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Improving the Current European Pharmacopoeia Enantio-Selective HPLC Method for the Determination of Enantiomeric Purity in Atorvastatin Calcium Salt Drug Substance

Francesca Romana Mammone, Daniele Sadutto, Giulia D'Ettorre, Antonina Mosca and Roberto Cirilli *

National Centre for the Control and Evaluation of Medicines, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; francescaromana.mammone@uniroma1.it (F.R.M.); daniele.sadutto@iss.it (D.S.); giulia.dettorre@iss.it (G.D.); antonina.mosca@iss.it (A.M.)
* Correspondence: roberto.cirilli@iss.it

Abstract: Atorvastatin (ATV) is a well-established lipid-lowering agent. ATV has two stereogenic centers, and of the four possible stereoisomers, only the (3*R*,5*R*) form is used therapeutically. The European Pharmacopoeia (EP) monograph 2022 for ATV calcium salt describes a normal-phase high-performance liquid chromatography (HPLC) method for the determination of enantiomeric purity in both drug substance and working standard samples, based on a 150 mm × 4.6 mm Chiralpak AD-H column. The main problems with this method are the very long analysis time and the high solvent consumption. Here, an alternative chromatographic protocol was developed using the Chiralpak AD-3 column (250 mm × 4.6 mm) packed with 3 µm silica particles instead of the 5 µm silica particles of the Chiralpak AD-H chiral stationary phase and characterized by the same polysaccharide selector, amylose-tris(3,5-dimethylphenylcarbamate). Using a mobile phase consisting of a mixture of n-hexane-ethanol-formic acid 90:10:0.1 (*v*/*v*/*v*) as the mobile phase and setting the flow rate and column temperature to 1.0 mL min^{-1} and 35 °C, respectively, a simultaneous stereo-selective separation was achieved within 35 min without observing any overlap between the enantiomeric impurity peak and peaks related to other ATV impurities. Compared to HPLC EP conditions, the analysis time to elute all the potentially related substances was faster and significantly less mobile phase volume was required. The linearity of the method has been demonstrated in the range of 4.4 µg mL^{-1} to 1000 µg mL^{-1} ($R^2 > 0.999$). At a concentration of 4.4 µg mL^{-1}, which is 0.075% of the test solution (5.8 mg mL^{-1}, as ATV free acid), the signal-to-noise ratio was found to be 20.

Keywords: atorvastatin calcium salt; chiral impurities; European Pharmacopoeia; enantio-selective HPLC; Chiralpak AD-3

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1. Introduction

The HPLC analytical methods described in the Pharmacopoeia monographs are widely used by the pharmaceutical industry to monitor the progress of the manufacture of active pharmaceutical ingredients and raw materials, and by regulatory agencies to control the quality of pharmaceutical products.

Such methods are continuously revised according to innovations in instrumentation and column technologies, and advances/optimizations in the analytical aspects of the separation process such as column, mobile phase, temperature, and sample preparation. Special attention is given to using more sustainable solvents to minimize environmental and human health impacts. This is particularly important given the millions of analytical tests performed daily in pharmaceutical and chemical laboratories around the world [1].

The process of reviewing and improving the current analytical procedures involves careful evaluation of their potential drawbacks and inadequacies and the development

of alternative key method features to move to a more consistent, robust, easy-to-use, and sustainable HPLC mode.

In this study, the European Pharmacopoeia (EP) HPLC method for the determination of enantiomeric purity in the atorvastatin (ATV) calcium salt drug substance [2] was considered for improvement.

ATV is a synthetic chiral statin designed to treat hypercholesterolemia and prevent cardiovascular disease caused by lipid disorders [3]. It is manufactured and used as a calcium salt (2:1). ATV has two stereogenic centers at C3 and C5 of the heptanoic acid scaffold. Thus, it can exist in four stereoisomeric forms: the two enantiomeric pairs (3*R*,5*R*)/(3*S*,5*S*) and (3*R*,5*S*)/(3*S*,5*R*). The active substance is the (3*R*,5*R*) stereoisomer, while the (3*S*,5*S*) enantiomer (designated IMP-E in the EP monograph), the (3*R*,5*S*) and (3*S*,5*R*) diastereomers (IMP-B) are considered impurities. Figure 1 shows the chemical structures of ATV and its stereoisomeric impurities.

