it by preparative HPLC. HPLC-MS analysis gave a molecular ion of the expected product $(M+H)^+$ 663 *m/z*. The most intensive ion in the mass spectrum was $(M+H-H_2O)^+$ 645 *m/z*. The ^{1}H NMR spectrum of the ATV-FX1 derivative in $CDCl_3$ also showed the missing signal of the exchangeable protons, altogether 7.6 (amide NH), 6.2 and 3.2 ppm.

We confirmed by COSY that the last peak corresponds to 3-OH and by HMBC that the peak at 6.2 ppm corresponds to the hydroxy group. HMBC correlations for the ATV-FX1 derivative convincingly indicate that the isopropyl and hydroxy groups are attached to the same carbon, i.e. C-5′ (Fig. 2).

The ethylchloroformate derivative of ATV-FXA was unstable and for that reason we were not able to isolate it.

^{13}C NMR spectra of ATV-FX1 and ATV-FXA were assigned by DEPT, HSQC and HMBC experiments. Major differences were observed for the pyrrole ring, carbons 6, 7 and isopropyl methyne carbons. The difference in the chemical shifts between carbons 3 and 5 increased in comparison to atorvastatin.

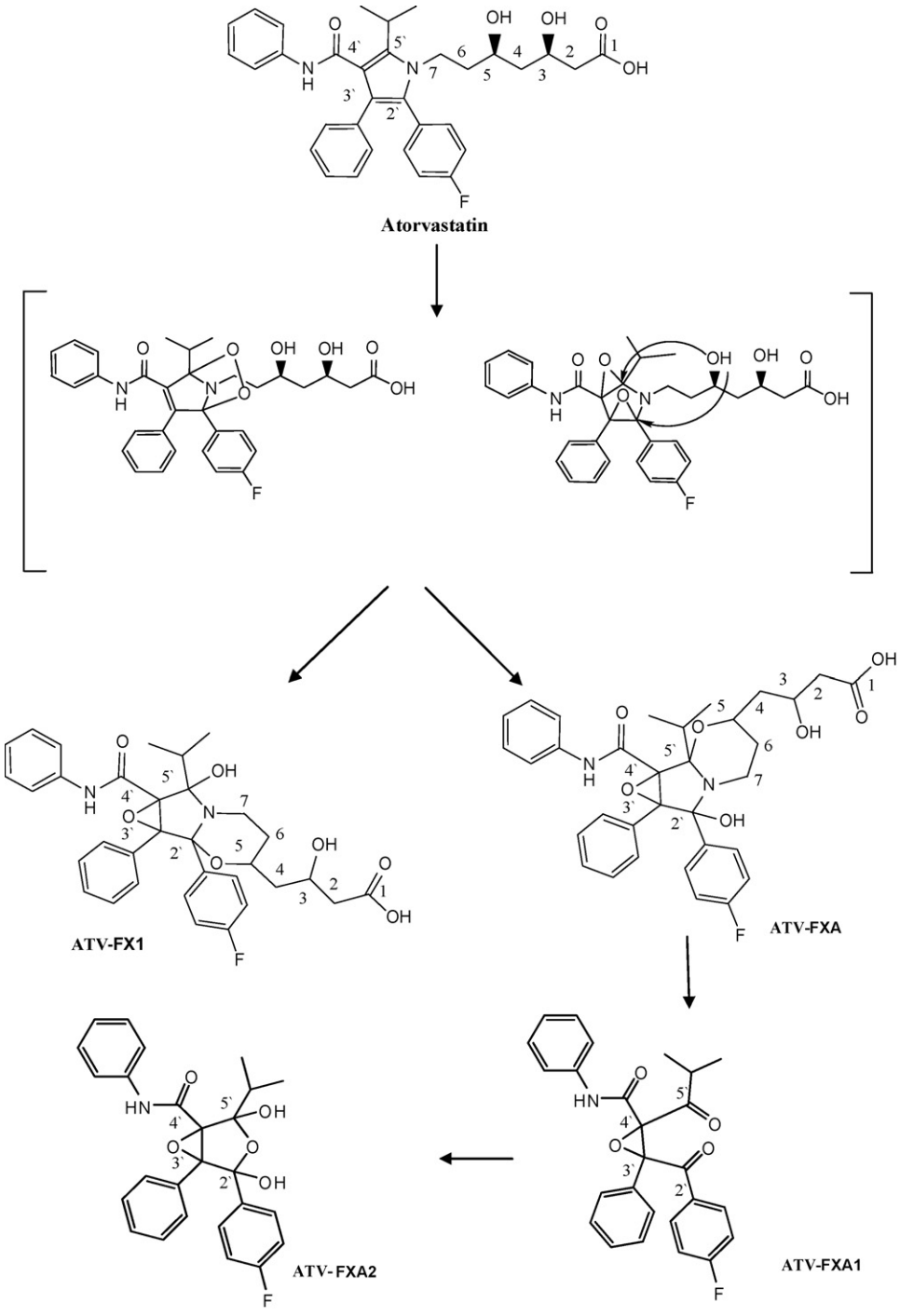
The appearance of new ^{13}C peaks in the spectral region between 70 and 100 ppm indicated loss of the aromatic character of the pyrrole ring. The chemical shifts of carbons 3′ and 4′ were identified as typical for quaternary carbons bearing the epoxide oxygen [12]. Consequently, carbons 2′ and 5′ had to form new bonds. We have established above for ATV-FX1 the formation of a new hydroxy group on the carbon 5′ that implies the cyclization of the side chain 5-OH group to the carbon 2′. The chemical shift of the isopropyl methyne group is more disturbed when the cyclization leads to the formation of the hydroxy group on the adjacent carbon 5′. Differences in the carbon chemical shifts of 6 and 7 arise from rigidity caused by cyclization.

