

## HPLC Autosampler Performance II: Improved Injection Precision of USP Methods With the Alliance™ iS HPLC System

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### Abstract

Method conditions and instrument characteristics can affect the autosampler performance of High-Performance Liquid Chromatography (HPLC) systems. Challenging method conditions, such as low injection volume and highly organic sample diluents, require high precision to meet strict suitability requirements. Instrument characteristics, such as sample aspiration mechanism and injector draw rate, often vary between vendors and may impact the precision performance of the autosampler. This can pose a challenge for analytical laboratories that desire to run methods with strict precision suitability criteria across HPLC systems from different vendors. In this study, the injection precision performance of the Alliance iS HPLC System and similar HPLC systems were compared using four compendial HPLC methods from the United States Pharmacopeia (USP) with strict precision criteria and challenging method conditions. Peak area reproducibility was evaluated as a proxy for autosampler performance of each system.

### Benefits

- High precision for a range of sample diluents and injection volumes on the Alliance iS HPLC System
  - Meets strict injection precision criteria for challenging USP methods on the Alliance iS HPLC System
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- Demonstrates improved precision on the Alliance iS HPLC System over similar HPLC systems for highly organic sample diluent

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## Introduction

For many regulated methods in the United States Pharmacopeia (USP), injection precision is a common system suitability criterion. Injection precision is a measure of the autosampler performance in a high-performance liquid chromatography (HPLC) system. The autosampler is responsible for delivering precise aliquots of sample to the HPLC system flow path.<sup>1</sup> This performance can be impacted by method conditions and instrument characteristics.<sup>2,3,4</sup> To ensure that acquired data is accurate and meets suitability requirements of the method, high performance is required from an autosampler.

Instrument characteristics, such as sample aspiration mechanism and injector draw rate, vary among vendors due to differences in system design. Sample aspiration is typically controlled by either a metering device or a sampling syringe.<sup>1</sup> Injector draw rate is a setting typically set to a default speed that often varies with instrument vendor.<sup>4</sup> These different characteristics can complicate running methods across systems from different vendors. A routine method that performs well on one HPLC system may not meet system suitability criteria on a different HPLC system. Therefore, it is important to consider how autosampler performance is affected by instrument characteristics.

In this study, four USP Assay monographs were used to examine the impact of instrument characteristics on injection precision of an Alliance iS HPLC System and comparable HPLC systems. The HPLC systems include sampling syringe and metering device aspiration mechanisms and utilize different injector draw speeds. The USP Assay methods have challenging method conditions: varying organic content in the sample diluent (20%–100% organic), low injection volumes (6.6  $\mu$ L–20  $\mu$ L), and narrow injection precision criteria (0.73% to 2.0%).

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## Experimental

Method parameters with asterisks (\*) indicate adjustments of the original monographs to accommodate for modern columns and sizes, per Chapter <621> Chromatography.<sup>5,6</sup> 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.<sup>5,6</sup> 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.<sup>5,6</sup> Changes were made according to USP <621> and using the Waters™ Columns Calculator.<sup>7</sup>

Per USP <621>, monographs with RSD requirements ≤2.0% require five replicate injections and ≥2.0% require six replicate injections.<sup>5</sup> The Fenofibrate monograph requires six replicate injections with an RSD requirement ≤1.0%.<sup>8</sup> Six replicate injections were used throughout to 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.

### Method Conditions

LC System:	Alliance iS HPLC System System X HPLC System System Y HPLC System Alliance e2695 System Arc™ HPLC System
Separation mode:	Isocratic
Detection:	UV for all systems (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 <sub>18</sub> 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.

### Method Conditions

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 system:	Alliance iS HPLC System
	System X HPLC System
	System Y HPLC System
	Alliance e2695 System
	Arc HPLC System

Separation mode: Isocratic

Detection: UV for all systems (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 <sub>18</sub> 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 assay was updated by the USP in August 2021, changing the sample diluent from 100% methanol to 40/60 Methanol/Water.<sup>1,8</sup>

## 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.

### Method Conditions

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 system:	Alliance iS HPLC System System X HPLC System System Y HPLC System Alliance e2695 System Arc HPLC System
Separation mode:	Isocratic
Detection:	UV for all systems (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 <sub>18</sub> 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.

## Method Conditions

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 system:	Alliance iS HPLC System System X HPLC System System Y HPLC System Alliance e2695 System Arc HPLC System
Separation mode:	Gradient
Detection:	UV for all systems (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/pkg (p/n: 186005666CV)
Column*:	XBridge™ Shield RP18 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 Management

Chromatography software: Empower™ 3.7

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## Results and Discussion

### Performance of Alliance iS HPLC System and Comparable HPLC Systems

To evaluate the performance of the Alliance iS HPLC System and comparable HPLC systems, four USP assay methods were run and peak area %RSD of standard solutions were compared. Each method was run continuously for three days to simulate system stability in a high-throughput environment. The HPLC systems were primed and purged prior to starting analysis on Day 1. The HPLC autosampler characteristics and default settings are described in Table 1.

