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DEVELOPMENT OF A STABILITY-INDICATING METHOD FOR EVALUATION OF IMPURITY PROFILE OF ATORVASTATIN FILM-COATED TABLETS USING CYANO COLUMN BASED ON CORE-SHELL TECHNOLOGY

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This research highlights the specificity of the new stability-indicating method developed to evaluate the impurity profile of Atorvastatin film-coated tablets. The proposed method has the ability to capture any possible changes that may occur during the stability studies over time and under different stress factors, and is selective enough to enable quality control of finished product from different suppliers of active pharmaceutical ingredient (API)/excipients.

Satisfactory critical peak resolution between specified and unspecified impurities was achieved using the fused-core shell technology and extensively endcapped diisopropyl-cyanopropylsilane stationary phase (Halo ES-CN 150 mm × 4.6 mm, 2.7 μm), with a 10 mM ammonium formate buffer pH 3.5 and acetonitrile as mobile phase. A potential worse case impurity profile was assumed by using retained samples combined with the data obtained for samples manufactured with APIs from different suppliers exposed to the forced degradation study. The mass balance for stressed samples demonstrated the stability-indicating capability of the proposed method.

Keywords: forced degradation study; Atorvastatin; stability-indicating method; cyano fused-core shell column

РАЗВОЈ НА МЕТОД ЗА ПРОЦЕНА НА ВИДОТ НА НЕЧИСТОТИИ ВО ФИЛМ ОБЛОЖЕНИ ТАБЛЕТИ АТОРВАСТАТИН СО КОРИСТЕЊЕ ЦИЈАНОКОЛОНА ВРЗ БАЗА НА ТЕХНОЛОГИЈА НА ОБВИВКА СО СПЛОТЕНО ЈАДРО

Ова истражување ја истакнува специфичноста на новиот метод за следење стабилност, развиен за да се процени видот на нечистотии во филм-обложени таблети Аторвастатин. Предложениот метод има способност да ги опфати сите можни промени што можат да се појават за време на испитување на стабилноста со текот на времето и под различни фактори на стрес, и е доволно селективен за да овозможи контрола на квалитетот на готовиот производ од различни добавувачи на активна фармацевтска состојка / помошни фармацевтски состојки.

Задоволителна критична резолуција помеѓу специфицираните и неодредените нечистотии беше постигната со користење на технологијата на обвивка со сплотено јадро и широко затворена стационарна фаза со диизопропил-цијанопропилсилан (Halo ES-CN 150 mm × 4,6 mm, 2,7 μm), со 10 mM пуфер на амониум формат со pH 3,5 и ацетонитрил како мобилна фаза. Можниот најлош случај за вид на нечистотиии беше претпоставен со користење на примероци со поминат рок на употреба, во комбинација со податоците добиени за примероците произведени со активна

фармацевтска состојка од различни добавувачи подложени на студијата за принудна деградација. Добиените вредности од студијата за рамнотежа на масата на состојките кај примероци изложени на принудна деградација ја покажаа способноста предложениот метод да е индикатор на стабилност.

Клучни зборови: студија за принудна деградација; аторвастатин; метод за следење стабилност; цијаноколона со обвивка со сплотено јадро

1. INTRODUCTION

Atorvastatin calcium, chemically (3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid calcium salt (2:1), is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which interferes with the early and rate-limiting step in cholesterol biosynthesis, thus effectively reducing the raised lipid levels of patients with hyperlipidemia or combined hyperlipidemia.[1,2] The high demand for this medicine has generated a lot of interest from generic medicine manufacturing companies. The active pharmaceutical ingredient (API) of a medicinal product is the primary constituent that governs the final cost of the medicine, guiding manufacturers to choose alternative API suppliers or change the existing API supplier either during the development phase or after development of the medicine. Each API manufacturing process holds a unique impurity profile that brings a different pattern of degradation products in the finished dosage form.[3]

From the listed impurities in the Ph. Eur. Monograph for Atorvastatin calcium trihydrate, suppliers have characterized Impurity A, Impurity B, Impurity C, Impurity G, Impurity E, and Impurity F as process related impurities, Impurity D and Impurity H as degradation impurities (Table 1).[1] Besides the listed impurities, Atorvastatin calcium was found to be a very unstable molecule, which can degrade in almost all types of stress conditions.[4]

Forced degradation studies (stress testing) are a very important tool in pharmaceutical research and development to predict long-term stability.[5] The pattern of the degradation profile can be predicted in a short period of time by exposure to environmental variables such as light, high temperatures, acidic or basic conditions, humidity, and oxidative environments, while exposing possible interactions with excipients from the formulation and materials from the container/closure system.[6–9] Additionally, the data provided by forced degradation studies brings valuable information for the development of a stability-indicating method, providing a generic approach to control the diverse quality attributes of APIs/excipients from different suppliers.[10–14] In order to develop such a generic stability-indicating method for Atorvastatin film-coated tablets at the manufacturer site, a research was conducted using retained samples, as well as forced degradation studies. During this research, the overlap of unspecified impurities co-eluting with impurity B was detected, revealing the risk of reporting false out of specification (OOS) results even though impurity B, as an in-process impurity, is not listed as a degradation impurity and would not therefore be expected to increase during formal stability studies.

