How sample acidification can save your PK assay

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INTRODUCTION

Design and optimization of sensitive and robust PK assays for large molecules is complex and can present many challenges. Different factors can interfere with assay performance. Sample matrices (urine, serum, plasma, saliva, etc.) can contain endogenous and exogenous factors that could affect assay precision and reproducibility. Drugs could also present stability issues such as aggregation, which affects the correct measurement of the drug and reducing the sensitivity of the assay.

In assay development and troubleshooting, it is crucial to adopt strategies to solve these challenges. The use of different buffers and sample pre-treatment can provide effective solutions.

Here, we describe two methods with poor robustness that we were able to optimize through acidification of the samples.

CASE STUDY N°1: BIOSIMILARITY ASSESSMENT

Analytical challenges:
The first method was a bridging ELISA PK assay to detect a Biosimilar biotherapeutic drug. The matrix to be analysed was human serum and the analytical range was from 40 - 2000 pg/mL.

During initial biosimilarity assessment, we observed a marked run-to-run variability when comparting calibration curves prepared with Biosimilar or Originator. Both curves overlapped in approx. 2 out 3 independent runs and showed acceptable biases when back-calculating the Originator curve towards the Biosimilar curve (Figure 1 A and 1 C, Table 1), indicating of a true biosimilar. However, in 1 out 3 independent runs, both curves diverged and showed biases > 20% (Figure 1 B and Table 1).

Due to the high variability between the different runs we were unable to develop a reliable assay. This also prevented a valid comparison between the calibration curves of Biosimilar and Originator, making it difficult to assess biosimilarity.