Standard solutions were aliquoted and stored refrigerated until use. Unpunctured vials of standard



solutions were used each day to minimize impacts from sample 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 HPLC systems running uninterrupted. Peak area relative standard deviation (RSD) was evaluated for each sample set and plotted. The injection precision performance of each HPLC system was compared to see if the systems could meet strict precision requirements consistently.

HPLC system	Aspiration mechanism	Injector draw rate setting (default)
Alliance iS HPLC System	Metering device	100 $\mu\text{L}/\text{min}$
System X	Metering device	300 $\mu\text{L}/\text{min}$
System Y	Metering device	100 $\mu\text{L}/\text{min}$
Alliance e2695 System	Sampling syringe	150 $\mu\text{L}/\text{min}$
Arc HPLC System	Sampling syringe	100 $\mu\text{L}/\text{min}$

*Table 1: HPLC system characteristics of injector draw rates and aspiration mechanisms. Injector draw rates were converted to  $\mu\text{L}/\text{min}$ .*

Injector draw rate of an autosampler is typically not a defined parameter in regulated methods. As such, many users often leave this method parameter with the instrument default value. However, this setting can be optimized based on the needs of the analysis. For example, in a high-throughput environment, it may be beneficial to increase the draw rate to expedite analysis time. While a fast draw speed can reduce cycle time, it may adversely impact peak reproducibility, as in the case for viscous samples.<sup>4</sup> In this study, instrument default draw rates were maintained for each system (Table 1).

The HPLC systems in this study use different mechanisms for sample aspiration: sampling syringes and metering devices (Table 1). To illustrate differences between the mechanisms, Aspirate/Inject stages are depicted in Figure 1. For a sampling syringe design, which the Arc HPLC System uses, a low-pressure syringe aspirates the sample (Figure 1A). When the sample is injected (Figure 1B), the syringe is isolated from the high-pressure flow path, which may accumulate air over the course of a run.

Potential for air accumulation is a drawback of the sampling syringe. Accumulated gas impacts accuracy and

precision of an autosampler, causing incorrect and inconsistent volumes of sample to be delivered. Therefore, it is important to purge the syringe prior to analysis. To mitigate this issue, some HPLC systems include a separate degassed purge solvent to more effectively clear the syringe for system startup.<sup>2</sup>

Another potential drawback for the sampling syringe is the precision of the syringe draw motor. On legacy HPLC systems, the syringe draw motor may be less fine-tuned. This can affect accuracy and precision for low injection volumes. This issue can be mitigated with different combinations of sample loops and syringe sizes, but the complexity in changing these out for different assays may be time-consuming for users.

The metering device design of the Alliance iS HPLC System addresses some of these drawbacks. Air accumulation is mitigated with a metering device that is in-line with the high-pressure flow path (Figure 1C). When the sample is injected, the metering device remains part of the flow line and is constantly flushed by mobile phase (Figure 1D). This prevents air from becoming trapped in the metering device. The motor mechanism controlling the metering device is more fine-tuned, which improves injection accuracy and precision.