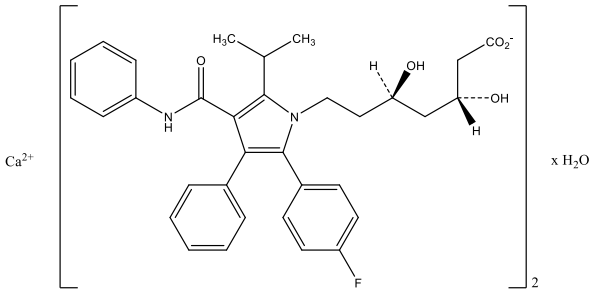
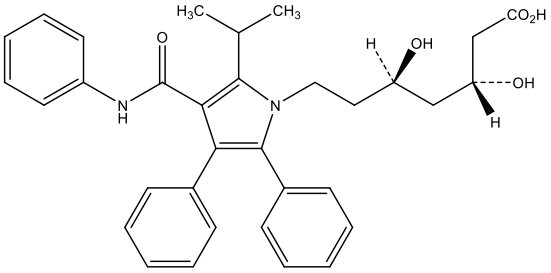
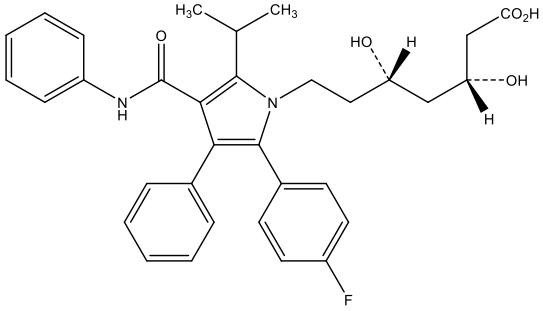
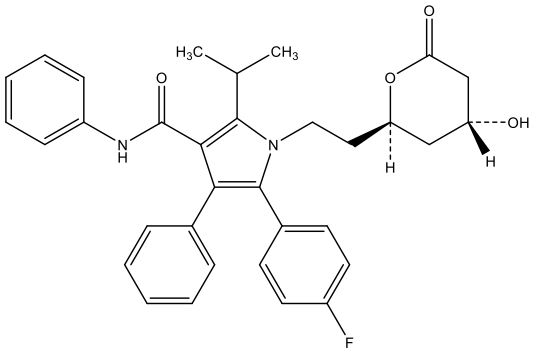
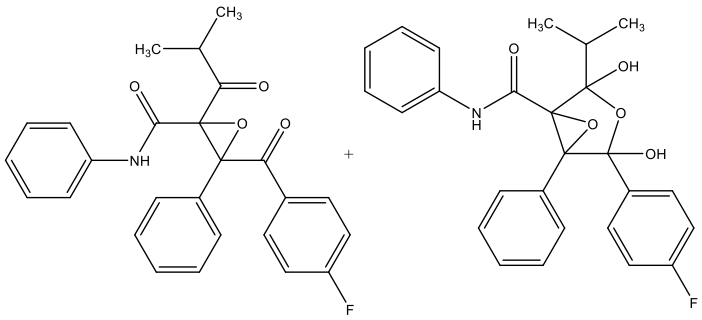
Numerous HPLC analytical methods have been reported for the determination of Atorvastatin and its known impurities. As a hydrophobic molecule, mostly columns recommended for the evaluation of Atorvastatin impurity profile are C18 or C8.[15–20] However, the overlapping peaks co-eluting with impurity B during the predictive stability indicating experiments could not be separated using the commonly used methods on C8 and C18 columns.

Given the promising and improved separation of acids, bases, and neutral compounds on fused-core technology stationary phases, where particle design exhibits very high column efficiency due to the shallow diffusion path in the 0.5-micron thick porous shell and the small overall particle size of 2.7-microns,[21] method development was set towards different stationary phases. The sterically protected, extensively endcapped diisopropyl-cyanopropylsilane stationary phase provides a stable, reversed-phase packaging that often exhibits different selectivity compared to straight-chain alkyl phases.[22]

The main goal of this research was to develop and validate a specific and selective analytical method to evaluate the impurity profile of Atorvastatin film-coated tablets, using a generic approach covering all risk scenarios that may arise during real-time stability, including changes of API/excipient suppliers.

T a b l e 1

Structures and names of Atorvastatin calcium and its related impurities

Anhydrous atorvastatin calcium	
A: (3*R*, 5*R*)-7-[2,3-diphenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid (defluoroatorvastatin)	
B: (3*RS*, 5*SR*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid	
H: (4*R*, 6*R*)-6-[2-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]ethyl]-4-hydroxyoxan-2-one	
D: Mixture of (2*E*,3*E*)-3-(4-fluorobenzoyl)-2-(2-methylpropanoyl)-*N*,3-diphenyloxirane-2-carboxamide (D1) and (1*E*,2*E*,4*E*,5*E*)-4-(4-fluorophenyl)-2,4-dihydroxy-*N*,5-diphenyl-2-(propan-2-yl)-3,6-dioxabicyclo[3,1,0]hexane-1-carboxamide (D2)	

2. MATERIALS AND METHODS

2.1. *Chemicals and standards*

Atorvastatin calcium trihydrate CRS (purity 95.2 %), Atorvastatin Impurity A, Atorvastatin Impurity B, Atorvastatin Impurity C, Atorvastatin Impurity G, and Atorvastatin Impurity D were provided by European Directorate for the Quality of Medicines and Health Care Council of Europe (EDQM- Strasbourg, France). Atorvastatin Impurity H (Atorvastatin Lactone) produced by LGC Im Biotechnologiiepark TGZ II D-14943 Luckenwalde, Germany, was used.

Atorvastatin active substance samples with certificates of suitability to the monographs of the European Pharmacopeia (CEP), received from DSM Sinochem Pharmaceuticals Pvt. Ltd., India, were used.

Retained samples of Atorvastatin film coated tablets 10 mg were used, with expired date of use at the time of analysis.

The analyzed pharmaceutical dosage form used for forced degradation study was film-coated tablets containing 10 mg of atorvastatin (Alkaloid AD Skopje, Republic of N. Macedonia).

HPLC grade acetonitrile, ammonium formate, formic acid, analytical grade sodium hydroxide, hydrochloric acid, and hydrogen peroxide were purchased from Merck (Darmstadt, Germany).

Water was purified by Werner water purification system, obtained in-house at Alkaloid AD Skopje, Skopje, Republic of N. Macedonia.

Regenerated cellulose membrane syringe filters with pore size 0.45µm, were purchased from Phenomenex (Torrance, CA. USA).

