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Beyond pass/fail: A procedure for evaluating the effect of carryover in bioanalytical LC/MS/MS methods

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Abstract

Eliminating carryover from bioanalytical methods can be a time and resource consuming process. While it is necessary to investigate root causes of the carryover and reduce problem areas, complete elimination of carryover may not be practical or even possible. The purpose of this paper is to suggest an avenue to investigate the effect of carryover within an analytical run rather than employ a simple pass/fail criterion. With more robust carryover information a risk threshold level can be established for individual injections based on the peak response of the previous injection. It is then possible to quickly evaluate the risk that any value in an analytical run has been adversely affected by a previous injection. Those samples which are identified as "at risk" can be reanalyzed to obtain a value that is not affected.

Keywords: LC–MS/MS; Bioanalytical methods; Carryover; Acceptance criteria; Injection sequence

1. Introduction

In HPLC analyses the appearance of an analyte of interest when a blank sample is injected is an undesirable situation. Peaks which appear in blank samples may be caused by analyte retained from previous injections (carryover), analyte that has been inadvertently added to the blank sample (contamination), or non-analyte related peaks which can arise either from a previous injection (late eluters) or the current injection (interfering endogenous peaks). A wide variety of suggested solutions exist to address the carryover issues and most bioanalytical method papers devote at least a paragraph to the characterization and elimination of carryover. Detailed systematic troubleshooting approaches have also been documented [1–3]. Vendors of analytical equipment and consumables promote their abilities to help the bioanalyst reduce carryover to lower and lower levels. Autosampler manufacturers have replaceable parts (rotors, seals, sample loops) made from a variety of materials and rinse pumps which can dispense large volumes of solvent at high rates. Sample vial or plate seals with "needle wiping" capability are suggested as a possible solution. Most analysts have "recipes" for their favorite all-purpose autosampler washes and/or autosampler injection and wash routines. Some HPLC column manufacturers even advertise lower carryover on their columns. Complete elimination of chromatography and reusable autosampler components have been proposed as making carryover a problem of the past [4–6]. Generally the last resort employed is to reduce the dynamic range of the assay or employ a dual range assay. However, all these remedies do little or nothing to affect contamination, late eluters or interfering endogenous peaks. The aim of this paper is to provide a procedure which will obviate the need for complete elimination of carryover while providing a mechanism for continual assessment of the assay performance in regard to analyte peaks in blank samples. The procedure also outlines a strategy to assign a rating for each injection based on the risk that the response obtained has been affected by carryover. Case studies illustrating the use of the procedure will also be provided.

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2. Discussion of current status

In bioanalytical assays the traditional carryover evaluation procedure is to inject a blank (no analyte has been added) sample following at least one injection of an upper limit of quantitation (ULOQ) concentration sample. One standard guideline for assessing the experiment is that any resulting peak in the blank should have an area less than 20% of the lower limit of quantitation (LLOQ). Often individuals developing these bioanalytical assays will attempt to obtain even lower amounts of carryover in order to avoid exceeding this 20% of LLOQ criterion as the bioanalytical assays move from development to the production realm because a high carryover result can be the basis to fail an analytical run. This can add significant time to the method development process as well as the individual analytical run cycle time [6,7].

In any discussion dealing with analyte peaks in blank sample, it is important to understand the basis for the traditional 20% of LLOQ criterion. From the FDA Guidance for Industry, Bioanalytical Method Validation [8] "the analyte response at the LLOQ should be at least 5 times the response compared to blank response" in order to qualify the LLOQ as the limit of quantification. This point is reaffirmed in Workshop/Conference Report from the 3rd AAPS/FDA Bioanalytical Workshop [9], "During validation, the operator should assess the analyte response due to blank matrix while eliminating or minimizing other contaminations. The analyte response at the LLOQ should be at least 5 times the response due to blank matrix." Also from the guidance on Method Validation in reference to the LLOQ: "Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%." Taken together it can be concluded that if a blank sample injected following the ULOQ standard injection meets the criterion of having no peaks with a response greater than 1/5th (20%) of the LLOQ peak response, then this "carryover" peak will not affect the accuracy and precision of the assay. However, this practice has several shortcomings.

A major concern of the traditional carryover test is that it can rely on a single measurement. The carryover measurement can be affected by its position in the sampling sequence due to adsorptive carryover issues or changes in instrument performance (e.g., consumption of autosampler rinse solvents, wear on seals, etc.). If an analyst is using a pass/fail approach to carryover, it is beneficial to measure the carryover as early in the sampling sequence as possible. This would ensure that as little time (and extracted sample) is wasted if there is a need for instrument maintenance to reduce the response obtained during the carryover measurement. However, carryover seen early in the injection sequence may not reflect the true carryover situation throughout the sequence, especially if it is due to a source that accumulates or an endogenous source present in study samples but absent in standard and QC samples. Similarly, measurement of the carryover at the end of the analysis may give a better indication of carryover during the entire course of the run, however much time and extracted sample has been consumed before remediation can be attempted if the entire analytical run must be failed based on the result of this measurement. A recent bioanalytical LC/MS/MS system suitability paper [10] discusses this need for carryover assessment at the beginning and end of an analytical sequence. Going a step farther, multiple measurements spread out during the course of an injection sequence give an even better determination of the scope and range of a carryover effect.