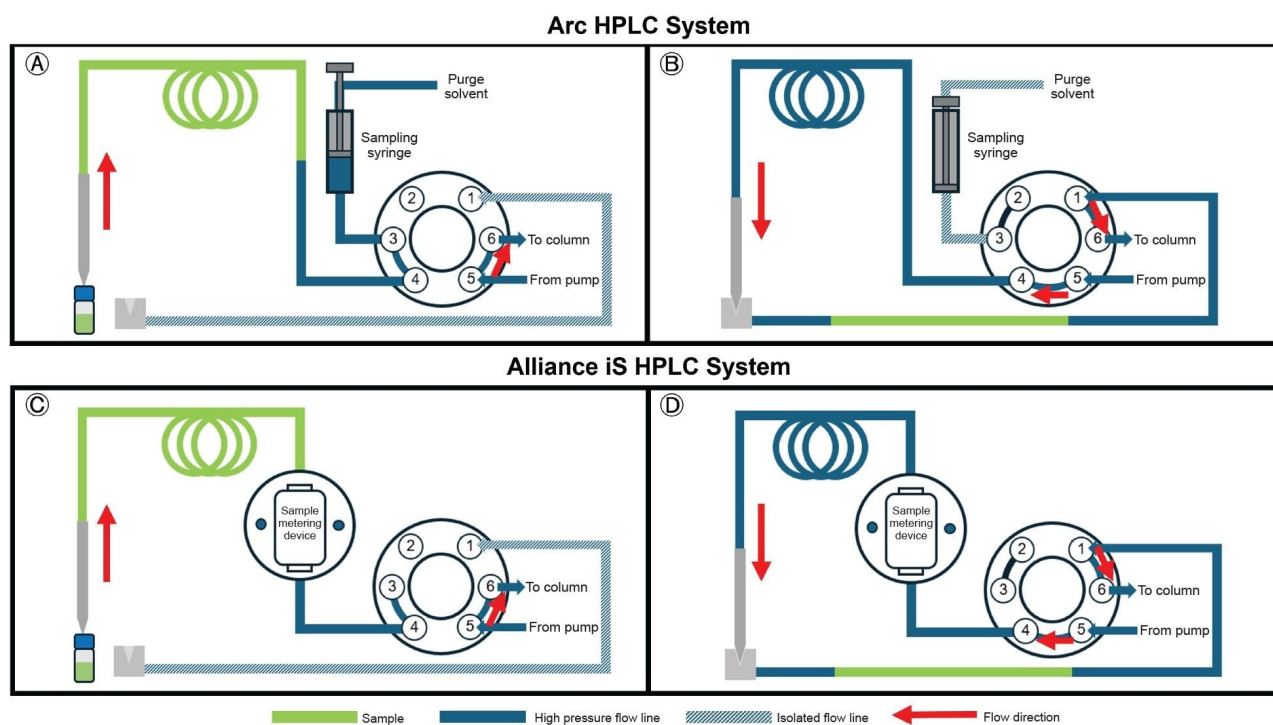


Figure 1: Aspiration mechanisms of sampling syringe and metering device. (A) Aspirate stage of the Arc HPLC System, (B) Inject stage of the Arc HPLC System, (C) Aspirate stage of the Alliance iS HPLC System, (D) Inject stage of the Alliance iS HPLC System. Red arrows indicate direction of flow.

## USP Fluconazole Monograph (20/80 organic/aqueous)

The fluconazole assay uses a predominantly aqueous sample diluent of 20% acetonitrile and 80% water. The assay was highly reproducible across all systems, with sample sets peak area RSD ranging from 0.02% to 0.37% (Figure 2). This is within the 2.0% peak area RSD requirement. HPLC systems with metering devices (the Alliance iS HPLC System, System X, and System Y) showed better precision performance compared to the systems with sampling syringes (the Alliance e2695 System and the Arc HPLC System), with no sample sets exceeding 0.2% peak area RSD. The Alliance iS HPLC System demonstrated the best individual performance overall, with peak area %RSD values between 0.02% and 0.05% over the three-day period.

