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Review Article

Atorvastatin and Related Compounds: Review on Analyses of Pharmaceutical, Blood and Environmental Samples

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Abstract

Statins have also anti-inflammatory and antimicrobial effects. According to literature, during the years 1999-2017 the research on atorvastatin with liquid chromatography has increased to 481 papers. However, atorvastatin studies with gas chromatography and capillary electrophoresis has both been reported in 15 papers during 2006-2017. The present paper compiles the most recent studies of these studies on atorvastatin made with chromatographic and electro aided separation techniques.

Keywords: atorvastatin, metabolites, chromatography, capillary electrophoresis, mass spectrometry

Abbreviations: API-MS/MS: Atmospheric Pressure Ionization Tandem Mass Spectrometry; ATOR: Atorvastatin; PFBBr: Pentafluorobenzylbromide; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; CE: Capillary Electrophoresis; EI: Electron Ionization; ESI-MS/MS: Electrospray Tandem Mass Spectrometry; FLU: Fluvastatin; GC: Gas Chromatography; HDL: High Density Lipoprotein; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HPLC: High Performance Liquid Chromatography; LDL: Low Density Lipoprotein; LDLR: LDL Receptors; LC: Liquid Chromatography; LLE: Liquid-Liquid Extraction; LOQ: Lowest Limit of Quantification; LOV: Lovastatin; MRM: Multiple Reaction Monitoring; MS: Mass Spectrometry; MS/MS: Tandem Mass Spectrometry; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; NCI: Negative Chemical Ionization; PKBS: Polyketide Biosynthesis; PRA: Pravastatin; QTOF-MS: Quadrupole-Time-of-Flight Mass Spectrometry; ROS: Rosuvastatin; SIM: Simvastatin; SPE: Solid-Phase Extraction; TMCS: Trimethylchlorosilane; TMS: Trimethylsilyl; UPLC: Ultra-Performance Liquid Chromatography; UV: Ultraviolet.

Introduction

Diseases and Statins

Statins, 3-hydroxy-3-methyl glutaryl-co-enzyme A (HMG-CoA) reductase inhibitors [1,2], are widely used compounds for decreasing blood cholesterol. They reduce effectively the level of LDL (Low Density Lipoproteins) by preventing the production of cholesterol in liver and by increasing the formation of LDL receptors LDLR [3,4]. In addition, they decrease triglyceride level and increase the amount of HLD (High Density Lipoprotein). Because of good chemical stability, statins are used as effective compounds in medicines for cholesterol disease [5,6]. According

to many studies, statins have also anti-inflammatory and antimicrobial effects [7-9]. Clinical studies have evidenced that statins significantly reduce the risk of heart attack and death in patients suffering coronary artery disease. They can also reduce cardiac events in patients with high cholesterol levels [7-14]. Cholesterol lowering statins include atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin (Figure 1). The most used of them in medicinal purposes are atorvastatin (ATOR), fluvastatin (FLU), lovastatin (LOV), pravastatin (PRA), and simvastatin (SIM). The small differences in their structures influence their solubility in solvents and evaporation in high temperatures, and their performance in analytical methods. The structure of the synthetic statin ATOR differs totally from that of the natural statins, which are formed by fermentation processes. ATOR is easy to identify with aid of the nitrogen and fluorine atoms in the molecule.

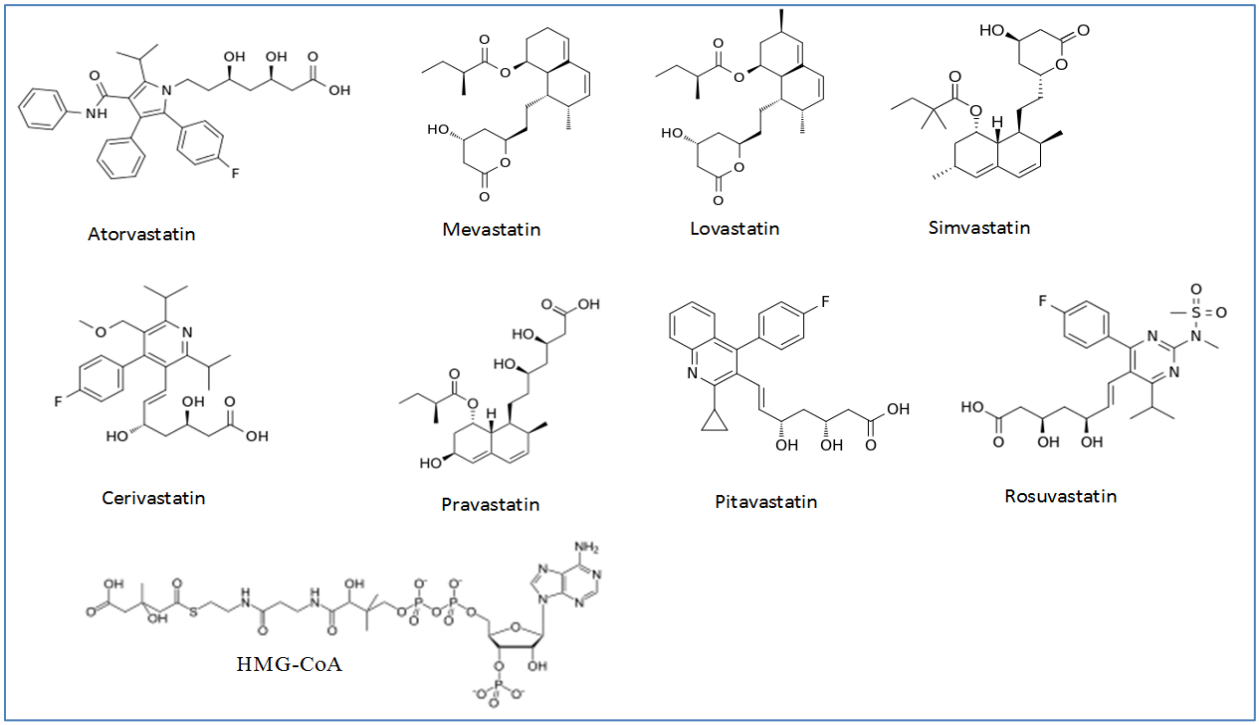

Figure 1: Cholesterol lowering statins and the structure of HMG-CoA (Chemical formula $C_{27}H_{44}N_7O_{20}P_3S$ and molar mass 911.661 g/mol). The boiling points of statins are 559.2°C-722.2°C

This review paper deals with atorvastatin [15,16] that is (βR, σR)-2-(4-fluorophenyl)-β,σ-dihydroxy-5-(1-methyl ethyl)-3-phenyl-4((phenylamino)carbonyl)-1H-pyrrole-1-hepatonoic acid. ATOR is a synthetic statin formulated in the pharmacological active open ring possessing a heptanoic acid side chain and two hydroxyl groups at the β and δ positions. The hydroxyl groups are recognized by HMG-CoA reductase (Figure 1) [17-21]. When statins decrease the LDL level, i.e. LDL-C, they inhibit the rate-limiting step in hepatic cholesterol synthesis and upregulate the LDL receptors (LDLR). However, statins also upregulate PCSK9 (proprotein convertase subtilisin kexin-like 9) expression [22]. PCSK9 is a protease, which binds to hepatic LDLR. It directs LDLR to the lysosomes for degradation and prevents recycling of LDLR to the cell surface. Therefore, PCSK9 levels correlate LDL-C in plasma [23-25]. Loss-of-function mutations in PCSK9 have shown to be in correlation with low LDLR degradation and low LDL-C level [24,26], whereas gain-of-function mutations (i.e. mutations that result in a new functional ability for a protein and are detectable at the phenotypic level) cause elevated LDL-C concentration with a higher cardiovascular risk [25].

LDL, IDL (Intermediate Density Lipoproteins), and VLDL (Very Low-Density Lipoproteins) are the major carriers of triglycerides and cholesterol in human [26]. They contain cholesteryl esters, triglycerides, fatty acids, and fat-soluble vitamins. The hydrophilic surface is made of apolipoproteins, phospholipids, and un-esterified cholesterol [27]. ApoB-100 is the major apolipoprotein in LDL, IDL and VLDL, and in Lp(a). ApoB-100 controls the structural integrity of the lipoproteins, as well as their receptor-mediated removal from the circulation and other cellular interactions [27]. Due to their complex nature, lipoproteins have different sizes, masses, and compositions [26-28]. Statins are deactivating apolipoproteins.

Natural and synthetic statins

Statins are divided into two groups based on the generation: natural and synthetic products. They are also categorized as natural (lovastatin), semi-synthetic (simvastatin and pravastatin), and synthetic compounds (fluvastatin, atorvastatin, cerivastatin, rosuvastatin, and pintavstatin) [29,30]. In nature, statins are fungal metabolites. They are produced by fermentation of the fungus to compounds with methyl groups at different positions in ring structure of the molecule and compounds with chemical differences in their side chains [31-35].

The mechanism of biosynthesis of statins was discovered in 1973, when mevastatin (MEV) was isolated from *Penicillium citrinum*. It was shown to exhibit cholesterol-lowering effects [36,37]. Soon after that, MEV molecule was noticed to be similar as 3-hydroxy-3-methylglutaryl coenzyme A (Figure 1), which was earlier invented and characterized to be a good target for cholesterol. The research showed that MEV decreased efficiently cholesterol. However, then it was not released to the markets [16].

Lovastatin (LOV) has observed to origin from Aspergillus terreus mold [16,34-41] and *Pleurotus ostreatus* [42]. After nearly one-decade lasting animal tests and clinical trials LOV became the first commercial statin in 1987 [8]. Within a few years, semi-synthetically processed PRA and SIM were commercialized. The first totally synthetically produced statin FLU was accepted to the USA markets in 1993, after which the statin drugs are merely synthetically produced [16,43]. One of them is cerivastatin, but it was withdrawn from the markets in 2001 because of the risk of *rhabdomyolysis*. Shortly after MEV, LOV was also identified from nature, because it was produced by *Aspergillus terreus* mould fermentation [16,44,45].