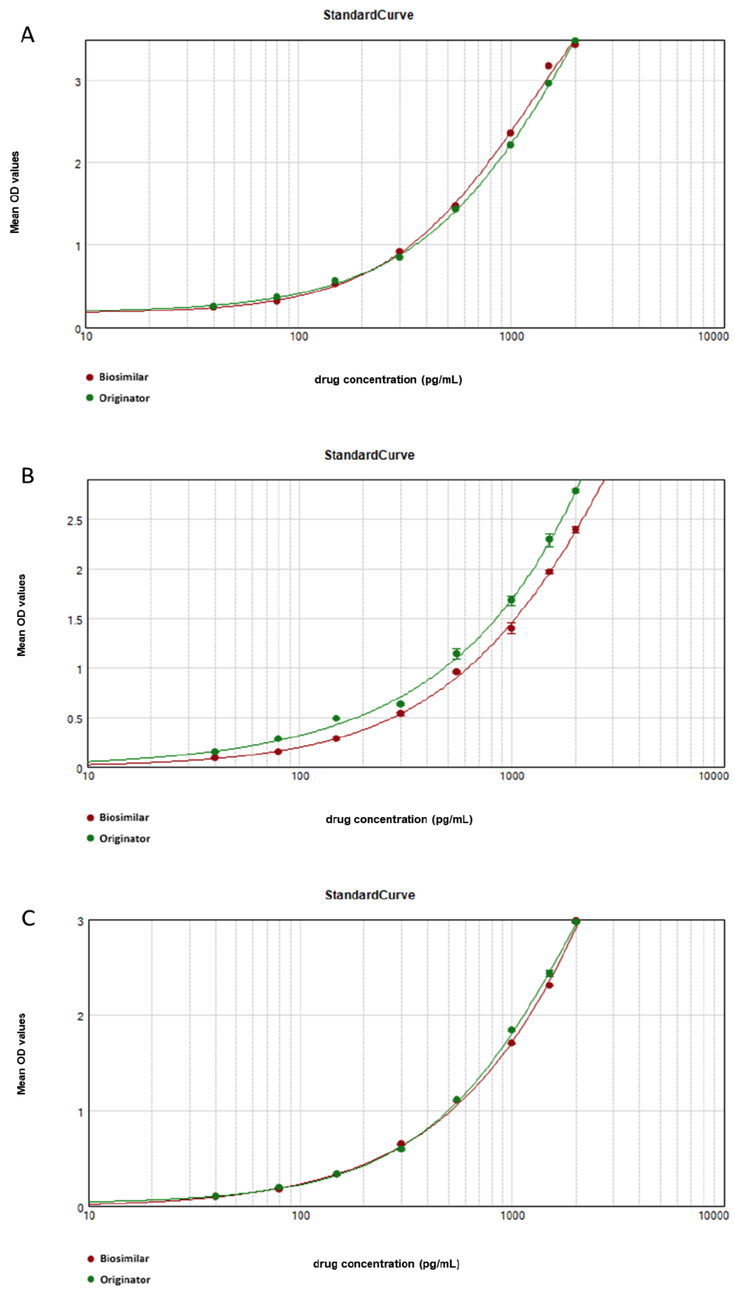

Figure 1: Visual comparison of calibration curves prepared with the Biosimilar and the Originator drugs. Figure 1 A, 1 B and 1 C show run-by-run variability and poor robustness of the assay.

% Bias	Nominal concentration [pg/mL]	A		B		C	
		Biosimilar	Originator	Biosimilar	Originator	Biosimilar	Originator
STD 1	2000	-4.60	-1.33	0.78	27.5	2.23	1.78
STD 2	1500	8.09	-4.72	0.77	25.4	-3.20	3.74
STD 3	1000	-1.50	-10.4	-5.22	20.8	-1.91	8.55
STD 4	550	-3.41	-6.68	6.61	32.7	5.33	5.72
STD 5	300	2.40	-7.35	-0.91	18.9	2.52	-6.23
STD 6	150	4.28	14.9	-0.71	79.2	-3.44	-1.07
STD 7	80	-7.19	19.7	-4.09	83.1	-7.18	31.4
STD 8	40	3.22	10.9	3.49	83.4	6.97	6.71

Table 1: Comparison of different calibration curves prepared with the Biosimilar and Originator drugs. The assay presented a high run-to-run variability. % Bias of the Originator curve was back-calculated towards the Biosimilar curve.

Troubleshooting:
Due to the suspected aggregation of the biotherapeutic drug after reconstitution, which could explain the observed run-to-run variability, we tested different buffers, diluents and sample pre-treatments, such as heat-inactivation, to prevent drug aggregation. However, none of these conditions improved the assay.

Through acidification and subsequent neutralization of the samples before adding them to the assay plate, we were able to improve drug solubility and increase out sensitivity by allowing the acid to dissociate immune complexes that were leading to drug aggregation (Figure 2 and Table 2). The acidification step in sample processing improved the precision and robustness of the assay and allowed biosimilarity assessment as seen by the now overlapping Biosimilar and Originator calibration curves.

