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Applikationsbericht

HPLC Autosampler Performance I: Challenging USP Methods on the Alliance™ iS HPLC System

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Abstract

Challenging method conditions can impact autosampler performance on High-Performance Liquid Chromatography (HPLC) systems. Methods with highly organic sample diluents, coupled with low injection volumes, can adversely impact injection precision of an autosampler, which may lead to failure to meet system suitability requirements for routine assays. In this study, the injection precision of an Alliance iS HPLC System was evaluated using four compendial HPLC methods from the United States Pharmacopeia (USP) with challenging method conditions and strict system suitability criteria. Peak area reproducibility of the selected compounds was evaluated as a proxy for autosampler performance.

Benefits

- Demonstrates high precision for a range of sample diluents and injection volumes
- Meets strict system suitability criteria for challenging USP methods

Introduction

Injection precision is a common system suitability criterion for many regulated United States Pharmacopoeia (USP) methods. System suitability requirements for a method ensure that the results acquired are acceptable and accurate. When using a High-Performance Liquid Chromatography (HPLC) system, the injection precision of the autosampler can be impacted by method conditions and instrument characteristics.[1,2] For example, highly organic diluents can become a concern for sample repeatability if volatile diluents evaporate and affect the concentration over the course of a run.[1] In addition, low injection volumes are a challenge for sample repeatability if the autosamplers are unable to accurately and consistently deliver the programmed volume.[1]

The Alliance iS HPLC System is the first Waters™ HPLC system using an in-line metering device as the aspiration mechanism. In this study, four USP Assay monographs were used to examine the impact of challenging method conditions on injection precision of an Alliance iS HPLC System. The methods were selected based on a few attributes: varied organic content for sample diluent (20%–100% organic), low injection volumes (6.6 µL–20 µL), and strict injection precision criteria (0.5%–2.0%). High precision is required of an autosampler, especially for methods with strict system suitability requirements.

Experimental

Method parameters with asterisks (*) indicate modifications of the original monographs to accommodate for modern columns and sizes, per Chapter <621> Chromatography.[3,4] Losartan Potassium and Fenofibrate monographs specify 4.0 × 250 mm columns. These were scaled to a modern column diameter of 4.6 × 250 mm, with adjustments to flow rate and injection volume.[3,4] The Ketoconazole monograph specifies a column with a 3 µm particle size. This was scaled to 3.5 µm particle size with adjustments to flow rate and gradient segments.[3,4] All changes were made according to USP <621> and using the Waters Columns Calculator.[5]

Per USP <621>, monographs with RSD requirements ≤2.0% require five replicate injections and ≥2.0% require six replicate injections.[3] The Fenofibrate monograph, however, requires six replicate injections with an RSD requirement ≤1.0%.[6] Six replicate injections were used remain consistent across all monographs.

USP Monograph for Fluconazole, Assay

Sample Description

A standard solution of USP Fluconazole RS (p/n: 1271700) was prepared at 0.5 mg/mL in 20/80 acetonitrile/water per the USP monograph.

LC Conditions

LC system:	Alliance iS HPLC System with TUV
Separation mode:	Isocratic
Detection:	260 nm, 10 points/sec
Vials:	TruView pH Control LCMS Certified Clear Glass, 23 x 32 mm, Screw Neck Vial, with Cap and pre-slit PTFE/Silicone Septum, 2 mL Volume, 100/pk (p/n: 186005666CV)
Column:	Atlantis® dC_{18} 4.6 × 150 mm, 3 µm (p/n: 186001342)
Column temperature:	40 °C
Sample temperature:	15 °C
Injection volume:	20 µL
Flow rate:	0.5 mL/min
Mobile phase:	Acetonitrile/Water (20/80)

USP Monograph for Losartan Potassium, Assay

Sample Description

A standard solution of USP Losartan Potassium RS (p/n: 1370462) was prepared at 0.25 mg/mL in 40/60 methanol/water.

Original monograph specifies a 4.0 × 250 mm, 5 µm column. Method was scaled to 4.6 × 250 mm, 5 µm with adjustments to injection volume and flow rate.

