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Analyte and internal standard cross signal contributions and their impact on quantitation in LC–MS based bioanalysis

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ABSTRACT

Cross signal contributions between an analyte and its internal standard (IS) are very common due to impurities in reference standards and/or isotopic interferences. Despite the general awareness of this issue, how exactly they affect quantitation in LC–MS based bioanalysis has not been systematically evaluated. In this research, such evaluations were performed first by simulations and then by experiments using a typical bioanalytical method for tiagabine over the concentration range of 1–1000 ng/mL in human EDTA K_3 plasma. The results demonstrate that when an analyte contributes to IS signal, linearity and accuracy can be affected with low IS concentration. Thus, minimum IS concentrations have been obtained for different combinations of concentration range, percentage of cross contribution, and weighting factor. Moreover, while impurity in analyte reference standard is a factor in cross signal contribution, significant systematic errors could exist in the results of unknown samples even though the results of calibration standards and quality controls are acceptable. How these systematic errors would affect stability evaluation, method transfer, and cross validation has also been discussed and measures to reduce their impact are proposed. On the other hand, the signal contribution from an IS to the analyte causes shifting of a calibration curve, i.e. increase of intercept, and theoretically, the accuracy is not affected. The simulation results are well supported by experimental results. For example, good inter-run (between-run) accuracy (bias: −2.70 to 5.35%) and precision (CV: 2.07–10.50%) were obtained when runs were extracted with an IS solution containing 1-fold of the lower limit of quantitation.

1. Introduction

Internal standards are commonly used in LC–MS based quantitative bioanalysis [1,2]. The main purpose of utilizing internal standards is to correct any variation other than that related to the amount of the analyte present in a sample, such as variability in dilution, evaporation, degradation, recovery, adsorption, derivatization, injection, and detection. Hence, an internal standard (IS) should have the same or very similar physico-chemical properties as its analyte, which means that they usually have similar molecular weights and synthesizing routes for their reference standards. Therefore, cross contributions in mass spectrometry (MS) responses are very common between an analyte and its IS due to chemical impurities and/or isotopic interferences [3–6].

Despite the general awareness of this issue [3–7], how exactly the cross contributions affect linearity, accuracy, the selection of an internal standard and the determination of its concentration in LC–MS quantitative analysis has not been fully evaluated. For example, Nilsson and Eklund have briefly demonstrated how cross signal contribution affects linearity at high concentration end and accuracy at the low concentrations by simulation, but lack of supporting experimental results. In addition, their focus was on the introduction of a new quantitation technique — direct quantification using internal calibration, instead of a more generally employed calibration curve approach.

Moreover, contradiction and ambiguity still exist in many closely related aspects among the literatures. For example, regarding the selection of IS concentration, Ansermot et al. proposed that IS concentration should be chosen at a relatively low concentration corresponding to about the first third of the calibration range [4] while others suggested the middle of the calibration curve [5,7] or even equal or higher than the upper limit of quantitation (ULOQ) [8]. Unfortunately, none of these were followed by more detailed theoretical considerations or supporting experimental data. Another example is regarding the molecular weight of a stable isotope labelled (SIL) internal standard in relation to that of the analyte (concerning isotopic interference). While Bakhtiar and Majumdar suggested at least 4 or 5 Da higher that of the analyte [5], Li et al. successfully validated a method for the determination of norethindrone over the concentration ranges of 2.5–500 pg/mL and

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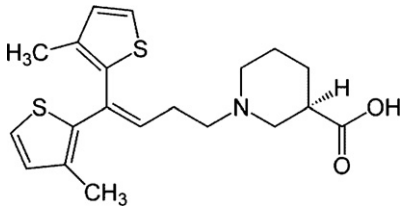

Fig. 1. Chemical structure of tiagabine (also known as (R)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidinecarboxylic acid); molecular formula: $C_{20}H_{25}NO_2S_2$; molecular weight: 375.55.

0.05–10 ng/mL using norethindrone-$^{13}C_2$ (only 2-Da difference) [9].

Based on the above, a continuous and more comprehensive research dedicated to this topic is clearly very much desirable. It was the purpose of this paper to perform such a research, first by simulations and then by experiments using a typical LC–MS/MS based bioanalytical method for tiagabine (Fig. 1) in human EDTA K_3 plasma. In addition, how the results obtained would impact and benefit method development, method validation, method transfer, cross-validation, and sample analysis in bioanalysis are explored.