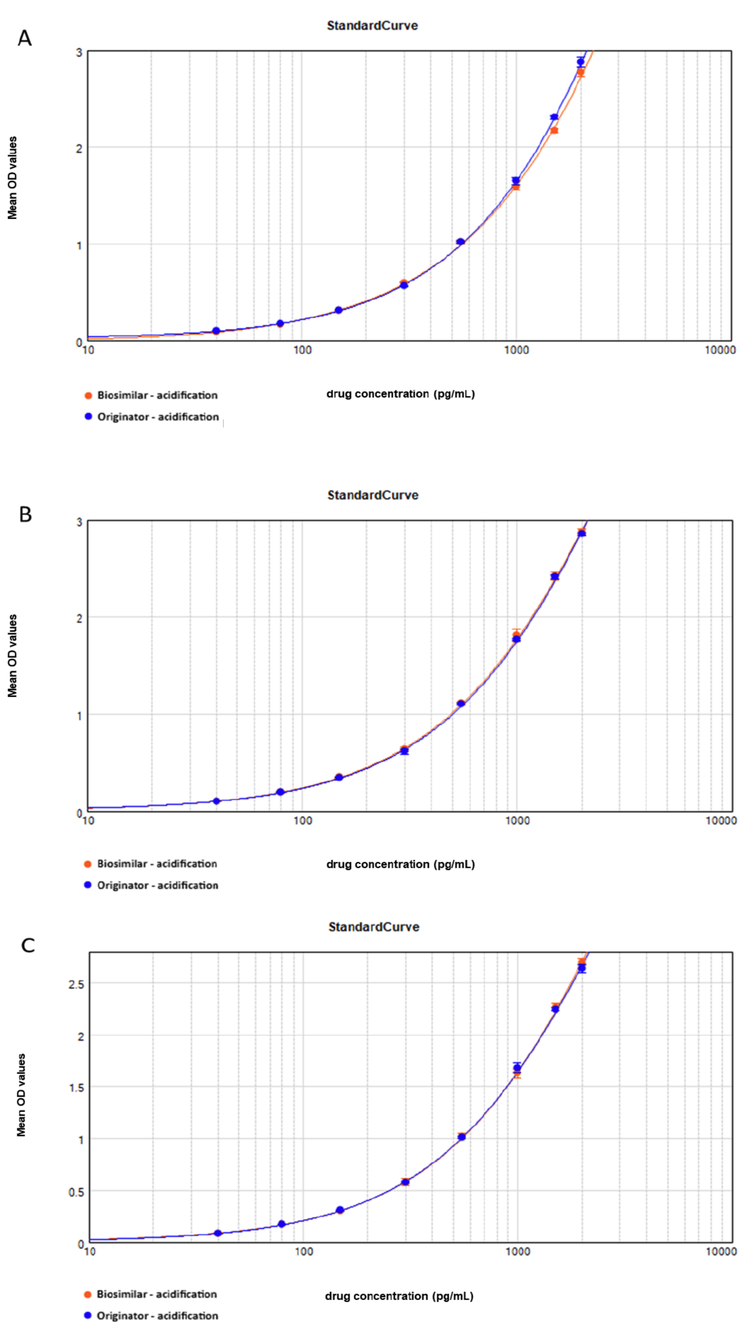

Figure 2: Visual comparison of calibration curves prepared with the Biosimilar and the Originator drugs after acid pre-treatment of samples (Figure 2 A, 2 B and 2 C).

% Bias	Nominal concentration [pg/mL]	D		E		D	
		Biosimilar	Originator	Biosimilar	Originator	Biosimilar	Originator
STD 1	2000	-7.42	-2.54	-1.70	-2.93	-1.03	-4.38
STD 2	1500	-10.6	-3.54	1.23	0.63	1.99	0.66
STD 3	1000	-10.1	-5.69	1.66	-1.21	-1.56	2.54
STD 4	550	-4.66	-4.99	0.64	0.07	1.98	0.32
STD 5	300	-5.40	-12.5	-2.80	-6.41	-1.22	-2.10
STD 6	150	-8.29	-8.23	0.35	-0.45	-0.94	-0.58
STD 7	80	-15.6	-7.34	1.48	-1.62	1.39	0.59
STD 8	40	-2.10	2.60	-0.55	-1.82	-0.37	-4.07

Table 2: Comparison of different calibration curves, pre-treating samples with acid. Statistical analysis of results presented acceptable results in terms of % CV (data not shown) and in terms of % Bias (≤ 20%).% Bias of the Originator curve was back-calculated towards the Biosimilar curve.

METHOD QUALIFICATION:

By incorporating acidification in the sample pre-treatment, the method could be successfully qualified.

The acceptance criteria, already established for comparative PK analysis in the industry, were applied to the statistical analysis of the intrabatch QCs (data not shown).

Bioanalytical similarity was confirmed by showing an absolute difference between Biosimilar and Originator mean biases (%RE) ≤ 20% for LQC, MQC and HQC and ≤ 25% for the ULOQ and LLOQ quality controls.

Furthermore, the 90% Confidence interval for the difference between the Biosimilar and Originator interbatch mean bias (%RE) was within the ±30 % for LQC, MQC and HQC and within the ±35 % for the ULOQ and LLOQ (Table 3).

