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# USP Method Case Study Part I: Understanding the Impact of Sample Preparation and Mobile Phase Stability

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## APPLICATION BENEFITS

- ACQUITY™ Arc™ System, a 9500 psi system that can accommodate HPLC 4.6 × 250 mm columns
- Control of mobile phase components with low vapor evaporation bottle caps
- Sample preparation control using appropriate vials for sample composition

## WATERS SOLUTIONS

ACQUITY Arc System

ACQUITY APC™ Reservoir Caps

Maximum recovery no-preslit screw caps vials

## KEYWORDS

USP, atorvastatin, ACQUITY Arc, APC bottle caps

## INTRODUCTION

Many established HPLC monographs have conditions or variables that can be sensitive to environmental factors, such as room temperature and humidity. While these contributors may vary from lab to lab and across continents, mitigating or reducing the impact often means that these methods require careful control of every aspect of an analysis, including mobile phase and sample preparation. For example, the USP monograph for the assay of atorvastatin[1] has a set of conditions that can pose a challenge to meeting the specified system suitability criteria. These include: a highly volatile sample diluent (1:1:2 acetonitrile: tetrahydrofuran (THF): water), a volatile component in the mobile phase (THF at 12%) and a long analysis time (2 hours). When these conditions are combined with system suitability requirements that include standard deviation of not more than (NMT) 0.6%, the method is particularly vulnerable and can fail system suitability with no relation to the column or instrument. Specifically, during the analysis of five replicate injections, which occurs over 10 hours, it is critical to ensure that no changes to the sample concentration or mobile phase occur as these impact the reproducibility of the method. To control these factors, sample preparation and mobile phase evaporation must be considered. In this example, we will review analysis of the method and steps taken to improve the reproducibility and therefore attain passing system suitability results for the USP assay.

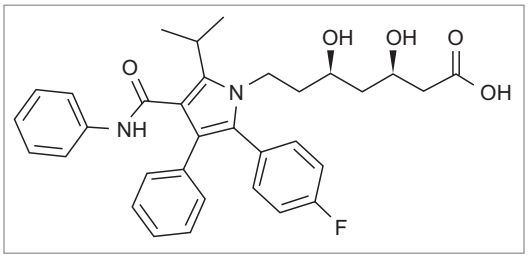


*Figure 1. Atorvastatin.*

## EXPERIMENTAL

### LC conditions

The monograph for the assay of atorvastatin calcium in the United States Pharmacopeia and National Formulary Compendium (USP39-NF34, 2016) was followed.

### Sample description

Sample prep was performed following the USP monograph.[1] Reference standards were purchased from USP (Rockville, MS) including atorvastatin (p/n 1044516) and atorvastatin related compound B (p/n 1044535). The diluent consisted of 1:1:2 acetonitrile/ stabilizer-free tetrahydrofuran/ water, which, as stated in the monograph, is permissible if significant fronting of the atorvastatin and atorvastatin related compound B is observed. All samples were prepared immediately prior to analysis. The system suitability standard was prepared from working stocks of atorvastatin calcium at 0.4 mg/mL and atorvastatin related compound B at 0.1 mg/mL in the diluent previously specified. The final concentration was 0.05 mg/mL in atorvastatin calcium and 0.06 mg/mL of atorvastatin related compound B. The standard solution was prepared at 0.4 mg/mL of atorvastatin calcium. A specific volume (300 µL) of standards and samples was aliquoted into each maximum recovery vials with both pre-slit and non pre-slit screw caps.

### Method conditions

### LC conditions

| | |
|---|---|
| LC system: | ACQUITY Arc with 30-CHC and 2998 PDA Detector |
| Seal wash: | 80:20 Water/methanol |
| Purge solvent: | Methanol |
| Wash solvent: | 1:1:2 ACN/THF/$H_2O$ |
| Wavelength: | 244 nm |
| Vials: | Maximum recovery, screw cap non pre-slit septa – lectra bond (p/n 186000326C) |
| Column: | Zorbax RX C8, 4.6 × 250 mm, 5 µm, L7 packing material |
| Column temp.: | 35 °C |
| Sample temp.: | 20 °C |
| Injection volume: | 20 µL |
| Flow rate: | 1.5 mL/min |
| Buffer: | 3.9 g/L Ammonium actetate in water, pH 5.0 adjusted with glacial acetic acid |
| Solution A: | 21:12:67 Acetonitrile/stabilizer-free tetrahydrofuran/buffer |
| Solution B: | 61:12:27 Acetonitrile/stabilizer-free tetrahydrofuran/buffer |
| Mobile phase bottle caps: | ACQUITY APC Reservoir Cap (p/n 205001152) |