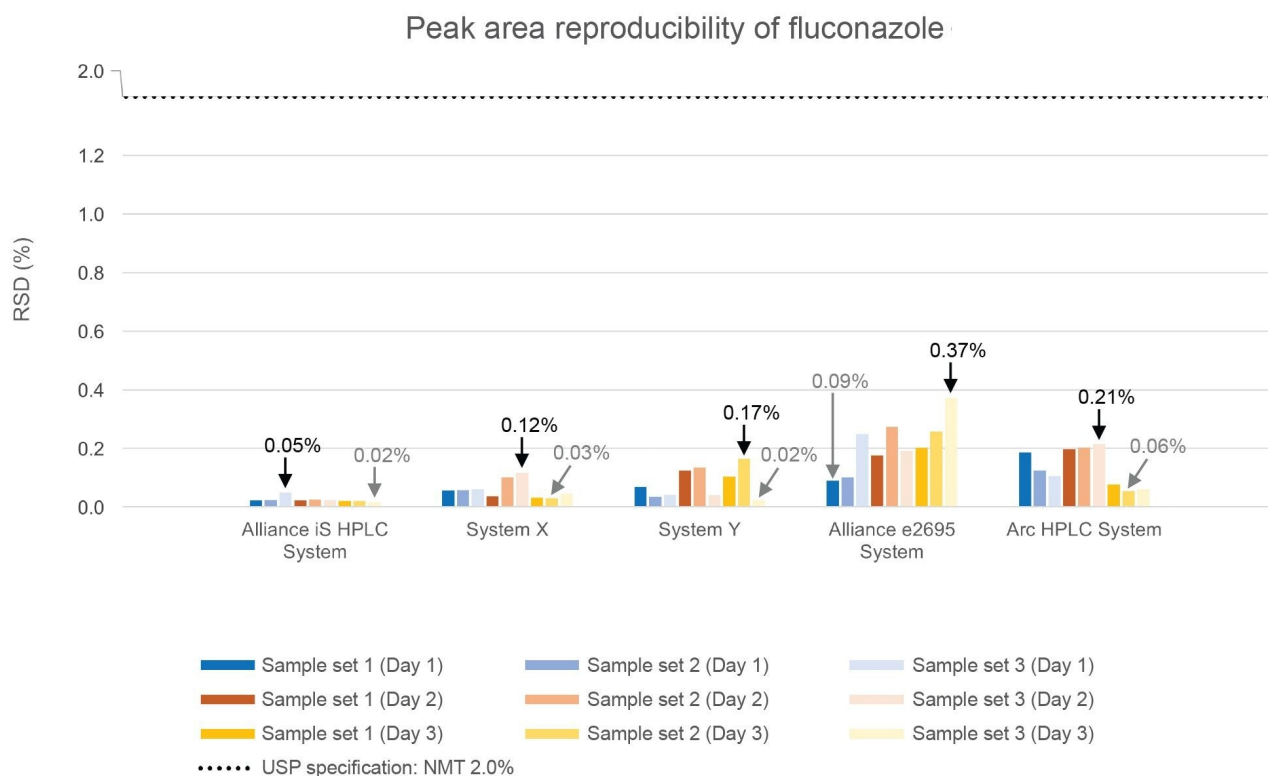


Figure 2: Sample set peak area %RSD of Fluconazole. Injection precision was assessed on five comparable systems over three days. N=3 sample sets were run daily. Sample set peak area %RSD minimum (gray) and maximum (black) are noted. The Alliance iS HPLC System, System X, and System Y use metering devices while the Alliance e2695 System and the Arc HPLC System use sampling syringes.

## USP Losartan Potassium Monograph (40/60 organic/aqueous)

The losartan potassium assay uses a majority aqueous sample diluent of 40% acetonitrile and 60% water. Like fluconazole, this assay was highly reproducible across the systems, with sample set peak area %RSD values ranging from 0.01% to 0.25% (Figure 3). All systems met the strict 0.5% peak area RSD requirement without significant performance differences between the aspiration mechanisms. A previous version of this method was tested in P. Hong (2020) and proved to be challenging, but new revisions by USP have changed the sample diluent from 100% organic to 40/60 organic/aqueous mixture.<sup>2</sup> The reduction in volatile organic component likely enabled the HPLC systems to meet this stringent requirement more easily.

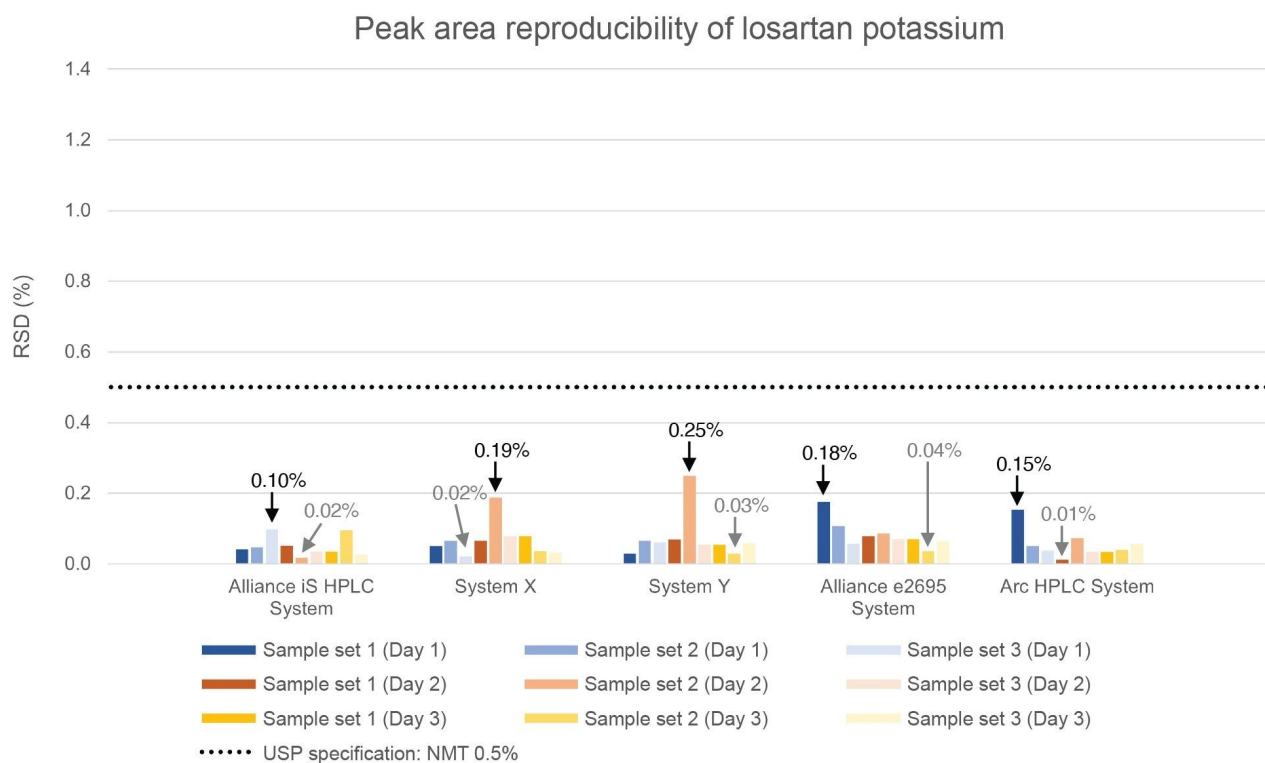


Figure 3: Sample set peak area %RSD of Losartan Potassium. Injection precision was assessed on five comparable systems over 3 days. N=3 sample sets were run daily. Sample set peak area %RSD minimum (gray) and maximum (black) are noted. The Alliance iS HPLC System, System X, and System Y use metering devices while the Alliance e2695 System and the Arc HPLC System use sampling syringes.