Another concern of a single measurement paradigm is the need to integrate a peak that is of much lower intensity than the LLOQ. Determining a baseline for this peak may be quite difficult and require manual intervention. It may be difficult to avoid bias when the fate of an entire analytical run is based on selecting the appropriate start and stop times for a peak which may not even be 3 times the noise level. By using multiple measurements and a more forgiving benchmark for assessing carryover impact, peak response measurements should become less subjective and anxiety producing.

Applying selectivity benchmarks to a blank sample following the ULOQ is an attempt to ensure that the accuracy and precision of any measurement in a run or batch is not significantly affected by the previous injection. In effect, the analyst is attempting to demonstrate a worst-case scenario. However, an injection sequence can be arranged so that samples with values approaching the LLOQ do not follow samples with values near the ULOQ, [1,9,11] in which case the 20% of LLOQ threshold is excessive. On the other hand, there can be unexpected concentration values which may result in peaks with intensities greater than the ULOQ in the sequence. In this case, the 20% threshold is inadequate [12].

Finally, carryover often greatly depends on the current condition of the analytical instrumentation. A new switching valve, a worn autosampler seal or new tubing, etc., can have a great impact on the amount of carryover observed. During validation the carryover should be minimized and characterized. However, it is equally important that the carryover and any risk it may pose to calculated results should be fully assessed during each analytical run.

In ideal terms, an analyst should see no analyte peaks when blank samples are injected. This ensures that each peak generated by an injection on the system is due to analyte contained only in the sample being injected. Being able to rely upon this principle allows creation of standard curves and quantitation of unknown samples. Unfortunately, as dynamic ranges are expanded, quantitation limits are lowered, thousands of samples are injected and complex column/flow switching techniques are employed, this ideal may not be realized. The question then becomes: how are those injections which have peak responses that have been adversely affected by previous injections distinguished and remediated?

The recent AAPS/FDA Bioanalytical Workshop Report [9] states "There is no standard acceptable magnitude of carryover for a passing bioanalytical run. Carryover should be addressed in validation and minimized" and "interference should not significantly affect the accuracy and precision of the assay". Moving beyond the traditional 20% of LLOQ guideline to determining whether carryover is significantly affecting the accuracy and precision of the assay has lately produced some interest [12,13]. There has also been recent work to decisively determine if peaks

in blank samples are due to carryover or contamination [14]. The purpose of this paper is to outline a procedure which will allow a user the freedom to minimize carryover without employing heroic measures to eliminate it, and tools to characterize the nature of analyte peaks in blank injections and continually assess the performance of an assay with regard to effect of sample injections upon subsequent sample injections. A risk-rating scheme to discern any results which should be remediated is also described.

3. Experimental details

Established validated LC/MS/MS methods (carryover has been minimized and characterized) were used to analyze extracted plasma samples. These samples consisted of matrix blanks, standard curve samples, quality control samples and unknown samples. Extracted matrix blanks are the preferred tool to measure carryover within these studies. This allows the carryover assessment injections to be of an identical composition to other injections in the sequence which may be an important factor in desorbing retained analyte from previous injections. Use of matrix blanks also provides an on-going selectivity assessment throughout the life of an assay. An underlying premise of this work is that it is important to control the injection sequence order with regard to concentration of the analytes of interest. Samples should be injected in order of increasing expected concentration, or at a minimum, the injection of low expected concentration samples should not follow high expected concentration samples. Part of control of the injection sequence order is also judicious placement of carryover measurements. For the experiments presented here, which consisted of no more than 96 extracted samples, 10 carryover injections were made. Some measurements followed expected high concentrations and some measurements followed lower expected concentrations in order to cover the assay range. Some measurements followed known concentration samples while other measurements followed unknown samples in order to observed differences between commercial matrix and study matrix. One of the carryover measurements did follow injection of the ULOQ in each experiment to allow direct comparison with traditional carryover assessments. The overall aim was to assess linearity of any carryover as well as establish confidence intervals around the carryover. By placing the measurements throughout the analytical run and at various concentrations the affects of non-contiguous injections can be evaluated indirectly. This manner of assessing carryover can also identify the magnitude and variability of analyte peaks in blank injections that are not due to carryover.

There were various options to consider when setting up multiple carryover injections. Since extracted sample volume allowed, all carryover injections could be made from the same extracted blank matrix sample. This would have increased the number of wells in an extraction plate that could be used to quantitate unknown samples. On the other hand, separate blank matrix samples could have been extracted for each carryover injection. This practice would have provided more diagnostic data around extraction variability and contamination issues. For the purpose of the following experiments, a hybrid of the two techniques was used: two blank matrix samples were extracted for each analytical run and each extracted sample was used for 5 carryover measurements.