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 40 | 100 | 0 |
| 70 | 20 | 80 |
| 85 | 0 | 100 |
| 100 | 0 | 100 |
| 105 | 100 | 0 |
| 115 | 100 | 0 |

*Table 1. Gradient.*

### System set up

Prior to analysis, each line (A, B, C, D) was primed for 5 min. The purge and wash lines were also purged for 20 cycles and 60 seconds, respectively. Buffer was prepared in a 3 L quantity. Mobile phase A was then prepared in a 3.5 L quantity and mobile phase B was prepared in 3 L. Prior to each test, a blank injection was performed.

### Data management

Chromatography software:
Empower™ 3 FR3

USP Assay System Suitability Criteria

## RESULTS AND DISCUSSION

The assay for atorvastatin calcium, an HPLC analysis with a run time of 115 minutes was evaluated. The method, which uses the same conditions as the organic impurities method 1, elutes the drug substance and the related compound B in the initial 40 minute isocratic hold. As described earlier, the volatility of the mobile phase and sample diluent components can impact the repeatability of the analysis, in two separate ways. The differing rates of evaporation of the mobile phase components could impact the elution or retention time of the analytes. Organic evaporation from the sample diluent could impact concentration and solubility of the analytes. Both of which can impact the system suitability requirements (Table 2). However, per USP <621>, since the method is gradient, there are no options for scaling or shortening the run.[2] Thus, the goal of the experimental plan was to devise a strategy to reduce variability of the method and to examine tools to mitigate environmental factors.

| | Criteria |
|---|---|
| Resolution | Not Less Than (NLT) 1.5 between peaks for atorvastatin related compound B and atorvastatin, System Suitability Solution |
| Tailing factor | Not More Than (NMT) 1.6%, Standard Solution |
| Relative standard deviation (n=5) | Not More Than (NMT) 0.6%, Standard Solution |

*Table 2. System suitability criteria for USP assay of atorvastatin calcium.*

## SAMPLE PREPARATION CONTROL

As mentioned previously, the presence of a highly volatile component in the sample diluent, can impact sample stability. To assess the impact of sample preparation on the assay, all samples were freshly prepared and sample aliquoting was tested. While the method did not specify a sample compartment temperature, 20 °C was used for minimal temperature control. In the first analysis, 500 µL of the standard was placed in a maximum recovery pre-slit septa vial. In the second set of analyses, a 300 µL aliquot of sample was placed into five separate vials, each vial capped with non-preslit septa. This ensured that there was no evaporation of the samples over the 10 hours required for the analysis. The results for atorvastatin are shown in Figure 2. Evaluating the area reproducibility of the two sets of injections, the repeatability of the atorvastatin injections from a single vial was 0.6%, which was at the system suitability limit for the assay of NMT 0.6%. For the analysis of standards in separate vials with non-preslit septa, the area RSD was 0.3% which met the system suitability requirements of the analysis. This approach is a viable option when handling samples with volatile components as per the USP.

Five injections from a single vial

**Name: Atorvastatin**

| | SampleName | Vial | Injection | RT | Area | Height | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|---|---|
| 1 | Atorvastatin Standard 1 | 1:A,5 | 1 | 31.69 | 12778814 | 294063 | 1.3 | 11669 |
| 2 | Atorvastatin Standard 1 | 1:A,5 | 2 | 31.70 | 12731596 | 293205 | 1.3 | 11699 |
| 3 | Atorvastatin Standard 1 | 1:A,5 | 3 | 31.73 | 12646266 | 291266 | 1.3 | 11721 |
| 4 | Atorvastatin Standard 1 | 1:A,5 | 4 | 31.73 | 12739342 | 293347 | 1.3 | 11717 |
| 5 | Atorvastatin Standard 1 | 1:A,5 | 5 | 31.75 | 12610331 | 290604 | 1.3 | 11756 |
| Mean | | | | 31.7 | 12701269 | 292497.0 | 1.3 | 11712 |
| Std. Dev. | | | | 0.0 | 70138 | 1481.2 | | |
| % RSD | | | | 0.1 | 0.6 | 0.5 | | |