MEV and LOV are formed in specific biofermentation processes. They are the secondary metabolites of fungus, which are formed via polyketide biosynthesis (PKBS). The PKBS is original to some bacteria, fungi, and plants. It exists because of fatty acid synthesis, which produces a group of compounds in a catalytic reaction by polyketidase enzyme. The PKBS demands genes that are clusters. According to investigations on natural statins, 18 genes affect the biosynthesis of LOV in cultivation solutions that are modified by minerals and pH [31-33]. LOV, PRA, and SIM are commercially available, but they have also been isolated from micromycetes (*Aspergillus*, *Penicillium*, and *Monascus*) [46,47]. Commercially available ATOR is produced by chemical synthesis. Usually, then multi-step chemical processes in its production (Figure 2) are preferred for allowing environmental friendly and energy efficient preparation. Because of the new requirements in pharmaceutical industry on its stereo chemical purity, new analytical methods are needed for measuring the product´s purity and content. In statin production, the use of biocatalysts has grown, because many of the fermenters enable to engineer statins by simple synthesis to directly stereo structures [49-51].

Especially, the synthesis to substitute β-hydroxyl acid of HMG-CoA reductase is done with biocatalysts [44,49]. The key statins, ATOR, SIM, and ROS can also be produced by biocatalytic methods [45]. ATOR (Figure 1) has a (3R,5S)-dihydroxyhexanoate side chain containing two chiral centres in its structure. It can be synthesized from chiral precursors by using metal catalysts. In addition, protein enzymes that result in either asymmetric molecules or a racemic mixture can process the structure. As an example, biocatalytic synthesis methods of production of chiral

ATOR intermediates have been made in presence of alcohol dehydrogenase, 2-deoxy-d-ribose 5-phosphate aldolase, nitrilase, lipase, ketoreductase and halohydrin dehalogenase [50,51].

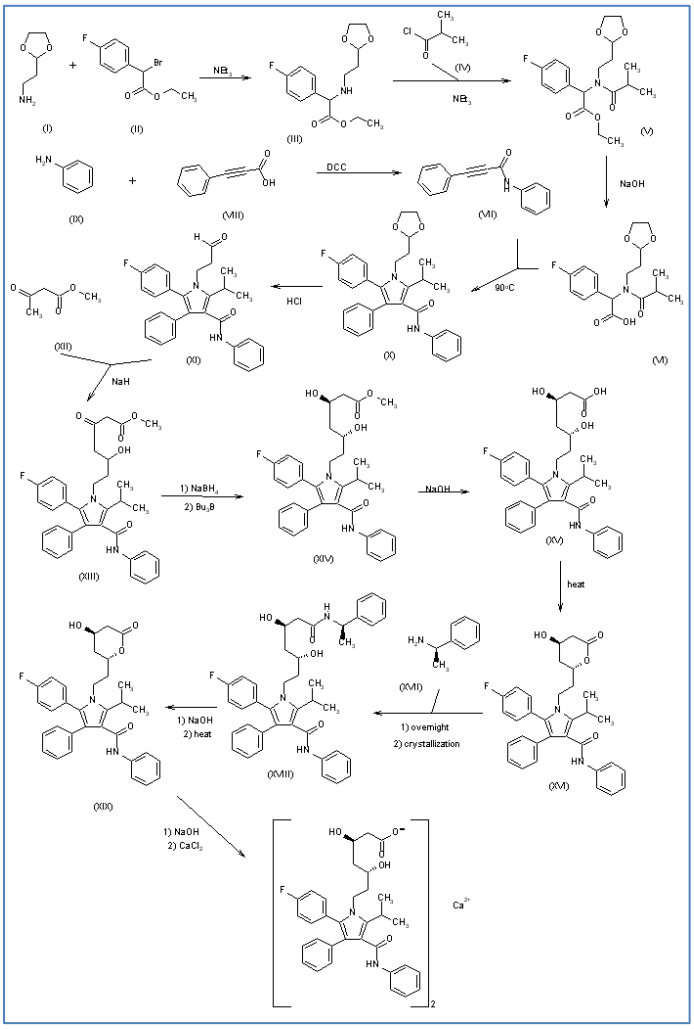

Figure 2: Synthesis of trans-6- [2-(3- or 4-carboxamido-substd. pyrrol-1-yl) alkyl]-4-hydroxypyran-2-one inhibitors of cholesterol [48]

Pharmacology of statins

When statin inhibits the activity of HMG-CoA reductase, formation of mevalonate (mevalonic acid) decreases, which causes decrease in cholesterol production [28]. Since statins effect in the beginning of cholesterol synthesis, they stop the cholesterol synthesis before the second step (Figure 3) to processed sterol compounds. Then the unused HMG-CoA is decomposed to smaller compounds in body [34,52,53].

The fermented statins (MEV, LOV, SIM) have a common hydroxyl substituted naphthalene structure with various kinds of independent substituents, whereas synthetic statins have no structural similarities. However, the mutual factor of the statins is the β-hydroxylactone side chain, which is similar as the HMG-CoA structure (Figure 1) causing HMG-CoA reductase inhibition [31,34] and decreasing cholesterol synthesis. When the side chain is lactone, it can be further formulated to nonpolar cyclic ester. On the contrary, when the statin is an acid, the structure is open and has polar carboxylic acid and hydroxyl groups. Usually, the pharmacologically active LOV and SIM are used as their lactone forms, which can be opened to active hydroxyl acid forms [54-56].

Statins combine with HMG-CoA reductase more intensively than with their natural substrates. When the binding coefficient K_m of HMG-CoA, i.e. Michaelis Menten coefficient [57], is 4 μM, inhibition coefficients K_{in} for statins are between 0.1-2.3 nM. The β-hydroxyl acid in the side chain of the statin is activated to interactions with HMG-CoA. However, that polar function has only a partial role in the interactions, since the strong binding properties are mainly due to the hydrophobic functionality of the whole statin structure [1].

Effect of statins

The mode of the statins behaviour on microbes is observed to vary [58]. Although SIM has noticed to decrease remarkably the growth of MSSA (*Methicillin-Sensitive Staphylococcus aureus*) and in some extent *in vitro* the growth of MRSA (*Methicillin-Resistant S. aureus*), the effect of FLU is known to be small [9]. On the contrary, FLU decreases the concentration of HCV RNA (*Hepatitis C. virus*, Ribonucleic acid) in chronic hepatitis C inflammation [43], but LOV decreases *in vitro* the growth of *Coxiella burneti* bacterium. PRA has not the similar effect [59]. Statins have shown effectiveness toward human immunodeficiency virus (HIV) in patients, but their way of acting and targeting molecules are not well known. However, the literature studies show that ATOR has a specific impact in phospholipids of the HI infected patients [60]. The unique study showed that e.g. ATOR has higher declines in total cholesterol, LDL-C, and non-HDL-C with similarly lower toxicity as PRA. Calza et al. showed that statins decreased total cholesterol level by 11-25% and triglyceride levels even up to 40% in HIV patients [61]. For the medication, the commercial calcium salt of ATOR (ATR-Ca) containing calcium at the ratio of 2:1 (mol/mol) is used [62]. The calcium salt is dosed at 10 mg - 80 mg per day [62,63].

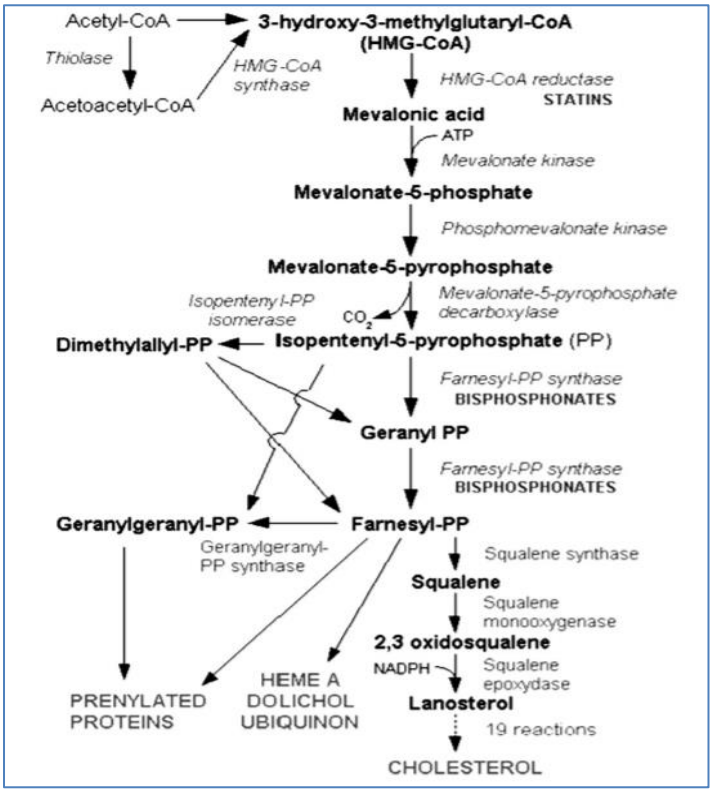

Figure 3: Cholesterol synthesis [64]

Atorvastatin (molecular weight 558.2530 g/mol, pK_a 4.5; log K_{OW} 6.36 [65] exists also as the ring-structural lactone and the open-structured hydroxyl acid (Figure 4). It has shown to degrade in both low acidic and high alkaline conditions. The first order kinetic degradation occurs in acidic conditions and the zero-order kinetic degradation in

alkaline medium. Under the latter conditions, ATOR tends to be less stable than in acidic solutions. The rate constants of the reaction (k) supports that since they were calculated to be 1.88×10^{-2} s^{-1} and 2.35×10^{-4} mol L^{-1} s^{-1} for degradation in low pH and high pH media, respectively [66].

