A Review of Current Trends and Advances in Analytical Methods for Determination of Statins: Chromatography and Capillary Electrophoresis

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1. Introduction

Statins are now among the most frequently prescribed agents for reducing morbidity and mortality related to cardiovascular diseases (Figure 1) and analysis of these drugs is a current problem. The major therapeutic action of statin drugs is reduction of circulating atherogenic lipoproteins as a result of inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [1]. The key enzyme catalyzes the conversion of HMG-CoA to mevalonate, a critical intermediary in the cholesterol biosynthesis. This mechanism was discovered in 1976, when Endo and co-workers isolated a compound mevastatin from *Penicillium citrinum* that exhibited cholesterol-lowering effects [2]. Clinical studies have shown that statins significantly reduce the risk of heart attack and death in patients with proven coronary artery disease, and can also reduce cardiac events in patients with high cholesterol levels [3]. Beside lipid-lowering activity, statins improve endothelial function, maintain plaque stability and prevent thrombus formation. There is also an increased interest in statins non-lipid activities such as an anti-inflammatory action [4].

Ischemic heart disease is the leading cause of death in middle- and high-income countries, killing over 7 million people each year. Cardiovascular disease has no geographic, gender or socio-economic boundaries, and will remain the leading cause of death globally in the future. Therefore, the development of new analytical methods for statin drugs is of great importance. Analytical methods are employed through entire life cycle of a drug, from design and manufacture, elucidating the mechanism of biotransformation, clinical trials, dosage scheme adjustment, its introduction into the marketplace, quality control and pharmacovigilance to drug recycling and disposal with emphasis on environmental protection.

Statins can be grouped into fermentation-derived and chemically synthesized. Lovastatin, also called mevinolin, was isolated as secondary metabolite of fermentation process of various fungi such as *Aspergillus terreus*, *Monascus ruber* and *Penicillium* species [5, 6]. Lovastatin was the first commercially available compound for treatment of hypercholesterolemia, approved for use in 1987. It is produced biosynthetically from the fungus *Aspergillus terreus*. Whereas lovastatin is a natural product, simvastatin and pravastatin are semi-synthetic. Simvastatin is obtained by synthesis from lovastatin by replacement of 2-methylbutyryl side chain with 2,2-dimethylbutyryl group, while pravastatin is produced by microbial hydroxylation of mevastatin by *Strepromyces carbophilus*. Fluvastatin, atorvastatin, pitavastatin and rosuvastatin are completely synthetic compounds. Although all statins share a common mechanism of action and structural component that is very similar to the HMG portion of HMG-CoA reductase, they differ in terms of their chemical structures (Figure 1). The statins differ from each other in the rigid, hydrophobic structures covalently linked to the HMG-like moiety. The naturally derived statins contain a substituted decalin ring structure. Only pravastatin has a hydroxyl substituent on the hexahydronaphthalene nucleus which causes higher hydrophilicity. Fully synthetic statins have fluorophenyl groups linked to the HMG-like moiety. Depending upon chemical structure, statins have different affinities for HMG-CoA reductase and different pharmacokinetic properties [7]. Clinical trials have demonstrated rosuvastatin to be the most effective in reducing LDL cholesterol. In addition to the standard statin pharmacophore, rosuvastatin molecule contains a polar methyl sulfonamide group that forms a unique interaction with the catalytic site of HMG-CoA reductase. Cerivastatin was a synthetic statin drug, approved in 1997. Unfortunately, due to its fatal rhabdomyolysis, as a sever side effect, it was voluntarily withdrawn from the market in 2001.

Statins exist in two forms, lactone and open-ring hydroxy acid forms. Lovastatin and simvastatin are administered as lactone prodrugs and subsequently transformed to active metabolites in contrast to other statins, which are formulated in the pharmacologically active β-hydroxy acid form. *In vivo*, lactone prodrugs are enzymatically hydrolyzed to their hydroxy acid pharmacophores in the liver to achieve pharmacological activity [8]. The lactone forms can be converted in aqueous solutions to their corresponding hydroxy acid equilibrium products. Such interconversion may occur even in the biological matrix before collecting aliquots of the sample, during sample preparation and analysis of the drug. Therefore, it is crucial to optimize the multiple steps of the analytical method in order to minimize the interconversion during the analysis. On the other hand, statins in β-hydroxy acid form possess two hydroxyl groups in an alkyl chain at the β and δ positions with respect to the carboxylic acid group. The carboxylic acid group and the hydroxyl group at the δ position are prone to lactonize. Therefore, all statins may exist in solutions in the free acid form or the lactone form or as an equilibrium mixture of both forms in a pH-dependent manner [9]. For samples of hydroxy acid and lactone forms, maintaining the pH of solution around 4-5 minimizes interconversion. Increasing the pH above 6 facilitates the conversion of lactone to acid, whereas lowering pH enables the conversion from acid to lactone or lactone to acid in the non-ionized form. Consequently, great care must be exercised when handling these compounds in order to isolate them in high yields and the analytical methods should be designed for the simultaneous quantification of two analytes that can potentially undergo interconversion during analysis.

Statins are considered for long-term therapy and thus the purity assessment of these drugs is of great significance. Development of selective methods for monitoring their potential impurities and degradation products is highly required. Identification and determination of drug-related substances is an important aspect because impurities and degradation products of drugs are often responsible for some side-effects. The estimation of the impurity profiles of bulk drugs or dosage formulations requires methods involving high sensitivity and resolution as well as acceptable analysis time. The hyphenated technique that incorporates the efficient separation using liquid chromatography and specific and sensitive detection by mass spectrometry has become indispensable tool for identification and structure elucidation of unknown impurities in statin drugs as well as quantification of trace impurity levels.

Various chromatographic methods for determination of statins and their related impurities in the bulk drug forms and pharmaceutical formulations were developed. Almost all methods used for the separation of statins are based on high-performance liquid chromatography. In pharmaceutical applications UV detection was most commonly used. Analytical methods for determination of statins were developed individually as expected from their different structural and chemical properties. This approach to the analysis was chosen most probably because statins are not used in combination with other statin molecules during therapy. However, the development of a rapid analytical procedure that is not limited to the analysis of only one statin can be considered as a very useful assessment in quality control. Numerous chromatographic methods for quantification of statins in different biological fluids were developed. The levels of statins in biological fluids are very low because only about 5% of dosed statin reaches the systematic circulation. The liquid chromatography coupled to tandem mass spectrometry has become the method of choice for therapeutic plasma level monitoring of statins and their metabolites in pharmacokinetic investigations [10]. Generally, hyperlipidemic patients are treated with multiple-drug regime which commonly leads to drug interaction. The simultaneous determination of statins and drugs usually combined in cardiovascular therapy in human plasma is important to get more insight in their possible interactions with a consequent increased risk to toxic effects. Due to different physical and chemical properties of co-administrated drugs development of methods for their simultaneous analysis is an over going challenge.

This chapter will present recent advances in chromatographic and capillary electrophoretic methods for the determination of statin drugs in various fields of application. Current trends in developing new methods for analysis of the most frequently used drugs will be discussed.

2. Pharmaceutical application

Pharmaceutical analysis provides information on the identity, purity, content and stability of starting material, excipients and active pharmaceutical ingredients (APIs). A distinction is made between analysis of the pure active ingredients and pharmaceutical formulations. Specification and test methods for the commonly used API and excipients are described in detail in pharmacopoeias.

Impurity profiling is of great importance in new drug substance and new drug product because of their potential unwanted pharmacological effects, possible toxicity, side effects, and their eventual impact on the activity, efficacy and the stability of the drug, its bioavailability and the results of the drug analysis. International Conference on Harmonization (ICH) gives strict regulatory guidelines for identification and quantification of trace impurities in drugs. Any compound that does not have the same chemical entity as the active substance, present at levels higher than 0.1% or 0.05% (depending on the daily dose), needs to be identified. Therefore there is a permanent need for developing new accurate, selective, and sensitive methods for the determination of drug impurities. Impurities can come from starting materials, they can be intermediars and by-products from the synthesis of the API (process related impurities), degradation products formed during manufacturing process and long-term storage, interaction products between API and other active ingredients and excipients or primary container.

Stability indicating methods are quantitative test methods that can detect changes of API and drug products during time and under certain conditions. Information on type and amount of degradation products over time is important for quality, safety and efficacy of the drug. Therefore, Food and Drug Administration (FDA), European Medicines Agency and other regulatory agencies, along ICH and good manufacturing practise require development and validation of stability indicating methods. General purpose of stability testing is to provide evidence on how the quality of an API or a finished pharmaceutical product changes during time under the influence of different environmental factors such as temperature, humidity and light. After these tests have been performed, recommendation on storage conditions and shelf life of the product can be given. ICH guidelines give detailed description of forced decomposition studies (stress testing). Stress testing of the API can help identify possible degradation products. It should include the effect of temperature (in 10 °C increments), humidity (≥ 75% relative humidity), oxidation, photolysis and hydrolysis of the API at a wide range of pH (acidic, neutral and alkali conditions).

In this section a review of chromatographic methods applied for identification and quantification of statins in bulk drug and pharmaceutical dosage forms will be given (Table 1). Each statin commercially available on the market will be covered in this review. Special emphasis will be given to stability indicating methods and papers describing impurity profiling.

Statins are often manufactured in combined pharmaceutical formulations together with ramipril, acetylsalicylic acid, amlodipine etc., and especially ezetimibe, a novel lipid-lowering agent that inhibits the absorption of cholesterol in the intestine by blocking Niemann-Pick C1-like protein cholesterol transporter. A synergic effect in reducing plasma concentrations of LDL cholesterol is achieved, mainly by the combination of statin and ezetimibe. Since statins are often co-administered with other drugs in therapy of cardiovascular disease, i.e. acetylsalicylic acid, antihypertensive medicines (ACE inhibitors, calcium channel blockers), but also in combined therapy of multiple disorders, e.g. antidiabetics, diuretics, nonsteroidal anti-inflammatory drugs and other analgetics, antibiotics etc. In order to avoid problems with patient compliance when a combination of acetylsalicylic acid, antihypertensives, lipid-lowering drugs and etc. is required, a polypill, a fixed-dose combination containing three or more drugs in a single pill, would be the solution. Methods describing simultaneous analysis of these combined pharmaceutical products will also be mentioned.

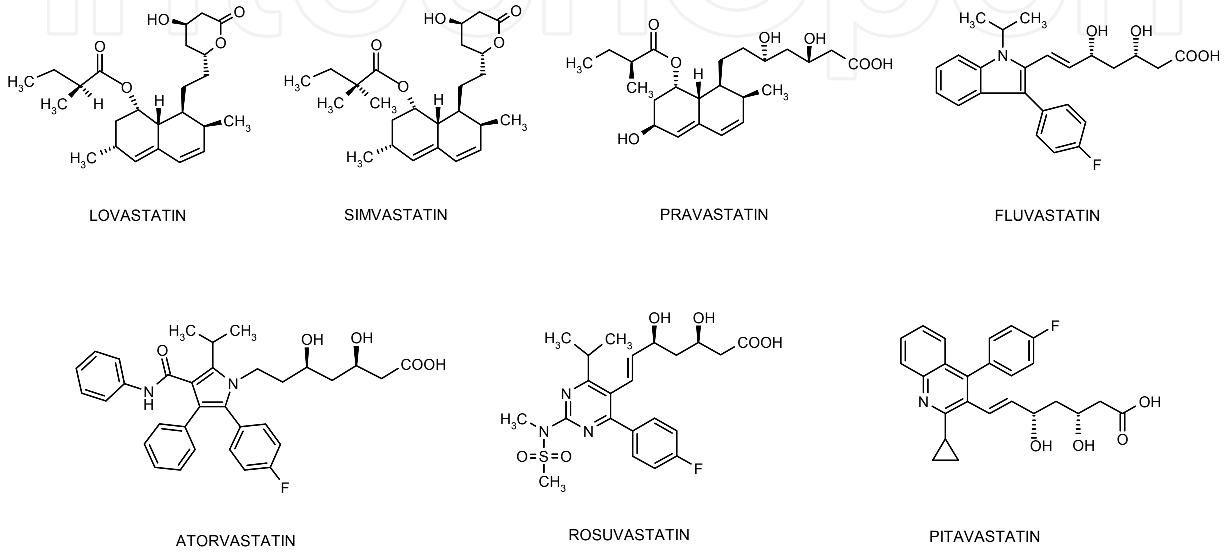

Figure 1. Chemical structures of statins

2.1. Lovastatin

The first statin registered as a drug was lovastatin. Nowadays, in therapy it is greatly replaced by new synthetic products, mainly atorvastatin and simvastatin. Therefore there are not many new methods for determination and quantification of lovastatin in bulk drug and pharmaceutical formulations.