2. Experimental

2.1. Chemicals and reagents

Tiagabine was purchased from United States Pharmacopeia (USP, Rockville, Maryland, USA). Tiagabine-d_9 was obtained from SynFine Research (Richmond Hill, Ontario, Canada). Methanol (Omnisolv) was obtained from EMD (Toronto, Ontario, Canada). Ammonium formate (AnalaR) and ammonium hydroxide (ACS) were obtained from Sigma (Oakville, Ontario, Canada). Human EDTA K_3 plasma was obtained from Valley Biomedical (Winchester, Virginia, USA). Water was produced in-house by a Milli-Q water system (Milford, Massachusetts, USA). High purity liquid nitrogen was supplied by Prodair (Mississauga, Ontario, Canada).

2.2. Stock solutions, calibration standards and quality control samples

The stock solutions were prepared in methanol/water (50/50, v/v) at the concentrations of 1.0037 mg/mL and 100.80 μg/mL (the decimals were due to difficulty in weighing an exact amount) for tiagabine and its internal standard (tiagabine-d_9), respectively. All intermediate and working solutions were prepared by the successive dilutions in methanol/water (50/50, v/v). Calibration standards were prepared in control human EDTA K_3 plasma at the concentrations of 1.00, 2.01, 20.07, 100.37, 200.74, 401.48, 802.96, and 1003.70 ng/mL. Quality control samples were prepared at the concentrations of 1.00, 3.01, 301.11, 702.59, and 1003.70 ng/mL. In addition, a second set of calibration standards and quality controls were prepared at the aforementioned concentrations but containing also the internal standard at concentrations equivalent to 5% of the analyte concentrations.

2.3. Sample processing

One hundred microliters (100 μL) of human EDTA K_3 plasma sample was aliquoted and mixed with 200 μL of internal standard working solution (methanol/water, 50/50, v/v). Then, 1.2 mL of methanol was added for protein precipitation. After centrifugation, 100 μL of the supernatant was transferred and mixed with 400 μL of methanol/water solution (50/50, v/v) by using a MultiPROBE II EX HT robotic liquid handling system (Perkin Elmer, Woodbridge, Ontario, Canada). The mixture was injected without further processing, such as evaporation and reconstitution.

2.4. LC–MS/MS conditions

The LC system consisted of a solvent delivery module (Hewlett Packard series 1100 from Agilent, Palo Alto, California, USA), an autosampler (PE series 200 of Perkin Elmer, Woodbridge, Ontario, Canada), and a Zorbax Extend-C18 column (30 × 3 mm, 3.5 μm, Agilent) operated at room temperature. The mobile phase was a mixture of methanol/water (57.5:42.5, v/v) with 1 mM ammonium formate and 0.1% (v/v) ammonium hydroxide. The flow rate was 1 mL/min and the injection volume was 10 μL.

Mass spectrometric detection was carried out with a Sciex API 4000 equipped with a TurboIonSpray interface (MDS Sciex, Toronto, Ontario, Canada). The ion source was operated in the positive mode. The MRM transitions were m/z 376.2 → 247.1 and 385.5 → 247.1 for tiagabine and the IS, respectively. The TurboIonSpray voltage and temperature were set at 4000 V and 550 °C, respectively. The declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) voltages were set at 60, 28, and 13 V, respectively for tiagabine. The same voltages were used for the IS except for DP, which was 65 V. The Analyst software (version 1.4.2, MDS Sciex) was used for data acquisition and processing.

2.5. Regression calculations

Calibration curves were constructed using analyte/IS peak area ratios with weighted ($1/C^2$ or $1/C$) or non-weighted least-squares linear regressions. The calculation was performed either using the Analyst software or an in-house built Excel program, e.g. for the regressions of simulated data. It should be noted that only the spiked analyte concentrations for calibration standards and quality controls were used in the regressions and the calculations of bias and precision. In other words, when a given amount of the analyte was brought in with the added impure internal standard solution, this extra amount was not added to the spiked analyte concentrations.

3. Results and discussion

3.1. Considerations on simulation and selection of bioanalytical model method

Generally speaking, there are three main causes for cross signal contribution between an analyte and its internal standard in MS detection, i.e. chemical impurity in the reference standards used, isotopic interference, and crosstalk inside a mass spectrometer. Since the crosstalk is usually instrument-dependant and variable [10] and it may not be strictly concentration-proportional, only the first two causes are considered in this paper. However, should it be reproducible and concentration-proportional in special cases, its impact on quantitation would be similar to that of isotopic interference. Furthermore, linear response between an analyte or an IS and its concentration is assumed in the simulations.