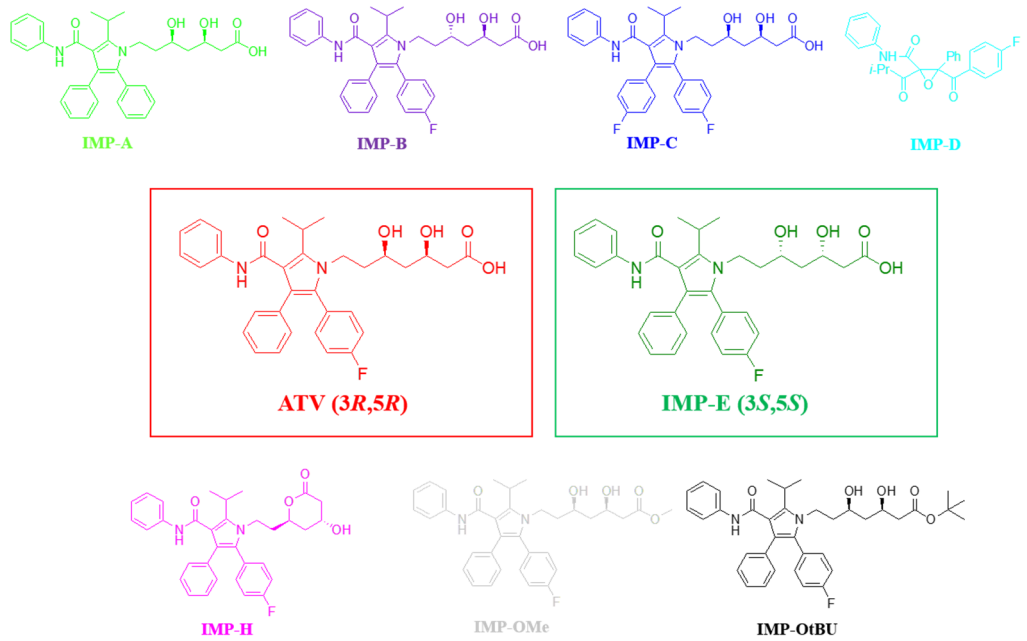

Figure 1. Chemical structures and abbreviations of atorvastatin and its potential organic impurities in the drug substance [2].

The HPLC enantioseparation described in the latest edition of the EP monograph 2022 for ATV calcium salt is performed on a 150 mm × 4.6 mm Chiralpak AD-H column using n-heptane-ethanol-formic acid (FA) 96:4:0.1 (*v*/*v*/*v*) as the mobile phase. The packing material of the Chiralpak AD-H column consists of amylose tris(3,5-dimethylphenylcarbamate) coated on 5 µm silica particles. The flow rate and column temperature are set to 1.8 mL min^{-1} and 40 °C, respectively [2]. The limit for enantiomeric impurity (IMP-E) in the drug substance is fixed at 0.15%. The chromatogram for the EP test for enantiomeric purity of atorvastatin calcium is shown in Figure S1 of the Supporting Information (SI).

The main problems of this method are the very long analysis time and high solvent consumption. The peak of ATV is expected to elute at approximately 35 min at a flow rate of 1.8 mL min^{-1}. Since the total run time is 1.2 times the retention time of ATV, 76 mL of the mobile phase is used for each analysis. Another critical aspect to consider is the potential presence of other impurities in the drug substance that could interfere with the analysis by coeluting with the enantiomer peak or eluting after the ATV peak, further increasing the analysis time.

Given the importance of the drug, it was surprising to find that only a few enantioselective HPLC methods for ATV have been described in the literature [4,5]. These methods have been developed in combination with normal-phase conditions without considering

the presence of other potential impurities and not verifying the chemo-selectivity and diastereo-selectivity of the analytical conditions.

A report from Daicel describes the normal-phase HPLC resolution of ATV on the amylose-based immobilized-type Chiralpak IA-3 chiral stationary phase (CSP) [6]. Unlike the coated-type Chiralpak AD-H CSP employed in the EP monograph, the polymeric selector (i.e., amylose tris(3,5-dimethylphenylcarbamate)) is immobilized on the 3 µm silica particles. The more robust anchoring of the amylose derivative to the silica ensures greater stability and versatility of the chiral stationary phase [7]. Using the 250 mm × 4.6 mm Chiralpak IA-3 column, the mobile phase n-hexane-ethanol-trifluoroacetic acid (TFA) 85:15:0.1 (*v/v/v*), setting the column temperature at 25 °C and flow rate of 1.0 mL min^{-1}, the enantiomeric peaks were separated from the baseline within 15 min. Again, neither the chemo-selectivity nor the diastereo-selectivity of the HPLC protocol was described, the latter referring to the ability to discriminate the diastereomeric impurity (IMP-B, Figure 1) potentially present in the drug substance [8–15].