There are scarce reports investigating the conversion of statins from lactone to their corresponding hydroxy acid forms. Yang and Hwang studied the conversion of lovastatin and simvastatin from lactone to corresponding hydroxy acid forms [11]. They concluded that the conversion of lactone forms to corresponding hydroxy acid forms would occur in water or 70% acetonitrile. However, this conversion could be retarded by addition of acetic acid to the solution. Hence a mobile phase with acetic acid added to the composition is recommended for HPLC analysis. Furthermore, lactone forms could only be transformed to their corresponding hydroxy acid forms in 0.1 M NaOH or 0.05 M KOH prepared in 25% or 50% acetonitrile in water. When alkaline methanolic solutions were used further transformation to methyl ester of hydroxy acid form would take place. Recently another paper was published investigating conversion of lovastatin [12]. The identity of all three forms, lovastatin, lovastatin hydroxy acid and its methyl ester was confirmed by electrospray ionization (ESI) mass spectrometry (MS). Their results imply that also under acidic conditions, with increase of storage time, lactone is converted to hydroxy acid form and further transformed to methyl ester form.

Bearing in mind the interconversion problem, special attention should be given to the choice of a mobile phase for HPLC analysis, the extraction procedure and sample storage time. Methanol in acidic conditions should be avoided because it induces the conversion and transformation of lovastatin forms. Hence, most recently developed LC methods utilize pH around 4.5.

Lovastatin is an active pharmaceutical ingredient in red yeast rice products, used as a dietary supplement. In such products lovastatin is mostly refered to as monakolin K, and is accompanied by 13 more monacolins naturally occurring in red yeast rice. These products are frequently used by millions of people as a complementary and alternative therapy for lowering total lipid and LDL cholesterol levels. Unfortunately dietary supplements do not follow strict quality control as medicines do, active ingredients are not standardized and published on labels, and considerable variations can be found among different manufactures even between lots of the same manufacture. Therefore there is a growing need for specific and precise methods for determination of lovastatin in red yeast rice dietary supplements in order to ensure standardization, efficacy and safety of these products.

Identification and chemical profiling of all 14 monacolins in red yeast rice and its formulated products was conducted using HPLC with photodiode array detecore (PDA) and MS [13]. Because red yeast rice has a complex matrix, sample extraction procedure was carried out with 75% ethanol. Chemical profiling was performed using electrospray ionization and ion trap mass analyzer. Since lovastatin content depends on the fermentation process of the rice by *Monascus purpureus*, an LC-PDA-ESI-ion trap method was published investigating differences in raw material powder and finished products [14].

A stability-indicating method for the stress test of red yeast rice was also performed [15]. An assay of seven main monacolins, monacolin K (lovastatin), monacolin J, monacolin L and their corresponding hydroxy acid forms and dehydromonacolin K, representing 97% of total monacolins, was determined. In order to shorten the analysis time Song et al. proposed a fast screening method of lovastatin in red yeast rice products by flow injection tandem mass spectrometry without LC separation [16].

2.2. Simvastatin

Simvastatin is along atorvastatin the most often used statin drug and there is a great number of analytical methods developed. Novakova et al. published a review paper on HPLC methods for the determination of simvastatin and atorvastatin [17]. An oversight on different areas of application, pharmaceutical formulations, clinical medicine (human plasma) and environmental (aqueous samples) was given. A more detailed overview will be given on papers not covered by this review.

A simple HPLC-UV method was optimized according to the USP chromatographic method for simvastatin [18]. By changing the column length from 30 cm to a Chromolith RP18 monolithic column, 10 cm in length and reducing the pH to 3.0, a reduction in elution time was about 60%, resulting in analysis time less than 4 min. Method was applied to determine the quality of 60 compounding simvastatin 40 mg capsules. The mean content and weight variation evaluation, content uniformity, determination of simvastatin concentration, determination of lovastatin as an impurity and the dissolution test were performed. Results were devastating. The mean content of the capsules varied from 70 mg to 316 mg. In ten Brazilian pharmacies more than one tested capsule was outside the range from 85-115%. Only three pharmacies presented content uniformity with values complying to reference ones. Capsules from all the pharmacies resulted in simvastatin content less than 100% of the declared value. In 6 of them the content ranged from 4-87% of the declared amount. These results do not meet the requirements for simvastatin contents, resulting in underdosing. These appalling results emphasize the need for the control of raw material, compounding process and finished products quality, efficacy and safety.

Tablet splitting is a somewhat controversial topic among pharmacy practitioners, patients, managed care organizations and many other associations involved in health care. However it has become increasingly common, especially within geriatric and psychiatry communities. There are many concerns surrounding tablet splitting program, mainly if there will be considerable weight fluctuations, will the daily dose be the same in two half's, and will tablet splitting deliver same clinical outcomes at a lower cost. Hill et al. presented an HPLC-UV method, taken from the USP monograph and adapted to half-tablets, for drug content and weight uniformity for half-tablets of six commonly split medications, including simvastatin [19]. There analysis found 38.80 mg as target drug content, while the measured drug content mean was 40.06 mg, with a RSD 4.29%. Target drug content ranges from 95.21% to 111.35%. These small changes in daily dose should have no significant impact on long-term clinical end points.

RP-HPLC method was developed and validated for simultaneous analysis of simvastatin and tocotrienol and tocopherols isoforms in simvastatin-tocotrienol nanoparticles manufactured as potential targeted therapy of breast cancer [20]. In order to obtain good resolution in short analysis time the separation was carried out on a Phenomenex Onyx C18 monolithic column (100 mm x 4.6 mm) with a gradient elution.

Preparation and evaluation of a high-dose nicotinic acid loaded sustained-release pellets coated with double polymer and immediate release simvastatin was introduced by Zhao and co-workers [21]. After the preparation of drug-loaded pellets, drug content analysis was performed by HPLC for both nicotinic acid and simvastatin. However, unnecessary, different methods, using similar columns and mobile phases, were employed.

There are a number of methods describing simultaneous determination of simvastatin and ezetimibe from their combination drug products [22-25]. Stability indicating studies on combined pharmaceutical products of simvastatin and ezetimibe have also been published [24, 25]. Different approaches to forced degradation study, chromatographic conditions and determination of degradation products were performed. Hefnawy and co-workers proposed a very fast and sensitive stability indicating method for simultaneous determination of ezetimibe and simvastatin in tablet dosage form [25]. Instead of traditional chromatographic columns packed with porous particles, they used a monolithic stationary phases, i.e. RP Merck Chromolith Performance column (RP-18e, 100 mm x 4.6 mm). Due to monolithic stationary phase, an elevated flow rate is possible, resulting in a run-time five-fold reduced (analysis time under 2 min), consumption of mobile phase about two-fold decreased, while the resolution between peaks remained unaffected.

Several methods have been developed for identification and quantification of known impurities, but many also studied fragmentation and structural determination of unknown simvastatin impurities [26-29]. Structural characterization and identification of a new compound, an unknown simvastatin by-product generated during the industrial synthesis starting from lovastatin was published [26]. After HPLC-diode array detector (DAD) analysis, ESI-ion trap mass analyzer was employed to obtain MS/MS spectra, followed by Fourier transform-infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) analysis.

Plumb et al. [27] proposed a method using high resolution sub 2 µm particle LC column together with hybrid quadrupole orthogonal time-of-flight (TOF) mass spectrometer used to profile and identify simvastatin impurities. All common impurites were identified in a single 10 min run. A new impurity of simvastatin was detected and identified as the saturated ring form of simvastatin. The same group published a paper on screening pharmaceutical products by ultra performance liquid chromatography (UPLC) coupled to TOF-MS [28]. Principal components statistical analysis was used for rapid classification of batches of simvastatin tablets according to their impurity profile.

Reddy et al. [29] performed HPLC separation of simvastatin and its two main impurities, anhydro-simvastatin and simvastatin dimmer. An unknown impurity was detected. MS/MS spectrum was obtained by ESI^+ and ion-trap mass analyzer and the structure of the so far unknown simvastatin impurity was proposed. Recently, a paper on synthesis, characterization and quantification of simvastatin's metabolites and impurities was published [30]. This method emphasizes use of non-compendial reference standards for quantification, with shorter analysis time and improved sensitivity. β-hydroxy acid and methyl ester of simvastatin were synthesized as non-compendial reference standards. After complete and detailed characterization by MS, FT-IR and NMR, they were used as reference standards in quantification of simvastatin impurities.

2.3. Pravastatin

An HPLC method for quantification of pravastatin in tablets was published [31]. However, an unnecessary complicated extraction procedure and linearity investigation was performed. Identification of an impurity in pravastatin was performed by application of collision-activated decomposition mass spectra both in positive and negative ionization mode [32]. The impurity is an analogue of pravastatin with an additional methyl group on ester side chain.

Two stability indicating studies of pravastatin under different forced degradation conditions were conducted [33, 34]. Forced degradation study was performed for neutral, acid and basic hydrolysis, chemical oxidation, photochemical degradation and thermal stress using HPLC-UV [33]. Under neutral hydrolysis a decrease in the peak area of pravastatin was observed accompanied by two additional peaks. In basic hydrolysis a 90% decrease of pravastatin peak was noted and an additional peak is obtained, while in acidic conditions pravastatin peak completely disappeared and two new signals appeared. Degradation of pravastatin was also observed under oxidative conditions, while under thermal stress no change was percived.

Results obtained by Brain-Isasi et al. [34] are somewhat different then those previously published [33]. They argue that the previously described method was to short (7 min) to observe all degradation peaks obtained by acid hydrolysis while all of them are eluting after pravastatin. This indicates they are more liphophyllic than the parent drug, probably formed after esterification and lactonization of pravastatin. By the use of MS/MS spectra obtained in the positive mode, one of the peaks was identified as pravastatin lactone form. In alkaline medium only one product was observed and after preparative TLC it was isolated and identified by ^{1}H-NMR and ^{13}C-NMR as the 8-hydroxy derivate of pravastatin.

2.4. Fluvastatin

Photodegradation study of fluvastatin has been studied and examined by high-performance-thin-layer chromatography (HPTLC) and spectrophotometry [35]. Photoproducts were separated by HPTLC on a nonpolar C18 stationary phase with a mixture of phosphate buffer and methanol as a mobile phase. Both in water and methanol solutions, photochemical decomposition led to the formation of three major products.

2.5. Atorvastatin

Of all seven statins, atorvastatin is the most often administered statin drug. In fact, it is one of the most often prescribed prescription drugs overall. Therefore many methods are developed for determination and quantification of atorvastatin in bulk drug and pharmaceuticals. Since Novakova et al. in 2008 [17] gave a review of HPLC methods for the determination of atorvastatin in pharmaceutical assays, only papers published afterwards will be presented.

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 RP18F(254) as the mobile phase consisted of methanol-water was used for determination of atorvastatin in bulk drug and pharmaceutical formulation [36]. Quantification was conducted densitometrically at 246 nm. Under acidic conditions drug underwent significant hydrolysis, while it was stable under alkali, oxidation, 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 [37]. 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 [38].