The LC–MS/MS systems consisted of a Shimadzu LC-10AD or a Shimadzu LC-20AD integrated pump system, a Shimadzu SIL-20AC autosampler or an HTC PAL autosampler, and an ABI Sciex API 4000 mass spectrometer equipped with the Turbo IonSpray® interface and optionally a six-port switching valve. The LC–MS/MS system was controlled using either Sciex Analyst Version 1.4.0 or 1.4.1 software.

Following analysis, either Sciex Analyst Classic or IntelliQuan integration algorithms were used to obtain peak response values. In many cases it was necessary to manually integrate the carryover sample peaks. An effort was made to use consistent starting and end points for those peaks which were difficult to distinguish from the baseline noise.

Within these tests peak area responses were used. This also allowed simultaneous measurement of internal standard (IS) carryover by using double blanks (matrix to which no analyte or IS was added) for carryover measurements. If it is desired to use area ratio responses instead, it would be necessary to use a blank with IS added to measure analyte carryover. In that case IS carryover would still need to be evaluated by either double blanks or samples with known concentration to which IS was not added.

The resulting carryover measurements for the analyte were evaluated by LabStats, an Excel add-in developed collaboratively by the Pfizer Global Research and Development Sandwich Nonclinical Statistics group, part of Biostatistics & Reporting at Sandwich Laboratories, UK and Tessella Support Services plc [15]. The peak response of the blank (carryover) injection sample was plotted against the preceding injection peak response. The best line fit was determined and 95% confidence intervals were calculated. Also the peak area of each sample within the injection sequence was divided into the peak area of the preceding sample. There was no line fit used to determine IS carryover. IS concentrations do not vary across a range, therefore it is more appropriate to simply assess carryover injection peak responses against non-carryover peak responses and IS carryover will not be discussed further in this paper.

4. Calculations

The slope of the line of carryover peak responses plotted against preceding injection peak response is the estimated carryover (expressed as a ratio, not a percent) for the analytical run, while the upper 95% confidence limit of the slope can be used to estimate an upper limit of carryover [16]. This upper limit can be used to obtain a boundary of the magnitude of the effect of any injection on subsequent samples. The needs, accuracy and precision of an assay can be used in combination with this maximum carryover effect to set a threshold for the ratio of the response sample against the response of the immediately preceding sample. The *y*-intercept obtained through this experiment shows the peak response that would be obtained by injecting a blank matrix sample following the injection of another blank

matrix sample. Theoretically, this value should be zero. If this value is greater than zero it is indicative that a mechanism other than carryover from previous samples is causing part, if not all the peak response obtained during the carryover measurements. If this value is greater than 5× the assay LLOQ, it will have an affect on the actual obtained LLOQ for the analytical run.

An explanation of the calculations used to assess the effect of carryover for the analytical run as well as evaluate the risk of compromised individual injections follows.

The contribution to the measured peak response of Inj_n that results from carryover from Inj_{n-1} can be calculated by:

$$P \times M = \text{contribution to peak response due to carryover from previous injections} \quad (1)$$

where P is the measured peak response of Inj_{n-1} and M is the upper 95% confidence interval value for the slope of the line of carryover peak responses against preceding injection peak response.

The percent change to a peak response due to carryover from the previous injections for any injection (n) is calculated by:

$$\frac{P \times M}{T} \times 100 = \text{peak response percent change due to carryover} \quad (2)$$

where T is the true peak response for Inj_n. This value must be less than the greatest acceptable percent change from the true value allowed by the needs, precision and accuracy of the assay. For further calculations this will be expressed as:

$$\frac{P \times M}{T} \text{must be} < u \quad (3)$$

where u is the greatest acceptable change expressed as a ratio rather than a percent.

The value of T can be calculated by:

$$T = A - (P \times M) \quad (4)$$

where A is the measured peak reponse of Inj_n. This is substituted back into Eq. (3) to obtain:

$$\frac{P \times M}{A - (P \times M)} \text{must be} < u \quad (5)$$

The ratio based on the Inj_{n-1} measured peak response (P) and the Inj_n (A) measured peak response is used to assign a "carryover risk (cR)" rating for any Inj_n:

$$\text{cR} = \frac{P}{A} \quad (6)$$

If we solve Eq. (5) for this carryover risk the result is:

$$\text{cR must be} < \frac{u}{M(1+u)} \quad (7)$$

and this maximal value is then set as the threshold value of acceptable carryover risk (acR):

$$\text{acR} = \frac{u}{M(1+u)} \quad (8)$$

The individual carryover risk values are calculated for each sample in the sequence and assessed against the acR.

For the bioanalytical assays presented as case studies in this paper, acceptance criteria call for percent relative error (accuracy assessment) of <±15% and percent relative standard deviation (precision assessment) of <15%. Therefore, the u value used to calculate the acR values has been assigned as 0.15. This value can be lowered if data sets are more sensitive to error or raised if the accuracy and precision criteria for a particular application allow for more error.