LC Conditions

LC system:	Alliance iS HPLC System with TUV
Separation mode:	Isocratic
Detection:	254 nm, 10 points/sec
Vials:	TruView pH Control LCMS Certified Clear Glass, 23 x 32 mm, Screw Neck Vial, with Cap and pre-slit PTFE/Silicone Septum, 2 mL Volume, 100/pk (p/n: 186005666CV)
Column*:	XSelect™ CSH™ C_{18} 4.6 × 250 mm, 5 µm (p/n: 186005291)
Column temperature:	35 °C
Sample temperature:	15 °C
Injection volume*:	13.2 µL
Flow rate*:	1.32 mL/min
Mobile phase:	Acetonitrile/0.1% Phosphoric Acid in Water (40/60)

Note: Losartan Potassium was updated by the USP in August 2021. The composition of sample diluent was

changed from 100% methanol to 40/60 methanol/water.[1,8]

USP Monograph for Fenofibrate, Assay

Sample Description

A standard solution of USP Fenofibrate RS (p/n: 1269447) was prepared at 1 mg/mL in 70/30 acetonitrile/water acidified to pH 2.5 with phosphoric acid.

Original monograph specifies a 4.0 × 250 mm, 5 µm column. Method was scaled to 4.6 × 250 mm, 5 µm column with adjustments to injection volume and flow rate.

LC Conditions

LC system:	Alliance iS HPLC System with TUV
Separation mode:	Isocratic
Detection:	286 nm, 10 points/sec
Vials:	TruView pH Control LCMS Certified Clear Glass, 23 x 32 mm, Screw Neck Vial, with Cap and pre-slit PTFE/Silicone Septum, 2 mL Volume, 100/pk (p/n: 186005666CV)
Column*:	XSelect CSH C_{18} 4.6 × 250 mm, 5 µm (p/n: 186005291)
Column temperature:	25 °C
Sample temperature:	15 °C
Injection volume*:	6.6 µL
Flow rate*:	1.32 mL/min

Mobile phase:	Acetonitrile/Water acidified to pH 2.5 with phosphoric acid (70/30)

USP Monograph for Ketoconazole, Assay

Sample Description

A standard solution of USP Ketoconazole RS (p/n: 1356508) was prepared at 0.1 mg/mL in 100% methanol.

Original monograph specifies a 4.6 × 100 mm, 3 µm column. Method was scaled to 4.6 × 100 mm, 3.5 µm column with adjustments to flow rate and gradient as indicated.

LC Conditions

LC system:	Alliance iS HPLC System with TUV
Separation mode:	Gradient
Detection:	225 nm, 10 points/sec
Vials:	TruView pH Control LCMS Certified Clear Glass, 23 x 32 mm, Screw Neck Vial, with Cap and pre-slit PTFE/Silicone Septum, 2 mL Volume, 100/pk (p/n: 186005666CV)
Column*:	XBridge™ Shield RP_{18} 4.6 × 100 mm, 3.5 µm (p/n: 186003044)
Column temperature:	25 °C
Sample temperature:	15 °C
Injection volume:	10 µL

Flow rate*:	1.71 mL/min
Mobile phase A:	Acetonitrile/3.4 mg/mL tetrabutyl ammonium hydrogen sulfate in water (5/95)
Mobile phase B:	Acetonitrile/3.4 mg/mL tetrabutyl ammonium hydrogen sulfate in water (50/50)

Gradient Table

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	1.71	100	0	–
23.33	1.71	0	100	6
29.17	1.71	0	100	6
30.33	1.71	100	0	6
35.00	1.71	100	0	6

Data Mangement

Chromatography software:	Empower™ 3.7

Results and Discussion

Injection Precision for USP Monographs, Assay

The assay tests of the USP monographs were executed on an Alliance iS HPLC System. These methods were selected for the organic content of the sample diluent (20%–100% organic), low injection volumes (6.6 µL–20 µL), and strict RSD criteria (0.5%–2.0%). For all monographs, the system suitability criteria are applied to the standard solutions and the peak area of the standard solutions was evaluated.