Another stability indicating method was proposed for simultaneous determination of atorvastatin and amlodipine alongside with their degradation products in commercial combined tablets [39]. An UPLC method using ethylene bridged hybrid C18 column (50 mm x 2.1 mm, 1.7 μm) was used for simultaneous determination and quantitation of atorvastatin, acetylsalicylic acid and their four known and six unknown degradation products in combined dosage forms [40].

Two LC-MS method were reported for structure determination and identification of atorvastatin degradation products. An LC method employing an atmospheric pressure chemical ionization (APCI) source in positive mode with TOF mass spectrometer for acquiring accurate mass and an ion trap analyzer for complete fragmentation pattern was introduced [41]. Six unknown atorvastatin degradation products formed under stress conditions of hydrolysis, oxidation and photolysis were identified. Preparative HPLC method with Luna prep C18(2) column (200 mm x 50 mm, 10 μm) was used for isolation of four oxidative degradation products [42]. HPLC coupled to MS, high resolution MS and NMR spectroscopy were applied for the structure elucidation. Quantitative NMR spectroscopy was used for assay determination of isolated oxidative atorvastatin degradation products. A fast UPLC method with analysis time of 3 min was employed for determination of atorvastatin, fenofibrate and their degradation products in combined dosage forms [43].

We have developed HPLC/DAD/ESI/MS^n method for separation and identification of atorvastatin and its four related impurities [44]. To avoid hydrolysis of the atorvastatin lactone and the lactonization of acid form, ammonium buffer pH 4.0 was used. In order to achieve separation between atorvastatin and its diastereomer, several mobile phases were examined. Finally, a gradient elution mode was chosen to achieve good separation between peaks adjacent to the drug components, as well as to keep short analysis time of lipophilic impurities (Fig. 2.). Mass spectra were obtained by ESI source in the positive ion mode and ion trap analyzer. For quantitative analysis of atorvastatin and its four known impurities multiple reaction monitoring (MRM) mode was employed. Several unknown impurities were identified through MS/MS fragmentation analysis, i.e. diamino-atorvastatin, photolytic oxo-product, photolytic degradation product and diastereomer of atorvastatin lactone. Method was successfully applied to bulk drug and pharmaceutical dosage forms provided by different manufactures (Figure 2).

HPLC-UV method was developed for simultaneous determination of atorvastatin and seven related compounds specified as process-related impurities and possible degradation impurities. Experimental design was used during method optimization and robustness testing [45]. Artificial Neural Networks were used for the modelling and prediction of chromatographic retention of atorvastatin and its impurities in micellar liquid chromatography [46].

Atorvastatin in combined dosage forms, e.g. with ezetimibe, fenofibrate, ramiprile was determined by HPTLC methods [47, 48]. HPLC methods were published for simultaneous determination of atorvastatin in combination with amlodipin [49], fenofibrate [50], ezetimibe [51] and ramiprile [47, 52]. An improved HPLC method, with higher sensitivity and shorter analysis time using a chemometric protocol (statistical experimental design and Derringer's desirability function) was developed for simultaneous analysis of amlodipine and atorvastatin in pharmaceutical formulations [53]. Three HPLC methods have been published for analysis of atorvastatin and acetylsalicylic acid in combination with clopidogrel [54] and ramipril [55].

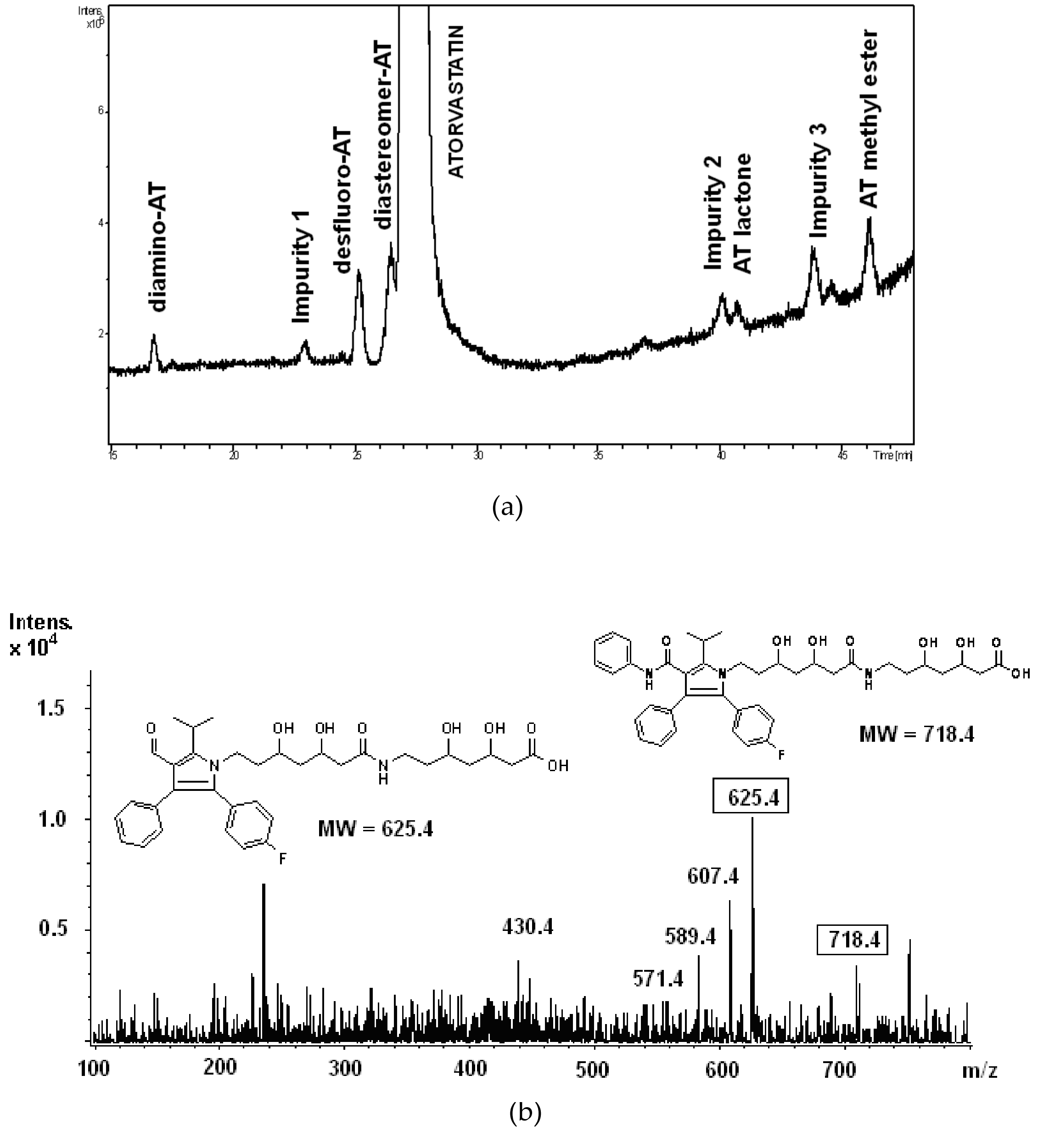

Figure 2. Total ion current chromatogram of atorvastatin pharmaceutical dosage form (A) and MS spectra of its process related impurity diamino-atorvastatin (B)

HPLC method was used for investigation of polypills for the treatment of cardiovascular diseases [56]. Seven drugs, i.e. lisinopril, aspirin, atenolol, hydrochlorothiazide and simvastatin/pravastatin/atorvastatin in the presence of their major interaction and degradation products were separated on a C8 column. In order to obtain mass spectra of the interaction and degradation products, ESI-MicroTOFQ mass spectrometer was employed. Atenolol, lisinopril, simvastatin and atorvastatin mass spectra were acquired in positive ESI mode, while hydrochlorotiazide and aspirin were ionized better in negative mode. Pravastatin gave good molecular ions in both modes. All the interaction and degradation products gave satisfactory mass spectra in positive ESI modes, except for two pravastatin related products which showed better molecular ions in negative mode. Results suggested that use of pravastatin in relate to other statins resulted in more interaction and degradation products, as well did the combination with atenolol by comparison with hydrochlorotiazide. This is a very nice approach that can be utilized for drug-drug interactions and stability studies of the polypill. Drawbacks of the proposed method are long analysis time of 90 min, replacement of the phosphate buffer with water for MS analysis and three different gradient methods for each of the statins.

2.6. Rosuvastatin

Far to our knowledge first HPLC method for the determination of rosuvastatin in bulk drug and in its dosage form was published by Mehta et al [57]. A forced degradation study was done at various pH values, under hydrolytic, oxidative, photolytic and thermal stress conditions. Developed method was able to resolve the degradation products formed during the stress study.

Not so commonly used in quality control analysis of pharmaceuticals, HPTLC method was proposed [58] for determination of rosuvastatin in its bulk drug and pharmaceutical preparations. Analysis was performed in a Camag twin-trough chamber on silica gel 60F(254) HPTLC plates. Aceclofenac was used as internal standard. Optimized mobile phase consisted of toluene-methanol-ethyl acetate-formic acid. Quantitation was performed densitometrically at 265 nm.

A paper employing both HPTLC and HPLC for determination of rosuvastatin and ezetimibe in combined tablet dosage forms was published [59]. HPLC analysis was performed on a Chromolith C18 column (100 mm x 4.6 mm) with PDA detector set at 245 nm. HPTLC separation was carried out on an aluminum-backed sheet of silica gel 60F (254) layers using n-butyl acetate-chloroform-glacial acetic acid as the mobile phase. Quantification of analites was performed with UV densitometry at 245 nm. A stability indicating method for simultaneous estimation of rosuvastatin and ezetimibe in their combination drug product was introduced [60]. Under oxidation, thermal and photodegradation conditions, both drugs were relatively stable. For rosuvastatin a high degree of degradation was observed in acidic hydrolytic conditions (0.1 M HCl at 80 °C for 1h), while ezetimibe was stable. On the contrary, ezetimibe was completely degradated with 0.1 M NaOH at 80 °C in 30 min, while rosuvastatin remained stable.

Simultaneous determination and quantification of atenolol, rosuvastatin, spironolactone, glibenclamide and naproxen sodium in bulk drugs, pharmaceutical formulations and in spiked human plasma was performed by HPLC [61].

2.7. Pitavastatin

Pitavastatin is the newest statin on the market available in Japan since 2003, and approved for use in US in 2009. Currently pitavastatin is under evaluation in Europe (in UK it was approved in 2010). Hence, not many methods have been reported for determination of pitavastatin in bulk drug and pharmaceutical formulations.

Two HPTLC methods were reported for the determination of pitavastatin in commercial pharmaceutical dosage forms [62, 63]. Validation was performed and both methods were shown to be selective, sensitive and accurate.

A HPLC method was proposed for determination of pitavastatin in pharmaceutical dosage forms by Kumar et al [64]. Separation was achieved on a Phenomenex C18 column (250 mm x 4.6 mm, 5 μm) in isocratic mode. Different mobile phases were tested and based on the best separation, analysis time, cost-effectives, sensitivity and suitability for the stability studies, a mobile phase consisted of 0.5% acetic acid:acetonitrile (35:65, *v/v*) was chosen. Four different drugs were tried out as the internal standard, and based on peak shape, resolution and elution time, paracetamol was chosen.

Several stability indicating methods have been published [65-68]. Panchal and co-workers proposed two different methods, using liquid chromatography and ultraviolet spectrophotometry for determination of pitavastatin in tablet dosage forms [66]. Additionally forced degradation study was conducted under acidic, basic, oxidative, thermal and photolytic conditions. No change in the area of pitavastatin peak and no additional peaks were detected under photodegradation conditions. Both acidic and basic hydrolysis and thermal conditions generated additional peaks. After oxidative degradation a significant decrease of pitavastatin peak and additional peaks were observed. Linearity range of the LC method was 0.1-2.5 μg/mL, while for the UV method it ranged from 2-20 μg/mL. The limit of detection (LOD) of the LC method was 0.0055 μg/mL, whereas for the UV method it was much higher, 0.4062 μg/mL. Statistical comparison between two methods by applying the paired t-test was performed and no statistically significant difference was observed.