In order to move the enantio-selective EP analysis of ATV toward faster elution times and less solvent consumption, a chromatographic method development was carried out in the present study. Two main factors were considered: the influence of the alcoholic organic modifier in the mobile phase on selectivity and retention, and column-related parameters such as column geometry and chiral material packing type.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC-grade solvents n-hexane, ethanol, n-heptane, 2-propanol, formic acid (FA) (99%), and trifluoroacetic acid (TFA) (99.5%) were supplied by Sigma-Aldrich (Milan, Italy). ATV calcium salt (99.99%) standard was purchased from the European Directorate for the Quality of Medicines & Healthcare (EDQM) (France). The impurities A (3*R*,5*R*, Ca salt, IMP-A), B (3*S*,5*R*, Ca salt, IMP-B), C (3*R*,5*R*, Ca salt, IMP-C), D (as a mixture of four stereoisomers, IMP-D), E (3*S*,5*S*, Ca salt, IMP-E), and H (IMP-H) were obtained by Sigma-Aldrich (Merck SpA) (Milan, Italy), while the impurities methyl ester (IMP-OMe) and t-butyl ester (IMP-OtBu) of ATV were kindly provided by Mylan S.p.A.

HPLC analyses were carried out on the Chiralpak AD-3 (250 mm × 4.6 mm, 3 µm), Chiralpak IA-3 (250 mm × 4.6 mm, 3 µm), Chiralpak ID-3 (250 mm × 4.6 mm, 3 µm), Chiralpak IG-3 (250 mm × 4.6 mm, 3 µm), and Chiralpak IH-3 (250 mm × 4.6 mm, 3 µm) columns (Chiral Technologies Europe, Illkirch-Graffenstaden, France).

2.2. Instruments

The HPLC system consisted of a Waters Alliance e2695 separation module, a 2998 photodiode array detector (PDA), and a Waters Alliance 30 cm column heater. Data acquisition was performed using the Empower software (version 2.0).

2.3. HPLC Operating Conditions and Partial Method Validation

HPLC analyses were carried out at a flow rate of 1.0 mL min^{-1}. The injection volume and the detection wavelength were 5 µL and 254 nm, respectively. During the preliminary screening of the enantio-selective conditions, fresh standard solutions of ATV and impurities were prepared by dissolving the analyte in 0.1 mL of methanol and diluting the solution to 1 mL with a mixture of n-hexane-ethanol 50:50 (*v/v*) (diluent) (concentration about 0.2–1.0 mg mL^{-1}).

The sensitivity of the enantio-selective HPLC method proposed in this work (column: Chiralpak AD-3 250 mm × 4.6 mm, 3 µm; mobile phase: n-hexane-ethanol-FA 90:10:0.1 (*v/v/v*); column temperature: 35 °C) was evaluated by calculating the signal-to-noise (S/N) ratio of solutions containing ATV prepared as follows: (i) an accurately weighed amount of ATV calcium salt, equivalent to about 96.7 mg of ATV free acid, was transferred into a 5 mL volumetric flask; (ii) after adding 3 mL of methanol, the resulting solution was maintained in an ultrasonic bath for 10 min and then diluted to volume with methanol (test solution,

concentration about 5.8 mg mL^{-1} as ATV free acid); (iii) 3 mL of the test solution was diluted to 100 mL with diluent (standard solution); (iv) appropriate dilutions were carried out to obtain final concentrations ranging from 4.4 µg mL^{-1} to 1000 µg mL^{-1} of standard ATV free acid. Linearity and S/N ratio were calculated in this concentration range. The concentrations of the solutions were plotted against the corresponding peak area responses of ATV free acid, and subsequently, linear regression equations were calculated. The LOD and LOQ parameters represent the concentrations of analyte that would result in S/N ratios of 3 and 10, respectively, according to the EP guidelines.

Selectivity was demonstrated to be unaffected by the presence of other impurities in the identification and/or quantification of the enantiomer impurity of ATV (IMP-E). The resolution factor (*Rs*) of adjacent peaks was the parameter evaluated for this purpose. To calculate the resolution between the peaks pertinent to the impurities E and H, 1 mg of each was dissolved with 1.0 mL of methanol. Then, 100 µL of both solutions were diluted to 10 mL with diluent (conc. = 10 µg mL^{-1}).

The precision of the method was assessed by measuring the repeatability (intra-day) expressed as relative standard deviation (RSD%). The parameters monitored were retention times and peak areas. This evaluation included multiple injections (n) of ATV samples at three different concentration levels, including the LOQ concentration (n = 6) and two other concentrations (n = 2) within the linearity range. In this way, 10 determinations were evaluated.

The robustness of the method was tested by deliberately varying conditions such as flow rate, column temperature, and ethanol content in the mobile phase.