The chemical shifts of ATV-FXA are comparable to ATV-FX1 with some minor variations. The analogy between the ^{13}C spectra indicates similar structures. It should be noted that the 'pyrrolic' carbons 2′ and 3′ interchanged the chemical shift positions with 5′ and 4′. Taking into account the NMR data and the MS results presented below in Table 4, we concluded that ATV-FXA is a regioisomer of ATV-FX1 and, therefore, confirmed the cyclization of 5-OH of the atorvastatin side chain to carbon 5′ and the formation of the hydroxy group on carbon 2′.

MS results together with some of the most intensive signals for ATV-FXA and ATV-FX1 are presented in Table 4.

ATV-FXA gave an intensive signal for the protonated molecule (Fig. 3). The molecular ion $(M+H)^+$ was observed in the HRMS spectrum at a mass of 591.2512 m/z. The calculated elemental composition was $C_{33}H_{36}N_2O_7F$. The mass shift between the calculated and the measured mass was 0.5 mDa. In comparison with atorvastatin, ATV-FXA contains two additional oxygen atoms in its molecular structure.

The most intensive ion of ATV-FX1 was 573.2 m/z in the MS spectrum shown below in Fig. 4. It is formed by the elimination of water from the molecule. The molecular ion was much less intensive in comparison to ATV-FXA. The molecular ion $(M+H)^+$ 591.2493 m/z



Scheme 1. Presumed oxidative degradation pathway of atorvastatin.

Table 4
MS results.

	M	Mol. formula	Redundant fragment[a]	*m/z* (%)	DBE[b]
ATV	558	$C_{33}H_{35}N_2O_5F$	/	559 (100)[c], 560 (40), 617 (30), 557 (25)	17
ATV-FXA	590	$C_{33}H_{35}N_2O_7F$	+2 × O	591 (100)[c], 592 (40), 649 (25)[d], 573 (15)	17
ATV-FX1	590	$C_{33}H_{35}N_2O_7F$	+2 × O	573 (100)[e], 574 (45), 575 (15), 613 (5), 591 (<5)	17

[a] Redundant fragments pertain to atorvastatin.
[b] Double Bond Equivalent—specifies the number of double bonds per molecule.
[c] $(M+H)^+$ molecular ion.
[d] $(M+ACN+NH_4)^+$.
[e] $(M+H-H_2O)^+$.

