

# **An Assessment of Autosampler Carry-Over Through The Determination of Ketoconazole in Rat Plasma by LC-MS/MS**

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## **OBJECTIVE**

To assess immediate and accumulative carry-over using autosampler configurations, typically employed in the field of quantitative bioanalysis. This was undertaken using a pre-developed assay for the determination of ketoconazole in rat plasma by LC-MS/MS, with the aim of achieving a calibration range of 1000 fold.

The objective of this assessment was not to show one autosampler configuration as “better” or “worse” than another, but to demonstrate the observed performance of each configuration within the context of achieving the desired calibration range. This investigation was undertaken from an “end-users” prospective, applying a basic set of generic autosampler conditions, with minimal optimization of the available operational features. It is recognized that the performance of each configuration could be further optimized through the implementation of an appropriate trouble-shooting strategy, with respect to this application.

## **INTRODUCTION**

Current LC-MS technology expedites the development of the fundamental components of a bioanalytical assay. However, with today's quantitative demands for sensitivity, over a wide dynamic calibration range, an inordinate amount of time can be spent trouble-shooting LC-MS system carry-over during assay development, to ensure that it is fit for its intended purpose. During development, a target calibration range is typically set at approximately 1000 fold, notwithstanding limitations with respect to the physicochemical properties of the analyte, the ionization, sampling and transmission efficiencies of the resulting ions, and/or electron multiplier saturation. This target range ensures a reasonable attempt at capturing the potential wide range of analyte concentrations present in toxicology study samples, therefore limiting the requirement for repeat sample analysis. This assay characteristic is particularly desirable for early preclinical studies, where the pharmacokinetic properties of the new drug entity under investigation may still be largely unknown. In addition, sample volumes may be limited as a consequence of the investigative model.

For a chromatographic bioanalytical assay to be considered GLP compliant, the FDA guideline<sup>2</sup> for the definition of an acceptable LLOQ, has, in part, been used to develop an industry-wide consensus on the acceptable performance of carry-over, as no direct guidance formally exists. The definition suggests, "The analyte response at the LLOQ should be at least 5 times the response compared to blank response.". This has unanimously been interpreted to mean, that in the assessment of carry-over, the observed response for a blank sample, injected immediately after the ULOQ, should be  $\leq 20\%$  of the LLOQ response. However, the extent to which carry-over is actually investigated during assay development, is left to the discretion and due diligence of the bioanalytical facility.

Generally speaking, carry-over from the LC part of the LC-MS system can be considered as either coming from the autosampler, and/or the LC column and plumbing between the autosampler and the MS. The extent and relative contribution of each aspect of LC carry-over is a composition of the following factors: the materials present in the sample flow path, autosampler design, its operational configuration and state of maintenance, the physicochemical properties of the analyte, and the chemical composition of the sample solvent. The contribution from "non-autosampler" components can often be limited through modification of the mobile phase and/or chromatography.

However, it is the reduction in the autosampler contribution that tends to be the most challenging, and therefore time-consuming factor.

LC carry-over can be further categorized into immediate and accumulative. Immediate carry-over can be considered as the observed response for a blank sample injected immediately after a high concentration standard. This is usually the ULOQ of the assay, and this is formally assessed during assay validation. Accumulative carry-over, on the other hand, can be considered as the observed response for a blank sample injected immediately after a series of high concentration standards. The definition and evaluation of this latter category is open to debate, and there is no guidance or requirement for its assessment. However, its evaluation does provide a more thorough investigation of carry-over to assure for the eventualities of study sample analysis. Here, a series of samples containing a high concentration of analyte may precede either a blank sample or a sample containing a low concentration of the analyte. This could potentially result in a false positive, or an artificially elevated quantitation, respectively. To limit such possibilities, carry-over should be monitored within every batch of samples assayed, although, it is important to understand that the presence of carry-over does not necessarily negate that particular batch. Prior consideration should ideally be given to the injection sequence, with careful evaluation of the resulting data to identify individual samples likely to have been affected by carry-over, enabling repeat analysis in duplicate to confirm the result. However, accumulative carry-over should be evaluated during assay development, to ensure the assignment of an appropriate calibration range, as well as the robustness of the assay.