### USP Fenofibrate Monograph (70/30 organic/aqueous)

The fenofibrate assay is predominantly organic with a sample diluent of 70% acetonitrile and 30% acidified water. Higher organic content in the diluent was expected to make sample repeatability challenging due to volatility. However, the assay was reproducible across systems, with sample set peak area RSDs between 0.04% to 0.43% (Figure 4). All systems were within the 1.0% peak area RSD requirement. The Alliance iS HPLC System, Systems X and Y, and the Arc HPLC System all performed similarly (peak area RSDs between 0.04% and 0.23%).

The assay was more challenging for the Alliance e2695 System, which showed higher peak area %RSD values (from 0.10% to 0.43%) compared to other systems. This is likely due to the low injection volume of the assay (6.6  $\mu$ L). In default configuration, the Alliance e2695 System uses a 250  $\mu$ L sampling syringe. The large volume of the

sampling syringe paired with the low injection volume would contribute to a loss in precision and higher peak area %RSDs.

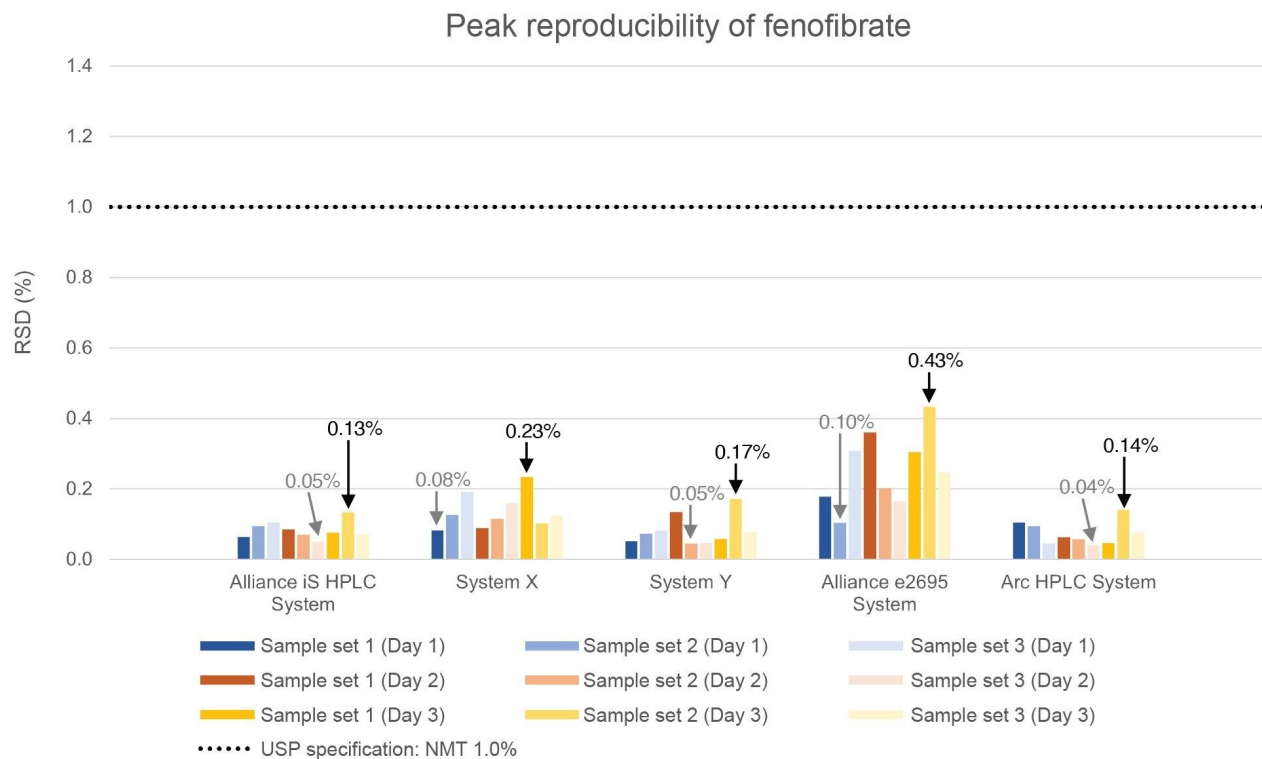
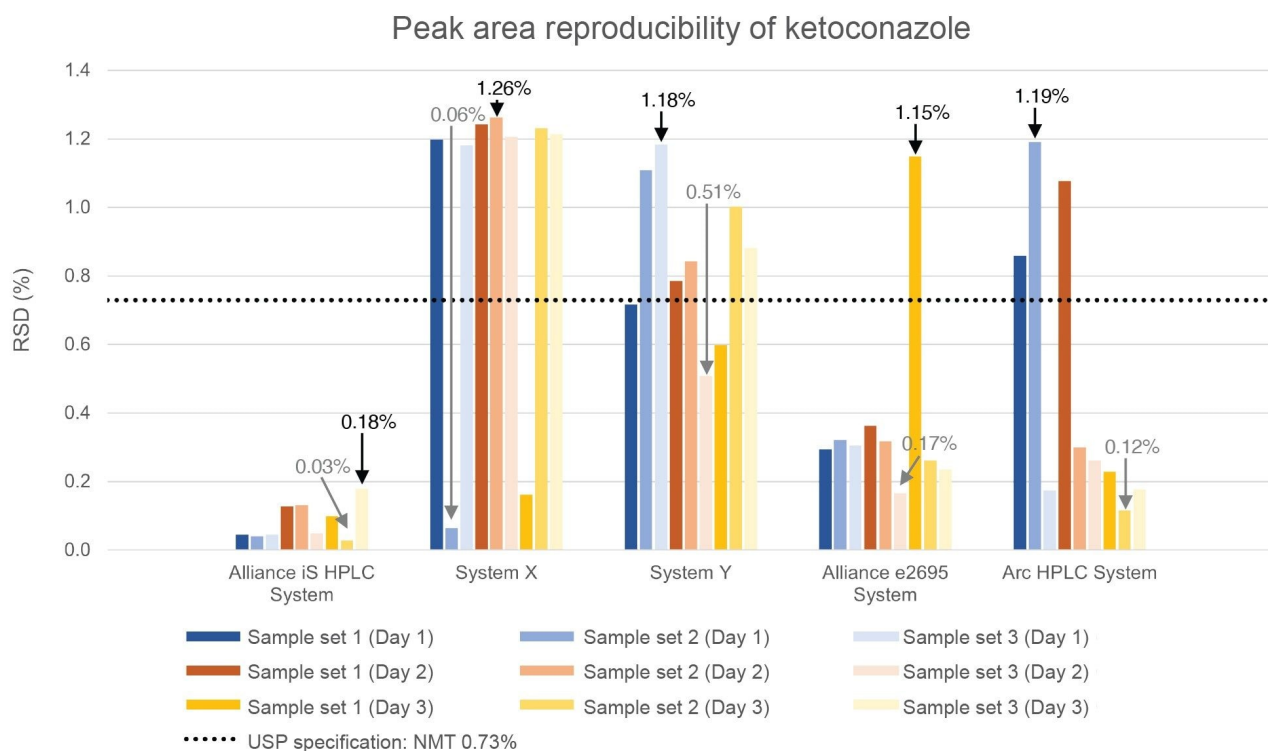