UPLC stability indicating method was developed for degradation study of pitavastatin [67]. Separation of pitavastatin and its degradation products and impurities was performed in less then 5 min. More detailed photodegradation study of pitavastatin was conducted by Grobelny et al. [68]. Pitavastatin solution was exposed to UV-A radiation. HPLC analysis was performed to monitor the changes of pitavastatin. Identification of four photoproducts was conducted by MS analysis.

A single method is reported for simultaneous determination of pitavastatin and ezetimibe [69]. After optimization and validation, the proposed method was successfully applied for determination of pitavastatin and ezetimibe in a prepared binary mixture. However, no real sample was tested.

Analyt	**Application**	**Separation technique and detector**	**Stationary phase**	**Mobile phase**	**Ref.**
LOV	stability indicating study	HPLC PDA 237 nm	Symmetry C18 (150 x 3.9 mm, 5 μm)	Gradient elution A: ACN B: 0.1% TFA	15
SIM, EZE	combined dosage form	HPLC UV 240 nm	Chromolith Performance monolitich column RP-18e (250 x 4.6 mm)	ACN:50 mM ammonium acetate (65:35)	25
SIM	impurity profiling	HPLC DAD 240 nm, ESI-ion trap	Symmetry Shield RP 18 (250 x 4.6 mm, 5 μm)	ACN:water (85:15)	26
SIM	combined dosage form	UPLC Q-TOF MS	Acquity BEH C18 (100 x 2.1 mm, 1.7 μm)	gradient elution ACN:ammonium acetate pH 6	28
PRA	impurity profiling	HPLC APCI-CAD MS	Betasil C18 (250 x 4.6 mm)	gradient elution A: 30%methanol+10 mM ammonium acetate B: 100% methanol + 10 mM ammonium acetate	32
PRA	stability indicating study	HPLC UV 238 nm	Alltima C18 (150 x 4.6 mm, 5 μm)	methanol:0.02 M phosphate buffer pH 7	33
ATO, AML	stability indicating study	HPLC UV 237 nm	Perfectsil Target ODS-3 (250 x 4.6 mm, 5 μm)	ACN:0.025 M sodium dihydrogen phosphate pH 4.5 (55:45)	39
ATO, ASA	stability indicating study	UPLC UV 247 nm	BEH C18 (50 x 2.1 mm, 1.7 μm)	ACN:0.1 M phosphate buffer	40
ATO, FEN	stability indicating study	UPLC UV 247 nm	BEH C18 (50 x 2.1 mm, 1.7 μm)	ACN:0.01 M ammonium acetate pH 4.7	43
ATO	related compounds	HPLC UV 248 nm	Zorbax XDB C18 Rapid Resolution HT (50 x 4.6 mm, 1.8 μm)	gradient elution A: Tetrahydrofuran:ACN (90:10) B: 0.025M phosphate buffer pH 3.5	45
ATO, RAM	combined dosage form	HPLC HPTLC	Phenomenex Luna C18 (250 x 4.6 mm, 5 μm) Silica gel 60F254	0.1% phosphoric acid:ACN (38:62) methanol-benzene-glacial acid (19.6:80.0:0.4)	47
ATO, FEN	combined dosage form	TLC UV 258 nm	Aluminum foil silica gel 60 F-254	toluene-methanol-triethylamine (7:3:0.2)	48
ATO, SIM, PRA	pharmaceutical dosage form	HPLC UV 225 nm ESI-QTOF MS	Supelco C8 (250 x 4.6 mm, 5 μm)	gradient elution A: ACN B: phosphate buffer pH 2.3	56

Analyt	Application	Separation technique and detector	Stationary phase	Mobile phase	Ref.
ROS, EZE	combined dosage form	HPLC HPTLC UV 245 nm	Chromolith C 18 (100 x 6 mm) Aluminium-backed silica gel 60F(254)	0.1% orthophosphoric acid pH 3.5:ACN (63:37)	59
ROS, EZE	stability indicating study	HPLC UV 242 nm	Hypersil C18 (150 x 4.6 mm, 5 μm)	0.05 M phosphate buffer pH 2.5:methanol (45:55)	60
PIT	pharmaceutical dosage form	HPTLC UV 245 nm	Aluminum backed Silica gel 60F(254)	ethyl acetate-methanol-ammonia+1drop formic acid (7:2:0.8)	62
PIT	photostability study	HPLC MS	LiChrospher RP-18 (250 x 4.6 mm, 5 μm)	gradient elution A:ACN B:10 mM phosphate buffer	66
ROS, ATO, SIM, LOV, PRA	pharmaceutical dosage form	HPLC GC UV 246 nm	Symmetry C18 (250 x 4.6 mm, 5 μm) HP-1 (30 m x 0.25 mm x 0.25 μm)	ACN:water (70:30) pH 2.5 1.2 mL/min 2.9 mL/min (GC)	123
PRA, FLU, ATO, ROS	stability indicating study	HPLC	RP C18	methanol-water (60:40)-PRA,ROS Methanol-water (70:30)-FLU,ATO	124

LOV-lovastatin, SIM-simvastatin, PRA-pravastatin, FLU-fluvastatin, ATO-atorvastatin, ROS-rosuvastatin, PIT-pitavastatin, EZE-ezetimibe, AML-amlodipine, ASA-acetylsalicylic acid, FEN-fenofibrate, RAM-ramiprile, ACN-acetonitrile

Table 1. Chromatographic methods for analysis of statin drugs in pharmaceuticals

3. Bioanalytical methods

There have been three reviews on analytical methods for the determination of HMG-CoA reductase inhibitors in biological samples. The first one, published by Ertürk and co-workers in 2003 [70], reviews bio-analytical methods for lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin. The second one, published in 2007, is focused only on chromatography-mass spectrometry methods for the quantification of statins in biological samples [10]. In 2008 Nováková and co-workers [17] have published a review on HPLC methods for the determination of simvastatin and atorvastatin in various fields of application, including bioanalytical assays. Since these reviews have been published, a number of bioanalytical methods have been developed for all HMG-CoA reductase inhibitors. Most of the methods published since 2007 were applied for investigation of HMG-CoA reductase inhibitors in human plasma or serum. Far to our knowledge since 2007 only two LC/MS/MS methods for determination of statins in human urine have been developed. The sample preparation procedures and analytical assays for quantification of statins in biological samples are listed in Tables 2 and 3.

3.1. Sample preparation

Sample preparation is a quite tedious but still unavoidable procedure in bioanalitical methods. The objective of this delicate and challenging step is to transfer analyte of interest into a form that is purified, concentrated and compatible with the analytical system. The extraction and enrichment of analytes from the sample matrix are often realized by procedures such as, protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE). These conventional sample preparation procedures are still dominating in the preparation of biological samples for determination of statin drugs as well as their metabolites.

In most of the methods protein precipitation reagent is used as a dilution solvent for internal standard in order to reduce the number of reagent additions [71-74]. Still, Apostolou and co-workers [75] suggested addition of protein precipitation reagent after the internal standard in order to ensure a more satisfying binding of internal standard molecules with plasma proteins, simulating the binding of proteins with analytes in real human plasma. A number of different protein precipitation reagents were tested [76]. Despite the good recoveries obtained with phosphoric acid, the authors recommended to avoid acidic precipitants due to degradation of fluvastatin in acidic conditions. The highest recoveries were obtained with organic solvents. Although no significant differences were observed between methanol and acetonitrile, the second one was used as it offered a more compact precipitate minimizing the risk of SPE cartridge obstruction.

The simplest way to concentrate the analyte is certainly LLE. Hence, Hamidi and co-workers [73] tested a wide spectrum of organic solvents from various physicochemical categories with different volume fractions as well as combinations for extraction of lovastatin from human plasma. The best extraction efficacy was obtained using diethyl ether as extraction solvent. The same solvent was used for extraction of pitavastatin from human plasma [77]. An addition of hidrocloric acid to the plasma samples afore the extraction procedure was shown to be necessary in order to obtain the non-ionized form of analyte which considerably improved extraction efficacy. Assays employing LLE with ethyl acetate [78] and ethyl ether [79] as extraction solvents for determination of rosuvastatin in plasma samples were already published with extraction recoveries of 74 and 69%, respectively. However, in the preliminary study by Lan and co-workers [80], it was found that the extraction recovery of rosuvastatin from plasma in most common organic solvents, such as above mentioned ethyl acetate and ethyl ether, was less than 20%, resulting in an insufficient, imprecise and inaccurate extraction procedure. The authors presumed that low extraction efficacy of rosuvastatin was due to its extremely low water solubility. However, a carboxyl group in its structure forms a salt with calcium ion which indicates that rosuvastatin was apt to ionization. The application of ion paring with tetrabutyl ammonium hydroxide was suggested for improvement of rosuvastatin solubility and subsequently extraction efficacy. Finally, using ion paring LLE, extraction efficacy of rosuvastatin in ethyl acetate was improved from around 10% to more than 50%. A somewhat unusual LLE method for determination of timolol maleate, rosuvastatin and diclofenac in human plasma and aqueous humor from the bovine eyes was proposed [81]. The mobile phase, consisted of acetonitrile and 0.2% triethylamine, was used as extraction solvent. The quite high extraction efficacy of all investigated compounds was obtained using this uncommon extraction solvent.

Although LLE is generally considered to be providing cleaner extracts and lower matrix effect than the SPE, lower recovery due to the transfer of a fraction of the organic extract after the extraction may be the main disadvantage of the LLE technique. Moreover, when low concentrations have to be detected it is necessary to use a large solvent volumes and sample preparation becomes time consuming and labor invasive. In order to reduce organic solvent consumption, sample volume and sample preparation time, Apostolou and co-workers [75] have presented a fully automated high-throughput two-step LLE-LC/MS/MS method for the quantification of simvastatin and its acid form using a robotic liquid handling workstation with 96-deepwell plates. Another fully automated high-throughput salting-out (SA) assisted LLE-LC/MS/MS method was introduced by Zhang and co-workers [82]. Due to the compatibility between SALLE and LC/MS/MS, the extracts of simvastatin and its acid from human plasma were injected directly into LC system immediately after sample extraction. In this way extract solvent evaporation was eliminated and consequently sample preparation procedure was simplified. Also, the exposure of the extracts to the room temperature was minimized and hence minimal interconversion between simvastatin and its acid was achieved.

Among the SPE methods, the reverse phase cartridges have been extensively used for extraction of statins from biological samples. Gonzalez and co-workers [76] have presented a nice work regarding the traditional one-variable-at a time optimization for SPE extraction of fluvastatin together with other drugs from human plasma. The optimization of conditioning and washing solution composition, pH for conditioning and washing step and elution solvent selection were described in details. The SPE procedure has also been used as sample preparation step for quantification of atorvastatin and simvastatin as well as their metabolites in serum from patients with end stage renal disease [83]. In order to obtain the satisfactory and repeatable extraction efficacy and to remove matrix effects, several different reversed-phase SPE sorbents have been tested. The best results were obtained using ZORBAX SPE C-18 (Agilent Technologies) and Discovery DSC-18 SPE (Supelco) cartridges. As ZORBAX SPE C-18 columns were withdrawn from commercial market circulation during optimization of method, further investigations were performed using Discovery DSC-18 SPE cartridges. For the purpose of minimization of the interconversion between lacton and open-ring hydroxy acid forms of simvastatin and atorvastatin, SPE sorbents were conditioned and analytes were eluated with solvents containing 0.1 M acetate ammonium buffer pH 4.5. In the work of Di and co-workers [84) SPE sample preparation procedure was used for determination of pitavastatin with rosuvastatin as internal standard in human plasma. The influence of pH on extraction efficacy of statin drugs was investigated in detail. The authors have pointed out importance of 0.5 M potassium dihydrogenphosphate buffer (pH 4.0) as conditioning reagent for cartridges. At pH lower than 4 both molecules were protonated, leading to a decrease in its partitioning in reversed-phase SPE and recovery. At pH higher than 4, the carboxylic group in both pitavastatin and rosuvastatin undergo ionization, which also resulted in a decrease in the recovery for the same reason. Furthermore, it was found that pitavastatin degradation was much faster at lower than at high pHs. Also, it was found that pitavastatin was sensitive to sunlight. It was recommended to minimize the exposure of samples to sunlight as well as to dissolve the dried extract rather in methanol and water than in mobile phase containing formic acid.