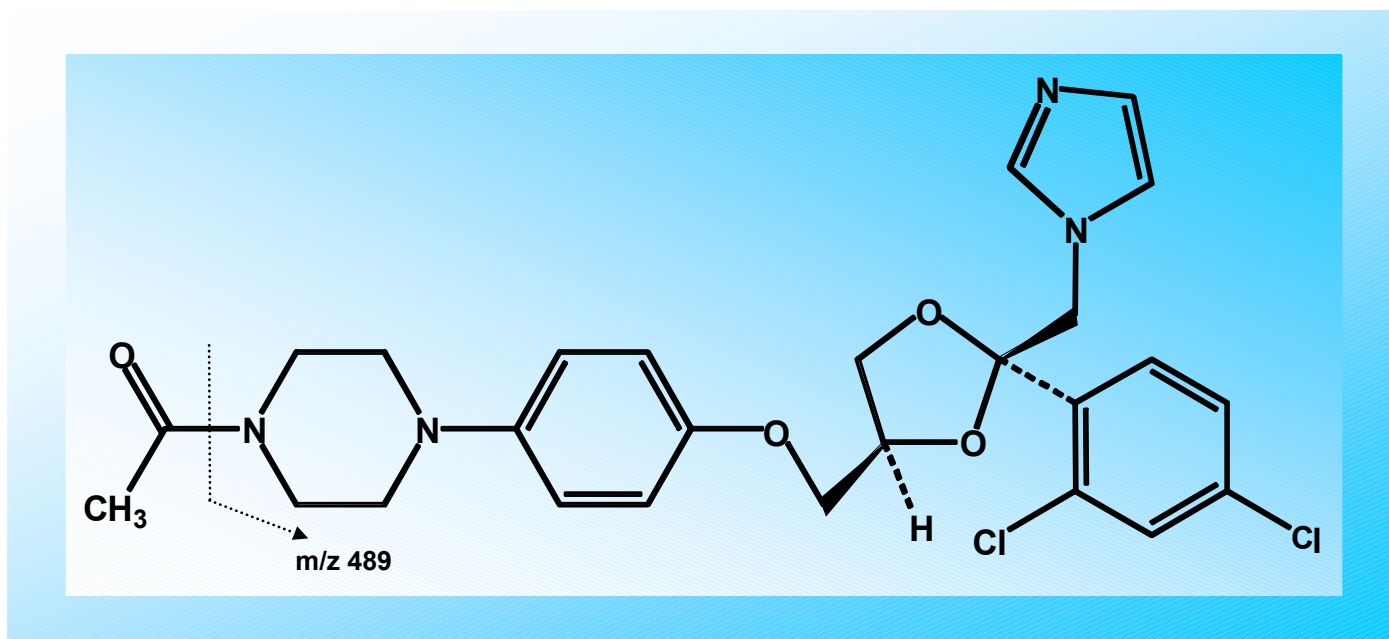
The purpose of this undertaking was to assess the immediate and accumulative autosampler carry-over for the operation configurations of four vendor designs, typically used in the field of quantitative bioanalysis, using a pre-developed bioanalytical protocol. Such designs included the three principal autosampler loop sampling modes<sup>1</sup>, namely, Pull-To-Fill, Push-To-Fill and Integral-Loop.

## METHOD

### Bioanalytical Protocol

The ketoconazole assay<sup>3</sup> involved off-line SPE followed by LC-MS/MS analysis, utilizing the sensitivity of an MDS Sciex API 5000™, to detect early signs of carry-over. Ketoconazole was ionized by (+) ESI to generate a positive precursor ion at  $m/z$  531, which was dissociated to a progeny ion at  $m/z$  489. The 1000 fold target assay calibration range was from 0.05 (LLOQ) to 50 ng/mL (ULOQ), in rat plasma. A 3-step LC gradient was applied to reduce the contribution of “non-autosampler” components, from the observed carry-over (Figures 1, 2 and 3).