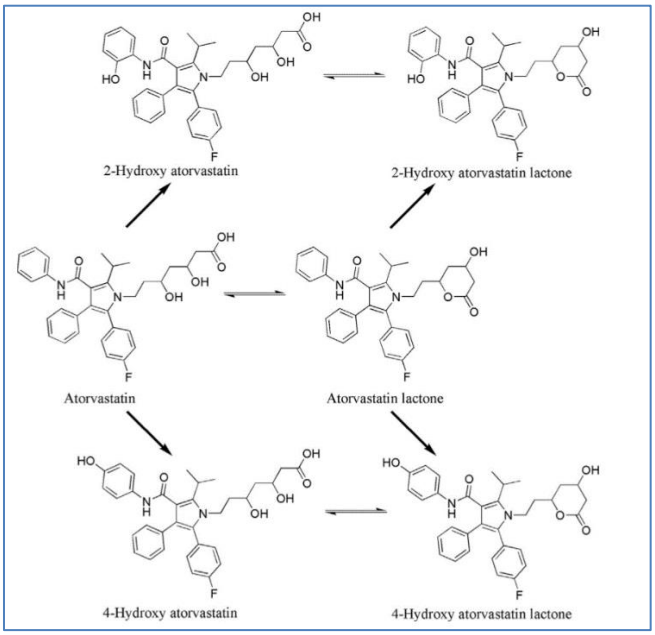

Figure 4: Chemical structures and metabolic pathways of ATOR and its metabolites [67]

Novelty Valued Progress in Atorvastatin Research with Analytical Methods

In statin research and in studies of its biological activity, analyses with biochemical methods are made to measure HMG-CoA reductase activity, cell growth, and antifungal activity. But, in the analytical point of view, that kind of research on statins does not give enough information about the drugs, metabolism, and their excretion from human body. Therefore, separation techniques are needed in drug research and purity analyses of the products. Thus, it is important to improve analytical separation methods to detect concentration variations, identification of the structure changes, and identification in complex formation, and targeting molecules [20]. There are not available any validated methodologies for patient samples assays done with all possible bioassays like protein electrophoresis, PCR, and Western blot analysis [35] comparable with analytical separation methods. The reason may be that chromatographic methods are used as direct methods targeting the behaviour of statin drug in body and biochemical methods are focused to monitor indirectly the effects of the abused statins via biochemical interactions.

Advantages in sample preparation and chromatography

Various analytical methods for the determination of ATOR have been dominated in the published papers [29,30,42,57,61-98]. During 1999-2017, research of ATOR has been described in 481 and in 15 papers made with liquid and gas chromatography, respectively. On the contrary, capillary electrophoresis analyses have been used only in 15 papers during the years 2006-2017 [57,61,62,90]. In HPLC research, mostly either UV detection (41%) or mass spectrometry (9%) have been used. This review compiles the recent studies focused to ATOR, its metabolites, and related compounds in pharmaceuticals, body fluids, and environmental water samples.

Commonly, ATOR and its hydroxyl metabolites (2-hydroxy, 4-hydroxy) have been analysed with HPLC and CE. However, GC have only used for analyses of the parent compounds. Because of the low concentrations of ATOR it has been detected with a triple-quadrupole electrospray MS detector. Then mostly, the selective mass fragment $[M+H]^-$ has been 559 Da and in MS/MS 466 Da and 440 Da were used [97].

In another study, either multiple reaction monitoring (MRM) or selective mass fragment-monitoring (SMFM) modes were used for its quantification. Studying ATOR metabolism in biological and clinical samples with LC the internal standard for qualifying accuracy has been another statin such as rovastatin (ROS), a deuterated (D5) analogue of ATOR, or another drug compound such as carbamazepine [98], β-naphthoflavone [99], or diltiazem [100]. Other internal standards are informed in the chapters describing the analytics with GC and CE.

Applications

Liquid chromatography of atorvastatin (HPLC, LC-MS): LC-MS has been preferred over traditional HPLC [76] due to the simplicity to handle samples for recognition of the compounds. There are several stability indicating methods for determination of atorvastatin using different techniques and detectors. A RP-HPTLC method using aluminium sheets precoated with silica gel 60 RP-18F (254) as the mobile phase consisted of methanol-water was used for determination of atorvastatin in bulk drug and pharmaceutical formulation [101]. Quantification was conducted densitometrically at 246 nm. Under acidic conditions drug underwent significant hydrolysis, while it was stable under alkali, oxidation, and dry heat and photodegradation conditions. HPLC method using fluorescence detector (282 nm excitation, 400 nm emission) was introduced for analysis of atorvastatin and its degradation products in bulk drug and tablet form [102]. HPLC method with UV detection at 247 nm was developed for determination of atorvastatin and its degradation products in bulk drug, marketed tablet and in-house prepared nanoemulsion formulation [103].

The LC-MS analysis needs to be fast and efficient for handling a large number of samples, such as plasma samples in pharmacokinetic studies at therapeutic dose levels of ATOR. Today, with tandem MS (MS^n) techniques seem to fulfil the demands (Figure 5, Table 1) [75,96]. Two LC-MS methods have also been published on determination of the ATOR structure and identification of its degradation products. The LC was on-line coupled with an MS with atmospheric pressure chemical ionization (APCI) source. It was applied in positive mode with TOF mass spectrometer for acquiring accurate masses. An ion trap analyzer for completing the fragmentation pattern was needed for accurate mass identification [96,104,105].

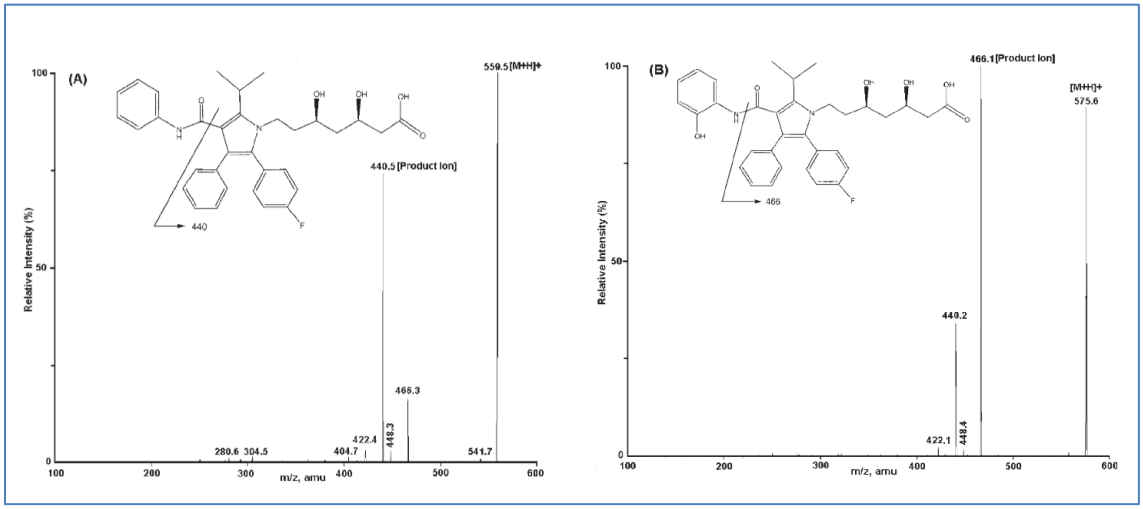

Figure 5: Mass spectra from LC-MS analyses. Full scan positive ion ionspray ionization. Product ion mass spectra and the proposed patterns of fragmentation of (A) atorvastatin and (B) ortho-hydroxy atorvastatin. The chromatographic separation on Waters symmetry® C18 column (5.0 µm, 100 × 4.6 mm i.d.) at 30°C. The isocratic mobile phase composition was a mixture of 0.03% formic acid-acetonitrile (30:70, v/v), which was pumped at a flow-rate of 1.0 mL/min with a split ratio of 10:90. In the mass spectrometry, an electrospray interface in positive ionization mode was used [96]

Atorvastatin in biological samples and drugs: Quite often sample preparation is made by liquid–liquid extraction (LLE) to isolate ATOR and its active ortho and para hydroxyl metabolites from plasma samples [96]. Actually, some studies show that ATOR can also be detected from blood serum, but its quantities in plasma and serum has not been compared. In plasma, the quantitative sensitivity (LOQ) of ATOR and its ortho- and para-hydroxyl metabolites has observed to be 100 pg/mL with LC-MS. Then, very good repeatability was achieved with overall CV less 8.0% [96].

Partani et al. have calculated [107] even lower LOQ values, which were 50 pg/mL for all the ATOR compounds. Negative ESI –MS and deuterated atorvastatin standards enhanced their selective detection (Figure 6). The initial sample volumes of plasma in refs. 96 and 107, were 1 mL and 200 µL, respectively. The low LOQ value was achieved by the systematically controlled sample preparation that prevented degradation. It has been examined that especially temperature and pH have effects on the degradation of ATOR [108]. In addition, oxidative and photolytic degradation can have an influence on their structures. Therefore, the whole sample preparation described in ref. 107 was carried out in ice-cold water bath (excluding the procedures of intensive mixing, SPE, and drying) and under low-lightness conditions to avoid the effect of temperature and UV-induced degradation. In addition, the pH of the plasma sample was adjusted to 4.1 for preventing degradation of ATOR in the initial sample but also that during pre-treatment.

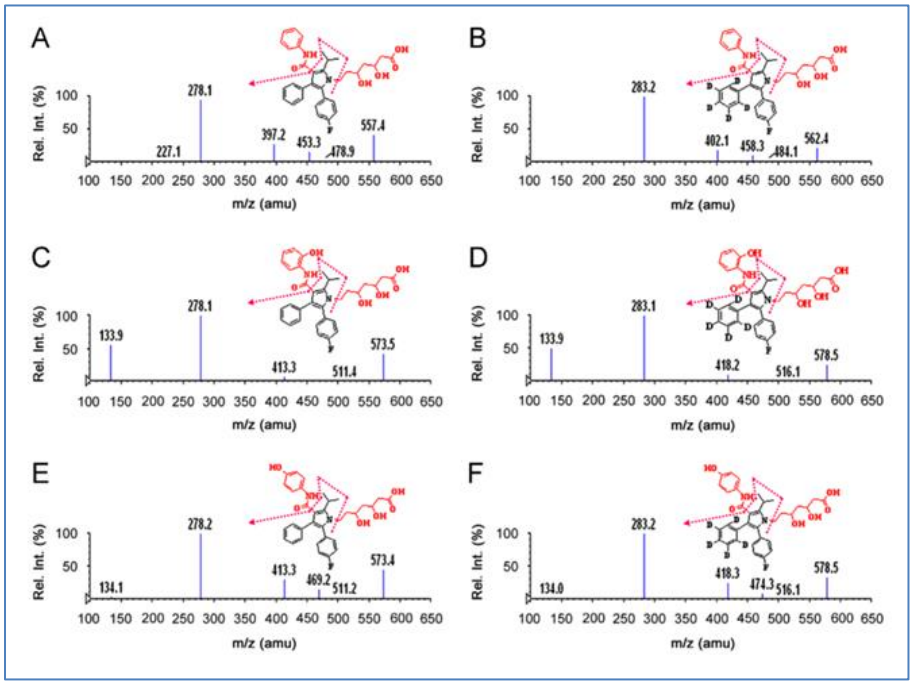

Figure 6: LC-ESI-MS spectra of ATOR compounds with negative electrospray ionization mode. The product ion spectra of (A) ATOR, (B) ATOR-D5, (C) orto-(OH-ATOR, (D) orto-OH-ATOR-D5, (E) para-OH-ATOR, and (F) para-OH-ATOR-D5 [107]

Positive electrospray ionization (ESI) mode was used to produce electrospray product mass spectra of the precursor ions of parent ATOR and its deuterated analogue. The mass fragment $[M+H]^+$ is 559.2 Da correlating the molar mass of ATOR, and 564.1 Da for the deuterated (D5) reference compound [109,110].