3. Results

3.1. Enantioseparation under Normal-Phase Conditions

Five columns were compared for the HPLC resolution of ATV. These were 250 mm × 4.6 mm in geometry and packed with 3.0 µm particles containing amylose derivatives as selectors. Two of them, Chiralpak AD-3 and Chiralpak IA-3 [16,17], have the same polymeric selector, amylose tris(3,5-dimethylphenylcarbamate). They differ in the way the polymer is anchored to the silica support, with the first being physically coated and the second immobilized. Columns based on amylose tris(3,5-dimethylphenylcarbamate), either adsorbed or immobilized on a silica matrix, are also commercially available under other trade names, including Lux Amylose-1 and Lux i-Amylose-1 [18]. Chiralpak ID-3, Chiralpak IG-3, and Chiralpak IH-3 are all immobilized-type CSPs containing amylose tris(3-chlorophenylcarbamate), tris(3-chloro-5-methylphenylcarbamate), and amylose tris[(*S*)-α-methylbenzylcarbamate], respectively. To investigate the enantioselectivity of the amylose-based CSPs, the mobile phases n-hexane/2-propanol/TFA 90:10:0.1 (*v/v/v*) and n-hexane/ethanol/TFA 90:10:0.1 (*v/v/v*) were selected. The presence of 0.1% TFA in the mobile phases was found to improve peak shape and efficiency due to the acidic nature of ATV. A comparison of the chromatographic data obtained with the column temperature set at 25 °C is shown in Table 1. The results of this study indicate the following:

(i) ATV was resolved at baseline (*Rs* > 1.5) only with Chiralpak IA-3 and Chiralpak AD-3 CSPs;

(ii) Only very poor enantiomer discrimination was observed with Chiralpak IG-3 (i.e., the maximum value of *Rs* was 1.20);

(iii) Chiralpak ID-3 and Chiralpak IH-3 CSPs showed no enantioseparation;

(iv) When switching from ethanol to 2-propanol as an alcohol modifier, the chiral resolution capability of Chiralpak IA-3 CSP and Chiralpak AD-3 was lost;

(v) Using the mixture n-hexane-ethanol-TFA 90:10:0.1 (*v/v/v*), the Chiralpak AD-3 CSP was superior to the Chiralpak IA-3 CSP (i.e., *Rs* = 4.10 vs. *Rs* = 2.62);

(vi) Increasing the ethanol content in the mobile phase from 10 to 20% dramatically decreased the retention and resolution on the Chiralpak AD-3 CSP (i.e., k_2 = 7.36 vs. k_2 = 1.27 and *Rs* = 4.10 vs. *Rs* = 1.99, respectively).

Table 1. Retention (*k*), enantioseparation (*α*), and resolution (*Rs*) factors of ATV in normal-phase conditions. Chromatographic conditions: column, as indicated in the table (geometry: 250 mm × 4.6 mm; 3 µm); mobile phase, as indicated in the table; flow rate, 1.0 mL min^{-1}; temperature, 25 °C; detection, UV at 254 nm. EtOH: ethanol; IPA: 2-propanol.

CSP	Mobile Phase	k_1	k_2	α	*Rs*
Chiralpak IA-3	n-Hexane-EtOH-TFA (90:10:0.1)	2.52	3.10	1.23	2.62
	n-Hexane-IPA-TFA (90:10:0.1)	3.70	-	1.00	-
Chiralpak AD-3	n-Hexane-EtOH-TFA (90:10:0.1)	4.11	7.36	1.79	4.10
	n-Hexane-EtOH-TFA (80:20:0.1)	0.72	1.27	1.75	1.99
	n-Hexane-IPA-TFA (90:10:0.1)	5.98	-	1.00	-
Chiralpak ID-3	n-Hexane-EtOH-TFA (90:10:0.1)	3.57	-	1.06	-
	n-Hexane-IPA-TFA (90:10:0.1)	7.14	-	1.06	-
Chiralpak IG-3	n-Hexane-EtOH-TFA (90:10:0.1)	4.87	5.49	1.13	1.20
	n-Hexane-IPA-TFA (90:10:0.1)	11.1	-	1.00	-
Chiralpak IH-3	n-Hexane-EtOH-TFA (90:10:0.1)	2.89	-	1.00	-
	n-Hexane-IPA-TFA (90:10:0.1)	4.13	-	1.00	-

The use of alternative mobile phases containing different percentages of n-hexane, ethanol, and non-standard cosolvents such as dichloromethane and ethyl acetate, in combination with the immobilized-type CSPs did not improve the performance in terms of enantioseparation and resolution. Thus, the potential advantage of the greater versatility of the chiral supports based on the immobilized polysaccharide derivative over the coated parent is lost in this case [8]. It is also worth noting that complete resolution of ATV could not be achieved using the Chiralpak IA-3 and Chiralpak AD-3 columns in reversed-phase mode (data obtained with aqueous mixtures containing acetonitrile or methanol are not shown).

3.2. Chemo- and Diastereo-Selectivity of the Normal-Phase Enantio-Selective Method

When developing an enantio-selective method for a chiral drug substance, care must be taken to ensure that it is capable of distinguishing not only the enantiomer impurity from the API but also from the other chiral and achiral related substances that may be present. A co-elution involving peaks related to these impurities can lead to misinterpretation of the drug enantiomer purity. The EP monograph on ATV describes a number of impurities that may be present in the drug substance. The structures of ATV impurities, A (IMP-A), B (IMP-B), C (IMP-C), D (IMP-D), E (IMP-E), H (IMP-H), and the methyl and t-butyl esters (IMP-OMe and IMP-OtBu) are shown in Figure 1.