**Figure 1. Ketoconazole Chemical Structure (C<sub>26</sub>H<sub>28</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>4</sub>, MW 531.4)**



## Figure 2. Schematic of Ketoconazole Bioanalytical Protocol

### Sample Pretreatment

Rat Plasma (K<sub>2</sub> EDTA; 50 µL) + Terconazole (Internal Standard; 2 ng/mL) in  
Water:Methanol:Ammonium Hydroxide (95:5:5, v/v/v; 300 µL)

### SPE Protocol

*Waters Oasis® HLB, (10 mg) - Gravity Elution*

**Condition 1:** Acetonitrile:Formic Acid (100:0.2, v/v; 500 µL)

**Condition 2:** Water:Methanol:Ammonium Hydroxide (95:5:5, v/v/v; 1000 µL)

**Load:** Sample Extract (~350 µL)

**Wash 1:** Water:Methanol:Ammonium Hydroxide (95:5:5, v/v/v; 400 µL)

**Wash 2:** Water:Methanol:Ammonium Hydroxide (40:60:5, v/v/v; 300 µL)

**Elution:** Acetonitrile:Formic Acid (100:0.2, v/v; 500 µL)

**Evaporation:** 40°C, N<sub>2</sub>

**Reconstitution:** Water:Acetonitrile:Formic Acid (60:40:0.2, v/v/v; 500 µL)

### Reverse Phase LC-(+)ESI-MS/MS Conditions

**Injection Volume:** 10 µL

**Autosampler Wash Solution:** 2-Propanol:Methanol:Trifluoroacetic Acid (80:20:0.1, v/v/v)

**Mobile Phase A:** Water:Formic Acid (100:0.2, v/v)

**Mobile Phase B:** Acetonitrile:Formic Acid (100:0.2, v/v)

**LC Gradient:** Step 1 - **A:B** 60:40, 1 mL/min, 0 to 2.5 min;

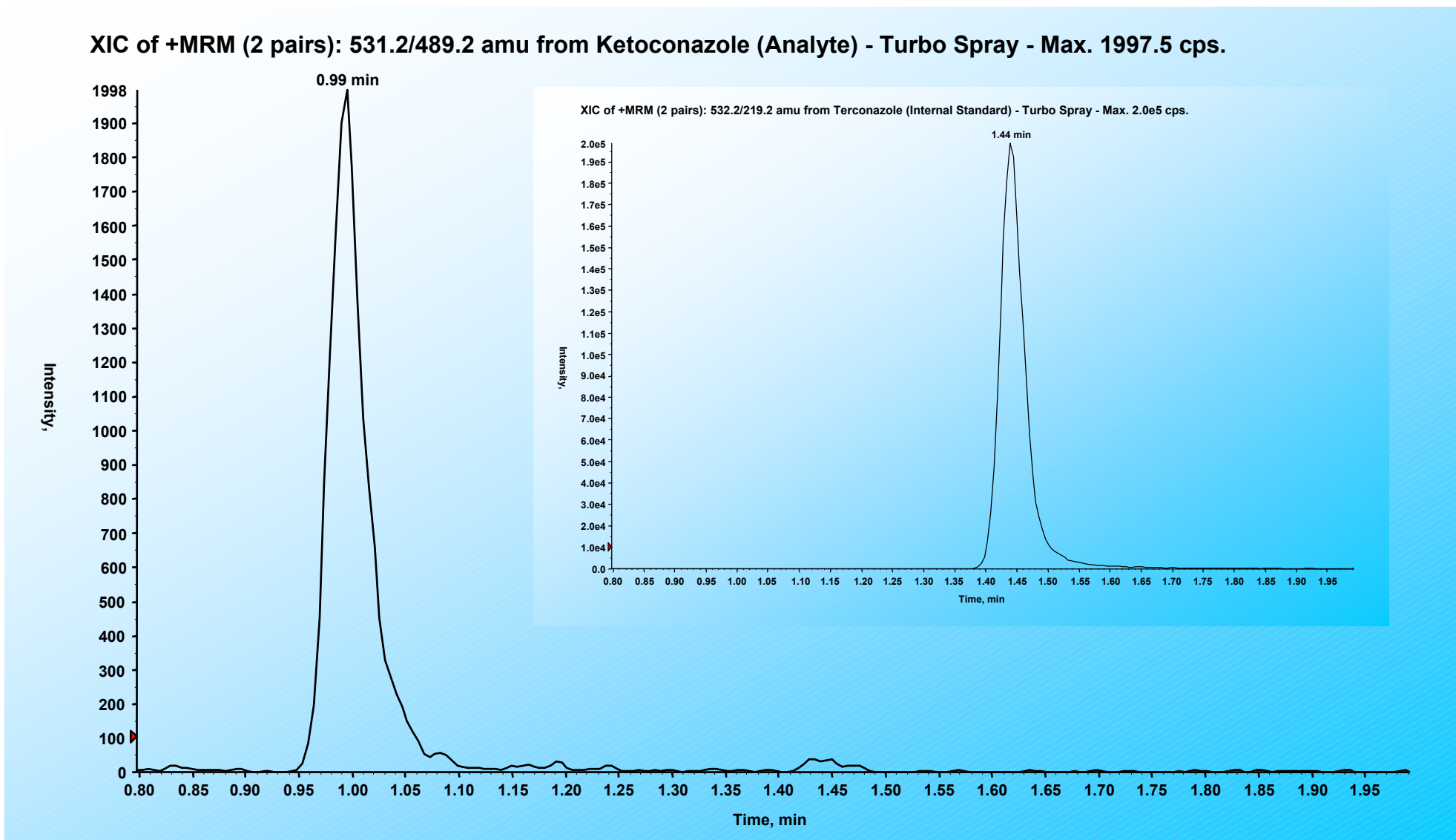
Step 2 - **A:B** 5:95, 3 mL/min, 2.5 to 5.5 min; Step 3 - **A:B** 60:40, 3 mL/min, 5.5 to 6.5 min