To reduce the time of sample preparation, Mertens and co-workers [85] have used an automated SPE on disposable extraction cartridges to isolate pravastatin and its metabolites together with fenofibric acid, another lipid-regulating agent, from the human plasma and to prepare cleaner samples before injection and analysis in the LC/DAD/MS/MS system. Different kinds of disposable extraction cartridges containing bonded silicas of different polarities (ethyl, endcapped ethyl, octyl, endcapped octyl, octadecyl, endcapped octadecyl and cyanopropyl) were tested. The best recoveries for all investigated compounds were reported when disposable extraction cartridges filled with octyl functionalized silica sorbent were used.

Unfortunately, conventional SPE and LLE approaches are multi-step, time-consuming and the sample required for analyses as well as the consumption of organic solvent are quite high, particularly in case of LLE. A solvent-minimized sample preparation approach has been popular in last decades, therefore Farahani and co-workers [71] have published liquid-liquid-liquid microextraction procedure (LLLME), a miniaturized format of LLE, for determination of atorvastatin in human plasma. A number of factors affecting the microextraction efficiency were studied in detailed and the optimized conditions were established. They have obtained quite high extraction efficacy of atorvastatin from human plasma using proposed sample preparation procedure. Vlčková and co-workers [86] have developed fast and simple extraction procedure using microextraction by packed sorbent (MEPS) for sample purification and concentration of atorvastatin and its metabolites from human serum. Briefly, MEPS is a miniaturization of conventional SPE, but it differs from commercial SPE by fact that packing is inserted directly into the syringe, not into a separate column. In addition, they have compared a previously described [83] SPE procedure for extraction of atorvastatin and its metabolites from human serum with newly developed MEPS approach. The results of samples treated by SPE and MEPS were compared by means of Student *t*-test. The difference between obtained concentrations was statistically not significant. Hence, MEPS procedure was found to be simpler and faster sample preparation technique using smaller volume of sample, which is regardful to the patients and smaller volume of solvents, which is environmentally friendly.

3.2. Liquid chromatography

3.2.1. High performance liquid chromatography

The high performance liquid chromatography has become the method of choice for bioanalytical methods. Generally, in the HPLC methods reversed-phase C18 chromatographic columns were used for analysis of statin drugs in biological fluids. The recently developed columns based on BEH particles technology were employed in several methods [83, 86, 87]. Only in one assay reversed-phase C8 chromatographic column was used [88]. Unusually, reversed-phase narrow bore phenyl column was employed for investigation of atorvastatin, rosuvastatin and their metabolites [74, 89]. The length and diameter of columns differed fairly from 50 to 250 mm and from 2.0 to 4.6 mm, respectively. Although in most of the cases columns with particle size 5 μm were used, several authors preferred columns with smaller particles in order to obtain better peak shapes, resolution and thus shorter analysis time [72, 82, 83, 86, 87]. Analytical run times have been very variable, the shortest 2 min, the longest about 20 min.

The selection of mobile phase was quite a challenging task in all investigations. In most of the methods acetonitrile or methanol were present in the mobile phase as organic solvent. The percentage of organic solvents was optimized such that the retention times of analytes were kept as short as possible. In most assays percentage of organic solvent was quite high, usually more than 70%. The majority of publications emphasize the pH as the most critical variable for separation of the statin drugs [76, 82, 84]. In order to minimize the interconversion, it is critical to maintain pH of mobile phase between 4 and 5.

The influence of mobile phase pH on retention of atorvastatin and rosuvastatin has been investigated [90]. Since both of the analytes are acidic compounds, their retention on the reversed-phase column was expected to be pH dependant. When pH of the mobile phase was decreased from 4.0 to 3.0, the retention times of the analytes decreased unexpectedly and with further decreases in the pH to 2.0 the retention times increased once again. This behavior was explained by a change in binding of the analytes to the stationary phase and also changes in the solubility of the analytes in the mobile phase. The pH 3.0 was chosen as optimum pH because of the reasonable retention times while the resolution between peaks, as well as peak shapes, were satisfactory.

The pH of mobile phase was also a critical variable for the separation of the fluvastatin from valsartan and its metabolite during the optimization of LC/PDA/FLD method [76]. The pH of the mobile phase was limited by the native fluorescence of valsartan and its metabolite, which disappears in the basic form (pK_a = 3.7). On the other hand, spectrophotometric studies showed that fluvastatin degradation was accelerated in acidic conditions. Mobile phases with different formic acid/formate proportions were tested in order to establish the range where fluvastatin was stable and valsartan and its metabolites kept their fluorescence. 0.01% formic acid/10 mM ammonium formate (pH 4.1) was finally chosen as appropriate buffer. Uncommon pH was used for quantification of lovastatin in human plasma [73]. Mobile phase consisted of acetonitrile and 0.05 M phosphate buffer with pH 7, adjusted with phosphoric acid.

The flow rate of the mobile phase was in range from 0.2 up to 1.5 mL/min. In all of the assays the flow rate did not change during the chromatographic analysis except in the reference [76] where the flow rate was gradually changed after three minutes.

The chromatographic separation of most of the methods was performed at room temperature. In order to shorten analysis time, in the several cases the column temperature

Extracted analytes	**Matrix**	**Sample preparation procedure**	**Stationary phase**	**PP reagent / LLE reagent / SPE eluent**	**Recovery (%)**	**Ref.**
ATO	plasma	PP, LLLME	-	methanol , HCl, trichloroacetic acid /1-octanol	91	71
SIM, MET IS=propranolol hydrochloride	plasma	PP	-	methanol:water (1:1)	83-91	72
ROS + metabolites IS=deuterium labeled	plasma	PP	-	0.1% acetic acid in methanol	88–106	74
FLU, VAL + metabolite, CLT IS=candesartan cilexetil	plasma	PP, SPE	Phenomenex Strata-X polymeric C18	ACN/methanol	78–91	76
ROS IS=hydrochlorothiazide	plasma	LLE	-	ethyl ether	69–72	79
ROS IS=estrone	plasma	ion pair LLE	-	ethyl acetate	47–63	80
ROS, TIM, DIC IS=naproxen	plasma, bovine aqueous humor	PP, LLE	-	methanol/mobile phase	95–99	81
SIM, SIM-acid IS=deuterium labeled	plasma	SALLE	-	ACN, 5 M ammonium formate buffer (pH 4.5)	71–79	82
SIM, ATO + metabolites IS= deuterium labeled	serum	SPE	Supelco Discovery DSC-18	ACN:0.1 M ammonium acetate buffer pH 4.5 (95:5)	65-100	83
PIT IS=ROS	plasma, urine	SPE	Supelco Superclean™ LC-18 SPE Tubed	methanol	plasma 84–88 urine 86–96	84
PRA + metabolites, FFA IS=triamcinolone	plasma	at-SPE	Disposable extraction cartridges C8 silica sorbent	methanol	50–77	85
ATO + metabolites IS=deuterium labeled	serum	MEPS	C8	ACN:0.1 M ammonium acetate pH 4.5 (95:5)	89–116	86
PIT, PIT-lactone IS=racemic *i*-prolact	plasma, urine	LLE	-	methyl-terc-butyl ether	plasma 70–75 urine 74–83	88
PRA, ASA IS=furosemide	plasma	LLE	-	tertiary butyl methyl ether	51–66	94

SIM-simvastatin, PRA-pravastatin, FLU-fluvastatin, ATO-atorvastatin, ROS-rosuvastatin, PIT-pitavastatin, FFA-fenofibric acid, TIM-timolol maleate, MET-metoprolol, DIC-diclofenac, VAL-valsartan, ASA-acetylsalycilic acid, CLT-chlorthalidone, ACN-acetonitrile, IS-internal standard

Table 2. Sample preparation procedures utilized for the determination of statins in biological samples

was maintained above 30 °C [76, 77, 81, 83, 86, 87]. The effect of column oven temperatures on the analysis of atorvastatin and rosuvastatin in the range 25 to 35 °C was investigated and best results were observed at 25 °C in terms of retention factor and resolution [90]. Increasing temperature above 25 °C resulted in the rapid elution of rosuvastatin close to the solvent front.

LC/DAD methods are rarely sensitive enough for quantification of statins as well as their metabolites in human plasma samples due to the poor UV-absorption properties of statin molecules. Furthermore, the levels of statins and their metabolites in biological fluids are very low due to low amount of drug reaching the systemic circulation. Their typical plasma concentrations are in ng/mL levels. However, several sensitive LC/DAD methods for determination of pravastatin [31], atorvastatin [71], lovastatin [73], rosuvastatin [81], and atorvastatin with rosuvastatin [90] have been developed with limit of quantification (LOQ) in range of 1 - 10 ng/mL. Less sensitive LC/DAD method for quantification of lovastatin in human plasma was developed [91]. The LOQ value for lovastatin was relatively high, 400 ng/mL. Another even less sensitive LC/DAD method for quantification of several HMG-CoA reductase inhibitors in human plasma was developed by Sultana and co-workers [92]. The LOQ values were between 376 and 1006 ng/mL. In fact, both of these methods were not used on real plasma samples.

Fluorescence detection has not been widely employed in the determination of HMG-CoA reductase inhibitors, as most of statins do not possess a natural native fluorescence. Still, Gonzalez and co-workers [76] have developed a SPE-HPLC/PDA/FLD method for determination of fluvastatin and valsartan in human plasma. Comparing results obtained with spectrophotometic and fluorimetric detector superior selectivity and sensitivity by fluorescence detection of fluvastatin could be perceived.

3.2.2. Ultra performance liquid chromatography

Recently UPLC is becoming a leading chromatographic technique in modern bio-analytical methods. Nováková and co-workers [83] have investigated its potential in combination with MS/MS detection for the fast, sensitive, reliable and selective detection of atorvastatin and simvastatin together with their main metabolites and interconversion products in human serum. Iriarte and co-workers [87] have investigated UPLC technique as a faster alternative to HPLC for simultaneous analysis of fluvastatin and other drugs usually prescribed in cardiovascular therapy. Acquity UPLC Columns Calculator software was used for transfer of previously developed HPLC method [76].

The UPLC technology has significantly improved the method optimization process since shorter analysis and re-equilibration times allowed a greater number of experimental testing conditions than with a conventional HPLC. The sample volume required was much lower than in HPLC method. Furthermore, shorter analysis time together with slower flow rates reduced the organic solvent consumption. The sharper and higher chromatographic peaks, thereby improved peak capacity, was obtained using UPLC technology. Still, the sensitivity of UPLC method was found to be analyte dependent as the improvement was not achieved for all analytes.

3.2.3. Liquid chromatography coupled to tandem mass spectrometry

In pharmacokinetic investigations of statins LC/MS/MS technique is unequivocally the method of choice. Recently, several procedures were described in the literature taking the advantages of the benefits of mass spectrometry. Both ESI and APCI sources as well as triple quadrupole analyzator were applied in most LC/MS/MS sample analysis.