Each USP assay method was run continuously for three days to simulate system drift in a high-throughput environment. For system preparation, the Alliance iS HPLC System was primed and purged once prior to starting analysis. The standard solutions were aliquoted and stored refrigerated until use. Unpunctured vials of standard solutions were used each day to minimize impacts from diluent evaporation. Three sample sets of N=6 replicate injections (for a total of N=18 injections) were made each day. Diluent blanks were added as needed to keep the system running uninterrupted. Sample set overlays for the USP assays are shown in Figure 1.

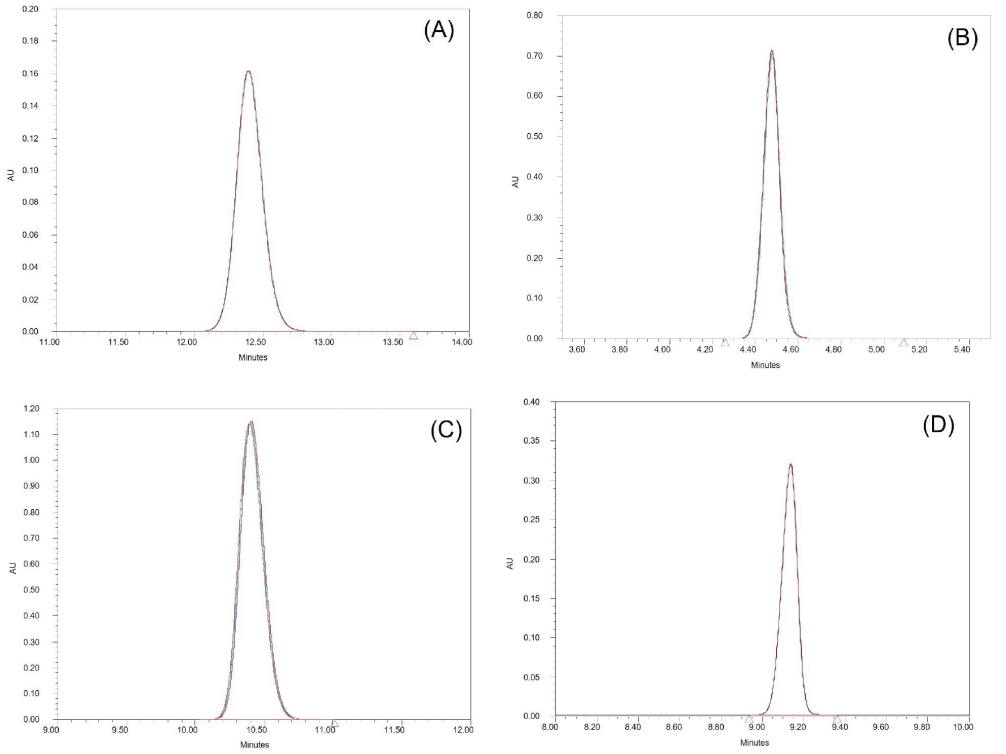

Figure 1. Chromatographic injection overlays of USP assays on the Alliance iS HPLC System. (A) Fluconazole, (B) losartan potassium, (C) fenofibrate, and (D) ketoconazole standards (N=6). Injections shown here are from Sample Set 1 of 3 acquired on Day 1.

Regulated methods often require sample diluents with high organic content due to concerns with compound solubility. High organic content can be a concern for sample repeatability if volatile diluents evaporate and impact sample concentration during a run.[1] To examine the impact of highly organic diluent on peak area repeatability, the methods use diluents ranging from 20% to 100% organic content (Table 1). The methods use

injection volumes from 6.6 µL to 20 µL, which are lower than typical for HPLC methods and can be a challenge for sample repeatability. The autosampler compartment was set to 15 °C to keep sample temperature consistent across the assays.

USP monograph	Diluent	Injection volume	Standard concentration	Amount on column
Fluconazole (assay)	Acetonitrile/water (20/80)	20 µL	0.5 mg/mL	10 µg
Losartan potassium (assay)	Methanol/water (40/60)	13.2 µL	0.25 mg/mL	3.3 µg
Fenofibrate (assay)	Acetonitrile/water pH 2.5 (70/30)	6.6 µL	1.0 mg/mL	6.6 µg
Ketoconazole (assay)	Methanol (100)	10 µL	0.1 mg/mL	1.0 µg

Table 1. Standard conditions for USP assays.