Five injections from five separate vials

**Name: Atorvastatin**

| | SampleName | Vial | Injection | RT | Area | Height | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|---|---|
| 1 | Atorvastatin Standard 1 | 1:A,5 | 1 | 31.69 | 12778814 | 294063 | 1.3 | 11669 |
| 2 | Atorvastatin Standard 1 | 1:A,6 | 1 | 31.76 | 12711391 | 291632 | 1.3 | 11657 |
| 3 | Atorvastatin Standard 1 | 1:A,7 | 1 | 31.78 | 12748526 | 292008 | 1.3 | 11628 |
| 4 | Atorvastatin Standard 1 | 1:A,8 | 1 | 31.80 | 12739480 | 291511 | 1.3 | 11620 |
| 5 | Atorvastatin Standard 1 | 1:B,1 | 1 | 31.82 | 12805184 | 292730 | 1.3 | 11608 |
| Mean | | | | 31.8 | 12756679 | 292388.8 | 1.3 | 11636 |
| Std. Dev. | | | | 0.0 | 36249 | 1049.8 | | |
| % RSD | | | | 0.2 | 0.3 | 0.4 | | |

*Figure 2. Data from five replicate injections of atorvastatin standard. The upper table is the data of five replicate injections of a sample from the same vial. The lower table is the data collected of five replicate injections from five separate vials with a non-preslit septa (p/n 186000326C), a single injection from each vial. Each run is 115 minutes; the lower table shows the stability of long term storage with proper sample placement.*

## CONTROL OF EVAPORATION OF MOBILE PHASE

The amount of time to complete testing of an assay can vary depending on number of samples, number of blanks among many variables. With regards to this, USP Chapter <621> on Chromatography describes the number of replicate injections required for a monograph. Specifically "Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation, %RSD, if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%."[2] Following these recommendations, the USP assay of atorvastatin calcium requires five replicate injections of the standards. However, this is in addition to any blanks, system suitability standards and samples that need to be analyzed. Thus based on the run time (115 minutes), testing of a drug substance requires a minimum of 8 injections (1 blank, 1 system suitability sample, 5 standards, and 1 sample injection) or approximately 16 hours.

Given the length of the analysis time and the mobile phase components (12% THF in A and B), control of the mobile phase composition is critical for retention time reproducibility. Therefore, the testing was evaluated for a longer period than was required to fully evaluate the mobile phase stability. Specifically the test was conducted over 38 hours and the data was compiled for 10 injections, (blanks and system suitability samples were injected along with standard injections). Standard reservoir or bottle caps were used in this experiment. The results show a significant shift in retention times as well as a retention time RSD of 1.2%, which is outside the system suitability requirement for this analysis (Figure 3). It is likely that evaporation of the organic portion of the mobile phase led to a change in mobile phase composition, a lower "strength" mobile phase with a higher aqueous content thus resulting in the retention time drift.

**Name: Atorvastatin**

| | SampleName | Vial | Injection | RT | Area | Height | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|---|---|
| 1 | Atorvastatin Standard 1 | 1:A,5 | 1 | 31.69 | 12778814 | 294063 | 1.3 | 11669 |
| 2 | Atorvastatin Standard 1 | 1:A,6 | 1 | 31.76 | 12711391 | 291632 | 1.3 | 11657 |
| 3 | Atorvastatin Standard 1 | 1:A,7 | 1 | 31.78 | 12748526 | 292008 | 1.3 | 11628 |
| 4 | Atorvastatin Standard 1 | 1:A,8 | 1 | 31.80 | 12739480 | 291511 | 1.3 | 11620 |
| 5 | Atorvastatin Standard 1 | 1:B,1 | 1 | 31.82 | 12805184 | 292730 | 1.3 | 11608 |
| 6 | Atorvastatin Standard 1 | 1:B,2 | 1 | 31.84 | 12770353 | 291763 | 1.3 | 11615 |
| 7 | Atorvastatin Standard 1 | 1:B,5 | 1 | 31.94 | 12840130 | 292545 | 1.3 | 11614 |
| 8 | Atorvastatin Standard 1 | 1:B,6 | 1 | 31.98 | 12848889 | 292623 | 1.3 | 11642 |
| 9 | Atorvastatin Standard 1 | 1:C,3 | 1 | 32.56 | 12844012 | 287521 | 1.3 | 11665 |
| 10 | Atorvastatin Standard 1 | 1:C,4 | 1 | 32.80 | 12716423 | 281960 | 1.3 | 11572 |
| Mean | | | | 32.0 | 12780320 | 290835.6 | 1.3 | 11629 |
| Std. Dev. | | | | 0.4 | 52247 | 3546.6 | | |
| % RSD | | | | 1.2 | 0.4 | 1.2 | | |