**LC Column:** Merck KGaA, Chromolith® Speed Rod RP-18e, 50 × 4.6 mm

**Mass Spectrometer:** MDS Sciex API 5000™

**MRM Transitions:** Ketoconazole *m/z* 531>489, Internal Standard *m/z* 532>219

Figure 3. Typical Chromatography for Ketoconazole - 1 ng/mL in Rat Plasma (*Internal Standard Insert*)



### Autosampler Configurations

Ten autosampler configurations were assessed, with respect to vendor design (Shimadzu SIL-HTc, Agilent Series 1100 WPALS, Perkin Elmer Series 200, Leap Technologies CTC HTS PAL and Spark Holland Reliance™), injection valve materials and valve toggling, through the application of a basic set of generic operational parameters. These were programmed using the standard features allowable through Analyst™, Version 1.4.1 (MDS Sciex) (Figure 4). Each configuration was semi-quantitatively evaluated within the context of immediate and accumulative carry-over, across the defined calibration range. An acceptance criterion of  $\leq 20\%$  of the LLOQ peak area response was set as a threshold, for an extracted blank sample injected immediately after an injection of an extracted ketoconazole calibration standard, or series of QC samples (n = 6), to observe the immediate or accumulative carry-over, respectively.

**Figure 4. Autosampler Configuration Operational Parameters**

Autosampler	Configuration	Operational Parameter											
		Loop Sampling Design	Loop Fill Mode	Injection Needle Material	Stator Material	Loop Material	Rotor Material	Wash Volume Inside Needle	Wash Volume Outside Needle	Valve Wash-1	Valve Wash-2	Valve Toggle	Valve Stem Wash
Shimadzu Sil-HTc	1	Integrated-Loop	n/ap	Stainless Steel/Platinum	Stainless Steel/PEEK®/Ceramic	Stainless Steel	PEEK®	LC Mobile Phase 14 mL	① 350 µL	LC Mobile Phase 14 mL	n/ap	No	n/ap
Shimadzu Sil-HTc	2	Integrated-Loop	n/ap	Stainless Steel/Platinum	Stainless Steel/PEEK®/Ceramic	Stainless Steel	PEEK®	LC Mobile Phase 14 mL	① 800 µL	LC Mobile Phase 14 mL	n/ap	LC Mobile Phase x4	n/ap
Leap Technologies CTC HTS PAL	3	Push-To-Fill	Full	Stainless Steel/Glass	Hastalloy®C	Stainless Steel	Valcon H	① 1.3 mL ② 0.3 mL	① 1.3 mL ② 0.3 mL	LC Mobile Phase 14 mL	n/ap	No	① 1.0 mL ② 0.3 mL
Leap Technologies CTC HTS PAL	4	Push-To-Fill	Full	Stainless Steel/Glass	PAEK®	PEEK®	Valcon E	① 1.3 mL ② 0.3 mL	① 1.3 mL ② 0.3 mL	LC Mobile Phase 14 mL	n/ap	No	① 1.0 mL ② 0.3 mL
Spark Holland Reliance™	5	Pull-To-Fill	Partial µL Pick-up	Stainless Steel/Teflon	Stainless Steel	Stainless Steel	Valcon H	① 1.5 mL ② 1.4 mL	① 0.7 mL	LC Mobile Phase 14 mL	① 1.5 mL ② 1.4 mL	No	n/ap
Spark Holland Reliance™	6	Pull-To-Fill	Partial µL Pick-up	Stainless Steel/Teflon	Stainless Steel	Stainless Steel	Valcon H	① 1.5 mL ② 1.4 mL	① 0.7 mL	LC Mobile Phase 14 mL	① 1.5 mL ② 1.4 mL	LC 95% Mobile Phase B x2	n/ap
Spark Holland Reliance™	7	Pull-To-Fill	Partial µL Pick-up	Stainless Steel/Teflon	PAEK®	PEEK®	Valcon E	① 1.5 mL ② 1.4 mL	① 0.7 mL	LC Mobile Phase 14 mL	① 1.5 mL ② 1.4 mL	No	n/ap
Spark Holland Reliance™	8	Pull-To-Fill	Partial µL Pick-up	Stainless Steel/Teflon	PAEK®	PEEK®	Valcon E	① 1.5 mL ② 1.4 mL	① 0.7 mL	LC Mobile Phase 14 mL	① 1.5 mL ② 1.4 mL	LC 95% Mobile Phase B x2	n/ap
Agilent Series 1100 WPALS	9	Integrated-Loop	n/ap	Stainless Steel	Stainless Steel	Stainless Steel	VespeI	LC Mobile Phase 14 mL	① 0.5 mL	LC Mobile Phase 14 mL	n/ap	No	n/ap
Perkin Elmer Series 200	10	Pull-To-Fill	Partial	Stainless Steel/Teflon	Stainless Steel/PEEK®/Ceramic	PEEK®	VespeI	① 10 mL	① 10 mL	LC Mobile Phase 14 mL	n/ap	n/ap	n/ap

n/ap = Parameter Not Applicable

① = n-Propanol:Methanol:Trifluoroacetic Acid (80:20:0.1, v/v/v)

② = Mobile Phase A:Mobile Phase B (60:40, v/v)