As it was mentioned above the selection of appropriate mobile phase composition for determination of statins in biological fluids is quite challenging task which is even more complicated when detection and quantification of statins is performed using MS. Only few additives could enable good stability at pH range 4 to 5 as well as volatility and sensitive mass spectrometric response. Therefore, Di and co-workers [84] have pointed out the importance of the formic acid in lowering the pH of mobile phase. In this way pitavastatin was obtained in non-ionized form and a symmetrical peak shape was observed. The concentration of formic acid was optimized not only to maintain a symmetrical peak shape in the chromatographic system but also to render good ionization and fragmentation of pitavastatin in the MS/MS detector. An addition of 0.025% formic acid to the aqueous phase was found to be an important factor for acquiring the high sensitivity of another LC/MS/MS method for determination of pitavastatin in human plasma [77].

Nováková and co-workers [83] have presented a nice example of optimization of the buffer pH and concentration in order to get the best signal to noise ratio of MS detector. Ammonium formate and ammonium acetate at pH 4.0 and 4.5 were tested at the concentration range 0.01 to 10 mM. The best response of atorvastatin and simvastatin was observed at 0.5 mM buffers. The concentrations higher than 5 mM significantly decreased the response of mass spectrometer. On the other hand, the concentrations lower than 0.5 mM were not sufficient to keep buffering capacity and thus had negative influence to the response of mass spectrometer. Ammonium acetate was preferred before ammonium formate because of better peak shapes. Finally, the optimized mobile phase composition was 70% of acetonitrile and 30% of ammonium acetate buffer 0.5 mM (pH 4.0). In most of bioanalytical methods isocratic elution has been utilized, still when more analytes with different polarities were separated, gradient elution had to be applied.

Tandem mass spectrometry detection for identification and quantification of simvastatin and atorvastatin together with their metabolites and lacton/hidroxy acid interconversion forms was employed [83, 86]. All analytes were monitored using electrospray positive ionization (ESI^+) mode and for all analytes protonated molecule $[M+H]^+$ was the most intensive ion in mass spectra. Quantification of all analytes was performed using selected reaction monitoring (SRM) and two specific transitions were optimized for each molecule in order to increase selectivity and sensitivity of the method. In the paper published afterwards simvastatin in its lactone form was determined in ESI^+ mode, while its hydroxy acid form was determined in ESI^- mode due to poor sensitivity of hydroxy acid form in positive ion mode [82].

LC/MS/MS method developed by Apostolou and co-workers [75] consisted also of two periods combining both negative and positive ionization modes. The mass spectrometer operated in the negative detection mode for 1.21 min until simvastatin and lovastatin hydroxy acid forms were eluted from chromatographic column. Afterwards a period of 0.69 min followed in the positive mode during witch simvastatin and lovastatin lacton forms were eluted. Comparing LOQ values for simvastatin acid obtained by these three methods it can be seen that lower LOQ values and thus better sensitivity were obtained in the last two methods. Unfortunately, simvastatin forms various adducts influenced by mobile-phase and matrix composition and such adducts sometimes give higher intensity than protonated molecule $[M+H]^+$, which is an ideal precursor ion for SRM transition and quantification studies. However, Senthamil Selvan and co-workers [72] have observed very high signal of $[M+Na]^+$ in the spectra of simvastatin next to the $[M+H]^+$. Consequently, it was used as precursor ion for quantitation of simvastatin. Also, Zhang and co-workers [82] have used the methylammonium aduct $[M+CH_3NH_4]^+$ as a parent ion for simvastatin because the adduct ion showed the best signal to noise ratio.

Rosuvastatin has a pyrimidine ring and a carboxylic group in its structure, hence it could be detected either in positive or negative ionization mode. However, the quantification of rosuvastatin in positive ionization mode is more common and was used for determination of rosuvastatin [80] and rosuvastatin together with its metabolites [74], respectively. In the both assays the major ion was protonated molecule $[M+H]^+$ in full-scan mode and principal product ion was at *m*/*z* 482. Macwan and co-workers [74] have also reported two minor fragments at *m*/*z* 300 and *m*/*z* 272.

During the method development, Gao and co-workers [79] also attempted to optimize ESI conditions under positive ionization mode. However, the observed signal intensity was not sensitive enough for determination of expected rosuvastatin's concentrations, especially for low dosage administration. Low sensitivity of positive ionization mode could be explained by a number of fragment ions produced in the product ion spectrum of $[M+H]^+$. In order to improve the sensitivity of the method, the negative ESI detection was taken into consideration. Under negative ESI mode, rosuvastatin produced abundant deprotonated molecule $[M-H]^-$ at m/z 480. In the product ion mass spectrum of $[M-H]^-$, fewer fragment ions were formed compared with that of $[M +H]^+$. Also, it was pointed out that negative ESI mode produced lower chemical background noise than positive. Comparing LOQ values obtained by these three methods, it can be observed that almost five times lower LOQ value for rosuvastatin was obtained using negative ESI detection.

Pitavastatin has similar structure to rosuvastatin. It contains alkaline nitrogen ion on the quinoline ring and a carboxylic group, therefore positive and negative ionization mode could be also employed. Both of ionization modes for determination of pitavastatin in human plasma and urine by LC/MS/MS method were applied [84]. The results showed that the response intensity of pitavastatin in negative mode was lower and furthermore the response was quite unstable. Pitavastatin was scanned under Q1 MS full-scan mode to determine the parent ion and under Q1/Q3 product ion scan mode to locate the most abundant production. The protonated molecular ion, $[M+H]^+$, was the predominant ion in the Q1 spectrum and was used as the parent ion to obtain the product ion spectra. The most sensitive mass transition was from *m*/*z* 422.0 to 290.1, which was similar to the MS/MS spectrum of pitavastatin reported in reference [88] and [93], while in the previously reported LC/MS/MS method the highest collision energy gave the most abundant product ion at *m*/*z* 318.0 [77].

Recently, two LC/MS/MS methods have been developed for determination of pravastatin in human plasma [85, 94]. Both methods utilized ESI but in different modes. In the method developed by Martens and co-workers [85] the mass spectrometer was operated in the positive mode. The MS/MS detection was set up in MRM mode. The full scan mass spectra of pravastatin and its metabolites were scanned. The collision energy in Q2 produced different significant fragment ions. The MS/MS ion transitions selected for quantification purpose were *m*/*z* 442.2 to 269.1, 442.2 to 269.1 and *m*/*z* 424.3 to 183.0 for pravastatin, 3-OH metabolite and its lacton form. On the contrary, Polagani and co-workers [94] have found high sensitivity and stability using negative ionization mode. Deprotonated form of pravastatin, $[M\text{-}H]^-$ ion was the parent ion in the Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transition was monitored from *m*/*z* 423.3 to 100.8.

Internal standards have been used in most of the assays leading to more corrected results. In some cases one of the statins has been used as internal standard [73, 75, 77, 84], while other works utilized internal standards of various structure, including hydrochlorothiazide [79], estrone [80], naproxen [90], gemfibrozil [91], pioglitazone [95] etc. The best internal standards for precise and accurate quantification in MS or tandem MS are stable-isotope-labeled standards. Only a few works employed deuterium labeled standards [74, 82, 83, 86]. In the case of atorvastatin, [d5] labeling usually occurs on the phenyl ring, which does not contain fluorine. [d3] labeling of simvastatin occurs on the side chain, while [d6] labeling of rosuvastatin occurs on isopropyl group attached to pyrimidin ring. In most of investigations only one compound was used as internal standard.

However, Mertens and co-workers [85] have used two different internal standards for quantification of fenofibric acid, pravastatin and its metabolites in human plasma by automated SPE-LC/DAD/MS/MS technique. To avoid the need for plasma dilution and two time-consuming analytical runs, the use of two internal standards was necessary as the concentration of fenofibric acid was too high and MS signal appeared saturated. Hence, the sulindac was selected for the quantification of fenofibric acid by UV-detector, while the triamcinolone was used for MS/MS quantification of pravastatin and its metabolites. As it was mentioned above, in the method developed by Zhang and co-workers [82], the LC/MS/MS data acquisition for simvastatin was conducted in positive ionization mode, whereas the data acquisition for simvastatin acid was conducted in negative ionization mode. Therefore, it was inevitable to use two internal standards deuterium labeled simvastatin and deuterium labeled simvastatin acid, respectively.

3.3. Gas chromatography

Several GC/MS methods for determination of statins in biological samples have been reported [10]. Unfortunately, these methods are limited and not recommended for routine applications as they include analyte derivatization step prior to anaysis in order to obtain volatile derivatives of the drug molecule and therefore a complicate sample preparation procedures.

Simultaneous determination of lovastatin, simvastatin and pravastatin in plasma using GC with chemical ionization mass spectrometry has been described [70]. The analytes were isolated from plasma by SPE procedure which separated the lactone and acid forms of the drugs. The lactone forms were converted to the corresponding acid forms, which were subsequently derivatized by pentafluorobenzylation of the carboxyl group, and trimethylsilylation of the hydroxyl functions. The method has sufficient sensitivity for the analysis of clinical samples containing the drugs administered at therapeutic doses with recoveries between 79 and 90%. In another method, simvastatine and its acid form were derivatized with ferroceneboranic acid.

Far to our knowledge since 2001 no method for determination of statin drugs in biological samples using gas chromatography has been published due to imprecise and time consuming derivatization procedures which is an unavoidable step in analysis of statin molecules and the biggest disadvantage of using this technique.

3.4. Pharmacokinetic studies

Since all HMG-CoA reductase inhibitors are given to the patients once daily, monitoring plasma concentrations over a period of 24 hours is necessary. In all published papers monitoring plasma concentration levels were performed at least over 24 hours, except in references [74, 89] were the blood samples were collected at various time points during a period of 12 hours after a single oral dose of rosuvastatin and atorvastatin, respectively. Also, in pharmacokinetic and bioavailability study of simvastatin in healthy volunteers and moderately hyperlipemic patients' drug plasma concentrations were monitored during 12 hours [96]. In the most of investigations pharmacokinetic parameters of statins were investigated after only one pharmaceutical tablet dosage.

Pharmacokinetic parameters of rosuvastatin have been investigated after single doses of 5, 10 and 20 mg [79]. The peak plasma levels obtained from this study were 8.32, 14.8 and 20.1 ng/mL, respectively. It was found that plasma exposure to rosuvastatin appeared increasing dose-proportionally and the plasma elimination half-lives were prolonged with increased doses. Not so many methods for determination of statins in human urine have been developed. The SPE-LC/MS/MS method was successfully applied to quantify the pitavastatin concentration in plasma and urine which were collected from Chinese volunteers [84]. The urinary excretion ratio of pitavastatin accounted for less than 0.6%, which suggested that pitavastatin was not excreted primarily by kidney. Quite similar data were obtained using LLE-LC/MS/MS method [88].