As reported in Table 1, the best resolution between ATV and impurity E was obtained using the Chiralpak AD-3 250 mm × 4.6 mm column with n-hexane-ethanol-TFA 90:10:0.1 (*v/v/v*) as the mobile phase. To verify the chemo- and diastereo-selectivity of the enantio-selective HPLC method, solutions containing the mixed enantiomer IMP-E and ATV, as well as the other individual impurities, were analyzed in sequence under the same chromatographic conditions. The retention times of the impurities were compared with that of the IMP-E enantiomer. From the chromatograms obtained and shown in Figure 2, it is clear that only IMP-H was critical for the evaluation of the enantiomeric excess. The incomplete resolution between the two impurities (*Rs* < 1.5) was confirmed by the analysis of a sample in which both impurities were present.

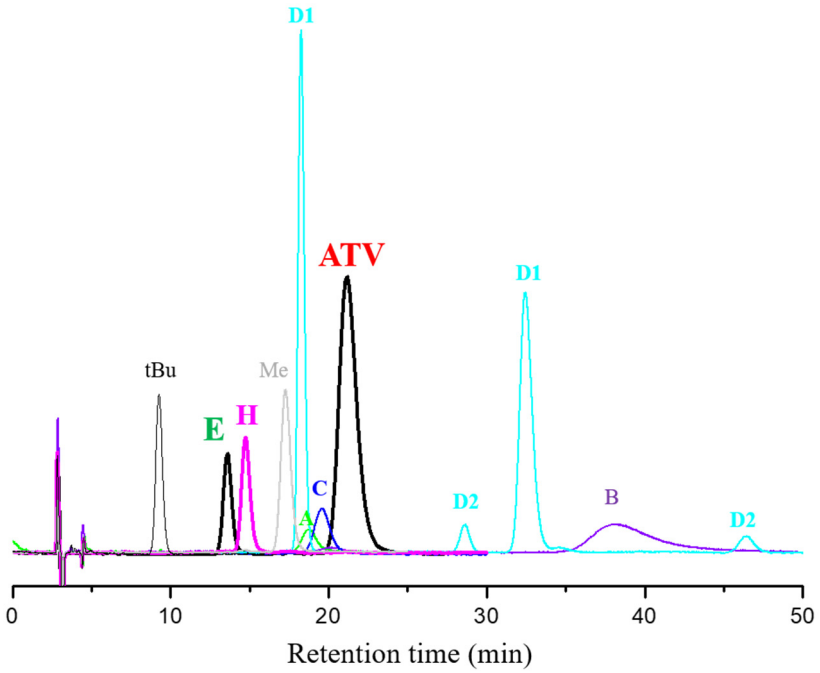

Figure 2. Chromatograms pertinent to the sequential HPLC analysis of the mixture IMP-E(E)/ATV and the single impurities IMP-A (A), IMP-B (B), IMP-C (C), IMP-D (D1 + D2), IMP-H (H), IMP-OMe (Me), and IMP-OtBu (tBu). Chromatographic conditions: column, Chiralpak AD-3 (250 mm × 4.6 mm, 3 μm); mobile phase, n-hexane-ethanol-TFA 90:10:0.1 (*v/v/v*); flow rate, 1.0 mL min^{-1}; temperature, 25 °C; detection, UV at 254 nm.

Any efforts to increase the resolution by increasing the column temperature were not successful. Referring again to Figure 2, the elution of two enantiomers took about 20 min, while the complete run time increased significantly to about 50 min due to the outstanding retention of the ATV diastereomeric forms, namely IMP-B and IMP-D. It should be noted that IMP-D has two stereogenic centers and was resolved into four well-separated stereoisomeric peaks. The more abundant enantiomeric couple is indicated as D1 and the less abundant as D2 according to the EP analysis data shown in the chromatogram supplied with atorvastatin for peak identification B CRS (Figure S2 of SI).