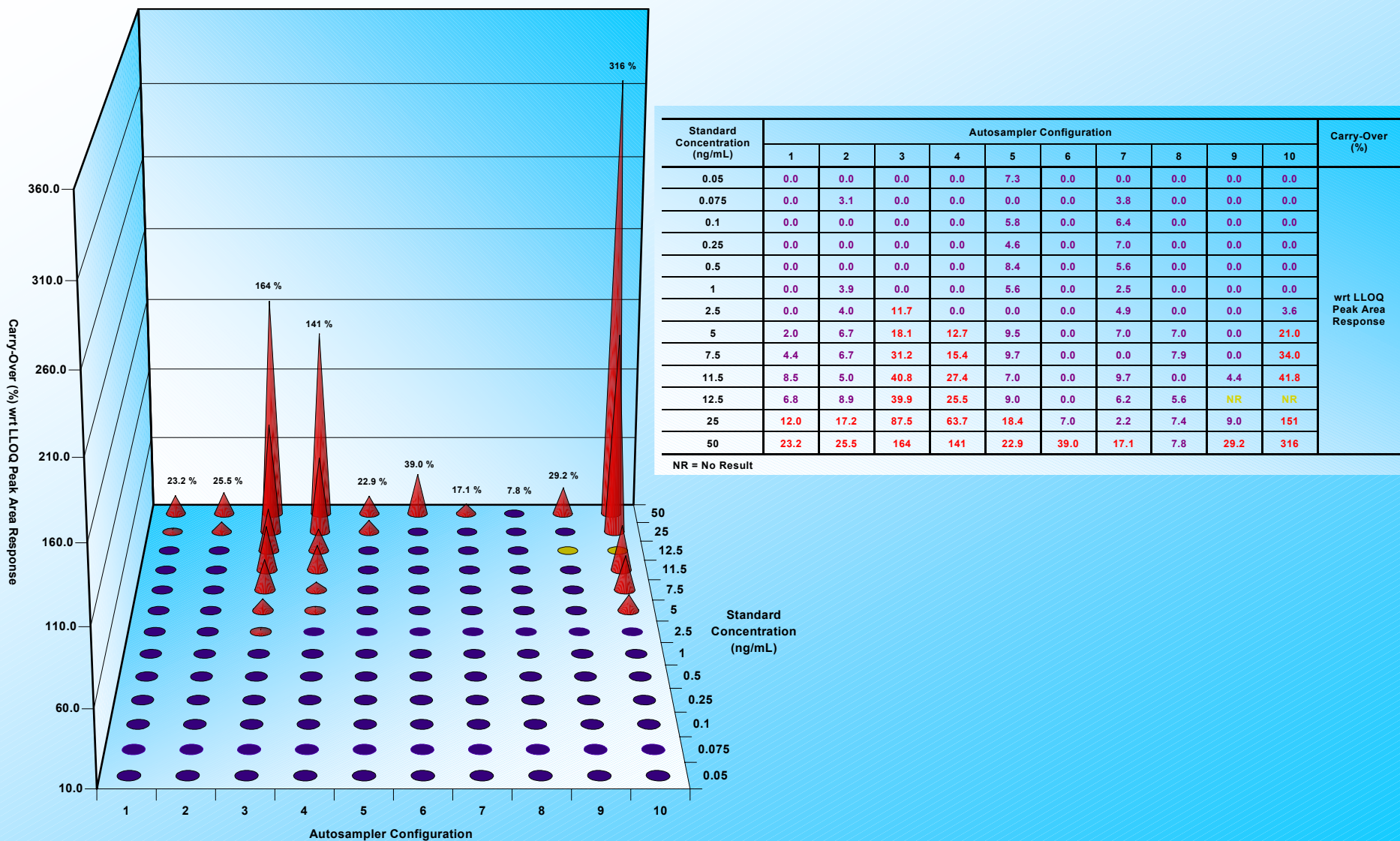
## **RESULTS AND DISCUSSION**

The lowest level of unacceptable carry-over was noted for each autosampler configuration. An arbitrary 10% “base” was used to separate carry-over that was considered insignificant ( $< 10\%$ ), from that, that was considered significant ( $\geq 10\%$ ). This was an attempt to “smooth” the data sets from the sporadic appearance of carry-over, resulting from the measurement of a peak area response  $\geq 5$  times the baseline noise, but less than the LLOQ response, for which the acceptance criterion for variability was already  $\pm 20\%$  (CV), (in accordance with FDA guidelines<sup>2</sup>). Therefore, the data sets presented should only be interpreted in a semi-quantitative manner, within the context of this assessment (Figures 5 and 6).