Table 5
MS results.

	M	Mol. formula	Missing fragment	*m/z* (%)	DBE[a]
ATV-FXA1	431	$C_{26}H_{22}NO_4F$	$-C_7H_{13}NO$	344 (100), 313 (40), 432 (35)[b], 345 (30)	16
ATV-FXA2	449	$C_{26}H_{24}NO_5F$	$-C_7H_{11}N$	344 (100), 313 (35), 432 (30)[c], 345 (30)	15

[a] See definition in Table 4.
[b] $(M+H)^+$ molecular ion.
[c] $(M+H-H_2O)^+$.

was observed in the HRMS spectrum. The calculated elemental composition for 591.2493 *m/z* was $C_{33}H_{36}N_2O_7F$. The mass shift between the calculated and the measured mass was −1.4 mDa. In comparison to atorvastatin, ATV-FX1 contains two additional oxygen atoms in the structure.

Considering both NMR and MS results ATV-FX1 was determined to be 4-[1b-(4-Fluoro-phenyl)-6-hydroxy-6-isopropyl-1a-phenyl-6a-phenylcarbamoyl-hexahydro-1,2-dioxa-5a-aza-cyclopropa[a]inden-3-yl]-3-(R)-hydroxy-butyric acid and ATV-FXA to be 4-[6-(4-Fluoro-phenyl)-6-hydroxy-1b-isopropyl-6a-phenyl-1a-phenylcarbamoyl-hexahydro-1,2-dioxa-5a-aza-cyclopropa-[a]inden-3-yl]-3-(R)-hydroxy-butyric acid.

3.2. ATV-FXA1 and ATV-FXA2

From proton and carbon NMR spectra it was clear that the side chain typical for statins is not present in the molecular structures of ATV-FXA1 and ATV-FXA2. On the basis of the MS results shown in Table 5, we concluded that the pyrrole ring nitrogen atom had been cleaved off as well.

The presence of the isopropyl group and 14 aromatic protons was confirmed in the proton and carbon NMR spectra. In Table 6 we present the most notable differences in the ^1H and ^{13}C chemical shifts between ATV-FXA1 and ATV-FXA2. The ^1H chemical shift difference between the isopropyl methyne groups in the two compounds amounts to 0.80 ppm.

^{13}C NMR spectra of ATV-FXA1 and ATV-FXA2 in the solvent mixture of $CDCl_3$:CD_3OD (1:2) were assigned by HMBC experiments. Significant changes between chemical shifts can be seen also in the ^{13}C spectra, especially for carbons of the preceding pyrrole ring and isopropyl $\underline{C}H(CH_3)_2$ carbons. In ATV-FXA1 two signals were observed at 206.0 and 191.4 ppm. They were assigned to carbons C-5′ and C-2′ indicating the formation of the carbonyl groups. Signals at 74.6 and 72.0 ppm were assigned to the quaternary carbons bearing the epoxide oxygen [12].

A molecular ion $(M+H)^+$ 432.1612 *m/z* with lower intensity was observed in the mass spectrum of ATV-FXA1. The calculated elemental composition was $C_{26}H_{23}NO_4F$. The mass shift between the calculated and the measured mass was 0.1 mDa. In comparison to atorvastatin, the fragment $C_7H_{13}NO$ is missing.

Table 6
A comparison of ^1H and ^{13}C chemical shifts (in ppm) between ATV-FXA1 and ATV-FXA2 in the solvent mixture of $CDCl_3$:CD_3OD (1:2).