During the selection of a model method for testing simulation results, the following factors were taken into consideration. Firstly, the method must be intrinsically linear over a wide range, e.g. 1000-fold. Secondly, the difference in molecular weight between the analyte and its internal standard is large so that the potential isotopic interference is negligible. Thus, the amount of cross contributions can be actively controlled by deliberate addition of the IS to the analyte solution or vice versa. Thirdly, the sample extraction should be based on protein precipitation, one of the three most commonly used extraction methods (i.e. protein precipitation, liquid–liquid extraction, and solid-phase extraction) yet with

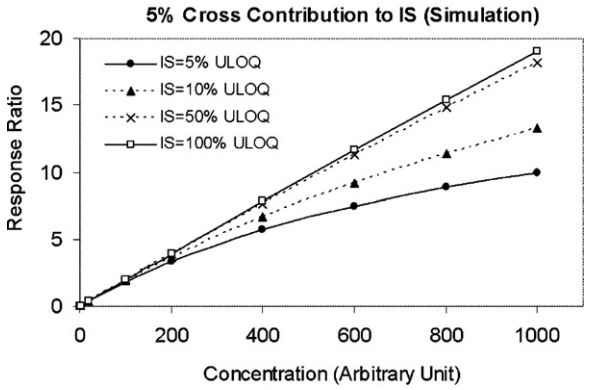

Fig. 2. Simulation results demonstrate that calibration curves become progressively non-linear with decreasing concentration of internal standard (cross contribution from the analyte to the internal standard is equivalent to 5% of the concentration of the analyte).

relatively less clean extract to mimic the worst scenario. Lastly, the sample processing can be automated to limit human errors as much as possible. After a non-exhaustive screening of our validated methods, it was found that a recently validated method for tiagabine (an anticonvulsive drug used to treat partial seizures, a type of epilepsy) satisfactorily meets the above criteria and it was therefore used with minor modifications, such as proportionally increase in the volume of sample, internal standard, and precipitating solvent, in order to minimize the impact of impression during pipetting on the interpretation of experimental results.

3.2. Signal contribution from analyte to internal standard

When an analyte contributes to the response of its internal standard either due to impurity in the reference standard used or isotopic interference, the linearity of a calibration curve and the accuracy of quantitation could be affected. The extent of this impact is dependent on the percentage of cross contribution, IS concentration, and weighting factor of regression. Summarized in Table 1 are the minimum internal standard concentrations needed in different scenarios to meet the accuracy criteria for calibration standards, i.e. bias within ±20% at the lower limit of quantitation and within ±15% for the rest. Generally speaking, the more severe cross contribution and the wider concentration range is, the higher IS concentrations are necessary. For a given situation, e.g. 5% signal contribution from the analyte and a 1000-fold concentration span, calibration curves increasingly curve down as the concentration of the internal standard is lowered (Fig. 2).

As expected, the experimental results from tiagabine method confirm those of simulations. When calibration standards contained the internal standard at concentrations equivalent to 5% of the analyte concentrations, significant non-linearity was observed when a low IS concentration (equivalent to 10% of the ULOQ) was used (Fig. 3a), which resulted in rejection of the calibration curve. While the same CS (calibration standard) samples were extracted using a higher IS concentration (equivalent to 50% of the ULOQ) in the same batch and injected side by side on the same LC–MS/MS instrument with the same LC and MS parameters, the linearity was significantly improved, i.e. meeting the acceptance criteria. On the other hand, when no cross contribution existed, no such linearity difference was noted in terms of IS concentration (Fig. 3b).