2.2. *Instrumentation*

Agilent Technologies 1260 Liquid Chromatography System (Agilent Technologies, USA) equipped with a Binary pump (G1312B), a column compartment (G1316A), autosampler (G1367E) and photo-diode array detector (G4212B) was used. Instrument control, data acquisition and processing were performed by using Thermo Scientific Dionex Chromeleon Chromatography data system version 7.2 SR5.

A reverse phase HALO 90 Å ES-CN (150 mm × 4.6 mm, 2.7 µm particle size) (Advanced Materials Technology, Wilmington, USA) was used for achieving the separation of all compounds. The photostability study was carried out in a photostability chamber (Suntest CPS+, Artisan Technology Group). The thermal stability study was carried out in a dry air oven (SANYO-MOV 212). Thermal/humidity study was carried out in pharmaceutical stability chamber (WEISS WK1500).

2.3. *Chromatographic conditions*

The chromatographic separation was achieved on HALO ES-CN column 150 mm × 4.6 mm, 2.7 µm particle size using buffer (10 mM ammonium formate, pH 3.5) as mobile phase A and acetonitrile as a mobile phase B. The LC gradient program was set as (*T* min/% acetonitrile) = 0/32, 35/38, 55/48, 75/70, 80/80, with a post run time of 10 minutes. The autosampler temperature was maintained at 4 °C and column compartment temperature was maintained at 30 °C. The detection wavelength was set at 244. Flow rate was 0.5 ml/min, and injection volume was 10 µl. A mixture of acetonitrile and water in the ration of 60:40 (*υ*/*υ*) was used as a diluent.

2.4. *Standard and sample preparation*

A solution of Atorvastatin calcium prepared at a concentration of 0.001 mg/ml was used for quantitative determination. Mixture of Atorvastatin calcium in concentration of 0.5 mg/ml and Atorvastatin Impurity B in concentration of 0.001 mg/ml was used as a resolution solution to determinate system suitability criteria. The stock solution of each impurity was prepared at a concentration of 0.05 mg/ml in the diluent. A mix solution of all impurities was also prepared at a concentration of 0.001 mg/ml. The concentration of Atorvastatin calcium in samples described in this study was 0.5 mg/ml.

The stress conditions, employed for degradation studies to verify the specificity and the stability indicating power of the proposed method, included acidic, alkaline, oxidative, thermal, thermal/humidity and photolytic conditions. For acid degradation, samples and placebo were treated with 1 M hydrochloric acid for 30 minutes at 60 °C, and then neutralized with sodium hydroxide. For alkali degradation, samples and placebo were treated with 1 M sodium hydroxide for 30 minutes at 60 °C and then neutralized with hydrochloric acid. Oxidative degradation was carried out at room temperature with 9 % hydrogen peroxide solution for 45 minutes. Samples and placebo were subjected to thermal degradation by keeping at 105 °C for 50 hours. After 50 hours, samples were cooled to room temperature and analyzed with proposed method. Samples and placebo were sub-

jected to thermal/humidity degradation by keeping directly exposed to 40 °C/75 % RH and after 30 days analyzed as per proposed methodology.

Photo stability study was conducted according to ICH guideline Q1B "Photo stability testing of New drug Substances and Products" – Option 1. Samples and placebo were exposed in a single layer, alongside with protected samples (wrapped in aluminum foil) used as a dark control to evaluate the contribution of the thermally induced change to the total observed change. Samples and placebo were exposed to light providing an illumination of 1.2 million lux hours (7.1 h) and an integrated near ultraviolet energy of 200 Wh/m^2 (2.9 h) and then exposed to five times increased irradiance dose (35 h).

Samples were analyzed for assay of API and assay of related and degradation products according to the optimized chromatographic conditions.

2.5. *Method validation*

The described method was validated according to ICH guidelines, to evaluate the method for specificity, linearity, accuracy, precision, recovery, limit of detection and quantification, robustness, and stability of solutions.[23]

Method specificity was established by peak purity determination using LC-UV (PDA detection).

Linearity was determined by series of three injections of Atorvastatin standard, Impurity A, Impurity B, Impurity C, Impurity G, Impurity H at seven different concentration that span 10 % (0.0001 mg/ml) – 500 % (0.005 mg/ml) of the standards working range. Linearity for Impurity D was determined by series of three injection of Atorvastatin Impurity D at six different concentrations that span 25 % (0.00025 mg/ml) – 300 % (0.003 mg/ml). The peak area versus concentration data was plotted for linear regression analysis. The correlation coefficient of regression, slope, intercept and percent *y*-intercept of the calibration curve were computed.

Relative response factor (RRF) was determined by series of three (and six) injections of mixed standard of impurities (Impurity A, Impurity B, Impurity C, Impurity G, Impurity H, Impurity D) and Atorvastatin, at four different concentrations that span from 25 % – 150 % of standard's working range. RRF was calculated as ratio of the responses of the impurity and Atorvastatin at the same concentration level as the ratio between the slopes obtained from individual linearity plots. For RRFs in the range from 0.8 and 1.2, no correction factor for impurity is used, and for RRFs out from 0.8 – 1.2 range, the correction factor of impurity is calculated as reciprocal value of the RRF.

Accuracy was determined using spiked placebo with known quantities of Atorvastatin, Impurity A, Impurity B, Impurity C, Impurity G, Impurity H and Impurity D at four different concentration levels (at limit of quantification, 50 %, 100 % and 150 % of working concentration), in triplicate. The percentage recovery at each level for each impurity was calculated.

The repeatability of the system was shown by six replicate injection of the standard solution in concentration of 0.001 mg/ml for Atorvastatin. Repeatability of the method was shown by six sample solutions prepared individually using single batch of tablets and analyzed as per analytical procedure. In order to express the precision between laboratories, the reproducibility was performed using six sample solutions prepared according to analytical procedure on single batch of tablets as per test method in different laboratories. The evaluation was made using Wätzig equivalence test.

The DL (limit of detection) and QL (limit of quantification) of Atorvastatin calcium and its impurities were determined by diluting their stock solutions to known concentration solutions that would yield a signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was carried out at the QL level by preparing six individual preparations of Atorvastatin and its impurities at the QL level, and by calculating the percentage RSD for the areas of Atorvastatin and its impurities.