The other most significant fragment ions of the parent ATOR are 466.1 Da and 440.2 Da (Figure 7). The fragments m/z 466.2 Da and m/z 440.2 Da are origin from the loss of aniline ($C_6H_5NH_2$) and phenyl isocyanate (C_6H_5-N=C=O), respectively. In addition, the fragments, which may be detected are 448 Da (m/z 446 Da after

cleavage of H_2O), 422 Da (m/z 440 Da after cleavage of H_2O), and m/z 250 Da, 292 Da, and 380 Da fragments from m/z 440 Da [109]. The ESI-MS spectra of hydroxyl metabolites of ATOR are shown in Figure 8.

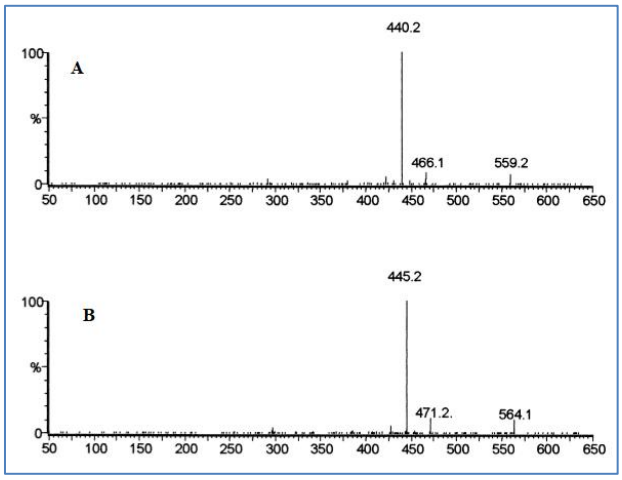

Figure 7: Mass fragmentation spectra of ATOR and its deuterated analogue. Positive electrospray ionization mode. Electrospray product ion mass spectra of the precursor ions of (A) ATOR ($[M+H]^+$ m/z of 559.2 Da and (B) of [D5]-ATOR ($[M+H]^+$ m/z of 564.1 Da [109]

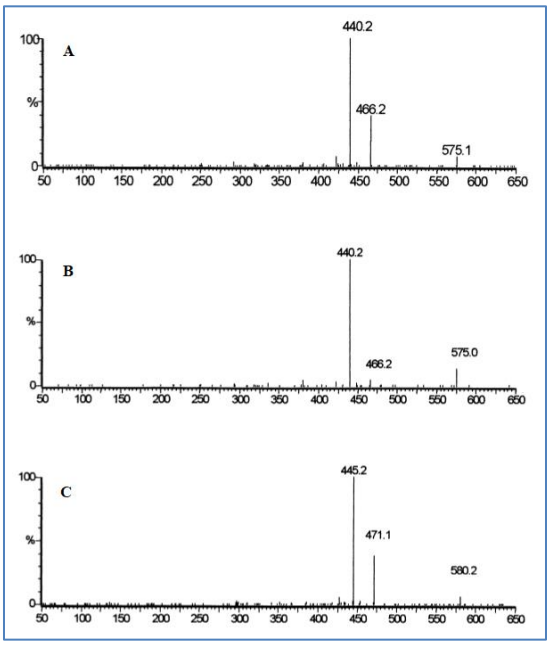

Figure 8: Mass fragmentation spectra of ATOR metabolites and the deuterated analogue of ortho-hydroxyl metabolite. Electrospray product ion mass spectra of ortho-hydroxyl ATOR ($[M+H]^+$ 575.3 Da) (A), and para-hydroxyl ATOR ($[M+H]^+$ 575.3 Da) (B), and [D5]-ortho-hydroxyl ATOR ($[M+H]^+$ 580.3 Da) (C) [109]

In LC-ESI-MS the deuterated ATOR drug (5 hydrogens replaced with deuterium, D5, Figures 7 and 8) has been used in the identification of ATOR and its human based ortho- and para metabolites in plasma samples. Deuterium isotopes are used in analyses to measure molecules in complicated matrices, when resolution in HPLC is not adequate for separation, or the metabolites cannot be identified due to their similar mass fragmentation [107]. In cases when the analyte concentration is very low and exact identification is needed, multiple reaction monitoring

(MRM) mode in MS is used to gain higher sensitivity and selectivity. In ref 92 (Figure SS) selectivity was enhanced with mass transitions of m/z 557.4→278.1 Da for ATOR, m/z 562.4→283.2 Da for ATOR-D5, m/z 573.5→278.1 Da for orto-OH-ATOR, m/z 578.5→283.1 Da for orto-ATOR-D5, m/z 573.4→278.2 Da for para-OH-ATOR, and m/z 578.5→283.2 Da for para-OH-ATOR-D5.

Recently, ATOR has been studied in human body samples [68,72,78,86,111]. Furthermore, the trend has been to validate methodologies for detecting only one statin at a time instead of simultaneous determination of statin metabolites and statins in mixtures, since only one statin is used at time for medication [112,113]. However, statins are at low concentration. Therefore, in many studies the samples were spiked with the studied analyte to increase its sensitivity in detection. Most often concentration enhancement was used, when the sample preparation was not efficient enough for improving the UV detection signal. This shows that the experimental conditions put restrictions for their determination of ATOR in real samples.

Lately, mass spectrometry detection has gained a growing interest as tandem MS method. Then, the detection limits and the justified concentrations could be decreased even to 0.05 pg/mL. In the case of laser induced fluorescence detection the reached level was ng/mL [114,115].

LC-MS/MS in the monitoring of ATOR and its metabolites in human plasma samples was published about studying the samples of 48 healthy volunteers [115]. It was noticed that plasma concentration of atorvastatin after oral administration were highly variable among individuals. The concentration range was 1.98-28.8 ng/mL (mean 8.50 ng/mL).

Ultra-performance liquid chromatography (UPLC) was used in a project on determination of 12 statins [ATOR, cerivastatin (CER), FLU, LOV, lovastatin acid (LOV-A), MEV, mevastatin acid (MEV-A), pitavastatin (PIT), PRA, rosuvastatin (ROS), SIM, and simvastatin acid (SIM-A)] in dietary supplements. They were isolated by ultrasonication into 50% (v/v) methanol. Then, the clean-up was performed using water-wettable polymer sorbents containing mixture of strong anion-exchange and reversed-phase materials. Elution was made with methanol and methanol containing phosphoric acid 0.2% (v/v). UPLC separation was performed in a reversed phase C18 column with phosphoric acid-acetonitrile gradient. The method was validated for dietary supplements spiked with the statins. The methodology was good achieving the recoveries between 89% and 101% [116].

Stability of statins /Sample pretreatment: Statin lactones become to hydroxyl acids in methanol - water solutions. Therefore, the conditions in sample preparation to isolate them separately from blood, water, or other matrices is important. The by-products of statins in synthesis and structural conversion are methyl esters of the hydroxyl acids. Especially, their production is high in presence of methanol. To prevent the unwanted products, usually the synthesis is done in alkaline acetonitrile [54]. To achieve repeatability in sample preparation and in analysis, changes in lactone ring can be prevented by stabilizing the pH of the sample [16,54,55] and by using adequate solvents. It has also been shown [54] that statins are stable in methanol, ethyl acetate, and acetonitrile-water (70:10, v/v) at ambient temperature. The above-mentioned solvents are also commonly preferred as eluents in SPE and in LC-MS. Based on the results, then the statin hydroxyl acids were also very stable. However, the lactones were more labile, since at acetonitrile – water solution (70:10, v/v) the ring structure could not be prevented for unfolding to hydroxyl configuration. However, the problem was solved by using 0.5% acetic acid.

Liquid-liquid extraction (LLE) or solid-phase extraction (SPE) on reversed phase sorbents C8 and C18 are used to handle ATOR samples for analyses. Usually, in LLE the sample solutions have been adjusted to acidic. The solvents in LLE extraction have been acetonitrile, dichloromethane, diethylene ether, and acetyl acetate [34,48,50,104,117]. Often, after LLE the extracts were evaporated, and the analytes concentrated to the final volume before LC-MS measurements. Polymer based nonpolar functionalities in SPE have enhanced the extraction recovery

of ATOR from plasma, when the pre-treatment has been made with acidified water [108]. SPE has also been used for purification of the native statins to organic extracts. Then, those as hydroxyl acids could be separated from lactones before the actual analysis [20,42,54,75,76,95,96,107,109].

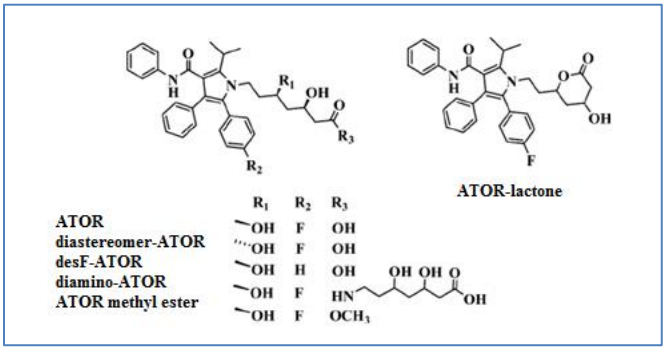

Figure 9: Chemical structures of ATOR, diastereomer-ATOR, desF-ATOR, diamino-ATOR, ATOR methyl ester, and ATOR lactone [118]

Atorvastatin and its hydroxyl metabolites have successively been extracted from human serum (107). LC-tandem-MS has been less used for identification of ATOR and various kinds of ATOR-substituents (Figure 9).

Much more information than in LC-ESI-MS (Figure 10) can be obtained with MS2 and MS3 ionization techniques, since 5, 10, or 9 m/z fragments based on the parent fragment (Figure SS) were observed, respectively. The results obtained using positive ion mode showed some diagnostic fragments that are useful for the identification of atorvastatin related impurities in real samples. Quantitative analysis of drug impurities was performed in the multiple reaction monitoring mode. Quantification limits for impurities were in the ranges 21.5–70.8 ng/mL [118].