*Figure 3. Retention time data from injections of atorvastatin standard for 10 injections which occurred over 30+ hours using standard reservoir caps. The retention time %RSD is 1.2% after 30+ hours, falling out of system suitability.*

To mitigate the retention time drift, the standard bottle caps were replaced with low vapor ACQUITY APC reservoir caps shown in Figure 4. The ACQUITY APC reservoir caps are designed with an outlet relief valve that releases any pressure build-up in the mobile phase bottle while also minimizing evaporation. In addition, any cap outlet that is not in use was plugged. Analysis of this test shows minimal retention time drift: The shift in retention time over the same time period was significantly lower than with the previous analysis, with an RSD of 0.5%.

**Peak Results**
**Name: Atorvastatin**

| | SampleName | Vial | Injection | RT | Area | Height | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|---|---|
| 1 | Atorvastatin Standard 1 | 1:A,3 | 1 | 31.36 | 12314326 | 284624 | 1.4 | 11551 |
| 2 | Atorvastatin Standard 1 | 1:A,6 | 1 | 31.38 | 12387565 | 286160 | 1.4 | 11556 |
| 3 | Atorvastatin Standard 1 | 1:A,7 | 1 | 31.39 | 12331181 | 284873 | 1.4 | 11565 |
| 4 | Atorvastatin Standard 1 | 1:A,8 | 1 | 31.40 | 12337083 | 284658 | 1.4 | 11544 |
| 5 | Atorvastatin Standard 1 | 1:B,4 | 1 | 31.44 | 12463129 | 286864 | 1.4 | 11514 |
| 6 | Atorvastatin Standard 1 | 1:B,5 | 1 | 31.46 | 12370381 | 284230 | 1.4 | 11488 |
| 7 | Atorvastatin Standard 1 | 1:C,1 | 1 | 31.54 | 12260247 | 281236 | 1.4 | 11519 |
| 8 | Atorvastatin Standard 1 | 1:C,2 | 1 | 31.56 | 12338974 | 282768 | 1.4 | 11509 |
| 9 | Atorvastatin Standard 1 | 1:C,7 | 1 | 31.72 | 12316973 | 280968 | 1.4 | 11521 |
| 10 | Atorvastatin Standard 1 | 1:C,8 | 1 | 31.77 | 12312317 | 280514 | 1.4 | 11526 |
| Mean | | | | 31.5 | 12343218 | 283689.6 | 1.4 | 11529 |
| Std. Dev. | | | | 0.1 | 54412 | 2212.2 | | |
| % RSD | | | | 0.5 | 0.4 | 0.8 | | |

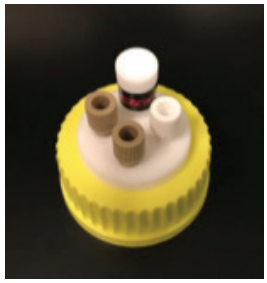

*Figure 4. Retention time data from injections of atorvastatin standard for 10 injections which occurred over 30+ hours. The mobile phase bottles were capped with ACQUITY APC reservoir caps. With this approach, the retention time RSD is 0.4% over 30+ hours, meeting system suitability of the method.*

The difference in retention time drift can be observed by comparing both the retention time and the %RSD for each set of analyses over time. The analysis included both a blank and a system suitability injection, therefore, the first injection of the standard occurred at 4 hours. As observed in the graph plot, the %RSD with the standard reservoir caps was approximately 1.2% at 38 hours. In contrast, analysis with the low vapor ACQUITY APC reservoir caps was <0.5% over the same time period, at which point the test was ended (Figure 5). This can also be verified with analysis of the retention time trend lines.