The robustness of the method was evaluated by conducting 11 experiments using Plackett-Burman design where four factors have been analyzed on two levels. The most important factors having the greatest impact on the system response identified were column temperature (in range of 28 °C–32 °C), mobile phase molarity (lower limit 9 and upper limit 11), pH of the mobile phase (in range from 3.3 to 3.7) and evaluating two different column suppliers.

The following criteria were taken to evaluate the robustness of the method: asymmetry (As), number of theoretical plates (NTP) of Atorvastatin peak used for quantification of samples (c = 0.001 mg/ml) and resolution between impurity B and Atorvastatin in resolution solution. MODDE® software (Umetrics, Sweden) was used to generate the experiments, statistically process the results and interpret them. The experiments were performed according to the experiment matrix generated by the software itself. For each of the 11 ex-

periments, 4 injections (diluent, standard solution, resolution solution and sample) were performed. Plan of the experiments defined by the Plackett-Burman design is given in Table 3, together with the obtained results.

The stability of the standard solution for quantification, resolution solution and sample solution was evaluated by analyzing the same sample immediately after preparation and at different time intervals by keeping the solutions in the autosampler at 4 °C.

3. RESULTS AND DISCUSSION

A new stability-indicating method to evaluate the impurity profile of Atorvastatin film-coated tablets was developed, intended for stability studies of the medicinal product over time, as well as the quality control of finished product manufactured using raw material from different suppliers of API/excipients.

The method development was performed in a generic way, covering risk-based scenarios of possible degradation profiles that may occur from APIs supplied by different manufacturers. This was achieved through the analysis of retained samples (with expired date), forced degradation studies (exposing samples to different stress conditions: thermal/humidity degradation, thermal degradation, photolytic degradation, acid hydrolysis, base hydrolysis and oxidative degradation), and using different batches of finished product produced using APIs from different suppliers.

3.1. *Method development and optimization*

The European Pharmacopoeia monograph of Atorvastatin calcium trihydrate[1] prescribes the HPLC method for impurities testing of atorvastatin active substance using an octylsilyl C8 column and a mobile phase composed of acetonitrile, tetrahydrofuran and ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid. There are also a few published HPLC analytical methods for the quantification of atorvastatin and its impurities using a C18 or C8 column, with tetrahydrofuran in the mobile phase composition,[15–20] which was to be avoided in our research due to its toxicity and instability. The method published by Vukkum[20] using simple gradient elution with 0.1 % trifluoroacetic acid and acetonitrile and a Zorbax Bonus RP column, reported good resolution between critical pairs in a short run time of 25 min. These high resolutions were achieved with peaks of impurities present at low concentrations and only between specified impurities of the Atorvastatin substance. However, these methods were not applicable of evaluating the impurity profile of Atorvastatin film-coated tablets during the shelf life of the product.

In the first stage of our research, method development started using different C18 columns such as Zorbax Eclipse XDB C18, Zorbax Bonus, Synergy polar RP, Restek Raptor ARC-18 and Poroshell 120 EC C18, with the best chromatographic result being achieved using the Zorbax Eclipse XDB C18 150 mm × 4.6 mm, 3.5 μm column. The initial focus was on choosing a selective and simple mobile phase, and it was decided to use acetonitrile as an organic phase, due to the better peak symmetry compared to methanol. Ammonium formate was chosen as a water buffer because of its low UV cut-off value, simple preparation, and ESI-MS compatibility. The effect of the molarity and pH of the buffer solution on separation efficacy were further investigated. It was observed that molarity variations between 8.0 mM and 15.0 mM did not significantly affect retention, thus it was decided to use 10 mM ammonium formate, which resulted in good chromatographic peak shape and baseline flatness. Considering the p*K*a value of Atorvastatin calcium ($pK_a = 4.6$) and the structural similarity of related compounds, the best resolution for specified degradation impurities was achieved with pH 4.0.

The required resolution between specified impurities was achieved when using this method to evaluate medicinal products with an expired date of use, but the unspecified and specified impurities were not separated. Furthermore, it was observed that the peak eluting on the expected retention time of impurity B (RRt 0.97) (Fig. 1) had an asymmetrical shape due to a shoulder peak, as well as interfering spectra within the peak, which indicated that there was another impurity/ies overlapping with this specified impurity. This overlapping of unspecified impurity/ies with impurity B explained the unexpected out of specification result (other unspecified impurities should be less than 0.2 %, in accordance with ICH guidelines), even though impurity B is a process related impurity and should remain stable during stability studies.