Several above described bioanalytical assays have been used in bioequivalence studies of statin drugs. The pharmacokinetic parameters derived from drug plasma concentrations, including maximum plasma concentration, area under the plasma concentration-time curve from 0 h to the last measured data, area under the plasma concentration-time curve from 0 h

Analytes	**Matrix / Sample preparation**	**Stationary phase**	**Mobile phase**	**Separation technique and Detection**	**LOQ**	**Ref.**
ROS + metabolites, IS=deuterium labeled	plasma	Agilent Zorbax-SB Phenyl, Rapid Resolution HT (100 x 2.1 mm, 3.5 µm)	gradient elution A: 0.1% glacial acetic acid in 10 % methanol in water B: 40% methanol in ACN	HPLC ESI$^+$ MS/MS MRM	0.1– 0.5 ng/mL	74
SIM, SIM acid IS= LOV, LOV acid	plasma	YMC ODS-A (50 x 4.0 mm)	ACN:5 mM ammonium acetate pH 4.5 (82:18)	HPLC APCI/ESI$^-$/ESI$^+$ MS/MS MRM	0.1 ng/mL	75
FLU, VAL + metabolite, CLT IS=candesartan cilexetil	plasma	Waters Atlantis dC18 (100 x 3.9, 3 µm)	gradient elution A: ACN, 0.01% formic acid, 10 mM ammonium formate B: 0.01% formic acid, 10 mM ammonium formate pH 4.1	HPLC UV 229, 254, 236 nm FD 254, 378 nm	UV: 31-85 µg/mL FD: 10-20 µg/mL	76
ROS IS=hydrochlorothiazide	plasma	Agilent Zorbax XDB-C18 (150 x 4.6 mm, 5 µm)	methanol:water (75:25), pH 6.0 with ammonia	HPLC ESI$^-$ MS/MS MRM	0.02 ng/mL	79
SIM, SIM-acid, ATO + metabolites IS=deuterium labeled	serum	Waters Acquity UPLC™ BEH C18 (100 x 2.1 mm, 1.7 µm)	gradient elution A: ACN B: 0.5 mM ammonium acetate buffer pH 4.0	UPLC ESI$^+$ MS/MS SRM	0.09–4.38 nM	83
PIT IS=ROS	plasma, urine	Shimadzu Shim-pak VP-ODS (150 x 4.6 mm, 5 µm)	methanol:water:formic acid (75:25:0.05)	HPLC ESI$^+$ MS/MS SRM	0.08 ng/mL	84
PRA + metabolites, FFA IS=triamcinolone	plasma	Phenomenex Synergi Max-RP (150 x 2 mm, 4 µm)	ACN:methanol:5 mM ammonium acetate buffer pH 4.5 (30:30:40)	HPLC ESI$^+$ MS/MS MRM	0.05–0.5 ng/mL	85
ATO + metabolites IS=deuterium labeled	serum	Waters BEH C$_{18}$ (100 x 2.1 mm, 1.7 µm)	gradient elution A: ACN B: 0.5 mM ammonium acetate pH 4.0	UPLC ESI$^+$ MS/MS SRM	0.08-0.66 nM	86
FLU, VAL + metabolite, CLT IS=candesartan cilexetil	plasma	Waters Acquity UPLC™ BEH C18 (50 x 2.1 mm, 1.7 µm)	gradient elution A: 10 mM ammonium formate, 0.01% formic acid B: ACN, 10 mM ammonium formate, 0.01% formic acid, pH 4.1	UPLC UV 220 nm	20-110 µg/mL	87
PIT, PIT-lacton IS=racemic i-prolact	plasma, urine	Thermo BDS Hypersil C8 (50 x 2.1 mm, 3 µm)	methanol:0.2% acetic acid in water (70:30)	HPLC ESI$^+$ MS/MS MRM	1 ng/mL	88

LOV-lovastatin, SIM-simvastatin, PRA-pravastatin, FLU-fluvastatin, ATO-atorvastatin, ROS-rosuvastatin, PIT-pitavastatin, VAL-valsartan, FFA-fenofibric acid, CLT-chlorthalidone, ACN-acetonitrile, IS-internal standard

Table 3. Analytical methods for the determination of statins in biological samples

to the infinity, the time to reach peak concentration, the apparent elimination rate constant, showed that there was no statistically significant difference between two investigated pharmaceutical formulations [72, 73].

Not so many chromatographic methods have been developed for the quantification of HMG-CoA reductase inhibitors in combination with their metabolites. They undergo quite extensive first-pass metabolism during witch active and inactive metabolites are produced. The actual plasma concentrations of both parent compounds and metabolites are of major interest in pharmacokinetics studies. Therefore, analytical methods for simultaneous determination of statins and their metabolites are quite valuable. Although simultaneous determination of statins and their metabolites was considered being difficult owing to the different polarities of the analytes, several methods have been published.

Recently, Apostolou and co-workers [75] published fast and fully automated LLE-LC/MS/MS method, while Zhang and co-workers [82] presented a high-throughput salting-out assisted LLE-LC/MS/MS method for simvastatin in lactone and acid form. Both of methods were very fast with analytical runs less than two min and fairly sensitive with LOQ values around 0.1 ng/mL. Nováková and co-workers [83] have developed fast selective and reliable SPE-UPLC/MS/MS method for simultaneous determination of simvastatin and atorvastatin as well as their active and inactive metabolites. The main advantage of the method was applicability of the method for determination of two clinically widely used statins using one sample preparation procedure and one chromatographic run, while the main limitation of study was slightly higher LOQ value obtained for simvastatin in open-ring hydroxy acid form.

More recently Vlčková and co-workers [86] have presented a new MEPS-UPLC method for determination of atorvastatin and its metabolites, faster and more sensitive comparing to previously published ones. A simple, fast and reproducible method for determination of rosuvastatin and metabolites in human plasma has been described [74]. The major advantages of the method were the requirement for small plasma volume and simple sample preparation procedure, protein precipitation. The major limitation of method was its inability to determine *N*-desmethyl rosuvastatin in the patient samples although its LOQ was quite low, 0.5 ng/mL. The patients included in the study took a single dose of rosuvastatin at 20 mg. *N*-desmethyl rosuvastatin is a minor metabolite that is present in much lower concentrations than rosuvastatin. Therefore, the authors anticipate that the methods should be sensitive enough to measure its concentration in patients receiving rosuvastatin on a routine basis.

A sensitive and accurate procedure based on solid-phase extraction coupled at-line to a LC/MS/MS for determination of pravastatin and its two metabolites in human plasma has been presented [85]. Optimized and validated LLE-LC/MS/MS method for determination of pitavastatin and its lacton form in human plasma as well as in urine is described [88].

Furthermore, a LC/MS/MS method for separation of fluvastatin from its *threo* isomers metabolites to support a bioequivalence study has been developed [97].

The advantage of the methods for simultaneous determination of several co-administered drugs is that the one sample preparation and one chromatographic run are required for monitoring therapeutic levels of several drugs. Therefore, these methods could be useful in daily routine sample handling, when many samples from patients taking different drugs together with HMG-CoA reductase inhibitors are analyzed in clinical laboratories. Recently, several chromatographic methods have been developed for the quantification of statin drugs in combination with other drugs, most of them are commonly used in treatment of cardiovascular disease: atenolol, spironolactone, glibenclamide [61], metoprolol succinate [72], valsartan and chlorthalidone [76, 87], timolol maleate, diclofenac sodium [81], fenofibric acid [85], ezetimibe [91], ceftriaxone [92], acetylsalicylic acid [94], amlodipine [98] and losartan, atenolol, acetylsalicylic acid [99].

Recently, several papers were published regarding prediction of statins' pharmacokinetics. In our work the usefulness of reversed-phase high performance chromatography in building models that would allow the prediction of pharmacokinetics parameters of statins was evaluated [100]. In order to get better insight into the nature of their chromatographic behavior, the retention times were measured using octyl and octadecyl chromatographic columns. Obtained chromatographic data were compared with pharmacokinetic parameters predicted by use of 17 different computer programs. Significant correlations were found between chromatographic data and lipophilicity of statins. In addition, with the combine set of descriptors (chromatographic data, solubility, quantum chemical and topological indices) the highly significant correlations with pharmacokinetic parameters have been found, which confirms the utility of HPLC technique for prediction of pharmacokinetic behavior of statin drugs.

In order to predict the bioavailability of statins, the association mechanism with phosphatidylcholine using immobilized artificial membrane high performance liquid chromatography technique was studied. Moreover, the thermodinamic driving forces for the statin molecules with phosphatidylcholine monolayers were analyzed in detail [101].

4. Capillary electrophoresis

Capillary electrophoresis (CE) is an alternative separation technique which is designed to separate species based on their size to charge ratio in an electric field in the interior of a small capillary filled with background electrolyte. Driving forces in CE are electrophoretic migration and the electro-osmotic flow (EOF). CE has become a useful tool in pharmaceutical analysis because of its advantages over other separation techniques, such as high resolution, high selectivity, simplicity, short analysis time, cost efficiency and low consumption of solvents and reagents [102]. Mainly employed CE modes for drug analysis are capillary zone electrophoresis (CZE) based on charge-to-mass ratio and micellar electrokinetic chromatography (MEKC) based on chromatographic partition of analytes between micelles and background electrolyte. MEKC is the most appropriate electrophoretic technique for impurity profiling because the neutral compounds and charged components that have similar electrophoretic mobilities can be separated simultaneously [103]. CE is currently recommended in several pharmacopoeias. Principal advantage of CE over well-established and widely used HPLC technique is its ability to deliver high efficiency in short analysis times [104]. However, CE methods proposed for the determination of statin drugs are scarce.

CE has been applied for determination of pravastatin in fermentation broth in order to optimize its production in bioreactors [105]. Pravastatin is produced in two-step fermentation. In the first step, mevastatin is produced by *P. citrinum*, and in the second step, bioconverted to pravastatin by *S. carbophylus*. The method successfully separated pravastatin from interfering matrix, mevastatin and 6-*epi* pravastatin. Its determination in production media was also performed using two HPLC methods. All three proposed methods had runtimes under 1 min. However two HPLC methods, performed on a particle and a monolithic LC column had superior sensitivity compared to MEKC, with LOD around 0.01 ng/mL, 0.2 ng/mL and 20 ng/mL, respectively.

We have developed CZE method for determination of pravastatin in pharmaceutical dosage form [106]. Rapid migration of negatively charged pravastatin molecule was obtained in alkaline buffer by the application of electric field of 30 kV. The alkaline buffer generated strong EOF that enabled determination of a fully charged drug molecule within 2.5 min. Pravastatin retention time is about 21 min in the assay procedure listed in European Pharmacopoeia (Ph. Eur.) using the HPLC with UV detection. Relatively short analysis time is the main advantage of the CZE method developed. Pravastatin is administered to patients in its active form as the hydroxy acid sodium salt. However, the drug exists in solution with its lactone equilibrium product reversibly formed at acidic pH. Pravastatin is also susceptible to an isomerization reaction which is relatively rapid [107]. The MEKC method was established to separate the drug and its degradation products in acidic media. Introduction of sodium dodecyl sulphate (SDS) in the background electrolyte solution plays a key role in the separation of negatively charged and neutral species. The proposed method allows baseline separation of pravastatin, C-6 epimer of pravastatin and their corresponding lactone forms that appear as interconversion products depending on the pH value. The migration times of degradation compounds ranged from 2.8 to 6.2 min. The above mentioned interconversion compounds of pravastatin represent its related impurities defined in Ph. Eur. and are also potential biotransformation products. CE has also been applied to the screening of anionic impurities in bulk drug [108].

The application of CE to rapidly quantitate lovastatin production levels by *Aspergillus terreus* mutants has been described [109]. The fermentation broths of thousands of mutated strains were efficiently and inexpensively screened for increased lovastatin production by the developed high-throughput method. Determination of lovastatin in the presence of its oxidation products after exposure to an oxidative atmosphere has been carried out using CE technique [110]. The method developed is suitable for the routine analysis of lovastatin.

The quantitative analysis of lovastatin in urine samples based on CE has significance for the control of clinical therapy [111]. The concentration sensitivity is poor in CE because of the short optical path length limited by the inner diameter of the capillary and small volume of sample injected. Such low sensitivity has hampered the use of this method in clinical drug monitoring. However, the sensitivity was enhanced by using a simple stacking method for the determination of trace lovastatin in biologic fluids.

The CZE method was developed for the separation and determination of lovastatin as active ingredient in the red yeast rice product [112]. Prior to determination, lovastatin was extracted from capsule by ethanol. In this study, high pH (10.5) was selected in order to convert lovastatin to its acidic form completely. However, earlier reported studies revealed that lovastatin and lovastatin hydroxy acid are the two main components which contribute to up to 90% of the total quantity of monacolins in the read yeast rice [13]. Hence, the main disadvantage of the proposed CE method is that the content of the main components contributing to the pharmacology effect in red yeast rice supplement was not determined individually.

Only one CE method for the analysis of simvastatin is available till date [113]. This method was developed for the quanitication of both lovastatin and simvastatin in pharmaceutical dosage forms.