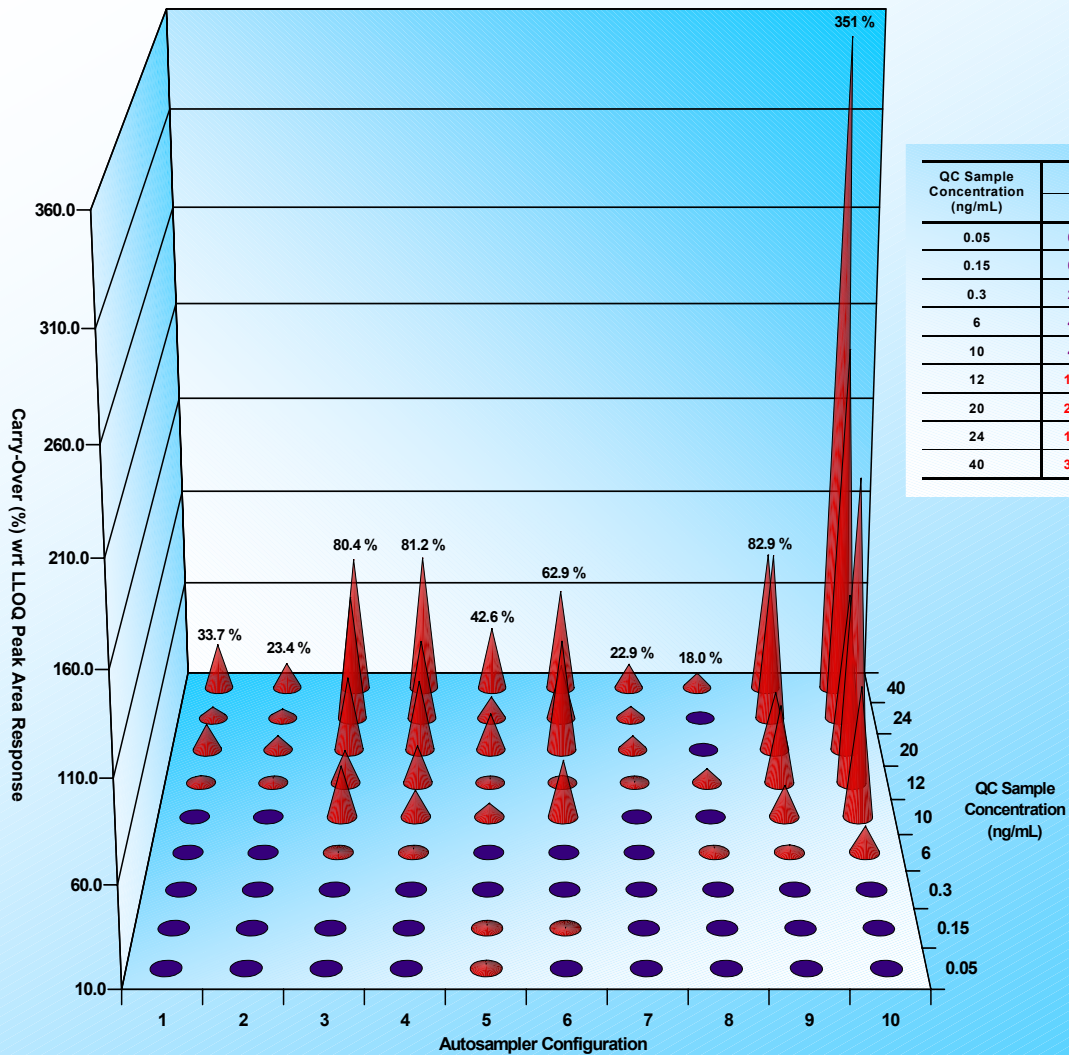
For the majority of autosampler configurations, the extent and prevalence of carry-over was greater for the accumulative situation, verses the immediate. The Reliance™ fulfilled the acceptance criterion at all concentrations evaluated, for both immediate and accumulative carry-over (50 ng/mL, ~8% and 40 ng/mL, ~18%, for configuration 8, respectively). Immediate carry-over was evident for the SIL-HTc and Series 1100 WPALS at 50 ng/mL (~26% and ~29%, for configurations 2 and 9, respectively), with greater accumulative prevalence at lower concentrations (40 ng/mL, ~23% and 10 ng/mL, ~26%, respectively). The CTC HTS PAL exhibited both immediate and accumulative carry-over at approximately 10 ng/mL (~26%, for configuration 4), while carry-over was observed for the Series 200 at approximately 5 ng/mL (~22%, for configuration 10).

Vendor design configurations utilizing PEEK®, PAEK® and Valcon E materials in the injection valve, appeared to exhibit lower carry-over than their respective metal and Valcon H configurations (configurations 3, 5 and 6, verses 4, 7 and 8, respectively). This was particularly apparent when used in combination with the option to toggle the injection valve (configuration 7 verses 8). Other than this, the ability to toggle the injection valve appeared to provide no significant improvements in the observed carry-over profiles (configurations 1 and 5, verses 2 and 6, respectively).