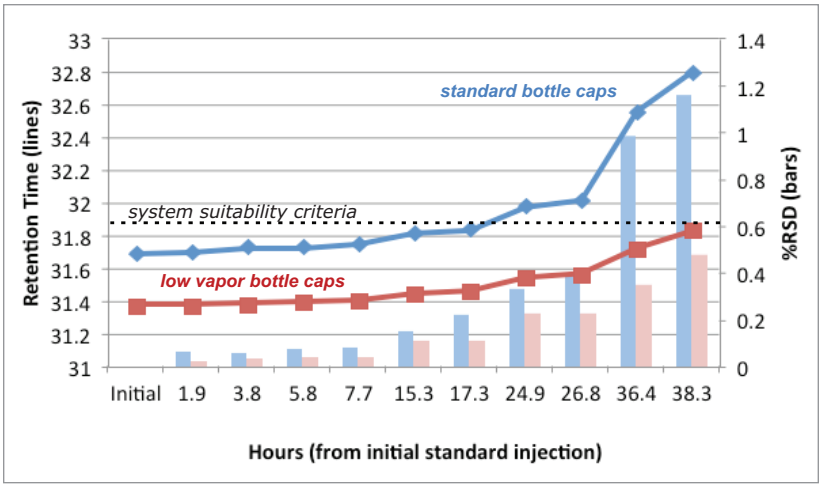


*Figure 5. Retention time data and relative standard deviation over 38 hours, with standard and low vapor reservoir caps. Tests with low vapor reservoir caps produced stable retention times (red line) and lower %RSD (red bar) as compared to standard caps (blue line and blue columns). Using the standard bottle caps, the retention time RSD (Blue bar) was greater than the system suitability criteria (grey line) after 30+ hours.*

## USP ASSAY RESULTS

Through careful control of the mobile phase and the sample preparation, the USP assay for atorvastatin was performed successfully. Specifically, the USP criteria were met for this analysis (Table 3), including peak area and retention time %RSD.

| | Criteria | Original results | New results* |
|---|---|---|---|
| Resolution | Not Less Than (NLT) 1.5 between peaks for atorvastatin related compound B and atorvastatin, system suitability solution | 2.3 | 2.3 |
| Tailing factor | Not More Than (NMT) 1.6%, standard solution | 1.4 | 1.4 |
| Relative standard deviation | Not More Than (NMT) 0.6%, standard solution | 1.2% Retention time<br>0.6% area | 0.5% Retention time<br>0.3% area |

*Table 3. System suitability criteria for USP assay of atorvastatin calcium with results. Without careful consideration of the variables that can affect long term sample and mobile phase stability, the assay did not meet the relative standard deviation requirements of the USP assay (original results). By aliquoting the sample into separate vials and using low vapor reservoir caps (new results*) the system suitability criteria were all met.*

## CONCLUSIONS

In the following study, a careful evaluation of the USP assay for atorvastatin was performed. The results demonstrate the impact of small changes in the composition of the sample and/or mobile phase over time. These changes, while slight, impact the method's ability to meet the system suitability criteria. Many of these changes are not due to the instrument or the column but instead are impacted by the lab environment. For example, both the room temperature and humidity can impact the volatility of the solvent and the sample diluent.

To minimize the impact of environmental conditions, both sample preparation and mobile phase composition were controlled. For example, initial analysis showed how aspiration of the standard multiple times out of a single vial could negatively impact peak area relative standard deviations. However, aliquoting the sample into separate vials increased the precision of the method over the 10 hours or five replicate injections due to minimal evaporation for the sample diluent. The long term stability of the mobile phase was also evaluated. Specifically, the impact of the reservoir cap on the evaporation and/or change in composition of the eluent was studied. In these studies low vapor reservoir caps provided more stable retention times over a longer period than standard reservoir caps. Both of these approaches are not prohibited within USP chapter 621.[2] These studies enabled a strategy to be devised, which was then used to minimize any variation in the method transfer of the USP method across multiple labs in different continents.

## References


1. Official Monographs, USP 39-NF34. United States Pharmacopeia and National Formulary (USP 39-NF34) Baltimore, MD: United Book Press, Inc.; 2016. p. 2627.
2. <621> CHROMATOGRAPHY. United States Pharmacopeia and National Formulary (USP 39 NF34 ) Baltimore, MD: United Book Press, Inc.; 2016. p. 459–71.


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