In the literature, CZE method has been reported for determination of atorvastatin [114]. The separation was optimized on capillary, but it was further miniaturized to a microchip platform with linear imaging UV detection. Even though CE is a rather good alternative for evaluation of impurity profile and enantiomeric purity of a drug, it is not enough applied. Therefore, we have developed a new MEKC method for separation and simultaneous quantitation of atorvastatin and its related substances diastereomer-atorvastatin, desfluoro atorvastatin, atorvastatin methyl ester and atorvastatin lactone [115]. The separation was carried out in an extended light path capillary in order to improve sensitivity at applied voltage of 30 kV using a background electrolyte consisting of 10 mM sodium tetraborate buffer pH 9.5, 50 mM SDS and 20% (*v/v*) methanol. Separation of neutral compounds from each other requires partitioning into charged micelles that migrate at a different rate from the EOF. The addition of methanol to the running buffer resulted in a very effective choice to achieve resolution between the peaks of charged substances adjacent to atorvastatin as well as the peaks of neutral drug-related substances. Linear calibration curves were established over the concentration range 100-1200 μg/mL for atorvastatin and 1.0-12.5 μg/mL for related substances. The applicability of the proposed MEKC method to the assay of atorvastatin in the presence of its related substances was investigated by analyzing the bulk drug provided by different manufacturers and various commercial formulations. The use of very low volumes of electrolyte (μL) and samples (nL) make the new MEKC procedures very interesting for determination of atorvastatin, purity evaluation and quantification of drug-related substances in a single analysis. The drawback of the proposed MEKC method is lower sensitivity compared to one obtained by RP-LC method for the same related substances [45]. The published UPLC method for simultaneous determination of atorvastatin and fenofibrate has better sensitivity and runtime of 3 min but the linearity, LOQ and LOD was established only for atorvastatin lactone [43].

CE method was also developed for the separation and simultaneous determination of atorvastatin and amlodipine in their combination formulations [116]. Degradation products produced as a result of stress studies did not interfere with the detection of both drugs and the assay can thus be considered stability indicating.

The CE method was developed for the enantiomeric purity determination of fluvastatin enantiomers [117]. Its principle involves the formation of diastereoisomer complexes after addition of neutral cyclodextrin to the running buffer. Fluvastatin enantiomers were separated on an uncoated fused silica with 100 mM borate solution containing 30 mg/mL of (2-hydroxypropyl)-β-cyclodextrin as running buffer and fenoprofen as an internal standard. The limit of detection and quantification for (+)-3R, 5S and (-)-3S, 5R-fluvastatin were 1.5 μg/mL and 2.5 μg/mL, respectively. Compared to chiral LC separations, CE analyses are cheaper (no chiral column, no solvent, low consumption of chiral selector) and peak efficiencies are higher by one order of magnitude.

There is only one CE method for quantification of rosuvastatin [118]. Currently, for rosuvastatin only a limited number of analytical methods are reported in literature. This is due to the fact that rosuvastatin is a new statin introduced in the EU in 2002 and approved in the US in 2003.

Using neutral β-cyclodextrin as chiral selector, the CZE method has been established for the chiral separation of pitavastatin calcium enantiomers [119]. Pitavastatin is a novel statin that potentially represents an important addition to the cardiovasculary therapy. In view of this, simple and efficient capillary electrophoretic methods for the determination of rosuvastatin and pitavastatin are highly required.

5. Simultaneous analysis of statin drugs

Since statins differ in their structure, analytical methods for their determination are developed individually. In fact, since statin drugs are never co-administered together during treatment of hyperlipimidemia, some authors even argued that there is no need for their simultaneous analysis. However, lately papers have been published that propose analytical methods that enable separation, identification and quantitative determination for two and even all six statins simultaneously in a single run. This kind of method would allow determination of any statin available on the market without the need of developing a new, separate, individual method for each statin, and could by used for simultaneous analysis of pharmaceutical dosage forms or in routine clinical monitoring.

A HPTLC method was published using precoated silica gel 60F 254 aluminum sheets and detection carried out at 239, 238 and 310 nm for determination of simvastatin, pravastatin and rosuvastatin in tablet dosage forms, respectively [120]. Far to our knowledge first HPLC-PDA method for simultaneous analysis of atorvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin was reported for determination in pharmaceutical formulations and *in vitro* metabolism studies [121]. An uncommon gradient method using 3 mobile phase reservoirs was employed. Downside of the method is relatively long analysis time of 40 minutes and fluvastatin not being included in the simultaneous analysis.

An interesting method for pharmaceutical analysis of atorvastatin, simvastatin and lovastatin using a charged aerosol detector (CAD) was published [122]. CAD is a universal detector for HPLC that operates regardless of the physiochemical and spectral properties of non-volatile analytes. It can provide data complementary to UV or MS detectors. The eluent from the HPLC column is first nebulized and then charged. A highly sensitive electrometer generates a signal proportional to the analyte quantity. Although CAD is considered as a non-linear detector, the authors found a perfectly linear response ($R > 0.9995$). Sensitivity of the CAD detector was two folds greater then the UV detector; LOD of atorvastatin measured with UV and CAD detectors was 0.17 μg/mL and 0.08 μg/mL, respectively.

A HPLC-UV method for quantification of rosuvastatin, atorvastatin, fluvastatin, lovastatin and simvastatin and four fibrates in pharmaceutical dosage forms was developed [123]. In this paper a simple GC-FID method was also proposed for identification of atorvastatin, lovastatin and simvastatin along four fibrates.

Two stability-indicating HPLC methods for quantitative determination of pravastatin, fluvastatin, atorvastatin and rosuvastatin in pharmaceuticals were developed [124].

Methods for their simultaneous determination in biological samples could provide easy quantification of drug level in human plasma without changes in the chromatographic procedures for individual statin. Despite the fact that these drugs seem to be structurally similar, development of the method for their simultaneous determination in complex biological samples is quite challenging task as they differ significantly in terms of solubility, polarity, stability as well as optic characteristics. Until now several analytical methods have been developed for the determination of statins in biological samples simultaneously, simvastatin and atorvastatin [83], rosuvastatin and atorvastatin [90], simvastatin, pravastatin, rosuvastatin and atorvastatin [92].

Investigation of statins in the environment has become an important issue in the last years due to their large worldwide consumption and their potential adverse effects on animal and human health. Three different preconcentration techniques including solid phase extraction, dispersive liquid–liquid microextraction and stir-bar sorptive extraction have been optimized and compared for the simultaneous analysis of statin drugs in wastewater and river water samples by HPLC coupled to quadrupole-time-of-flight mass spectrometry [125].

Due to low sensitivity of CE, three on-line preconcentration strategies were investigated for the analysis of charged and neutral statins by MEKC [126]. A background electrolyte consisting of 20 mM ammonium bicarbonate buffer (pH 8.50) and 50 mM SDS was used for the separation of all statin molecules including mevastatin. The methods were applied for the analysis of statin analytes in wastewater samples. The more frequently prescribed statins are of environmental concern. Consequently, sensitive methods for investigation of distribution of statin drugs in the environment are very valuable.

We have introduced a universal MEKC method with diode-array detection for the simultaneous and short-time analysis of lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin and rosuvastatin in a single run [127]. Base hydrolysis was used to open lactone ring of lovastatin and simvastatin, administered as lactone prodrugs, in order to transform these compounds to the corresponding acid forms before analysis. This approach offered shorter analysis time due to a decrease of the migration times of negatively charged statin drugs in comparison to neutral lactone forms. The first step in CE method development for optimizing the separation of ionisable statin molecules was the selection of the buffer pH, which determined the extent of ionization and mobility of each drug. As reported in the literature, statins with β-hydroxy acid forms have pKa values between 4.1 and 4.6 [128] and statin molecules are completely in anionic forms above pH 6. Surfactant was added to the electrolyte to improve the selectivity of the separation. With SDS, negatively charged statins molecules were not strongly attracted to the micelles, and drug molecules were separated as a result of differences in their electrophoretic mobilities and lipophilicity. The addition of an organic modifier in the presence of SDS in the electrolyte solution played a key role in the separation of statin molecules. The addition of an organic modifier changes the selectivity and migration times due to the change in electrolyte viscosity, dielectric constant and the zeta potential. The SDS micelles and methanol in the concentration of 10% *v/v* added to the borate buffer (pH 9.5) were employed in order to reduce the analysis time while maintaining good resolution between all six statins. The new developed MEKC method enabled a powerful separation and simultaneous, simple and rapid determination of six statins in 5 min (Figure 3). The method developed was successfully applied to analysis of six different pharmaceutical dosage forms of statin drugs.

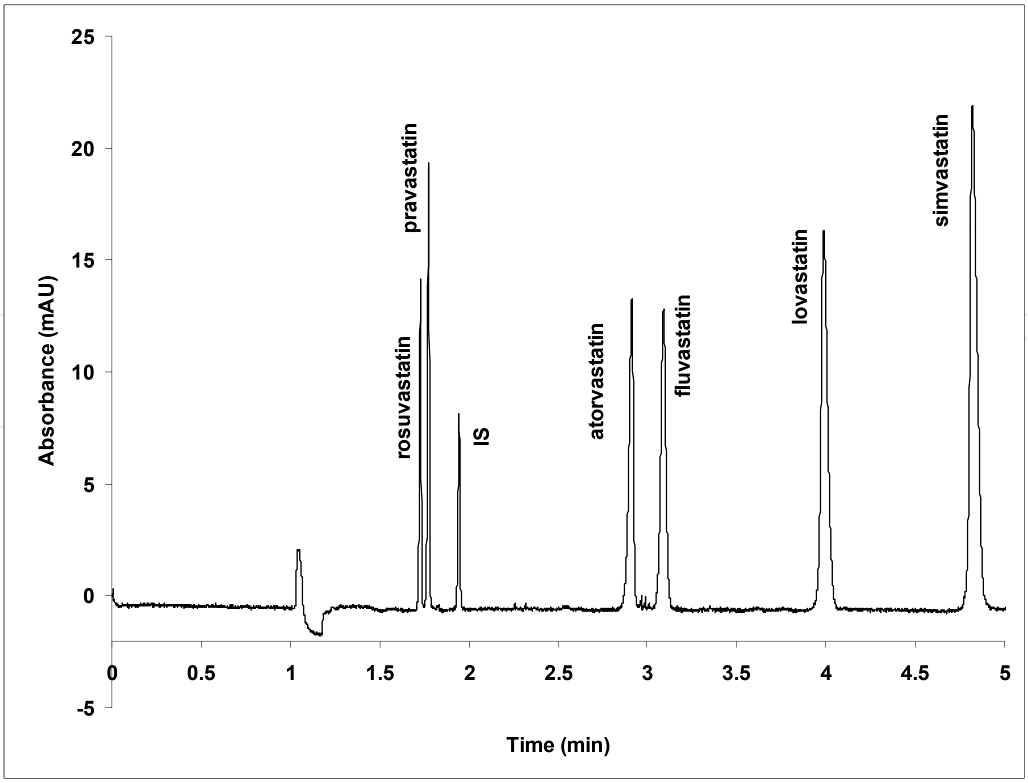

Figure 3. Electropherogram of a simultaneous analysis of statins

6. Conclusion

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. This review includes trends and advances in separation methods developed for the analysis of statin molecules with different physical and chemical properties. The chapter surveys the application of chromatographic techniques for the determination of statins in pharmaceutical dosage forms and biological samples.

Stability indicating methods and papers describing impurity profiling are discussed in this review. Special emphasis is given to sample preparation as unavoidable and delicate step in bioanalitical methods for quantification of statins and their metabolites. 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. 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 and compared with chromatographic methods.

The use of statin drugs has augmented in recent years and is expected to increase further in the years ahead because high cholesterol and cardiovascular diseases are being diagnosed more frequently. Therefore, the development of new analytical methods for commercially available statins as well as novel upcoming statin drugs will be a future challenging task for many analysts.

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