Sample sets were evaluated according to system suitability criteria of individual monographs. Suitability parameters were peak area %RSD (Table 2), peak retention time %RSD (Table 3), and USP tailing factor (Table 4). System suitability was met for all assays across three days of testing.

As organic content of the sample diluent increased, peak area variability increased (Table 2). Fluconazole (20/80 acetonitrile/water) showed the lowest peak area variability, with peak area RSD values between 0.019% to 0.032%. Losartan potassium (40/60 methanol/water) and fenofibrate (70/30 acetonitrile/water) showed increases in peak area RSD values, between 0.05% to 0.06% for losartan potassium and between 0.07% to 0.09% for fenofibrate. Ketoconazole had the highest organic content (100% methanol) and greatest peak area variability, with RSD values of 0.102% for Day 2 and 3.

For injection volume, no clear trend could be determined with peak area repeatability. Fluconazole uses the largest injection volume of 20 µL and demonstrated low peak area RSDs. Losartan potassium and ketoconazole use similar injection volumes (13.2 µL and 10 µL, respectively), but the peak area RSDs were dissimilar. Fenofibrate uses the smallest injection volume of 6.6 µL, but this did not correlate to an increase in peak area variability. This can indicate that the challenge of small injection volumes is mitigated on the Alliance iS HPLC System.

Injection precision (average peak area RSD)				
USP monograph	System suitability criteria	Day 1	Day 2	Day 3
Fluconazole (assay)	NMT 2.0%	0.032%	0.024%	0.019%
Losartan potassium (assay)	NMT 0.5%	0.06%	0.05%	0.05%
Fenofibrate (assay)	NMT 1.0% (n=6)	0.09%	0.07%	0.09%
Ketoconazole (assay)	NMT 0.73%	0.043%	0.102%	0.102%

Table 2. Peak area %RSD (average of three sample sets) over three days for USP assays on Alliance iS HPLC System. RSD system suitability criteria were met.

Average retention time RSD				
USP monograph	System suitability criteria	Day 1	Day 2	Day 3
Fluconazole (assay)	NMT 2.0%	0.0%	0.0%	0.0%
Losartan potassium (assay)	NMT 0.5%	0.0%	0.0%	0.0%
Fenofibrate (assay)	NMT 1.0% (n=6)	0.1%	0.1%	0.1%
Ketoconazole (assay)	NMT 0.73%	0.02%	0.01%	0.01%

Table 3. Retention time %RSD (average of three sample sets) over three days for USP assays on Alliance iS HPLC System. RSD system suitability criteria were met.

Average USP tailing factor				
USP monograph	System suitability criteria	Day 1	Day 2	Day 3
Fluconazole (assay)	NMT 2.0	1.1	1.1	1.1
Losartan potassium (assay)	NMT 1.4	1.0	1.0	1.0
Fenofibrate (assay)	0.8–1.8	1.1	1.1	1.1
Ketoconazole (assay)	NMT 2.0	0.9	0.9	0.9

Table 4. USP tailing factor (average of three sample sets) over three days for USP assays on Alliance iS HPLC System. Tailing system suitability criteria were met.

Based on these results, organic content is the method parameter that primarily impacts the autosampler performance on the Alliance iS HPLC System. To further characterize this issue, the absolute peak area of ketoconazole was evaluated. Ketoconazole, which uses 100% methanol as diluent, showed the greatest peak area variability of the four USP monographs. The high organic content of the diluent means that the standard solution is volatile and, therefore, sample concentration is more easily impacted by evaporation.