When these procedures are employed, samples with a cR exceeding the acR should be considered to be adversely affected by carryover from a previous sample. If these adversely affected samples are standard curve points or QC evaluations, they should not be used to establish the curve or evaluate the success of the analytical run. If blank matrix without IS samples are being used to assess carryover, they will quite likely result in very high cR values which can be ignored because the calculated concentration of these carryover samples are not used. If the adversely affected sample is unknown with a calculated concentration above the LLOQ, the obtained value should be considered suspect. These "at-risk" unknown values can be remediated by reinjection with corresponding extracted standard curve and QCs or re-extracted and reanalyzed, as directed by appropriate governing procedures. Adversely affected samples with calculated concentrations below the LLOQ do not need to be re-evaluated.

If there are concerns about the choice of an appropriate greatest acceptable percent change, it is also possible to calculate the greatest observed percent change U_{obs} for the analytical run by using the highest observed cR and upper 95% confidence interval value for the slope.

$$U_{obs} = \left(\frac{\text{cR} \times M}{(1 - (\text{cR} \times M))} \right) \times 100 \quad (9)$$

No predetermined criteria were set regarding use of U_{obs} for the case studies which follow. Therefore, Eq. (9) will not be discussed in assessing those studies.

A second parameter obtained from this line fit exercise is the y-intercept. Theoretically this value should be zero, indicating that a blank matrix sample injected following a blank matrix sample should not have a peak response. The y-intercept may not be zero if a source of analyte peak in blank matrix samples is due to contamination, interfering endogenous compounds, late eluting peaks or adsorptive carryover. These issues may be present alone or in combination with one another and/or carryover. It is important to evaluate these y-intercept values in relation to the assay LLOQ. If the upper 95% confidence interval value for the y-intercept is less than 20% of the assay LLOQ peak response value, then the influence of the y-intercept can be considered to be negligible across the assay. However, if the upper 95% confidence interval value for the y-intercept is greater than 20% of the assay LLOQ peak response all sample injections with responses less than 5× the response due to blank matrix (upper 95% confidence interval of y-intercept) should be considered to be suspect.

Table 1
Peak responses and "traditional" carryover assessments

Compound	Carryover peak area[a]	LLOQ peak area	ULOQ peak area	Carryover as a percent of LLOQ	Carryover as a ratio of ULOQ
A	4,316	9,291	9,614,391	46.5%	0.000449
B	774	2,482	2,057,129	31.2%	0.000376
C	1,584	1,644	1,401,332	96.4%	0.00113
D	404	1,895	599,361	21.3%	0.000674

[a] Blank matrix sample injection following injection of ULOQ sample.

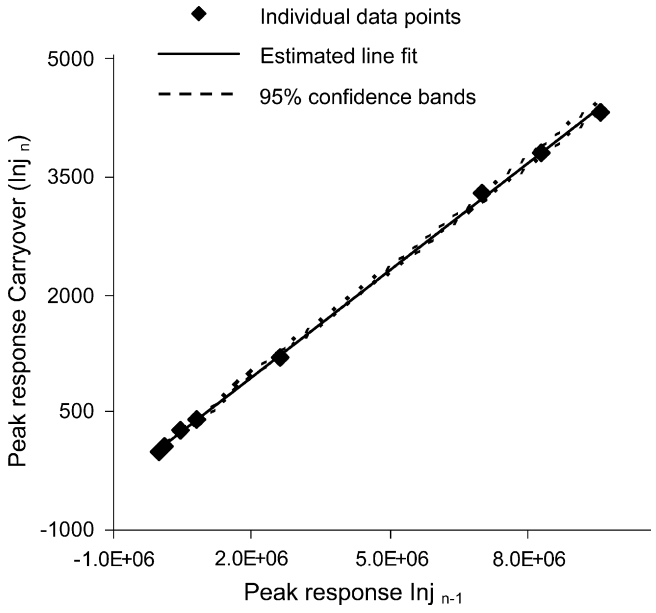

Fig. 1. Carryover determination Compound A.

5. Case study #1

Compound A is a particularly "sticky" compound. All efforts to eliminate carryover were unsuccessful. The current assay has been documented with instructions to inject samples in order of increasing expected concentration and carryover exceeding 20% of the LLOQ is expected. Table 1 shows the peak responses of the carryover injection which followed the ULOQ injection, the LLOQ injection and the ULOQ injection. From these responses the "traditional" carryover measurement as a percent of LLOQ was found to be 46.5%. The carryover measurement was also calculated as a ratio of the ULOQ measurement (0.000449) which allows direct comparison to the line fit determination of carryover. Fig. 1 shows the graphical result of the line fit from the 10 carryover measurements, while the numerical details are presented in Table 2. Compound A presents a classic case of linear carryover as is evidenced by the very narrow 95% confidence intervals of the slope of the line. The *y*-intercept 95% confidence interval does show some deviation, however the values are quite low in comparison to the LLOQ value (as shown in Table 3) and the interval does include 0. This indicates that carryover is responsible for any increased peak responses and therefore use of an acR threshold to monitor cRs as an indication of affected values is appropriate. Because carryover is an ongoing issue with this compound, the injection order was carefully determined prior to injection and it was possible to achieve a very low maximum cR of 2 for this injection sequence. Table 4 is presented as an example of the risk review that should be conducted with each analytical run. This review table will not be presented for the other case studies. The maximum cR value is much lower than the threshold limit of 282 for this run. If any samples with cR above 282 and calculated concentrations above the LLOQ had been identified, it would have been possible to create a sequence which would reinject those affected samples along with their co-extracted standards and QCs to obtain unaffected values.