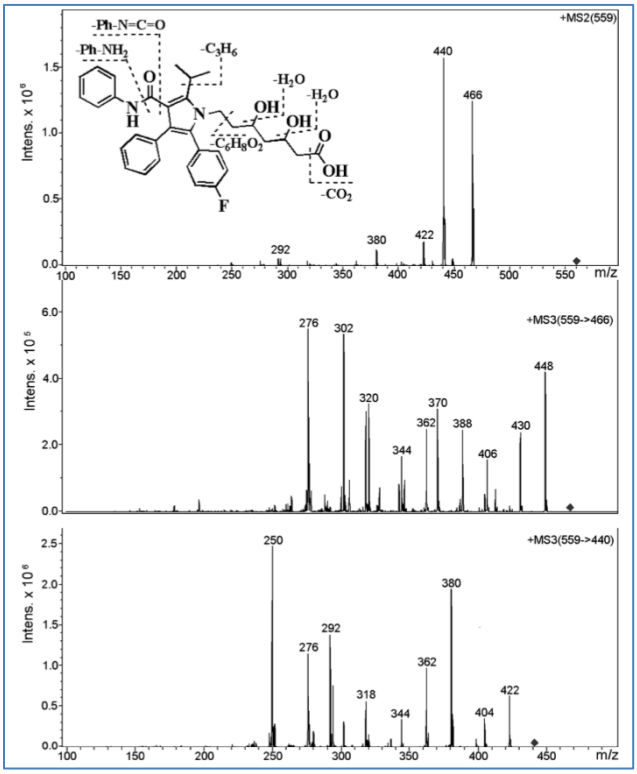

Figure 10: ESI-MS^2 spectra of ATOR ion at m/z 559 Da from MS, MS^3 of the ion at m/z 466 Da and 440 Da from MS^2 [118]

The analysis of the ESI-MS3 spectra of the fragment ions at m/z 466 Da and m/z 440 Da yielded good information on the origin of generated ions indicating the same fragmentation pattern as found for ATOR in the literature [105,119]. Reliability to identification of ATOR has shown to be achieved using both the positive and negative ionization modes, when deuterated compounds are not available (Figure 11).

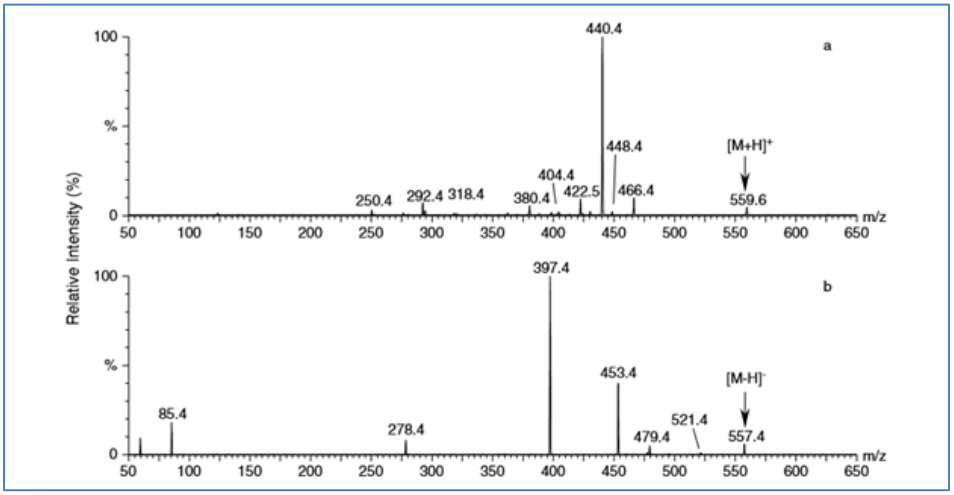

Figure 11: Mass spectra of atorvastatin. (a) $[M+H]^+$ and (b) $[M-H]^-$.The ion species subjected to CID is indicated with a vertical arrow. The applied laboratory collision energies were (a) 24 and (b) 26 eV [119]

Monitoring of atorvastatin in water samples: The research on pharmaceutically active compounds in the environment conditions has grown a lot during the last years. Especially, effluents of wastewater treatment plants have been studied a lot, since they are observed to contain many kinds of pharmaceuticals and drugs. Due to the high persistence and widespread occurrence of lipid-regulating agents in aquatic environments, their presence in drinking water has also been observed. Only a few papers have dealt with statin class (cholesterol-reducing agents) drugs in environmental waters [120-123]. Selected methods have been validated for the determination of statin drugs, especially ATOR, among other pharmaceuticals in environmental water systems [97,121-124].

ATOR has been found in wastewaters and in hospital effluents [121]. Its concentration in the samples has been notable or even high in nearly all paper, since it varied from ng/L to μg/L. Concentrations of 18.8 μg/L to 35.3 μg/L were measured in Brazil, South America [79]. As the other statins, ATOR has also been analysed from surface water. Then the analytes were concentrated with SPE treatment before LC-MS or LC-UV measurements [107,125]. In one recent paper, water samples were sampled from groundwater and surface waters of four rivers in Serbia. Then, ATOR was LLE treated (recovery ~30%) at pH 7.5 into methanol-dichloromethane mixture (1:1, v/v). The LOD and LOQ values of ATOR in groundwater and in wastewater were more than 1000 times lower than in the Ref. 79, namely 0.76 ng/L and 2.53 ng/L, respectively [97,117].

It has also been observed that the influent water of a wastewater pre-treatment plant contained ATOR at 36 ng/L, but the effluents were clean showing that the purification process could remove the drugs completely in the process. Unfortunately, the groundwater samples needed fortifying the samples by ATOR standard with 10 ng/L concentration. In that study the recovery of ATOR was 26%. Consistently, the recovery from wastewater was also low (45% with addition of 100 ng/L ATOR into the solution) [121,122].

Recently, a new multi-residue analytical method was introduced and applied to determine the concentration of ECs in crude wastewater and final effluent from a trickling filter (WwTWs) (population equivalent ~105000) in South-West England [124]. Several compounds which have not been previously studied in the UK were poorly removed. The extraction procedure developed is documented in Figure 12.

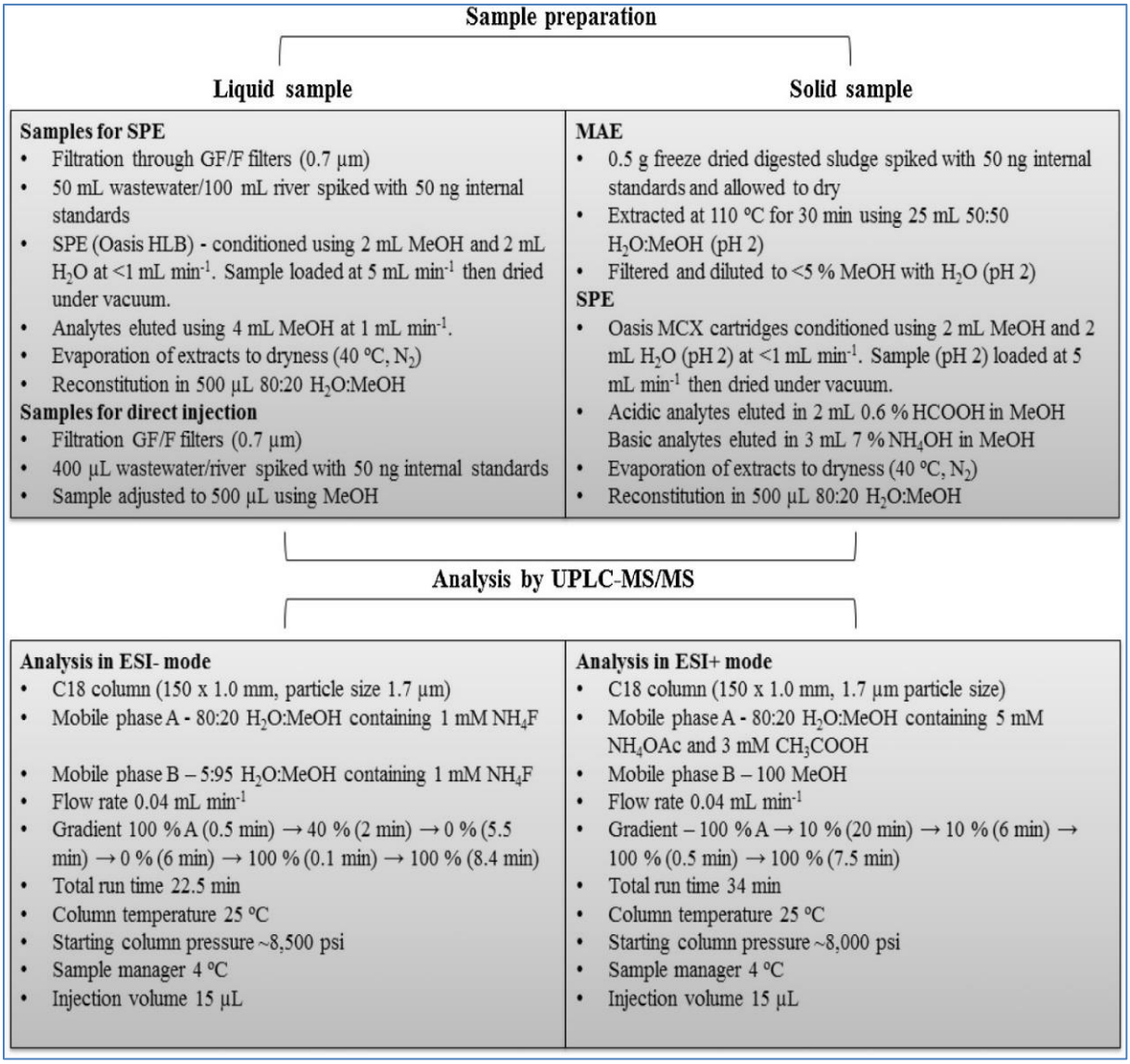

Figure 12: Overview of analytical protocol used to determine target ECs in crude wastewater, final effluent, river water and digested sludge [125]

Among the 90 contaminants ATOR was analysed with UPLC-ESI -MS/MS [125]. In validation, the method detection limit (MDL) and method quantification limit (MQL) values in crude wastewater, final effluent, river water, and digested sludge its concentration was 0.17, 0.85, 0.17, 0.84, 0.14, and 0.70 ng/L, respectively. ATOR in crude wastewater, final effluent, and river water was 188 ± 12.5 ng/L, 60.5 ± 3.5 g/L, and 7.0 ± 1.1 ng/L, respectively. In digested sludge it was not detected. The removal was 68 %.