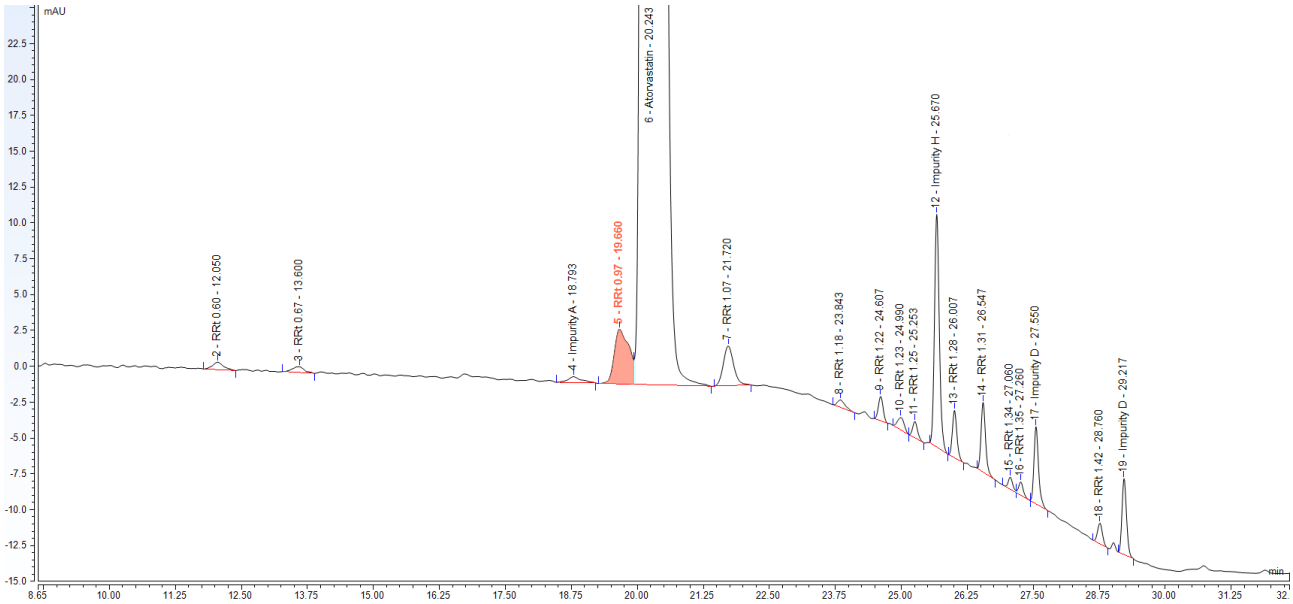


Fig. 1. Chromatogram of Atorvastatin 10 mg film-coated tablets with expired date analyzed with Zorbax Eclipse XDB C18

The same overlapping was observed with several tested C18 and C8 columns, and other types of column were therefore checked. Because of the inadequate separation on common reverse phase columns, different CN columns (Zorbax SB CN, Supelco ES CN, Halo ES-CN) were investigated. It was decided to continue the investigation on a HALO ES-CN 150 mm × 4.6 mm, 2.7 µm particle size column, which achieved the best selectivity for the unspecified impurities. HALO ES-CN is a high-speed, high-performance liquid chromatography column based on fused core particles. This particle design exhibits very high column efficiency due to the shallow diffusion path in the 0.5-micron thick porous shell and the small overall particle size of 2.7-microns. Other advantages of using fused core technology include the improvement of kinetic performance as faster separation, higher resolution, efficiency, reduced backpressure and also improving selectivity, retention, loading capacity and peak shape. The sterically protected, extensively endcapped diisopropylcyanopropylsilane stationary phase of this column provides a stable, reversed-phase packaging that often exhibits different selectivity compared to straight-chain alkyl phases, such as C8 and C18, and can be used for the separation of acid, bases and neutral compounds.

Using the same chromatographic conditions as in the first stage of the research, the separation of unspecified impurities from impurity B was noted, but with critical resolution. Considering the pK_a value of Atorvastatin calcium (pK_a = 4.6) and the structural similarity of related compounds, changing the pH value of the buffer solution was the most critical for unknown degradation impurities and the best resolution between all detected impurities was achieved with pH 3.5. The column temperature also had an impact on the separation of the peaks, thus, the column compartment temperature was set to 30 °C for the best resolution between unspecified from specified impurities. A gradient elution was necessary and played a vital role in achieving sufficient resolution between all impurities. The gradient program was optimized as (*T* minutes / % acetonitrile) = 0/32, 35/38, 55/48, 75/70, and 80/80, with a post run time of 10 minutes. In the optimized conditions (Fig. 2), Atorvastatin calcium was well separated from impurity B and impurity B was well separated from the two unspecified degradation impurities that occur due to thermal/humidity degradation, which was the critical reason for using a fused core cyano column.

Comparing the results obtained with both methods it was confirmed that the overlapping peak with RRt 0.97 in the chromatogram obtained with the first method (Fig. 1) using the Zorbax Eclipse XDB C18 column, which comprised the sum of four peaks, was separated on the HALO ES CN column (RRt 0.88, 0.89, 0.94, and Impurity B) (Fig. 2).

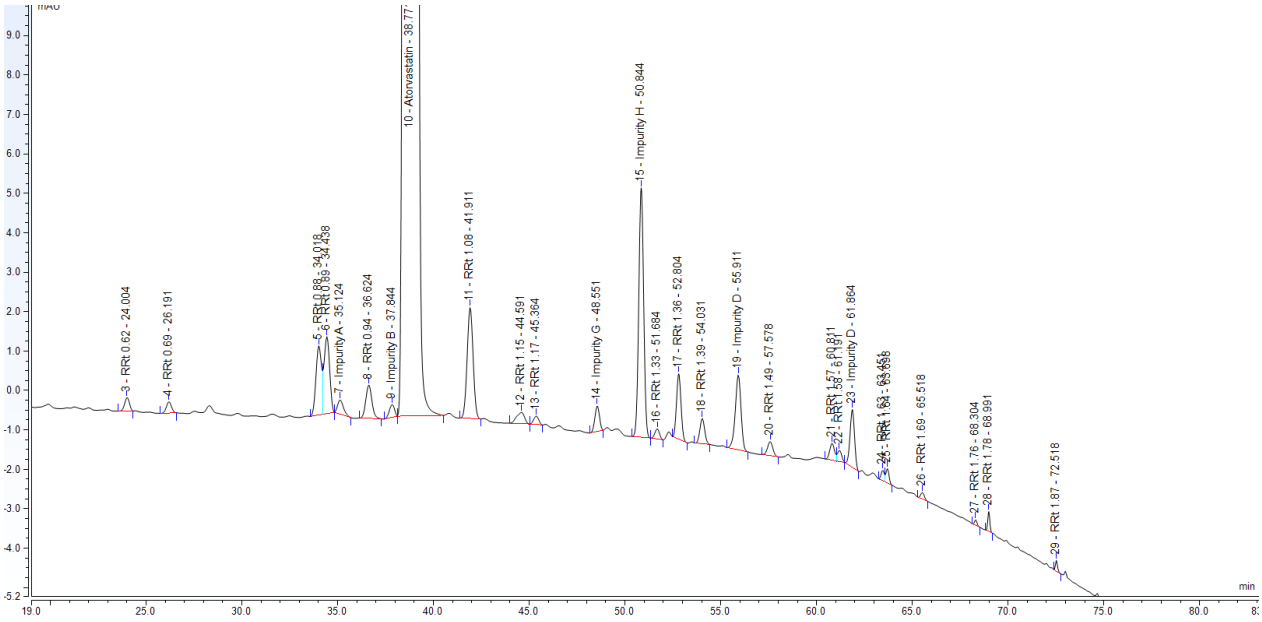


Fig. 2. Chromatographic impurity profile of Atorvastatin 10 mg film-coated tablets with expired date of use analyzed with fused-core CN column