Though this type of non-linearity occurs at the high concentration end, apparently it is not due to detector saturation. In fact, similar non-linearity was even observed in a much narrower range from 1 to 100 ng/mL when low IS concentration was used (other conditions were kept the same as those for 1–1000 ng/mL range,

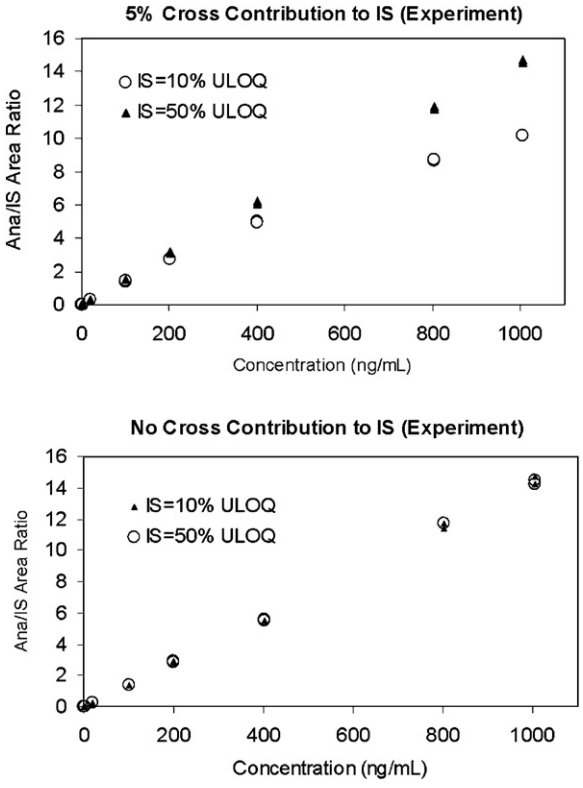

Fig. 3. Experimental results demonstrate the presence of non-linearity when there was cross signal contribution from the analyte and a low internal standard concentration (100 ng/mL) was used (a, upper panel). On the other hand, both low (100 ng/mL) and high (500 ng/mL) internal standard concentrations led to linear calibration curve in the absence of cross signal contribution to the internal standard from the analyte (b, lower panel). For a good visual comparison, the area ratios associated with high internal standard concentration were multiplied with a factor of 5.

data not presented here). Therefore, during trouble-shooting of non-linearity at high concentrations in method development or application, one should not focus one's efforts only on the possibility of detector saturation. The cross-contribution from the analyte and the appropriateness of the IS concentration used should also be checked.

3.3. Signal contribution from internal standard to analyte

When an internal standard contributes to the response of the analyte, theoretically, the linearity of calibration curves and the

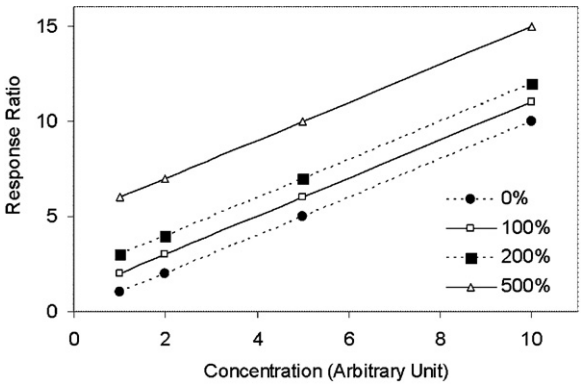

Fig. 4. Simulation results show the upward shifting of calibration curves with increasing amount of analyte in the internal standard.

Table 1
Minimum internal standard concentrations required for different situations when an analyte contains its internal standard.

Concentration range	Weighting factor	Cross signal contribution from analyte					
		0%	0.5%	1%	2.5%	5%	10%
1000-fold	$1/C^2$	>0	1.6	3.2	7.9	16	32
	$1/C$	>0	3.5	6.9	18	35	69
	None	>0	168	339	840	1679	3358
500-fold	$1/C^2$	>0	1.6	3.1	7.7	16	31
	$1/C$	>0	3.3	6.6	17	33	66
	None	>0	84	168	418	836	1671
250-fold	$1/C^2$	>0	1.5	3	7.4	15	30
	$1/C$	>0	3	5.9	15	30	59
	None	>0	42	83	207	414	828
100-fold	$1/C^2$	>0	1.5	2.9	7.2	15	29
	$1/C$	>0	2.8	5.6	14	28	56
	None	>0	18	36	88	176	352

Note: The minimum internal standard concentrations are expressed as the % of the upper limit of quantitation of the analyte.

Table 2
Calibration curve parameters and the results of lower limit quality control when the internal standard contributed to the analyte.