6. Case study #2

Compound B is a compound which has demonstrated variable carryover during use in production. Frequent maintenance of the autosampler is used to keep the carryover below 20% of LLOQ. Table 1 shows the peak responses of the carryover injection which followed the ULOQ injection, the LLOQ injection and the ULOQ injection. From these responses the "traditional" carryover measurement as a percent of LLOQ was found to be 31.2%. The carryover measurement was also calculated as a ratio of the ULOQ measurement (0.000376) which allows direct comparison to the line fit determination of carryover. Fig. 2 shows the graphical result of the line fit from

Table 2
LabStats fit line results

Compound	Slope estimate	Slope confidence intervals		*y*-Intercept estimate	*y*-Intercept confidence intervals	
		Lower 95%	Upper 95%		Lower 95%	Upper 95%
A	0.000454	0.000445	0.000463	17.7	−24.3	59.7
B	0.000298	0.0000458	0.000551	231	146	317
C	0.000125	−0.0000207	0.000270	1396	1307	1485
D	0.000257	−0.000179	0.000694	121	28.7	214
D1	0.000267	0.0000570	0.000476	36.5	−21.3	94.2
D2				199	143	255

Table 3
Analytical run risk calculations

Compound	Acceptable carryover risk (acR) for analytical run	Highest measured carryover risk (cR) for analytical run[a]	*y*-Intercept confidence intervals as a % of LLOQ	
			Lower 95%	Upper 95%
A	282	2	−0.3%	0.6%
B	237	44	5.9%	12.8%
C	Not appropriate	Not appropriate	79.5%	90.3%
D	188	6	1.5%	11.3%
D1	273	6	−1.1%	5.0%
D2			7.6%	13.5%

[a] Excluding carryover measurements and BLQ measurements.

Table 4
Compound A carryover risk review—acR = 282

Injection order #	Sample name	Analyte peak area (counts)	Carryover risk (cR)
1	CO test SYS SUIT 1 1	1,143	
2	CO test SYS SUIT 2 1	9,783	0
3	CO test D G std.15 1 1	8,323,904	0
4	CO test Carryover 2 5	3,786	2,199[a]
5	CO test DOUBLE BLANK 1 1	631	6
6	CO test blank_with_IS 1 1	102	6
7	CO test D A std.09 1 1	9,291	0
8	CO test D B std.10 1 1	25,119	0
9	CO test LQC.06 1 1	29,102	1
10	CO test Day 1 0h PLM-1 1	65	448[b]
11	CO test Day 1 0h PLM-1 1	44	1
12	CO test Day 1 0h PLM-1 1	60	1
13	CO test Carryover 1 4	0	0
14	CO test LQC.06 2 1	28,202	0
15	CO test Day 1 0h PLM-1 1	77	366[b]
16	CO test Carryover 2 3	0	0
17	CO test Day 8 0h PLM-1 1	770	0
18	CO test Day 8 0h PLM-1 1	102	8
19	CO test D C std.11 1 1	100,116	0
20	CO test Carryover 1 3	54	1,854[a]
21	CO test D D std.12 1 1	249,202	0
22	CO test Day 8 0h PLM-1 1	120	2,077[b]
23	CO test Day 8 0h PLM-1 1	130	1
24	CO test Carryover 2 4	0	0
25	CO test Day 1 6h PLM-1 1	58	0
26	CO test MQC.07 1 1	427,600	0
27	CO test MQC.07 2 1	439,708	1
28	CO test Carryover 1 5	254	1,731[a]
29	CO test Day 1 6h PLM-1 1	311,457	0
30	CO test Day 1 6h PLM-1 1	1,958,296	0
31	CO test Day 1 6h PLM-1 1	2,644,428	1
32	CO test Carryover 2 2	1,206	2,193[a]
33	CO test D E std.13 1 1	1,006,120	0
34	CO test Day 8 6h PLM-1 1	448	2,246[b]
35	CO test Day 8 6h PLM-1 1	794,528	0
36	CO test Carryover 1 2	389	2,042[a]
37	CO test D F std.14 1 1	2,451,424	0
38	CO test HQC.08 1 1	6,718,528	0
39	CO test HQC.08 2 1	7,018,370	1
40	CO test Carryover 2 1	3,300	2,127[a]
41	CO test Day 8 6h PLM-1 1	4,140,465	0
42	CO test Day 8 6h PLM-1 1	2,401,263	2
43	CO test D H std.16 1 1	9,614,391	0
44	CO test Carryover 1 1	4,316	2,228[a]

[a] Carryover measurements are not candidates for remediation.
[b] BLQ values do not require remediation.