	ATV-FXA1	ATV-FXA2
Proton		
$(CH_3)_2\underline{C}H$	3.20	2.40
$(\underline{C}H_3)_2CH$	1.20 and 1.02	1.20
Carbon		
$\underline{C}$-5′	206.0	107.1
$\underline{C}$-2′	191.4	104.1
$\underline{C}$-3′, 4′	74.6 and 72.0	75.9 and 70.4
$(CH_3)_2\underline{C}H$	38.3	35.6
$(\underline{C}H_3)_2CH$	18.5 and 17.7	17.9 and 17.0

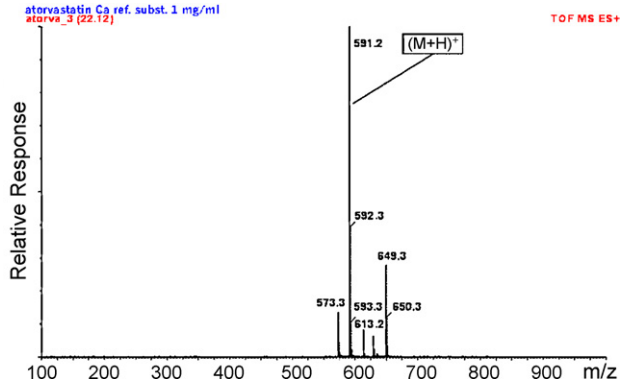


Fig. 3. Mass spectrum of isolated ATV-FXA eluting in chromatographic peak at 22.1 min with marked protonated molecular ion $(M+H)^+$ = 591.2 *m/z*.

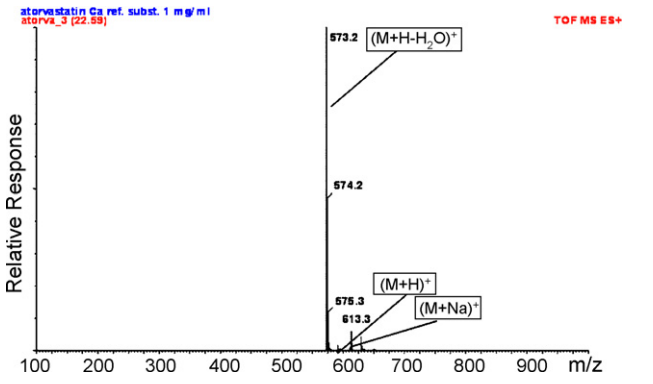


Fig. 4. Mass spectrum of isolated ATV-FX1 eluting in chromatographic peak at 22.6 min with marked protonated ion $(M+H-H_2O)^+$ = 573.2 *m/z*.

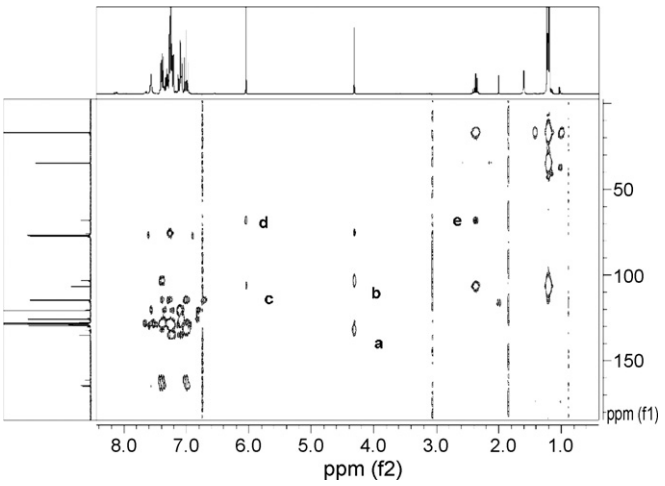


Fig. 5. 2D HMBC NMR spectrum of ATV-FXA2 in $CDCl_3$ with the assigned key correlations: (a) C_{ph}, 2′-OH; (b) C2′, 2′-OH; (c) C5′, 5′-OH; (d) C4′, 5′-OH; (e) C4′, $(CH_3)_2\underline{CH}$.