Figure 4: Sample set peak area %RSD of Fenofibrate. Injection precision was assessed on five comparable systems over three days. N=3 sample sets were run daily. Sample set peak area %RSD minimum (gray) and maximum (black) are noted. The Alliance iS HPLC System, System X, and System Y use metering devices while the Alliance e2695 System and the Arc HPLC System use sampling syringes.

## USP Ketoconazole Monograph (100% organic)

The ketoconazole assay is entirely organic for the sample diluent, using 100% methanol. This method proved challenging across the HPLC systems due to its strict precision requirement of 0.73% peak area RSD. Among the tested HPLC systems, only the Alliance iS HPLC System met criteria over the three-day period (peak area RSD values between 0.03% and 0.18%). The remaining four HPLC systems failed to consistently meet criteria (Figure 5). System X and the Arc HPLC System showed wide variances between sample sets. Peak area %RSD ranged

from 0.06% to 1.26% on System X and ranged from 0.12% to 1.19% on the Arc HPLC System. System Y produced consistently high peak area %RSDs, ranging from 0.51% to 1.18%. The Alliance e2695 System had peak area RSD values between 0.17% to 0.36% and one high RSD sample set at 1.15%.



*Figure 5: Sample set peak area %RSD of Ketoconazole. Injection precision was assessed on five comparable systems over three days. N=3 sample sets were run daily. Sample set peak area %RSD minimum (gray) and maximum (black) are noted. The Alliance iS HPLC System, System X, and System Y use metering devices while the Alliance e2695 System and the Arc HPLC System use sampling syringes.*

No clear trend related to sample aspiration mechanism could be determined from the ketoconazole data. Although metering devices were expected to provide better injection precision performance over sampling syringes, the results here were inconclusive. The Alliance iS HPLC System, System X, and System Y showed no consistent trend in precision performance, while Systems X and Y failed to meet precision criteria for majority of the sample sets.

Data collected from the systems using metering devices was evaluated to determine what additional factors may

impact autosampler performance, such as sample diluent and injector draw rate. The ketoconazole assay uses 100% methanol as sample diluent, which is highly volatile. Throughout the study, sample compartment temperatures were controlled at 15 °C to minimize sample evaporation. Unpunctured vials of standard solution were used each day (N=18 total injections produced from the same vial). Carryover was ruled out as a contributor to peak area increase because post-standard blanks showed no carryover peaks across all HPLC systems (Figure 6). If diluent evaporation is a main factor impacting peak area variability, the absolute peak area of ketoconazole would increase over time as diluent evaporates and sample solution becomes more concentrated.