3.2. *Results of forced degradation study*

The purpose of the degradation studies was to challenge the ability of the proposed stability-indicating method to separate, detect, identify and quantify degradation products. The medicinal product was exposed to the following stress conditions: *photolytic degradation, thermal degradation, acidic, basic, oxidative degradation and thermal/humidity degradation.* The results obtained from this study demonstrated the specificity and the stability-indicating power of the optimized method.

Figure 3 is presenting overlaid chromatograms with impurity profile of Atorvastatin 10 mg film-coated tablets for all performed stress conditions: initial analysis, thermal degradation, acid hydrolysis, oxidation with H_2O_2, base hydrolysis, thermal/humidity degradation and photo degradation.

Thermal degradation led to a higher level of Ph. Eur. impurity H and Ph. Eur. impurity D, as well as unspecified impurities with RRt 0.73, 0.88, 0.89, 0.94, 1.17, 1.25, 1.33, 1.36, 1.37, 1.38, 1.55, 1.57, and 1.61. On exposure to *acidic* solution, the finished product degradation showed Ph. Eur. impurity H as the main degradation product, as well as impurities with RRt 1.53, 1.29, 1.12, 0.88, 0.89, and 0.95. A significant increase of impurities was observed with *oxidative degradation:* impurities with RRt 0.67, 0.73, 0.85, 0.88, 0.89, 1.32, 1.33, 1.45, 1.64, and Ph. Eur. impurity D were increased after exposure to H_2O_2, while Ph. Eur. impurity H remained unaffected. Following *base degradation*, the most evident unspecified impurities had a RRT of 0.88 and 0.89, while Ph.Eur. impurities H and G remained unaffected and Ph. Eur. impurity D was not detected. After exposure of *thermal-humidity* conditions, the Ph. Eur. H and D increased, along with the unspecified impurities with RRt 0.88, 0.89, and 0.95. During *photo degradation* with 35 h overexposure, Ph. Eur. impurity D (sum of both isomers) increased and unspecified impurities formed with RRt 0.67, 0.73, 0.88, 0.89, 0.94, 1.07, 1.18, 1.33, and 1.35. Atorvastatin impurity A, impurity B and impurity G remained stable through all stress conditions, and Atorvastatin impurity C was not detected at all. The impurities with RRt 0.88 and 0.89 were degradation products in almost all of the forced conditions: after exposure to H_2O_2 significant degradation was observed and the concentration of these impurities rose above the specification limit, while these impurities were slightly degraded upon thermal/humidity and base hydrolysis conditions. No changes in the physical properties (appearance and color) were observed at any of the stress conditions.

The *peak purity UV-match value* for all impurities was above 950, which indicated the selectivity of the method.

The *mass balance* in the stressed samples (sum of assay value and levels of degradation products) reached the required criteria of 98–102 %, thus the suitability of the analytical method for the examination of degradation products was confirmed.

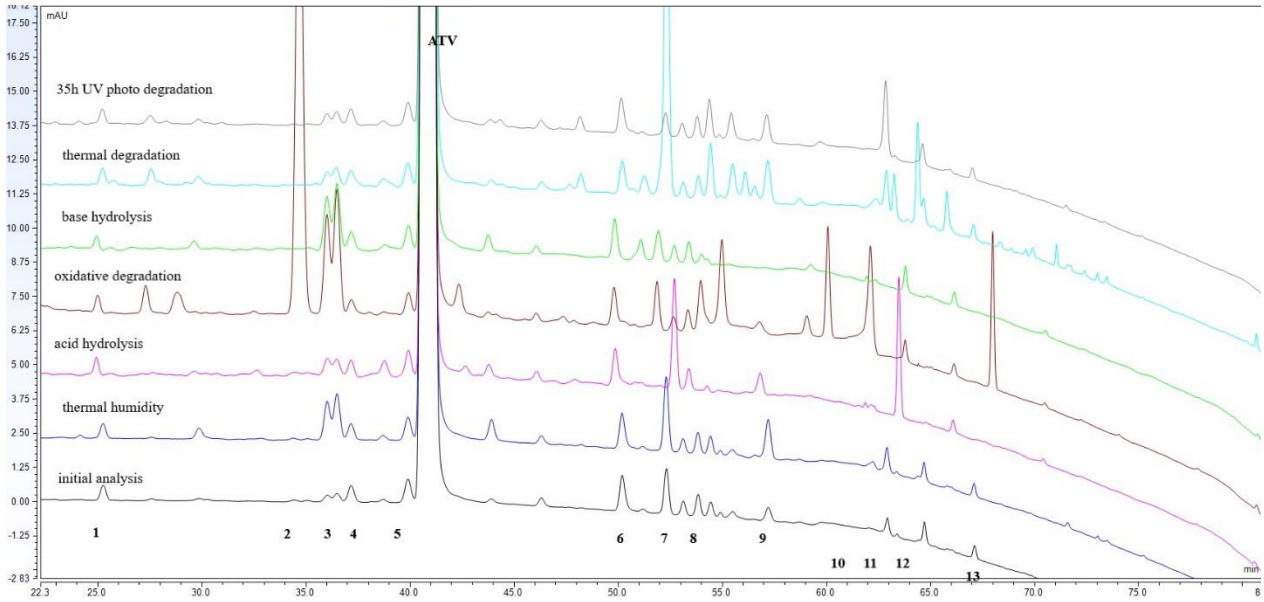


Fig. 3. Overlaid chromatograms from all degradation conditions where 1 – Atorvastatin imp. F; 3 – RRt 0.88 and 0.89; 4 – Atorvastatin imp. A; 5 – Atorvastatin Imp B; 6 – Atorvastatin Imp. G; 7 – Atorvastatin Imp. H; 9 and 11 – Atorvastatin Imp. D; 2, 8, 10, 12, 13 unknown degradation impurities