3.3. Optimization of the Normal-Phase Enantio-Selective EP Method

At this stage, it is useful to make a comparison between the method based on the Chiralpak AD-3 250 mm × 4.6 mm column described above (mobile phase: n-hexane-ethanol-TFA 90:10:0.1 (*v/v/v*); column temperature: 25 °C; flow rate: 1.0 mL min^{-1}) and that based on the Chiralpak AD-H 150 mm × 4.6 mm column reported in the EP monograph for ATV. The official method has some minor but important differences: (i) the column used is a shorter AD-H column packed with 5.0 μm particles instead of 3 μm Chiralpak AD-3 CSP; (ii) the column temperature and flow rate are set at 40 °C and 1.8 mL min^{-1}; (iii) the mobile phase is a mixture of n-heptane-ethanol-FA 96:4:0.1 (*v/v/v*). Under these conditions, the resolution between IMP-E and IMP-H is >1.5. The use of FA as an acid additive and n-heptane as a hydrocarbon eluent component is intended to make the mobile phase less damaging to the environment and safer for the analyst [19,20]. However, this effort is in strong contrast to the large volume required for each chromatographic run. Considering the possible presence of impurities D and B in the drug substance and their delayed elution, at about 2.5 times the retention time of ATV (i.e., about 87 min) and a flow rate of 1.8 mL min^{-1}, about 160 mL is required to complete the analysis. Thus, the EP approach provides chemo- and enantio-selective conditions, but it suffers dramatically from very high solvent consumption.

In order to improve the separation speed and reduce the mobile phase volume, the normal-phase conditions used with the Chiralpak AD-3 column were modified according to some indications of the EP method. Thus, n-hexane and TFA were replaced by n-heptane and FA, maintaining the percentages of the two solvents at 90% and 10%, respectively, and the temperature of the column was set at 40 °C. However, this attempt failed because the resolution between the critical IMP-E/IMP-H pair did not reach the minimum value of 1.5.

A new analytical strategy was then tried. A sample containing the impurities E and H was analyzed at temperatures of 25, 30, 35, and 40 °C keeping FA as an acid additive while n-hexane was used as a hydrocarbon solvent. From the chromatograms and data presented in Figure 3, it can be observed that the resolution factor between impurities E and H always remained above 1.5 over the temperature range investigated.

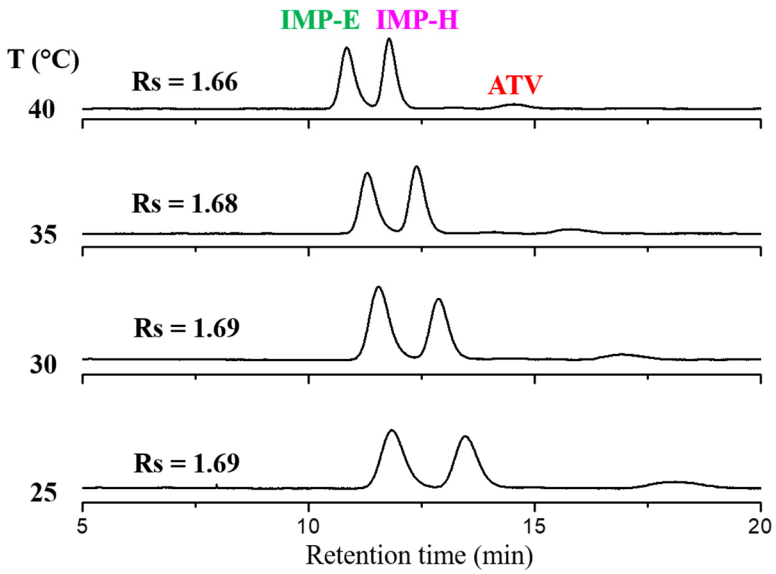

Figure 3. Variable temperature HPLC resolution of impurities E and H. Chromatographic conditions: column, Chiralpak AD-3 (250 mm × 4.6 mm, 3 µm); mobile phase, n-hexane-ethanol-FA 90:10:0.1 (*v/v/v*); flow rate, 1.0 mL min^{-1}; temperature, 25, 30, 35, and 40 °C; detection, UV at 254 nm.

Three samples were then analyzed sequentially using the same mobile phase with the column temperature fixed at 35 °C, one containing only ATV at the nominal concentration of 6.0 mg mL^{-1}, (corresponding to 5.8 mg mL^{-1} as ATV free acid), the second containing the impurities E and H (concentration = 13.2 µg mL^{-1} for each impurity), and the last containing ATV and the other related substances (concentration range 13.1–19.3 µg mL^{-1}).

The chromatograms shown in Figure 4 indicate the following: (i) ATV and impurities did not interfere with the quantification of the enantiomeric purity; (ii) the resolution between IMP-E and IMP-H was 1.68, a higher value than that required in the suitability test of the EP monograph (i.e., 1.5); (iii) the total run time was reduced to 35 min with a consumption of 35 mL of mobile phase. It should be clarified that since the quantitative determination of the enantiomer E is performed using the external calibration curve of ATV, the partial resolution between impurity D1 and ATV and the co-elution of impurity C with the drug substance do not affect the determination of the enantiomeric purity.

Looking again at Figure 4, the chromatogram of the impurity mixture shows four additional peaks marked with an asterisk. The appearance of these peaks about 20 h after sample preparation is due to the partial degradation of the stereoisomers of IMP-D that occurred in the autosampler at 25 °C. Under these conditions, about 18% of the impurity D is degraded. To avoid the comparison of such interfering peaks, samples containing ATV should be prepared before use.