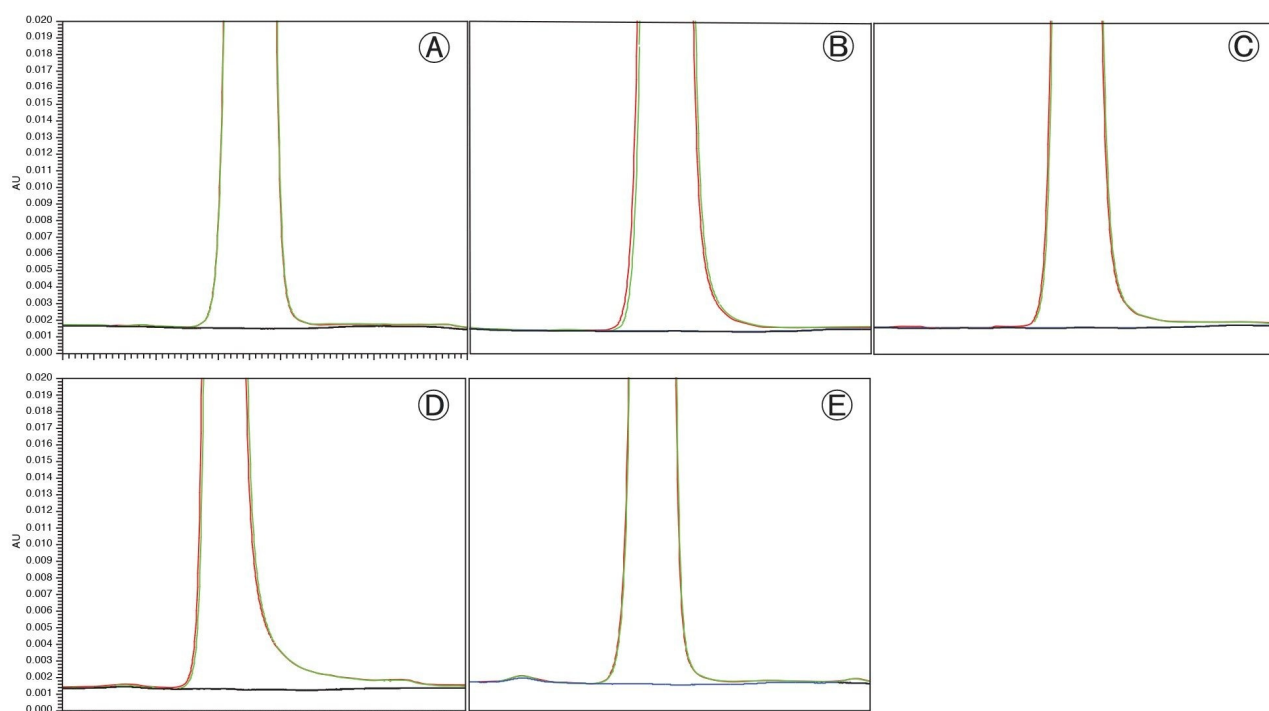


Figure 6: Chromatographic overlays of ketoconazole standard solution (red/green) and post-standard blank (black/blue) on five HPLC systems. For each set of injections, standard solution injections #5 and #6 were overlaid with the post-standard blanks immediately following to verify that there was no carryover. Injections shown here are from Sample Set 3 of 3 on Day 3 across all five systems: (A) Alliance iS HPLC System; (B) System X; (C) System Y; (D) Alliance e2695 System; (E) Arc HPLC System.

Absolute peak areas of ketoconazole for N=18 individual injections on the Alliance iS HPLC System, System X,



and System Y are visualized in Figure 7. Overall, the Alliance iS HPLC System showed the least peak area variability between injections. Sample peak area on the Alliance iS HPLC System trended upwards (yellow trace in Figure 7), which indicates some sample evaporation, but performance was not impacted, and peak area RSDs were low (0.3% to 0.18%). Systems X and Y (orange and blue traces in Figure 7, respectively) showed no trend of evaporation; sample peak area did not increase but tended to alternate between low and high peak area values, which resulted in high peak area RSDs. These peak area fluctuations occurred intermittently throughout the testing and indicates that sample evaporation is not the main cause of peak area variability on these systems.