QCs	Mean difference in %RE	90% Confidence Interval on mean Difference in %RE	
		Lower Limit	Upper Limit
ULOQ	8.87	4.30	13.4
HQC	10.8	5.80	15.8
MQC	10.2	5.31	15.1
LQC	9.05	3.39	14.7
LLOQ	3.17	-1.04	7.38

Table 3: Statistical evaluation of interbatch QCs data to assess bioanalytical similarity between Biosimilar and Originator.

CASE STUDY N°2: SELECTIVITY CHALLENGE

Analytical challenges:
The second case study was a bridging ELISA assay to detect a biotherapeutic drug in animal serum matrix.

During method development, we encountered selectivity issues. A subset of individual samples spiked at both LLOQ and HQC levels did not fulfilled the acceptance criteria. In addition, we observed a low assay response even at high drug concentrations (4660 RLU at ULOQ), resulting in a narrow dynamic range (approx. 30-fold between LLOQ and ULOQ) (Figure 3 A).

Troubleshooting:
We tested different strategies to improve selectivity, while maintaining the requested sensitivity. Since we were limited in the number of individuals and volume of matrix available, we focused our troubleshooting analysis on the individual samples, which failed the selectivity test. Again, sample pre-treatment with acid was able to solve the selectivity issues in the challenging individuals.

In brief, the samples were first diluted in acid at a dilution factor of 2.5, incubated for 20 – 30 min and then diluted again with neutralization buffer to reach the final MRD of 5 in a deep well plate. After this procedure, samples were transferred to the assay plate. Impact of acidification was clear: acidification significantly reduced the biases of the challenging individuals spiked at LLOQ level (Figure 3 B).

Remarkably, acidification also increased the detected RLU signals (18300 RLU at ULOQ), thereby increasing the dynamic range of the assay to approx. 70-fold.

A

QCs

Sample ID	Concentration ng/mL	MeanBackCalc Concentration	Mean RLU Value	% Bias
ULOQ	1000	1060	4660	6.24
HQC	750	745	3150	-0.657
MQC	140	144	573	2.79
LQC	60.0	61.5	282	2.41
LLOQ	20.0	20.5	155	2.64

LLOQ – spiked samples

Sample ID	Concentration ng/mL	MeanBackCalc Concentration	Mean RLU Value	% Bias
Sample1	20.0	24.0	165	20.1
Sample2	20.0	29.9	183	49.8
Sample3	20.0	13.5	136	-32.7
Sample4	20.0	22.9	162	14.9
Sample5	20.0	31.8	188	59.0
Serum pool	20.0	22.8	162	14.0

B

QCs

Sample ID	Concentration ng/mL	MeanBackCalc Concentration	Mean RLU Value	% Bias
ULOQ	1000	949	18300	-5.13
HQC	750	745	13500	-0.648
MQC	140	142	1810	1.41
LQC	60.0	59.3	683	-1.26
LLOQ	20.0	20.9	268	4.73

LLOQ – spiked samples

Sample ID	Concentration ng/mL	MeanBackCalc Concentration	Mean RLU Value	% Bias
Sample1	20.0	21.4	272	7.2
Sample2	20.0	19.8	257	-0.95
Sample3	20.0	15.9	222	-20.8
Sample4	20.0	20.6	265	3.4
Sample5	20.0	21.3	272	6.9
Serum pool	20.0	19.6	255	-2.0

MeanBackCalcConcentration: "Mean of the Back Calculated Concentration" for the duplicates of a sample. It is the predicted concentration for a particular response based on the calibration curve's algorithm.

Figure 3: Impact of acidification on selectivity samples and raw response values. Results obtained using the canonical procedure without acidification (Figure 3A), compared to results obtained using the modified procedure with acid pre-treatment (Figure 3 B).

DISCUSSION & CONCLUSIONS

Different factors affect performance of bioanalytical methods.
Decreasing the pH of a complex protein solution through acidification could break the weak bonds between molecules, reducing aggregation, unspecific bindings or matrix interference. All these factors can prevent a precise measurement of the target, limiting the reliability, accuracy and robustness of the assay.
In these two studies, we demonstrate that sample pre-treatment with acid has a strong impact in improving the performance of an assay and can increase the magnitude of the signal detected, as in case study n°2.

REFERENCES

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