Table 1: Selection of the latest papers and conditions in ATOR sample analyses with HPLC. More studies discussed in the text

Analyte	Sample	Experimental data	Analysis method	Reference
ATOR-Ca	pharmaceuticals	Kromasil 100 column C18, (250 mm x4.6 mm); Mobile phase:10 mM potassium dihydrogen phosphate – acetonitrile (41:59, v/v)	HPLC-UV	[80]
ATOR and other statins	waters from wastewater treatment systems, hospital effluent	Luna C18 column (150 x 4.6 mm, 5 mm particle size) with a C18 precolumn (4 x 3.0 mm); Mobile phase: 20 mM ammonium acetate (pH 3.5) - acetonitrile (25:75, v/v)	HPLC-DAD	[79]
ATOR and other statins	deionized water, wastewater, urine, plasma	The stationary-phase column was an octadecyl silane column (5-µm particle size, 250 mm × 4.6 mm inner diameter); Mobile phase: acetonitrile - 0.05M phosphate (pH 3) (59:41, v/v)	HPLC-UV	[78]
ATOR and nine cardiovascular drugs	dried blood spot (DBS) samples	Zorbax Eclipse Plus C18 rapid resolution HD column (100 mm × 2.1 mm i.d., 1.8 µm particle pore size); Mobile phase: 0.1% (v/v) formic acid (eluent A) and acetonitrile cont. 0.1% (v/v) formic acid (eluent B) TOF mass spectrometer: Mass range: 100–1000 m/z; recording rate: 1 Hz. HRMS reference masses: 121.0508 m/z and 922.00979 m/z.	HPLC-HRMS	[126]
ATOR and related compounds	degradation products from active ingredients	Column: XDB C18 150×4.6 mm, 5µm particle size column; Mobile phase: phosphate (pH3.3) - acetonitrile (30:70, v/v).	HPLC-UV	[19]
ATOR and metabolites	biological samples	HPLC fractions were directly electrosprayed (+ESI) to produce IR/mass spectra. Column: 150 × 3 mm Uptisphere Strategy C18-2 (Interchim, Montluçon, France) packed with 2.2 µm particles; Mobile phase:A (0.1% formic acid in water) - 5% solvent B (acetonitrile-methanol 80/20, v/v)	HPLC-FTMS-MS/MS	[76]
ATOR	tablets	Column: ZORBAX SB-C18 (150 × 4.6 mm, 3.5 µm); Mobile phase: acetonitrile-distilled water (85:15, v/v) at pH 4.5 (adjusted with phosphoric acid).	HPLC-UV	[127]

ATOR and metabolites	human plasma	Column: Acquity UPLC HSS T3 column (3.0 mm, 100 mm, 1.8 mm); Mobile phase: 0.05% (v/v) formic acid in water-acetonitrile (25 : 75, v/v) ESI: metabolites ortho-hydroxy atorvastatin, para-hydroxy atorvastatin, atorvastatin lactone, ortho-hydroxy atorvastatin lactone, and para-hydroxy atorvastatin lactone were optimized at the m/z 559.4 / 440.1, m/z 575.4 / 466.2, m/z 575.5 / 440.5, m/z 541.3 / 448.3, m/z 557.3 / 448.3, and m/z 557.3 / 448.3 transitions, respectively.	UPLC-MS/MS	[128]
ATOR and ortho-hydroxy-ATOR	human plasma	Lichro CRART 55-2, Purospher STAR RP-18e ACQUITY UPLC system Mobile phase: water-acetonitrile (45:55 v/v) both containing 5% methanol and 0.01% formic acid	HPLC-ESI-MS/MS	[75]
ATOR	human plasma	Agilent Eclipse-plus C18, 100 × 4.6 mm, 3.5 μm; Mobile phase: 0.2% formic acid in water–acetonitrile (30:70, v/v) MS: MRM transitions measured at positive mode at m/z 559.0 → 440.0 for ATOR	LC-MS/MS	[129]
ATOR, p-OH-ATOR, o-OH-ATOR		Column: Luna C18, (100 mm × 4.60 mm, 5 μm); Mobile phase: acetonitrile-0.20% formic acid (65:35% v/v)	HPLC-UV	[130]
ATOR	drug-free plasma, post-spiked drug-free plasma	Columns: (50 mm×4.6 mm, 5-μm and 50 mm×2.1 mm, 5-μm); Mobile phase: 0.1% (v/v) aqueous formic acid with 1.0 mM ammonium formate) - (0.1% (v/v) formic acid with 1.0 mM ammonium formate in acetonitrile MS: atorvastatin 559.6 → 440.3	LC-APCI-MS/MS	[131]
ATOR and ATOR metabolites	spiked samples	Column: C18 reverse-phase fused-core®; Mobile phase: 0.005% formic acid in water-acetonitrile-methanol (35:25:40, v/v/v)	LC-ESI (neg)-MS/MS	[107]
ATOR	human plasma	Column: Alltima HP C18; Mobile phase acetonitrile - 10 mM ammonium acetate (pH 3.0) (60:40, v/v)	LC-MS/MS	[132]

ATOR and glimepiride	human sample	Column: ACE5C18 (50 × 4.6 mm); Mobile phase: 0.1% formic acid: acetonitrile (30:70, v/v) MS: m/z 559.4 precursor ion to the m/z 440.1 product ion m/z 445.1 product ion for ATV-IS	LC-MS/MS	[133]
ATOR, related impurities	grapefruit juice, serum samples	Column: Symmetry C18 (150mm,4.6 mm, particle size 3.5 μm); Mobile phase: 10 mM ammonium formate (adjusted to pH 4 with formic acid) - acetonitrile (60:40, v/v) MS: range m/z 100–800 Da; collision energy at 27%	LC-tandem-MS MS^2; MS^3	[99]
		HPLC-MS/MS: Column: 4MC J-sphere H80 S-4 2.0 15 μm; Mobile phase: acetonitrile - 0.1% formic acid (70 : 30, v/v) m/z 559 Da (parent ion for atorvastatin), m/z 564 Da (parent ion for deuterated atorvastatin).		

Gas chromatography of atorvastatin (GC, GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is used widely in metabolomics research [134]. At the moment, ATOR has the highest boiling point of the statin drugs. Therefore, its pretretament has been tried to optimize in many ways, for example by handling standards and calibrators (the studied chemicals) with acid before the main pretreatment, by adding pyridine in derivatization, by freeze-drying the samples, and by increasing the reaction time. GC-MS is considered as one of the most effective tools for urine analysis, because most metabolites in urine have polar groups that facilitate trimethylsilylation and subsequent GC-MS analysis. Therefore, it is odd that only few GC-MS studies about statins have been reported. Most probably, the reason for that is the multi-step sample preparation and derivatization, which are needed to volatilize the high-boiling ATOR (512°C) [135].

Overall, in GC the sample preparation plays even a more important role in quantitative analyses than in LC when it is used. Figure 13 shows an example of a procedure when sample preparation was used to process statins in the presence of wood materials to extracts, to isolate statins with microbe fermentation, and to finish them for analysis with GC-MS. The requisite sensitivity and selectivity in determination of ATOR and its hydroxyl metabolites in plasma and serum samples has been achieved at therapeutic levels by using pentafluorobenzyl bromide (PFBBr) for derivatizing of polyfunctional thiols and preparation of pentafluorobenzyl esters from organic acids. In addition, as commonly known silylation with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide (Figure 14) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) [42,51,54] also fulfil the requirements. Inspite of that, sample purification is needed to minimize the matrix effects in electron impact (EI) and negative chemical ionization (NCI) methods. Internal standards, like other statins, isotopically labelled statins and different kind of pharmaceuticals and phenols have been used in the analyses [48,52,54].

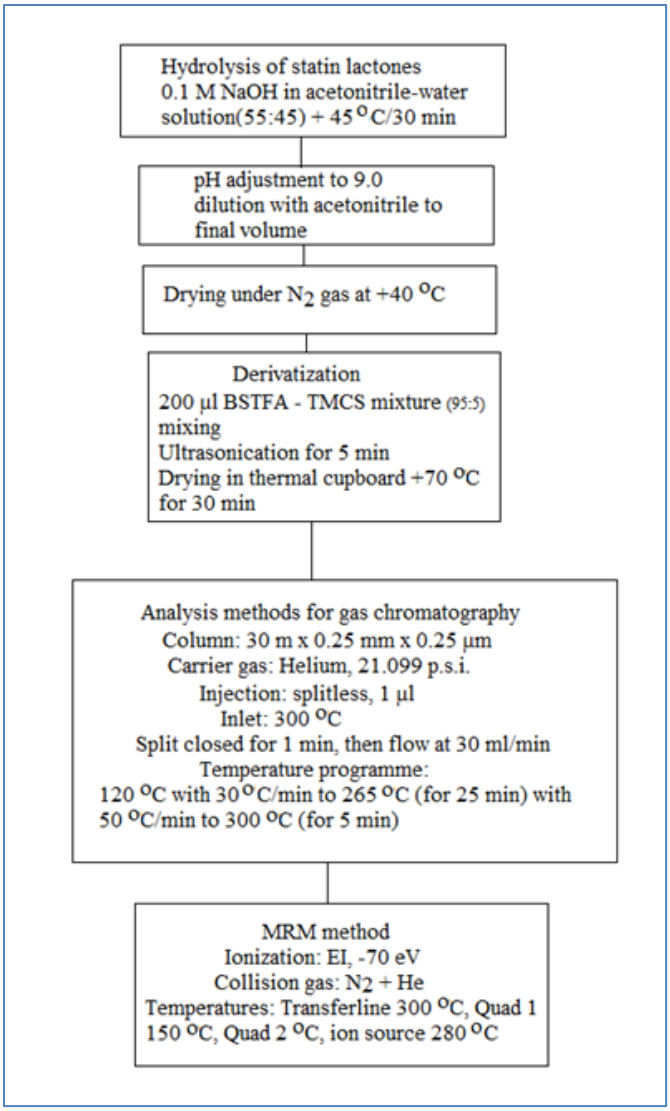

Figure 13: Scheme of the experimental procedures used to study statins from wood hydrolysis with fungi microbes. Separation (incl. ATOR) analysis made with GC-MRM-MS/MS after silylation of statins [42]