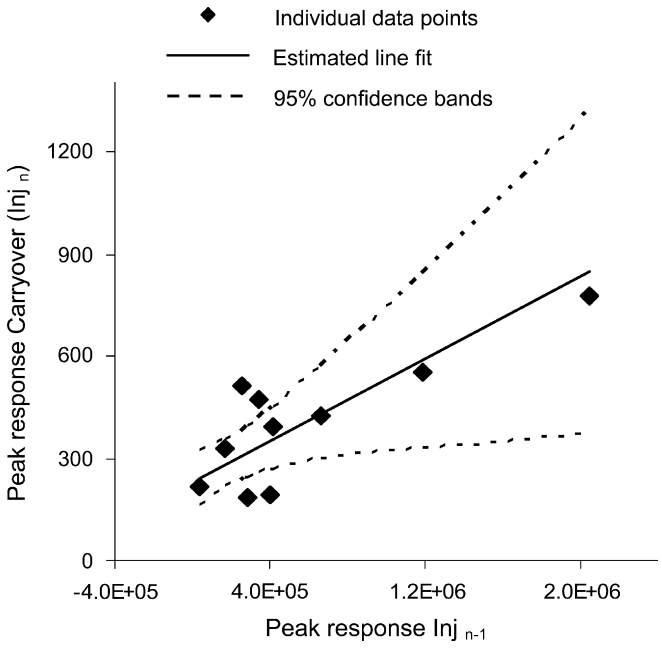

Fig. 2. Carryover determination Compound B.

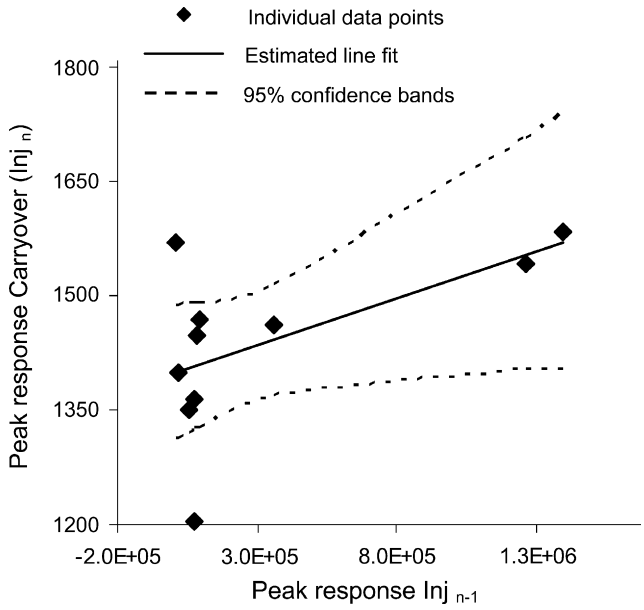

Fig. 3. Carryover determination Compound C.

the 10 carryover measurements, while the numerical details are presented in Table 2. Compound B presents a much more variable carryover as is evidenced by the divergent 95% confidence intervals. This is perhaps due to additional variable sources of peak response in the extracted blank matrix such as late eluters or interfering endogenous peaks. (Contamination would provide additional constant peak response due to the use of only 2 extracted blank matrix samples-see Case study #4.) The *y*-intercept 95% confidence intervals indicate that there would be an inherent peak response even following a blank matrix injection. However, the upper 95% confidence interval indicates that this value is still less than 20% of the LLOQ value (Table 3). This indicates that carryover is responsible for increased peak responses of concern within the range of this analytical run of the assay and use of the acR threshold to monitor cR's as an indication of affected values is acceptable. The maximum cR for this injection sequence is 44 which is lower than the threshold limit of 237 for this run. However, this does not indicate the wide margin of safety that was demonstrated for Compound A. While no calculated values for this analytical run are flagged as being affected, autosampler maintenance, more careful arrangement of the injection sequence or both are indicated before more samples are analyzed. As indicated in Case study #1, if any affected samples had been indicated, they could have been remediated.

7. Case study #3

Compound C is a compound which did not exhibit carryover during method development or validation. Table 1 shows the peak responses of the carryover injection which followed the ULOQ injection, the LLOQ injection and the ULOQ injection. From these responses the "traditional" carryover measurement as a percent of LLOQ was found to be 96.4%. The carryover measurement was also calculated as a ratio of the ULOQ measurement (0.00113) which allows direct comparison to the line fit determination of carryover. Fig. 3 shows the graphical result of the line fit from the 10 carryover measurements, while the numerical details are presented in Table 2. The slope estimate of the line fit is a quite low value and at the lower 95% confidence interval it is not significantly different from zero. This is a very good indication that the problem with this analytical run is not caused by carryover. Examination of the *y*-intercept 95% confidence interval (Table 3) shows that the issue is an inherent peak response which is in the range of 79.5–90.3% of the LLOQ. Because carryover is not responsible for any increased peak responses, use of an acR threshold to monitor cRs as an indication of affected values is not appropriate. With the appropriate governing procedures in place, it is possible to set a threshold level at 5× the upper 95% confidence interval level for the *y*-intercept. All standard and QC samples with peak responses below this threshold level should not be used to establish the analytical curve or evaluate the success of the analytical run. Because it may still be possible to meet the analytical run acceptance criteria, unknown samples with peak responses greater than 5× the upper 95% confidence interval would have acceptable calculated concentrations. This particular case study was prepared using an intentionally contaminated extraction solvent. All unknown samples were also analyzed with non-contaminated extraction solvent. Table 5 shows a comparison of those results, only the sample identified with the peak response less than 5× the upper 95% confidence interval differed from the non-contaminated assay by more than ±15%. In the contaminated analytical run, removal of the affected standards and QCs resulted in the QCs not meeting a strict interpretation of the in-house procedure governing sample analysis. This further illustrates the need for additional governing procedures to be in place prior to using the procedures outlined here to accept or reject analytical results.