In the ^{13}C spectrum of ATV-FXA2 the quaternary carbons bearing the epoxide oxygen were evident at 70.4 and 75.9 ppm. Carbons C-5′ and C-2′were assigned to 107.1 and 104.1 ppm, as saturated and substituted with a hydroxy group. In the ^1H spectrum of ATV-FXA2 in $CDCl_3$ exchangeable protons were detected at 7.6 (amide NH) and at 6.1 and 4.3 ppm. The latter two signals were assigned to the hydroxyl group protons. HMBC spectra confirmed the correlation of the two ^1H signals to carbons 5′ and 2′, respectively (Fig. 5). The chemical exchange was also confirmed by the addition of CD_3OD. With regard to the ^1H and HMBC spectral data given above we propose that ATV-FXA2 has a five-membered central ring with the oxygen atom as the heteroatom. HRMS experiment gives an ion with mass of 432.1606 *m/z* that should correspond to the ion $(M+H-H_2O)^+$. The calculated elemental composition was $C_{26}H_{23}NO_4F$. The mass shift between the calculated and the measured mass was −0.5 mDa. The molecular ion $(M+H)^+$ of isolated ATV-FXA2 was not observed in the mass spectrum due to the fast elimination of water from the molecule.

Based on the NMR and MS results, ATV-FXA1 was identified as 3-(4-Fluoro-benzoyl)-2-isobutyryl-3-phenyl-oxirane-2-carboxylic acid phenylamide and ATV-FXA2 as 4-(4-Fluoro-phenyl)-2,4-dihydroxy-2-isopropyl-5-phenyl-3,6-dioxa-bicyclo[3.1.0]hexane-1-carboxylic acid phenylamide.

Afshaq et al. reported that degradation of atorvastatin calcium in methanol and hydrogen peroxide set aside for 2 months resulted in the crystallization of the compound (1*R*,2*S*,4*S*,5*S*)-4-(4-fluorophenyl)-2-hydroperoxy-4-hydroxy-2-isopropyl-*N*,5-diphenyl-3,6-ioxabicyclo[3.1.0]hexane-1-carboxamide ($C_{26}H_{24}FNO_6$), the 5′-peroxy analog of ATV-FXA2 [33].

An HPLC separation technique connected to UV detection is the most frequently employed procedure for the determination of the purity of pharmaceutical substances and products. The degradation products of active substance can exhibit different structural characteristics that contribute to the absorption of UV light and, therefore, affect quantitation. For that reason the response factor for each compound should be determined. As an assay determination procedure we employed the quantitative ^1H NMR measurements for each isolated OXDP substance. Response factors were then established by HPLC-UV analysis. It was found that three substances of isolated OXDP (ATV-FX1, ATV-FXA and ATV-FXA2) possess response factors lower than atorvastatin (Table 7). The UV absorption maxima of these substances were only slightly or even not changed with regard to atorvastatin (246 nm). It is perhaps surprising that the UV maxima of the degradants remain so close to those of the parent atorvastatin. It seems that the dominant contribution to the position of UV maxima comes from phenyl rings and that conjugation with the pyrrole ring in the intact atorvastatin is not very strong. The carbonyl groups of ATV-FXA1 shifted the absorption maximum to the higher wavelength at 256 nm and the response factor to a value of 1.20.

Table 7
UV response factors of isolated OXDP at the wavelength of 248 nm.

ATV	1.00
ATV–FX1	0.48
ATV–FXA	0.41
ATV–FXA1	1.20
ATV–FXA2	0.72

4. Conclusions

Experimental conditions were established which stimulate the production of atorvastatin derivatives, which are the same compounds that appear due to the degradation of atorvastatin in the solid form exposed to normal air conditions. We developed methods for the isolation of four compounds by preparative HPLC and determined their structures. Two compounds were structural isomers, the molecular mass increased by two oxygen atoms with regard to atorvastatin, formed by the cyclization of the 3,5-dihydroxyheptanoic chain to the pyrrole ring. The other two compounds arose from the rearrangement after splitting off of the heptanoic fragment: one an open ring diketone derivative and one a closed, furan ring derivative. The assays of the isolated oxidative degradation products of atorvastatin were determined by quantitative NMR spectroscopy. At the end UV response factors were determined in order to improve the HPLC-UV analytics of atorvastatin.

Acknowledgement

Mr. Sandi Borišek is gratefully acknowledged for his skillful assistance with the NMR measurements.

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