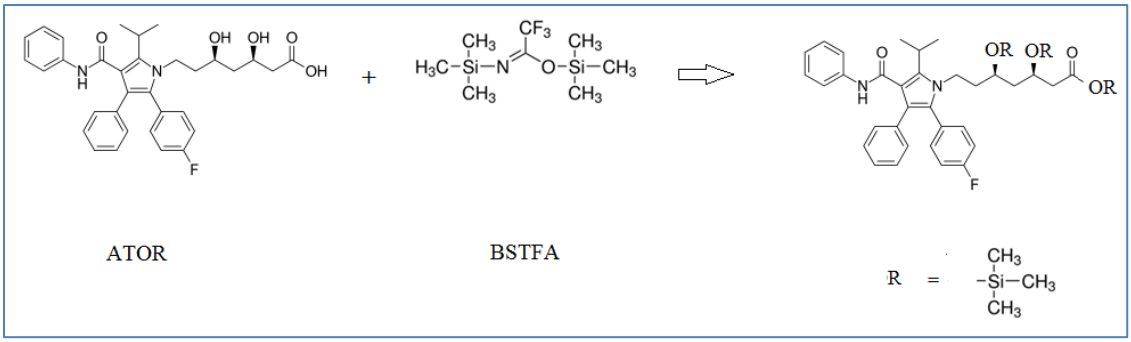

Figure 14: Scheme of silylation of atorvastatin with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) for GC analysis

Recently, only one GC-MS/MS study has been reported [42]. It described about statin analyses of pine wood samples and about quantification of statins in fluids processed with oyster mushrooms (*P. ostreatus*) in aqueous wood pulps during fermentation (Figure 13). The ATOR analysis was used for validation of the method, since only natural statins were studied. Then MRM detection of the silylated LOV, PRA, SIM, FLU, and ATOR was needed due to the low concentrations in the complicated matrices. The developed method was suggested to consider as a universal method, because it could be utilized for various statins from very diverse matrices like bark, phloem, and hardwood [42].

Aforementioned study was a special case, since nearly without exception, the statins have been isolated from water containing samples with either LLE or SPE into non-polar and medium polar organic solvents and from solid samples by combination of hydrolysis and solid-liquid extraction (SLE) treatments into water. In SPE the sorbents have been silica or polymer-based materials which have been deactivated with octyl (C8) or octadecyl (C18) functional groups [136]. Before extraction, the samples have been modified with mineral acids by total conversion of hydroxyl acids to lactone (Figure 1). In addition, multistep extraction processes have been used to enhance the purification as in the case to receive wood sample extracts [42]. The samples are usually dried and concentrated, or they are purified further with SPE that instead of LLE has advantages such as to separate statin hydroxyl acids from lactones [19,20,48]. Therefore, in many cases SPE is preferred to prevent the modification of lactone ring. Then, the pH stability has been cared, before the different structural forms could be isolated, as informed also in the HPLC section. In GC analyses the suitable pH to keep the stability is also pH 4-5, but pHs 7-9 correlating the physiological pH have accepted [42, 137,138].

Anyhow, in GC-MS the LOQ of ATOR has shown to be twice as high as the concentrations of the other statins [42]. Yang et al. (54) have noticed that the stability of ATOR in methanol, ethyl acetate and acetonitrile - water mixture (70%, v/v) opened partly the lactones to hydroxyl acid, exept in the presence of acetic acid stabilizer. In GC, the determination limits of derivatized statins in the methods have been noticed to be ng/mL [121]. Table 2 lists the selected publications on this field.

Table 2: Selection of the published data and experimental conditions and ATOR samples analysed with GC or with GC and HPLC. More studies discussed in text

Analyte	Sample	Experimental data	Analysis method	Reference
ATOR	medicinal product	GC: HP1 column: 30 m, 0.25 mm x 0.25 μm; detector temperatures: 320°C; injection chamber temperature: 300°C; column temperature: programme initial temp. 240°C for 1 min; increment of 10°C/min to the final temp. of 295°C staying for 2 min; gas flow: 3.9 mL/min; injection volume: 1.0 μL; split: 10 : 1. HPLC:Column: Symmetry C18 (250 x 4.6 mm, 5 μm);Mobile phase: acetonitrile-water (70 : 30, v/v), adjusted to pH 2.5 with 85% orthophosphoric acid	GC-FID HPLC-UV	[137]
ATOR and other statins	artificial samples and patient samples: urine, plasma	GC: Column HP-5 fused silica capillary column (30 m, 0.32 mm id, 0.25 μm) HPLC: Column Eclipse XDB-C18 (250mm, 4.6 mm, 5 μm); Mobile phase: 30% MeOH - 30 mM phosphate buffer (pH 6.5)	GC-FID HPLC-UV	[138]
ATOR and fatty acids	rat liver plasma	GC column: HP5-MS (30 m, 0.25 mm, 0.25 μm), MS: m/z 40 Da to 500 Da at a rate of 2 s in full scan mode	GC-MS	[139]

ATOR and other statins	standards, wood samples	Column: HP-5 capillary column (30 m × 0.250 mm × 0.25 µm) Derivatization was made with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) modified with trimethylchlorosilane (TMCS, 5%, v/v).	GC-MRM-MS/MS	[42]
ATOR, LOV	tablets capsules	Column: CP-SIL 5 CB (or HP-1) (length 25 m × 0.25 mm)	GC-FID	[140]
ATOR and diazepam (IS)	plasma	GC: Column DB5-MS column (length 10 m × 0.18 mm i.d., 0.18 µm bonding) Silylation with MSTFA MS: SCAN ***m/z*** 50–680 at a rate of 30 spectra/s. LC–ESI-MS/MS : SRM precursor ions to product ions: 559.2 → 440.4 (m/z) for atorvastatin and 285.2 → 154.2 (m/z) for the IS (diazepam)	GC-TOF-MS LC-MS/MS	[115]

There are not many fragments in EI ionization pattern of ATOR in MS (Figure 15). Anyhow, the sensitivity improvement with MRM allows identification of the analyte, although in MS mode it is not selective enough for quantification. The precursor ions chosen for ATOR in EI-MS mode have been m/z 398, 306, and 262 Da. The primary product ions have been 306.2 Da (qualifier ion), 264 Da (quantifier ion), and 232.8 Da (qualifier) [141].

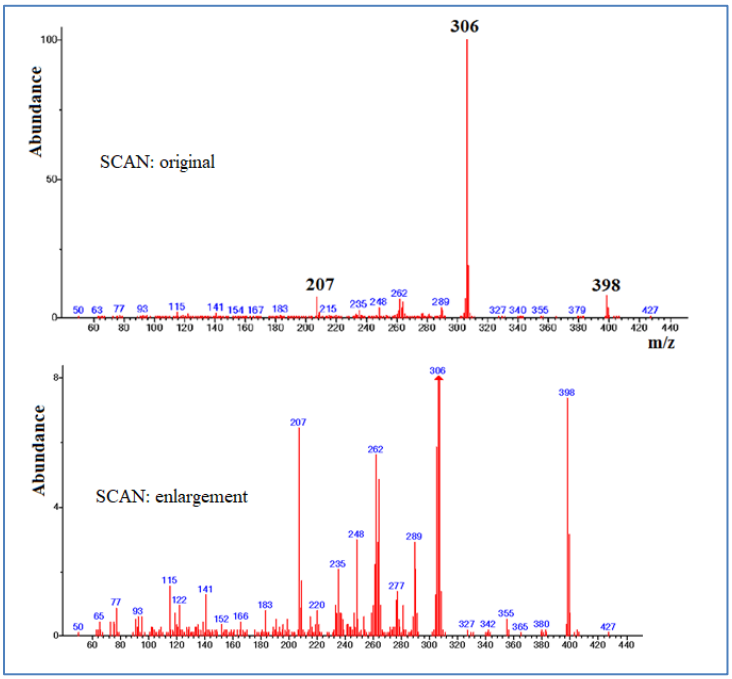

Figure 15: ATOR-TMS (EI Scan 28.298-28.278 min, 11 scans). Silylated ATOR-A has m/z 631.3 Da (exact mass 558.2530 Da, trimethyl silyl group 73 Da). The fragmentation could not be detected at higher abundance than 10%. Therefore, the m/z below 450 Da were used [141]

Capillary electrophoresis of atorvastatin (CE, CZE, MEKC)

Capillary electrophoresis (CE) is a useful separation technique in pharmaceutical analyses, since high resolution and selectivity can be obtained by paying attention to analyte´s stucture and ionization. It is designed to separate compounds, species, and ions based on their sizes, charges, or their ratios under an electric field modified

inside a capillary filled with conducting electrolyte solution. Driving forces in CE are electrophoresis, electrolysis and electro-osmosis. One of the CE methods is micellar electrokinetic capillary chromatography (MEKC) that has been applied to determination of ATOR in multicomponent samples containing also the other statins and some pharmaceuticals [20,95,142-144]. Another method used is capillary zone electrophoresis (CZE), which has been applied for example in quantitation of parent ATOR in pharmaceutical tablets. That method was also further optimized to the microchip platform for electrophoresis separation of multicomponent drug mixtures [145] and simultaneous determination of ATOR and amlodipine from dose formulations [146].

Sample preparation is needed before CE as in LC and GC for real samples to concentrate the analytes to the quantitative limits and ranges of the CE method. For impurity profiling, MEKC is the most appropriate electrophoretic technique, since neutral and charged compounds are allowed to differentiate with micelle interactions during the separation process [147]. Nigović et al. have made a fundamental work in validating one simple MEKC method for achieving simultaneous quantitation of ATOR, desF-ATOR, diastereomer-ATOR, atorvastatin methyl ester (ATOR methyl ester) and atorvastatin lactone (ATOR lactone) (Figure 16, Table 3) [95]. The MEKC analysis allowed simultaneous separation and quantification, but also purity measurement of ATOR and its related substances in 10 mM sodium tetraborate buffer (pH 9.5) containing 50 mM sodium dodecyl sulphate (SDS) and 20% (v/v) methanol. The method was successfully applied for purity evaluation of ATOR in native samples and in commercial tablet formulations. Very low concentrations of diastereomer-ATOR and desfluoro-ATOR at <1.0 % and 0.2 %, respectively, were found. The other ATOR compounds, methyl ester and lactone form could not be detected. In spite of all, the negative feature of the developed MEKC method was that the concentrations of ATOR needed to be 100 - 1000 times higher in order to determine the related substances [95]. When the total amount of statins was interesting, alkaline hydrolysis was used to open lactone ring in lactone drugs, in order to transform these compounds to the corresponding acids before analysis. In CE, this approach offers shorter analysis time due to a decrease of the migration times of anionic statins in comparison to neutral lactone forms. Linear calibration ranges were established over the concentration range 100–1200 μg/mL for AT and 1.0–12.5 μg/mL for related substances. As a comparison with HPLC-ESI-MS method the quantification limits for those impurities were in the range of 21.5–70.8 ng/mL [95].