Table 5
Comparison of contaminated and uncontaminated analytical run results

Sample name	Factor[a]	Contaminated value calculated concentration (ng/mL)	Uncontaminated value calculated concentration (ng/mL)	% Difference from uncontaminated concentration
Unknown010 24h	3	1,660	2,280	−27%
Unknown005 24h	7	5,360	5,980	−10%
Unknown025 24h	7	5,700	5,930	−4%
Unknown015 24h	8	7,220	6,820	6%
Unknown020 24h	8	7,410	6,950	7%
Unknown030 24h	11	9,480	9,550	−1%
Unknown008 4h	12	11,700	12,900	−9%
Unknown016 1h	12	11,100	12,200	−9%
Unknown009 7h	22	21,800	21,400	2%
Unknown006 1h	29	29,400	28,100	5%
Unknown001 1h	34	33,700	32,200	5%
Unknown026 1h	36	36,100	35,600	1%
Unknown002 2h	41	42,300	41,300	2%
Unknown011 1h	41	41,500	39,800	4%
Unknown021 1h	41	42,100	41,400	2%
Unknown007 2h	43	44,900	41,900	7%
Unknown029 7h	45	45,000	43,300	4%
Unknown004 7h	50	51,400	49,300	4%
Unknown024 7h	51	51,600	51,200	1%
Unknown022 2h	52	52,900	52,300	1%
Unknown027 2h	52	54,200	52,900	2%
Unknown028 4h	54	55,800	53,700	4%
Unknown019 7h	56	58,700	57,100	3%
Unknown023 4h	58	57,500	56,400	2%
Unknown014 7h	59	59,700	56,400	6%
Unknown003 4h	63	67,500	63,800	6%
Unknown018 4h	65	69,800	67,400	4%
Unknown017 2h	69	70,300	67,400	4%
Unknown012 2h	76	78,100	75,400	4%
Unknown013 4h	83	82,200	79,300	4%

[a] Factor calculated by dividing peak area by upper 95% confidence interval for *y*-intercept.

8. Case study #4

During the development of a method for Compound D the range was reduced in order to achieve low to no levels of carryover. Table 1 shows the peak responses of the carryover injection which followed the ULOQ injection, the LLOQ injection and the ULOQ injection. From these responses the "traditional" carryover measurement as a percent of LLOQ was found to be 21.3%. The carryover measurement was also calculated as a ratio of the ULOQ measurement (0.000674) which allows direct comparison to the line fit determination of carryover. These values were higher than usually seen and quite unexpected. Fig. 4 shows the graphical result of the line fit from the 10 carryover measurements, while the numerical details are presented in Table 2. Although the variability of the results for Compound D resembles that of Compound B, the lower 95% confidence interval for the slope for Compound D actually falls below zero, which indicates that carryover is not the cause of peak response in the blank matrix samples. A more careful examination of the carryover results reveals the cause of the variability. As indicated earlier, the carryover measurements were provided by two extracted blank matrix samples which were each injected 5 times. The LabStats Excel add-in is able to compare two lines and indicate if they have shared slopes and/or *y*-intercepts. The two sets of data obtained do share a slope; however they do not share

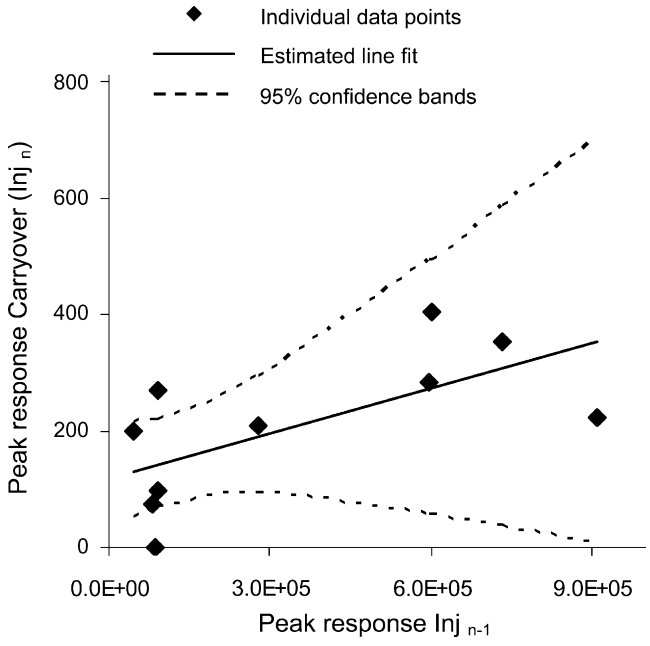

Fig. 4. Carryover determination Compound D.