**Figure 5. Ketoconazole Immediate Carry-Over Data Sets - Table and Chart**



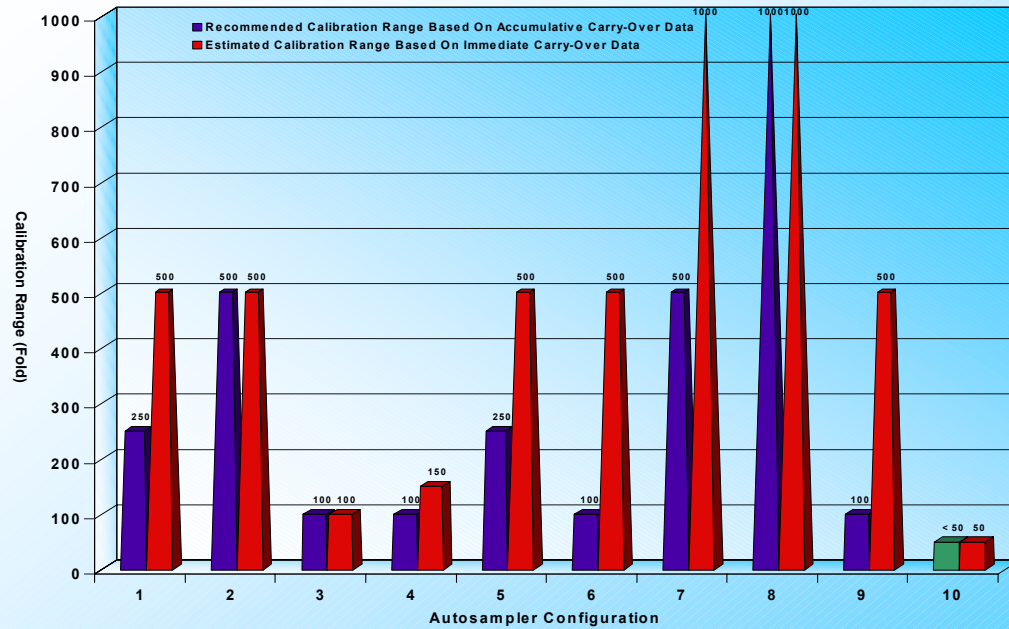
**Figure 6. Ketoconazole Accumulative Carry-Over Data Sets - Table and Chart**



QC Sample Concentration (ng/mL)	Autosampler Configuration										Carry-Over (%)
	1	2	3	4	5	6	7	8	9	10	
0.05	0.0	0.0	0.0	0.0	12.5	0.0	1.9	0.0	0.0	0.0	wrt LLOQ Peak Area Response
0.15	0.0	0.0	0.0	0.0	11.9	10.5	7.6	5.8	8.1	0.0	
0.3	2.3	0.0	0.0	0.0	5.8	0.0	4.1	0.0	0.0	0.0	
6	4.9	6.3	10.4	12.3	0.0	0.0	9.5	12.8	14.0	23.5	
10	4.7	8.5	36.4	23.9	17.2	39.4	0.0	0.0	26.3	77.0	
12	13.8	12.9	27.0	29.5	12.4	13.9	10.8	17.4	50.4	167	
20	23.7	17.6	48.4	46.7	29.2	67.9	17.5	0.0	40.7	92.0	
24	16.1	15.0	75.4	51.7	21.6	38.5	16.4	9.8	97.6	205	
40	33.7	23.4	80.4	81.4	42.6	62.9	22.9	18.0	82.9	351	

From these data sets, estimated and recommended calibration ranges could be proposed for each autosampler configuration, demonstrating the potential for calibration range, ULOQ and/or LLOQ misassignment, if immediate carry-over is considered without an assessment of accumulative carry-over (Figure 7).

**Figure 7. Estimated and Recommended Calibration Range, ULOQ and LLOQ Assignments Based On Immediate and Accumulative Carry-Over Data, Respectively - Table and Chart**



Autosampler Configuration	Estimated ULOQ Based On Immediate Carry-Over Data (ng/mL)	Recommended ULOQ Based On Accumulative Carry-Over Data (ng/mL)	Estimated LLOQ Based On Immediate Carry-Over Data (ng/mL)	Recommended LLOQ Based On Accumulative Carry-Over Data (ng/mL)
1	25	12.5	0.1	0.2
2	25	25	0.1	0.1
3	5	5	0.5	0.5
4	7.5	5	0.3	0.5
5	25	12.5	0.1	0.2
6	25	5	0.1	0.5
7	50	25	0.05	0.1
8	50	50	0.05	0.05
9	25	5	0.1	0.5
10	2.5	Φ < 2.5	1	Φ > 1

Φ = Estimate Due To Data Gap In Accumulative Carry-Over Data For Autosampler Configuration 10

## **CONCLUSIONS**

Due diligence is required during bioanalytical assay development, to ensure that the prevalence of immediate and accumulative carry-over is adequately evaluated, and limited. This will allow for the assignment of realistic and robust calibration ranges, based on available autosampler configurations, assuring for the eventualities of study sample analysis. In addition, more formal guidance on carry-over is required within the industry, as this phenomenon will inevitably prevail, as LC and MS technologies in the field advance.

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