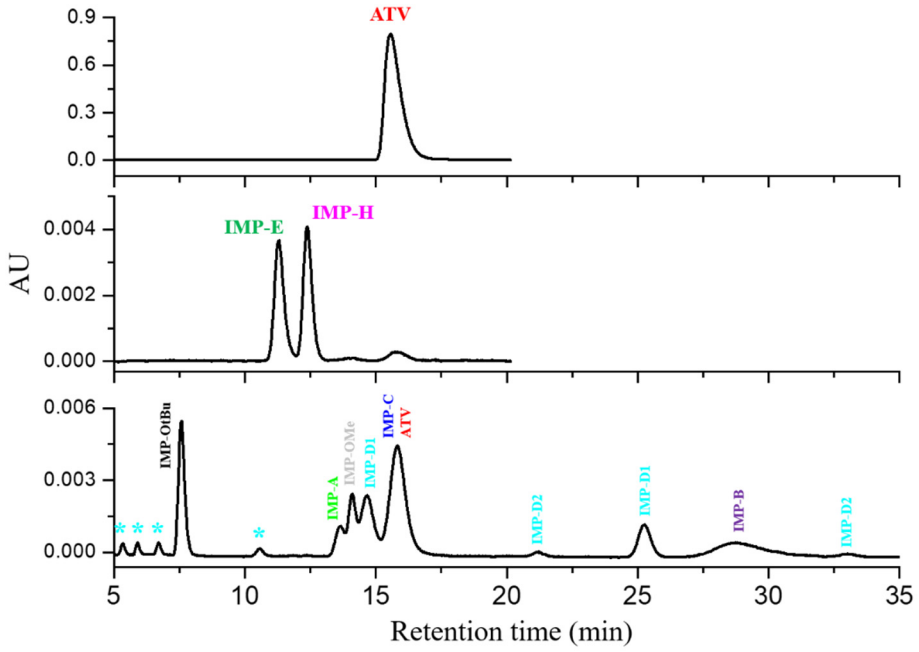

Figure 4. Typical HPLC chromatograms pertinent to (top) ATV (conc. = 5.8 mg mL^{-1}), (middle) mixture IMP-E/IMP-H (conc. = 13.2 μg mL^{-1}), and (bottom) mixtures of ATV, IMP-A, IMP-B, IMP-C, IMP-D (IMP-D1 + IMP-D2), IMP-H, IMP-Ome, and IMP-OtBu (conc. range = 13.1–19.3 μg mL^{-1}). Chromatographic conditions: column, Chiralpak AD-3 (250 mm × 4.6 mm, 3 μm); mobile phase, n-hexane-ethanol-FA 90:10:0.1 (*v/v/v*); flow rate, 1.0 mL min^{-1}; temperature, 35 °C; detection, UV at 254 nm. *: Peaks marked with an asterisk are degradation products from the stereoisomers of IMP-D.

In accordance with the EP monograph, the linearity of the enantio-selective method was evaluated on the ATV. The optimized method was shown to be linear from 4.4 μg mL^{-1} to 1000 μg mL^{-1} of ATV free acid (correlation coefficient, r^2, values ≥ 0.9999), with an S/N ratio of 20 at 0.075% (conc = 4.4 μg mL^{-1}) of the test solution concentration (5.8 mg mL^{-1}, as ATV free acid).

The results for method precision showed acceptable repeatability (intra-day) at the three concentration levels evaluated. The retention times of the ATV peak recorded an RSD% of 0.07% in 10 determinations, while the RSD% of the peak areas at each concentration level studied was <1.0%.

The robustness of the method was evaluated by varying the chromatographic parameters affecting the separation. The flow rate was varied from 0.90 to 1.10 mL min^{-1}, the column temperature from 30 °C to 40 °C, and the percentage of ethanol in n-hexane from 9.5% to 10.5%. The resolution always remained above 1.5.

3.4. Influence of Ethanol Percentage on Elution Order of Impurities E and H

The last part of our work was dedicated to the investigation of the influence of changes in the ethanol percentage in the mobile phase on the elution order of the impurities E and H. This is because in the EP method, using the 4% alcohol modifier, the impurity H is eluted before the impurity E, while in our method, using 10%, the elution order is reversed. Thus, the critical IMP-H/IMP-E pair was analyzed at 35 °C using the mixtures n-hexane-ethanol-FA 95:5:0.1, 93:7:0.1, and 89:11:0.1 (*v/v/v*) as mobile phases. Examination of the chromatograms in Figure 5 shows that a peak coalescence occurs at 7% ethanol and a reversed elution order of two impurities is observed with the other two mobile phases.