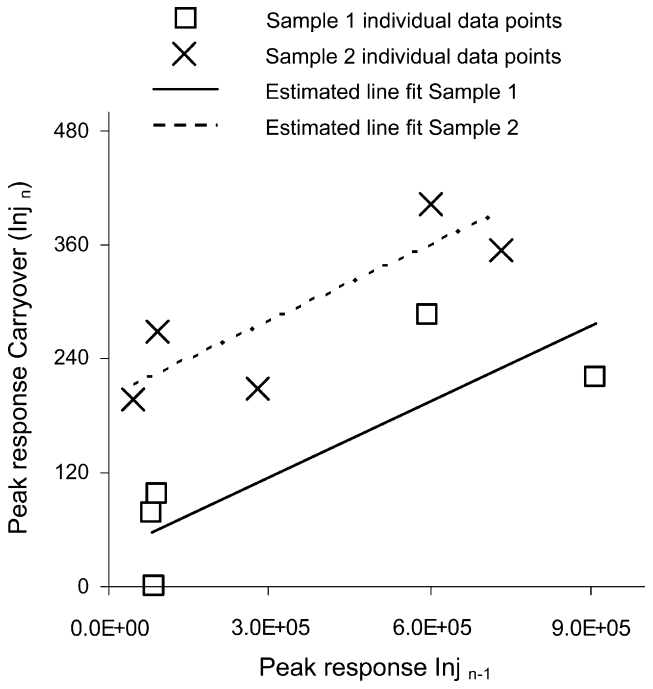

Fig. 5. Carryover determination Compound D separated by individual sample.

a *y*-intercept (Table 2 and Fig. 5). Separating the data out by extracted sample narrowed the 95% confidence interval for the slope (and it no longer includes zero). The *y*-intercept 95% confidence intervals are shown in comparison to the LLOQ value in Table 3. The first extracted sample which was used to measure carryover has a much lower *y*-interval than the second sample. In either case, the upper 95% confidence interval for the *y*-interval is less than 20% of the LLOQ which indicates inherent analyte is not primarily responsible for the peak response found in the blank matrix samples. The upper 95% confidence interval of the shared slope is used to calculate the acR for the analytical run. The maximum cR for this injection sequence is 6. This is much lower than the threshold limit of 273 for this run which indicates that no sample peak responses were adversely affected by carryover from a previous injection. If any samples with cR above 273 and calculated concentrations above the LLOQ had been identified, it would have been possible to create a sequence which would reinject those affected samples along with their co-extracted standards and QC's to obtain unaffected values. The non-shared *y*-intercepts may be due to cross-contamination during extraction. In this particular case, 10 extracted blank matrix samples may have given a better indication of the level of cross-contamination throughout the plate. All other acceptance criteria for this analytical run were met which is used as the evidence that no individual samples were affected by cross-contamination during sample extraction.

9. Discussion

The results of the case studies highlight some of the advantages of using multiple carryover determinations during an analytical run and across the analytical range. Using a pass/fail criterion based on a carryover limit of <20% of LLOQ would have failed each of these analytical runs. At a minimum, repeat injections following instrument maintenance would have been required. Using the procedures outlined in the paper, it was possible to demonstrate that no analytical results were compromised by carryover from previous injections. However, governing procedures regarding the use of carryover assessment in this manner and criteria involving the rejection and remediation of affected samples are a very necessary element in the use of cR and acR, specifically in regard to gaining acceptance of the method by regulatory bodies, such as the FDA. The carryover results also illustrate the importance of carefully controlled injection sequence order with respect to expected concentrations. If it is not possible to arrange the injection sequence with regard to concentration of the analytes, it is still possible to use the procedures outlined within this paper. Unfortunately, since no effort is made to control the risk of carryover from one sample to the next, many more samples may be affected and need to be remediated. It was demonstrated that it is possible to salvage valid calculated concentrations for analytical samples in an analytical run that is affected by a relatively consistent level of contamination. Again, the need for pre-established criteria regarding use of the *y*-intercept and its confidence intervals for analytical run and individual sample acceptance or rejection in governing procedures is emphasized.

10. Conclusion

Advances in bioanalytical methods allow for faster, more robust analyses with wider dynamic ranges. Time and other laboratory resources are constrained in an effort to improve productivity. There is a growing awareness of environmental costs of excessive measures resulting from harsh solvents and solvent consumption. These factors and others have inspired bioanalytical laboratories to look beyond the traditional <20% of LLOQ requirement for carryover. Use of the procedures presented in this paper improves the understanding of the source and effect of analyte peaks in blank matrix samples and clearly demonstrates how any interference does or does not adversely affect precision and accuracy. This directly addresses the goals regarding the effect of interference stated in the recent AAPS/FDA Bioanalytical Workshop Report [9]. By statistically determining carryover from previous sample injections as well as inherent analyte peak responses within an analytical run it is possible to establish risk thresholds for the run. Evaluating individual risks against this threshold and remediating values that exceed these thresholds adds robustness to the assay while reducing carryover elimination method development time, increased autosampler cycle time and unnecessary analytical run repeats.

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