Indeed, a trend of increasing absolute peak area was observed for N=18 consecutive ketoconazole standard injections (Figure 2). N=18 standard solution injections were drawn from the same vial, which indicates that the standard was becoming more concentrated over time. Carryover was ruled out as a contributor to peak area increase since post-standard blank showed no carryover peaks (Figure 3). Therefore, this peak area increase is likely due to diluent evaporation affecting the sample concentration

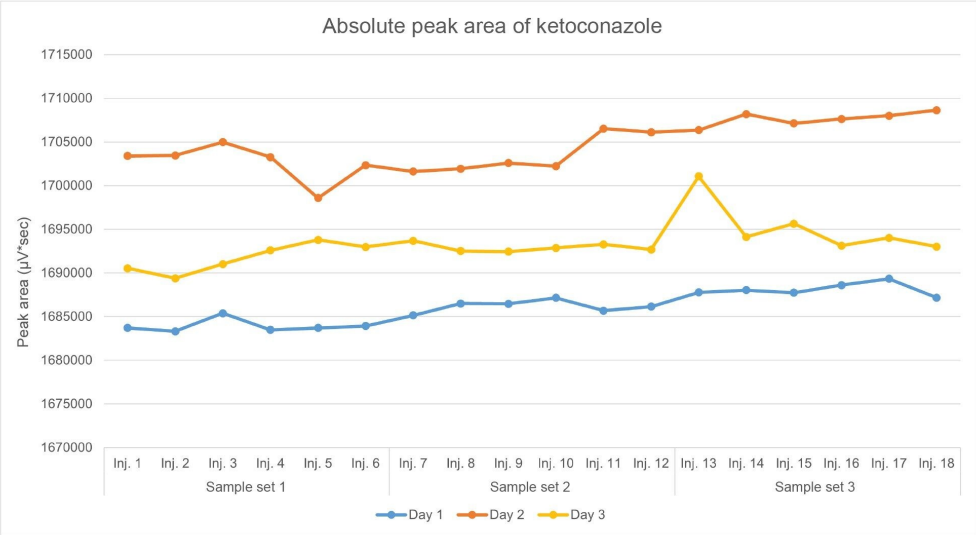

Figure 2. Absolute peak area of ketoconazole on Alliance iS HPLC System for three days (N=18 total injections). A trend of increasing peak area over injections was visualized. Vials of the ketoconazole standard solution were aliquoted and stored refrigerated until use. Unpunctured vials of standard solution were used each day. Day 1 (blue), Day 2 (orange), and Day 3 (yellow).