Furthermore, by decreasing the ethanol content in the mobile phase from 11% to 5%, a ~5-fold increase in the magnitude of the retention time of the more retained impurity was observed.

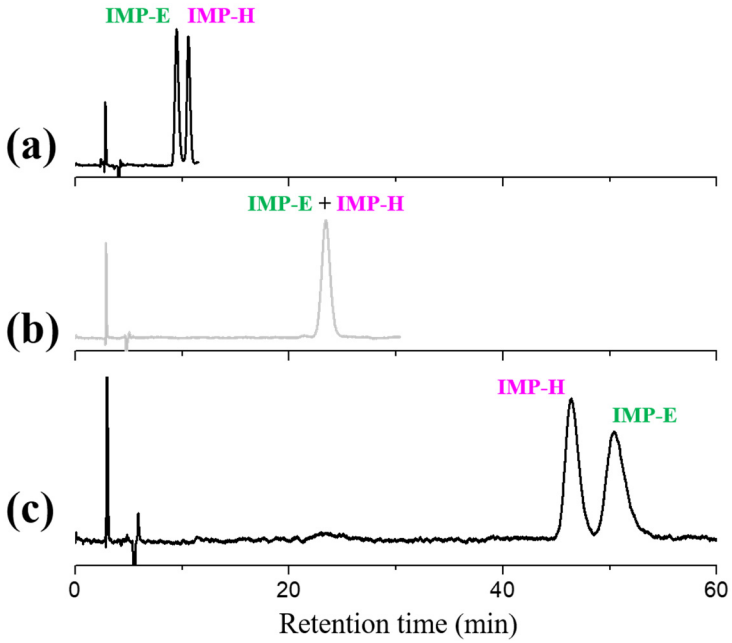

Figure 5. Typical HPLC chromatograms showing the inversion of the elution order of the impurities E and H. Chromatographic conditions: column, Chiralpak AD-3 (250 mm × 4.6 mm, 3 µm); mobile phase, (**a**) n-hexane-ethanol-FA 89:11:0.1 (*v/v/v*), (**b**) n-hexane-ethanol-FA 93:7:0.1 (*v/v/v*), (**c**) n-hexane-ethanol-FA 95:5:0.1 (*v/v/v*); flow rate, 1.0 mL min^{-1}; temperature, 35 °C; detection, UV at 254 nm.

4. Conclusions

In this work, an enantio- and diastereo-selective normal-phase HPLC method based on the Chiralpak AD-3 column (250 mm × 4.6 mm, 3.0 µm) was developed to evaluate the enantiomeric excess of the blockbuster drug ATV without interference from other related substances. Optimization of the simultaneous stereo-selective and chemo-selective system for ATV was not straightforward. Most of the amylose-based commercially available CSPs show no resolving power and all of those analyzed are ineffective in the reversed-phase mode.

The improvement of the official EP analytical conditions has required a careful study of the effects of mobile phase composition and temperature on the selectivity of the system. The EP enantiomeric purity test for ATV was published in 2022 and uses a 5 µm Chiralpak AD-H as the CSP and the mixture n-heptane-ethanol-FA 96:4:0.1 (*v/v/v*) as the mobile phase. At a temperature of 40 °C and a flow rate of 1.8 mL min^{-1}, the ATV retention time is approximately 35 min, while the critical impurity H and enantiomeric impurity E have a relative retention time to ATV of 0.57 and 0.66, respectively. The run time is set at 1.2 times the ATV retention time (i.e., 42 min) but should be shifted to about 2.5 times the ATV retention time (i.e., 87 min) to allow elution of potential impurities D and B. These conditions require about 160 mL of mobile phase to complete the analysis. The selectivity of the isocratic HPLC method on the Chiralpak AD-3 column was ensured using the mobile phase n-hexane-ethanol-FA 90:10:0.1 (*v/v/v*) and a column temperature of 35 °C. At a flow rate of 1.0 mL min^{-1}, the volume of the mobile phase that is required to elute all ATV-related compounds is approximately 35 min. The EP method is used to determine enantiomeric purity limited to not more than 0.15%. Using the presented method, the signal-to-noise ratio was found to be 20 at 0.075% test solution concentration. Thus, the variations introduced into the official method, although subtle, have greatly improved the performance of the analysis and reduced the analysis time and solvent consumption, with a significant impact on the environmental and economic cost of the analysis. This feature, together with the recognized selectivity and sensitivity, makes this protocol reliable and attractive for the pharmaceutical testing of the content of enantiomeric impurity in the drug substance and in the ATV working standard.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11050154/s1, Figure S1: The Chromatogram for the EP test for enantiomeric purity of atorvastatin calcium: a 7% atorvastatin calcium, 0.3% impurity E and 0.15% impurity H solution. Figure S2: Atorvastatin for peak identification B CRS.

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