3.3. *Discriminating ability of the proposed method*

The proposed method was used for the analysis of three batches of finished product, which were produced using APIs from different suppliers (marked as A, B and C on Figure 4). The comparative impurity profile data showed different degradation pattern and the selectivity of the method was confirmed with the high values obtained for the peak purity match. The impurity profile of the medicinal product with API from supplier C was significantly different to that of the products with APIs from suppliers A and B. It is notable that impurity B is detected only in the impurity profile of the product with API from supplier C, while the impurity with RRt 1.89 (declared to be process related and a degradation impurity) is only present in the impurity profile of product with API from supplier B. The impurity profile around the peak of impurity H is also different between different API suppliers.

The ability of the proposed method to reveal different impurity profiles from products manufactured with APIs from different suppliers confirms the discriminatory power of the proposed method.

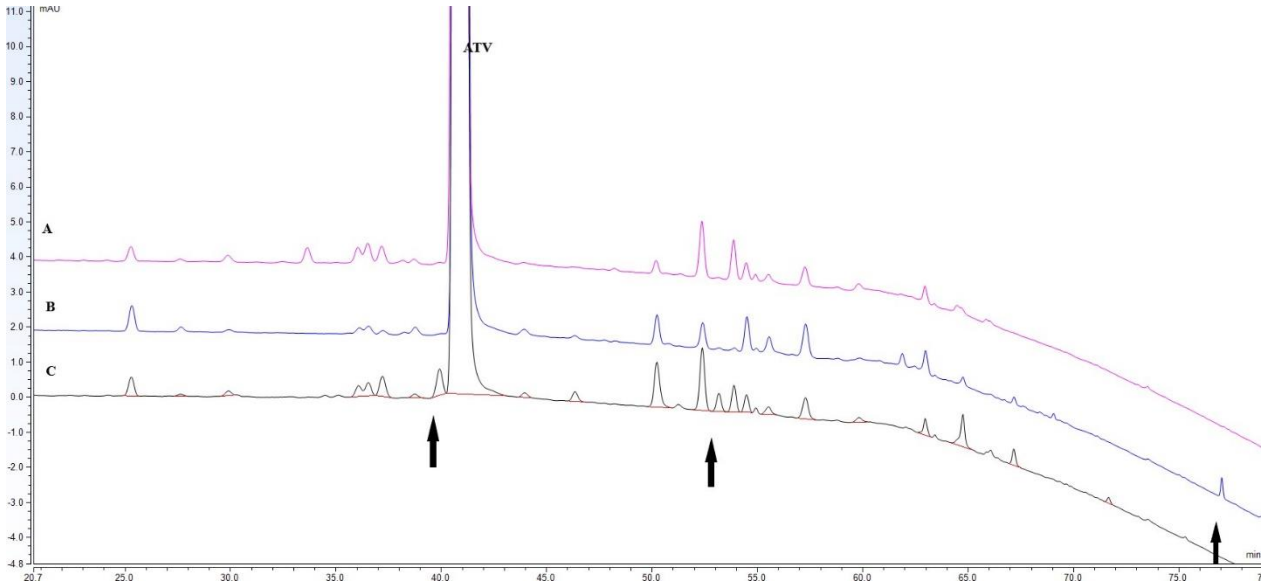


Fig. 4. Comparative data on impurity profile of Atorvastatin film coated tablets manufactured with API from three different suppliers: A, B and C

3.4. *Validation*

The proposed method was validated in terms of specificity, linearity, range, accuracy, quantification limit, detection limit (Table 2); precision; robustness (Table 3, Fig. 5), and the stability of solutions and was found to meet the predetermined acceptance criteria.

There was no interference due to diluent and placebo, and no peaks were observed at the retention times of known related substances or the atorvastatin peak, so the method was found to be specific.

The reproducibility of the method was confirmed, the *F* values for impurity H, impurity D, any other unspecified impurities and the total impurities were: 0.30, 2.24, 0.11 and 0.08, respectively ($DF_1 = n_1–1$ and $DF_2 = n_2–1$); $F = 5.05$ for $\alpha = 0.005$)

The robustness of the method was assessed by making small, deliberate changes to the chromatographic conditions and observing the effect of these changes on the system suitability parameters. The evaluation involved 11 experiments using the Plackett-Burman design with software MODDE® Go (Umetrics) where 4 factors were analyzed on two levels. The results obtained for each experiment are shown in Table 3.

T a b l e 2

Results obtained from testing different parameters during validation of the analytical method

Parameter	**ATV**	**Imp. A**	**Imp. B**	**Imp. C**	**Imp. G**	**Imp. H**	**Imp. D**
Linearity							
Concentration range μg/ml	0.1–5	0.1–5	0.1–5	0.1–5	0.1–5	0.1–5	0.25–3
Slope:	742.027	643.077	619.167	706.173	669.224	834.519	707.870
Intercept:	–0.01	–0.02	–0.01	–0.01	–0.01	–0.01	–0.04
Correlation coefficient:	1	1	1	1	1	1	1
Accuracy							
QL	96.24	100.22	95.54	98.92	97.30	96.58	92.44
50 %	99.41	101.35	101.68	101.22	100.57	99.33	92.17
100 %	104.26	103.86	103.66	104.09	103.95	98.27	92.39
150 %	101.96	102.64	102.02	103.36	103.01	97.41	99.48
Sensitivity							
DL (μg/ml):	0.05	0.05	0.05	0.05	0.05	0.05	0.2
QL (μg/ml):	0.1	0.1	0.1	0.1	0.1	0.1	0.25
System suitability							
RRt	1	0.91	0.98	1.06	1.23	1.28	1.40/1.54
As	1	1	1	1	1	1	1
N	87229	71880	81987	117428	221175	235646	215015/452337
Rs	4.82	4.89	1.77	14.59	5.08	10.63	13.23