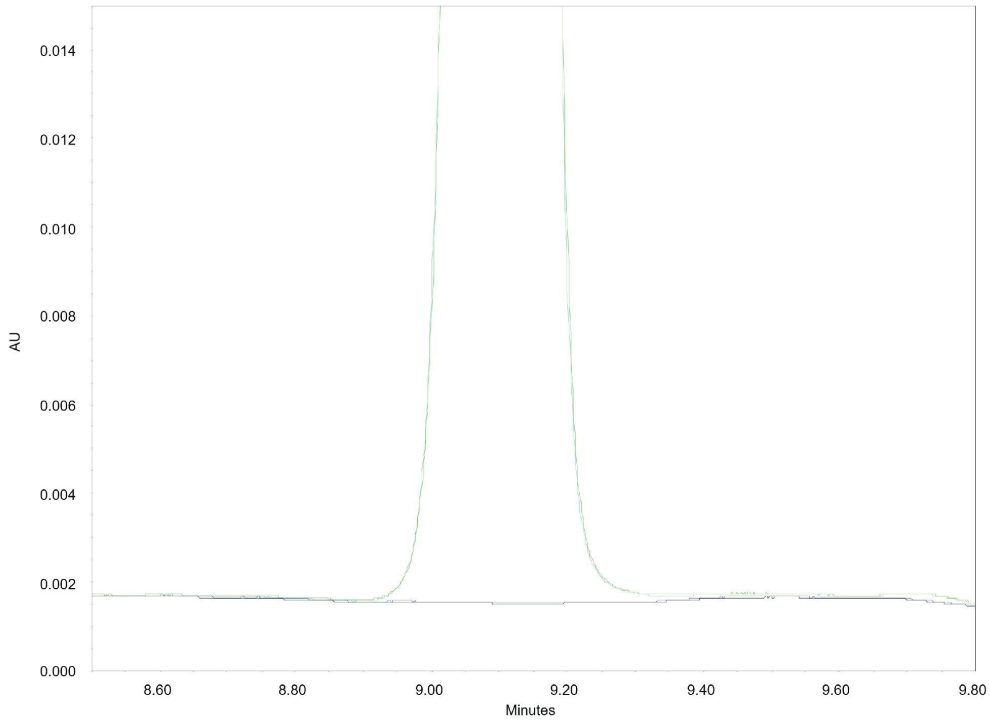

Figure 3. Chromatographic overlays of ketoconazole standard solution (green/teal) and post-standard blank (black/blue). For each set of injections, standard solution injections #5 and #6 were overlayed with the post-standard blanks to verify that there was no carryover. Injections shown here were from Sample Set 3 of 3 on Day 3.

Conclusion

The ability of an autosampler to meet injection precision requirements can be impacted by method conditions and instrument characteristics. In this study, four challenging USP assay methods were used to evaluate autosampler performance of the Alliance iS HPLC System, using peak area repeatability as a proxy. The methods were run continuously for three days to simulate system drift that would be encountered in a high-throughput environment. The Alliance iS HPLC System demonstrated high precision and met all system suitability requirements for the USP methods tested.

Organic content of the sample diluent and injection volume were assessed as two method conditions that could impact autosampler performance. As organic content in the diluent increased, peak area variability also increased. This indicates that the volatility of organic diluent can impact autosampler repeatability. By tracking the absolute peak area of ketoconazole, this variability was found to be caused by diluent evaporation. Injection volume did not significantly impact injection precision and no trend between injection volume and peak area repeatability was observed. The problem with small injection volumes is possibly mitigated due to the new metering device aspiration design for the Alliance iS HPLC System. Further testing can include assessing the performance of other comparable systems with different aspiration mechanisms.

References

1. Hong P. Improved Injection Precision of USP Method with an Arc HPLC System. Waters Application Note. 720007043. 2020.

2. Waters Corporation. ACQUITY Arc, ACQUITY Arc Bio, and Arc HPLC Sample Manager Flow Through Needle-R: Overview and Maintenance Guide. 715004749 < https://www.waters.com/waters/support.htm?cid=135068659&lid=134854304> Version 04, September 2021.

3. General Chapter USP <621> Chromatography. Official: December 1, 2022. https://www.usp.org/sites/default/files/usp/document/harmonization/gen-chapter/harmonization-november-2021-m99380.pdf <https://www.usp.org/sites/default/files/usp/document/harmonization/gen-chapter/harmonization-november-2021-m99380.pdf>

4. Layton C, Rainville P. Achieving Method Modernization with the New Liquid Chromatographic Gradient Allowances Provided by USP General Chapter <621> Chromatography and the Alliance™ iS HPLC System. Waters Application Note. 720007865. 2023.

5. Waters Corporation. Columns Calculator Version 2.0. 667005222 < https://www.waters.com/waters/support.htm?lid=134891632> , March 2016.

6. Monograph: USP. Fenofibrate. In: USP-NF. Rockville, MD: USP; 01 May 2020. DOI: https://doi.org/10.31003/USPNF_M32710_04_01 <https://doi.org/10.31003/USPNF_M32710_04_01>

7. Monograph: USP. Fluconazole. In: USP-NF. Rockville, MD: USP; 01 May 2020. DOI:

https://doi.org/10.31003/USPNF_M33240_03_01 <https://doi.org/10.31003/USPNF_M33240_03_01>

8. Monograph: USP. Losartan Potassium. In: USP-NF. Rockville, MD: USP; 01 Aug 2021. DOI: https://doi.org/10.31003/USPNF_M45933_05_01 <https://doi.org/10.31003/USPNF_M45933_05_01>
9. Monograph: USP. Ketoconazole. In: USP-NF. Rockville, MD: USP; 01 May 2020. DOI: https://doi.org/10.31003/USPNF_M43990_04_01 <https://doi.org/10.31003/USPNF_M43990_04_01>
10. Steiner F, Paul C, Dong M. HPLC Autosamplers: Perspectives, Principles, and Practices. LCGS North America. August 1, 2019. Volume 37, Issue 8. Pages: 514–529.

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