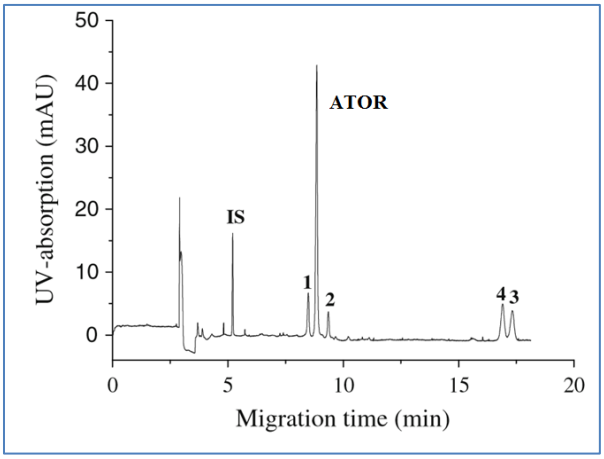

Figure 16: Optimized MEKC separation of atorvastatin and drug-related substances. Peaks: IS pravastatin; 1 desfluoro atorvastatin, ATOR = atorvastatin, 2 = diastereomer of atorvastatin, 3 = atorvastatin methyl ester and 4 = atorvastatin lactone. Separation conditions: 10 mM borate buffer, pH 9.5, 50 mM SDS, 20% (v/v) methanol, voltage 30 kV, 25°C, detection wavelength 214 nm, hydrodynamic injection 50 mbar, 4 seconds [95]

CZE was used for analysis of ATOR in authentic human plasma samples that were spiked with 5.0 mg/L of amiodipine besylate (AML) and with the studied compound ATOR. The electrolyte solution was phospate buffer at pH 6.5. The LOD was 0.05 μg/mL, while the LOQ was 0.10 μg/mL for both drugs [94]. In another CZE study, pravastatin (PRA) was used as the IS (Figure 16) in measuring ATOR in a lipitor tablet. Then the electrolyte was sodium acetate at pH 6. Even though the detection wavelength was 214 nm and extended optical path length was needed, the LOD was very high (1 μg/mL).

The LOD values of ATOR, desfluoro-ATOR, diastereomer-ATOR, ATOR-lactone, and ATOR methyl ester were 18.35, 0.48, 0.26, 0.32, and 0.59 μg/mL, respectively. ATOR could be calibrated till 1.2 mg/mL concentration, although the other ATOR compounds gave linearity only to 10 μg/mL.

The authors did not comment why ATOR concentration needed to be as high, although the Figure 17 informs that lower concentrations are possible.

When the sensitivities are not high enough for detection in CE there are on-line concentration methods available in electro aided techniques, like stacking and sweeping. Stacking is based on the analyte velocity in capillary, and it can be optimized by modifying chemically sample and electrolyte solutions. Simply, in stacking the analyte has higher velocity in the sample zone than in the BGE zone meaning that the analyte concentrates at the boundary of the electrolyte solutions before the analytes are moved into the electrolyte. In sweeping, the situation is contrary. The analyte has a slower velocity in the sample than in the electrolyte, which contains surfactant for micelle formation. Sweeping is the most effective technique for hydrophobic and cationic compounds [148]. Wei et al. used sample stacking by field-amplified sample injection and sweeping for simultaneous analysis of ATOR and other anionic and basic drugs in biological samples and tablets. [149].

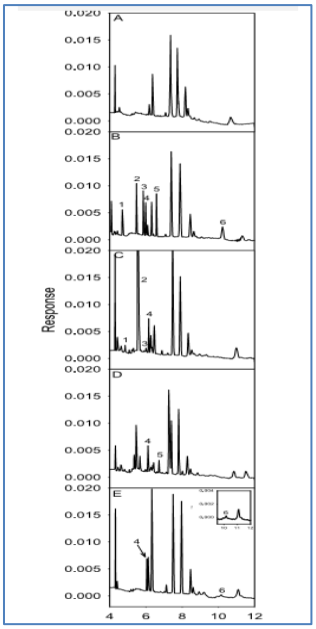

Figure 17: Electropherograms of the separation of drugs in biological samples. (A) plasma blank; (B) plasma spiked 100 ng/mL for AM and DO, 200 ng/mL for ATOR, IR, and RO with 200 ng/mL IS; (C) plasma sample from a patient receiving 5 mg of AM (Norvasc R) orally, 5 mg of DO (Aricept R), and 150 mg of IR (Aprovel R); (D) plasma from a patient receiving 40 mg of ATOR (Lipitor R) orally at steady state; (E) oral administration of 20 mg RO (Crestor R) with single dosing after 4 h in healthy volunteer plasma. Peaks: 1, amiloride; 2, irbesartan; 3, donepezil; 4, IS diclofenac; 5, atorvastatin; 6, rosuvastatin [149]

The method was suitable for the analysis of studied drugs in plasma that were collected during pharmacokinetic investigations from patients after oral administration of the commercial preparations. Example on results are shown in Figure 17, which shows that the spiked analytes separated from the plasma matrix compounds (A-E). ATOR from plasma of a patient, who received orally 40 mg of ATOR separated quantitatively from two compounds of the matrix. The quantitative results in plasma were 49.62 ng/mL and 12.01 ng/mL, when 40 mg and 10 mg tablets were used for medication. When compared the concentrations in normal and sample stacking by field-amplified injections, one thousandth lower values could be measured even though 50 mM Tris buffer with 41% MeOH and 0.1% PEO (pH 3) and micellar stacking solution made of 25 mM Tris buffer with 60 mM SDS (pH 3) were used.

Micelle to solvent stacking (MSS) is a new on-line sample concentration technique for charged analytes in capillary zone electrophoresis (CZE). A 10-fold peak height enhancement factor or a magnitude improvement in concentration detection sensitivity compared to typical injection was achieved with acceptable reproducibility and linearity (150).

Table 3: Selection of electrolyte compositions and ATOR samples analysed with CE (CZE, MEKC). More studies discussed in paper

Analyte	Sample	Experimental data	Analysis method	Reference
ATOR	pharmaceutical tablets	25 mM sodium tetraborate-25 mM SDS (pH 9.5) 25 mM tetraborate (pH 9.5)	MEKC: UV 240 nm CZE:UV 240 nm selective max ATOR 237 nm	[73]
ATOR-Ca	a commercial lipitor tablet	25 mM phosphate (pH 6.5)-methanol, (80:20, v/v)	CZE-UV	[151]
ATOR	tablets plasma	25 mM phosphate (pH 6.5)	CZE-UV	[94]
ATOR	tablets	HPLC: Mobile phase (isocratic): acetonitrile-methanol-phosphate buffer (pH 3.0) (45:30:25, v/v/v), pH was adjusted to 2.5 ± 0.1 with orthophosphoric acid CZE: phosphate and borate buffers (pH 8.0)	HPLC-UV, CZE-UV	[152]
ATOR and related substances	bulk drug and formulated products	10 mM sodium tetraborate buffer pH 9.5, 50 mM sodium dodecyl sulphate and 20% (***v/v***) methanol	MEKC-UV	[125]
	purity evaluation of bulk drug and formulated products			
ATOR-Ca	pharmaceutical tablets	6 mM potassium hydrogen phthalate - 1.2 mM CTAB (pH 6.5)	MEKC-UV	[153]
ATOR	pharmaceutical tablets, plasma	25 mM phosphate (pH 6.5)	CZE-UV	[94]
ATOR	standards	30 mM ammonium acetate (pH 6), 10 mM CTAB - 20 mM NH_4HCO_3 (pH 9.5), 50 mM NH_4HCO_3 - 50% MeOH (pH 9.7); 50 mM NH_4HCO_3 - 25% MeOH, (pH 9.5)	CZE	[154]
ATOR	standards	50 mM NH_4HCO_3 and 50% methanol	CZE	[155]
ATOR	drug formulations	25 mM phosphate (pH 6.5)-methanol, (80:20, v/v)		[151]

ATOR, impurities (diastereomer-ATOR and desfluoro-ATOR)	lipitor tablets	25 mM sodium acetate (pH 6)	CZE	[71]
ATOR		2.5 mM phosphate buffer (pH 6.7)-methanol (70:30, v/v)	CZE	[156]
ATOR in mixtures	commercial preparations, plasma samples	rinsing electrolyte differs from the separation electrolyte: 50mMTris buffer with 41% MeOH and 0.1% PEO (pH 3); electrolyte in separation: 25 mM Tris buffer with 60 mM SDS (pH 3)	MEKC	[149]

Conclusions

The hyphenated technique that incorporates the efficient separation using liquid chromatography and sensitive detection by mass spectrometry has become an indispensable tool for quantification of statins in biological fluids and pharmacokinetic studies. Development of the analytical methods for identification, purity evaluation and quantification of statin drugs has received a great deal of attention in the field of pharmaceutical analysis in recent years. Methods describing simultaneous analysis of different statins as well as drugs in combined pharmaceutical products and other co-administered drugs in therapy of cardiovascular disease are also described. The application of capillary electrophoresis as alternative separation technique for statins is considered. Even CZE with UV detection has shown to be able to determination of ATOR and simultaneously separate the parent compound from degradation products and related organic impurities present in the formulations.

The future trend is to overcome the sensitivity problem of atorvastatin. It has been met in all chromatographic techniques and especially with capillary electrophoresis. In most cases concentration is the solution, but in capillary electrophoresis various kind of on-line methods and focusing processes, like the FASI sweeping MEKC method and trancient isotachopheresis with acetate as leading and hydroxide as the terminating ions, have improved the sensitivity of statins.

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