RRt – relative retention time, As – symmetry factor, N – number of theoretical plates, Rs – resolution, DL – detection limit, QL – qualification limit

T a b l e 3

Plackett-Burman design for verification of robustness of proposed method

Experiment No	Column temperature (*T*)	Molarity of mobile phase (ammonium formate)	Column*	pH	Resolution ImpB/Atorvastatin	Assymetry	NTP
N8	28	9	A	3.3	1.60	1.01	71918
N9	28	9	A	3.3	1.61	1.03	73364
N10	28	9	A	3.3	1.61	1.02	72284
N11	28	9	A	3.3	1.61	1.03	73069
N2	28	11	A	3.7	1.71	1.02	68915
N1	28	9	B	3.7	1.66	1.01	64738
N6	28	11	B	3.3	1.58	1.03	67059
N7	32	9	A	3.3	1.58	1.02	69010
N3	32	11	A	3.7	1.67	1.03	66374
N5	32	9	B	3.7	1.62	1.00	63554
N4	32	11	B	3.3	1.57	1.01	65898

*Column A: Halo ES-CN 150 mm × 4.6 mm, 2.7 μm from Advanced technologies
Column B: Supelco ES-CN 150 mm × 4.6 mm, 2.7 μm from Merck

The model was validated by the analysis of variance (ANOVA). The statistical analysis showed that the model is fitted (multiple linear regression) and thus useful in predicting the effects of the factors on the selected responses. The percent of variation of the response explained by the model $R^2 > 0.5$ in all cases and the prediction ability of the model $Q^2 > 0.5$ in all cases indicate that the model reasonably fits the experimental data. Model validity bars were > 0.25 for all three responses (resolution Impurity B/Atorvastatin, Asymmetry, NTP), indicating that the method had a good validity in the proposed setting range (the model error is in the same range as the pure error).

The qualitative contribution of each factor on the responses were analyzed by Pareto charts as shown in Figure 5. Pareto charts establish the critical value of the effect and coefficients with value of effect above one are designated as certainly significant. In this case all investigated effects are considered as insignificant.

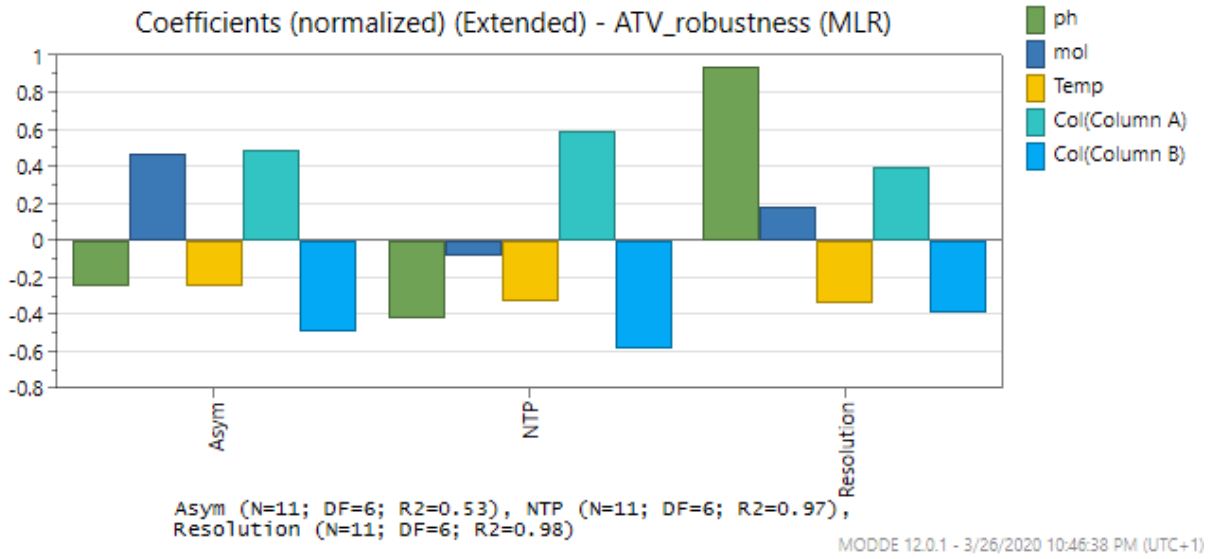


Fig. 5. Pareto chart showing the significant effects based on the observation of Plackett–Burman design for the investigated responses

According to the analysis of the values of the coefficients, the most important factors that influence the robustness of the method are column compartment temperature and the pH of the mobile phase. The temperature of the column is inversely proportional to the resolution between the peaks of impurity B and Atorvastatin, *i.e.* an increased column temperature results in decreased resolution, but not significantly. The pH value inversely affects the asymmetry and the number of theoretical plates, but is proportional to the resolution between impurity B and Atorvastatin, *i.e.* increasing the mobile phase pH value increases the resolution between the peaks. The molarity of the buffer has very little, almost insignificant impact on the parameters being monitored. Columns from different suppliers also show no change in any of the parameters being monitored.

The stability study showed that the standard solution and system suitability solution are stable for at least 138 hours at 4 °C, and the sample solution is stable for at least 12 hours at 4 °C.

4. CONCLUSION

The newly developed analytical method using an endcapped diisopropyl-cyanopropylsilane stationary phase based on fused core particles for the determination of related and degradation products in Atorvastatin film coated tablets achieves satisfying separation, spacing, and resolution between impurities and the API. The specificity and selectivity of the method is confirmed with expired samples giving the most reliable impurity profile and with forced degradation studies conducted on samples at the beginning of their shelf life and manufactured with APIs from different sources. The validation of the method and the forced degradation studies show that the method is fully stability-indicating and could be used for the impurity profile evaluation in formal stability studies of atorvastatin film coated tablets produced with different API/excipient suppliers.

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