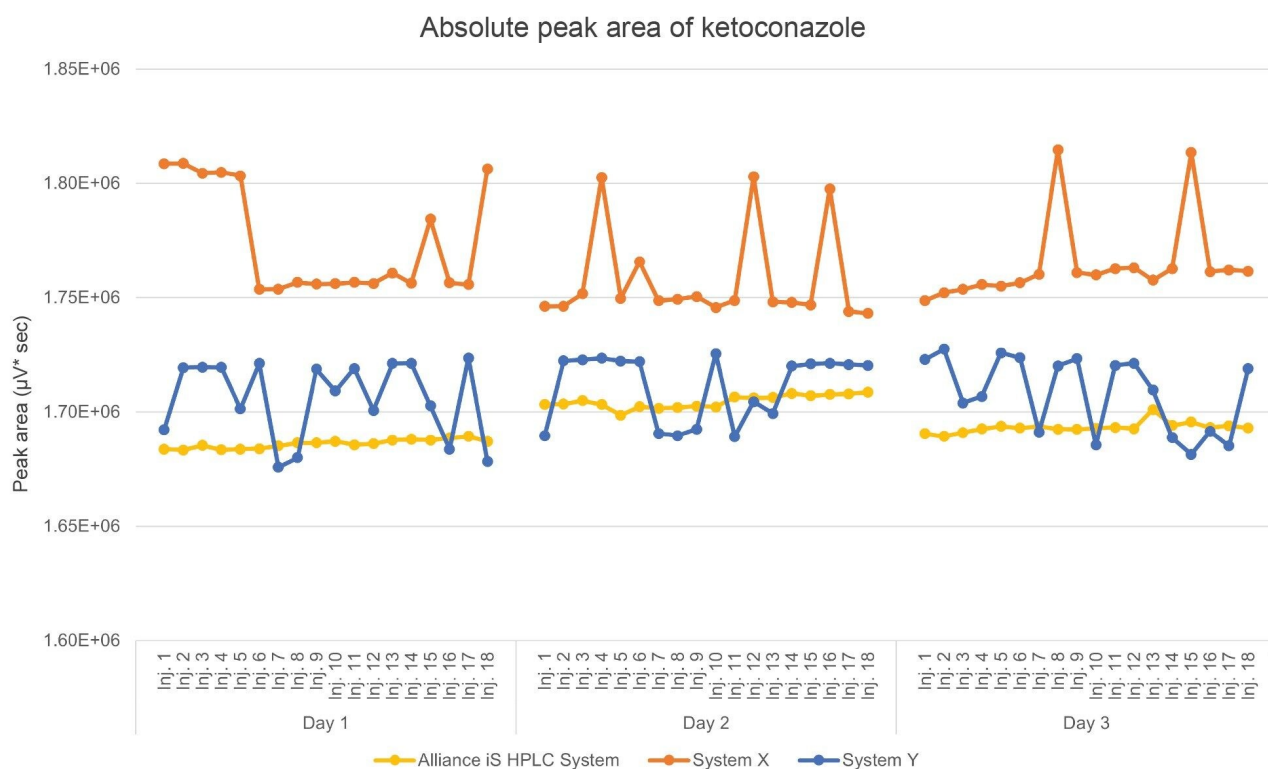


Figure 7. Absolute peak area of ketoconazole of three HPLC systems across three days. N=18 injections were made from one vial each day. Alliance iS HPLC (yellow), System X (orange), and System Y (blue) use metering devices.

Another factor to consider is injector draw rate, which may need to be optimized for the sample diluent. As shown in Table 1, default draw rate values of the HPLC systems can vary. The draw speeds of Alliance iS HPLC System and System Y are 100 µL/min while System X uses 300 µL/min. System X uses the fastest draw speed of

the three systems and demonstrates the greatest variability among the three systems. If the draw speed is not optimized for the diluent, this could account for the inconsistency in peak area between injections. On the other hand, Alliance iS HPLC System and System Y both use 100  $\mu\text{L}/\text{min}$  draw speed, but the precision performances were vastly different. The injector draw rate and its impact on peak area reproducibility may warrant more investigation.

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## Conclusion

The ability of an HPLC system to acquire consistent, repeatable data is critical and requires high performance from an autosampler. This study examined the impact of instrument characteristics, such as sample aspiration mechanisms and injector draw rates, on HPLC performance in meeting precision requirements. Autosampler performance is complex and was found to vary with the method and the system. The metering device has benefits for precision performance over the sampling syringe, using a more finely tuned syringe draw motor and being located in-line with the high-pressure flow path.

For fluconazole and ketoconazole USP assays, the Alliance iS HPLC demonstrated the best performance overall, with the lowest peak area %RSDs. The ketoconazole assay had particularly challenging method conditions and produced widely varying results among the HPLC systems. Alliance iS HPLC System demonstrated high precision and produced consistent and reproducible results across the three-day period, meeting the stringent 0.73% peak area RSD requirement.

The ketoconazole assay produced disparate results among the HPLC systems using metering devices, which was unexpected. Upon tracking the absolute peak area of ketoconazole for each system, evaporation and carryover were ruled out as causes of the peak variability. Additionally, despite identical injector draw rates on two of the HPLC systems, their performance varied significantly. While aspiration mechanism and injector draw rate are important factors, they do not fully explain the observed variations in autosampler performance.

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