Analyte in IS (% LLOQ)	Intercept	Slope	Regression coefficient	LLQC		
				Mean ($n=6$)	CV (%)	Bias (%)
0	0.00020	0.003974	0.9994	0.98	5.2	−1.5
50	0.00232	0.004035	0.9986	0.95*	5.9	−4.6
100	0.00405	0.003845	0.9971	1.05	9.6	5.0
200	0.00851	0.003893	0.9977	0.92	12.7	−8.3
300	0.01269	0.003849	0.9977	1.05	18.2	5.1
400	0.01672	0.003889	0.9985	1.15	16.4	14.8
500	0.02192	0.004024	0.9992	0.92	19.4	−8.5
800	0.03431	0.004110	0.9988	1.15	14.8	15.2
	Mean	0.003952				
	CV (%)	2.48				

Notes: IS, internal standard; LLOQ, lower limit of quantitation; LLQC, lower limit quality control.
* $n=5$ due to an outlier caused by contamination, which was confirmed by an NMR (Maximum Normal Residual) test.

accuracy of quantitation are not impacted, except for an upward shifting of the whole calibration curve (increase of intercept) because an equal amount of internal standard is added to all samples (Fig. 4).

This was also confirmed by experiments. As shown in Table 2, when different amounts of analyte were added to the internal standard working solutions (up to 8-fold of the LLOQ), the calibration curves remained linear ($r \geq 0.9971$) with constant slopes but linearly increasing intercepts ($r = 0.9997$, intercept = 4.289×10^{-5} (% LLOQ in IS) − 9.3×10^{-6}). The quantitation of the lower limit quality controls was all acceptable. However, either CV or bias approaches the limit of acceptance criteria for the contamination level of 3-fold LLOQ and above. To reserve sufficient "wiggle" room, it is therefore better to limit the contamination within 2-fold of the LLOQ for this bioanalytical method. At this contamination level, between-run (inter-run) accuracy and precision meet the acceptance criteria for all quality control samples (Table 3).

Table 3
Between-run accuracy and precision when the internal standard contributed to 2-fold of the LLOQ.

Sample type	Nominal conc. (ng/mL)	Mean measured conc. (ng/mL)	CV (%)	Bias (%)
LLQC	1.00	1.084	13.3	8.4
QC1	3.01	3.097	4.3	2.9
QC2	301.11	307.800	2.3	2.2
QC3	702.59	716.109	1.9	1.9

Notes: LLQC, lower limit quality control; QC, quality control; Conc., concentration; LLOQ, lower limit of quantitation; $n = 18$ (3 runs, 6 replicates/run) for all samples.

3.4. When mutual contributions exist

From a theoretical point of view, when an analyte and its internal standard contribute to each other's response, the final outcome is a linear combination of the two factors discussed in the above, i.e. linearity and accuracy issue as mentioned in Section 3.2 but with increasing intercept as detailed in Section 3.3.

However, due to the unavoidable existence of experimental variations, experimental results start deviating from the theoretical predication when the contamination level in working internal standard solution is 5-fold of the LLOQ or above (Table 4). By comparing the results of Table 4 with those in Table 2, it is evident that the slopes in Table 4 are slightly more variable and the intercepts are also slightly less linear in relation to the % level of contamination in the IS ($r = 0.9993$, intercept = 7.092×10^{-5} (% LLOQ in IS) − 2.8×10^{-5}). These differences may be due to a combination of experimental variations and the intrinsic non-linearity nature of the calibration curve, though it was mitigated by a relatively high IS concentration (50% of the ULOQ in this case). While in Table 2, only the experimental variation factor was present.

Nevertheless, based on the results in Table 4, it is reasonable to assume that reproducible and reliable results could be obtained while keeping the analyte contamination level in the internal standard solution within 1-fold of the LLOQ. Accordingly, between-run accuracy and precision were evaluated by using both pure and contaminated IS (equivalent to 1-fold of the LLOQ). The results are acceptable for both cases and are comparable (Table 5).

These results demonstrate that a distinction should be made between the known and stable cross signal contribution from an

Table 4
Calibration curve parameters and the results of lower limit quality control when the analyte contributed to the internal standard (equivalent to 5% of the analyte concentration).

Analyte in IS (% LLOQ)	Intercept	Slope	Regression coefficient	LLQC		
				Mean (n = 6)	CV (%)	Bias (%)
0	0.00030	0.006190	0.9978	1.02	5.2	2.3
50	0.00369	0.006835	0.9989	0.98	8.8	−2.5
100	0.00637	0.006878	0.9986	1.14	11.3	13.6
200	0.01345	0.007261	0.9987	1.09	10.4	8.6
500	0.03730	0.006841	0.9987	0.79	22.6	−20.6
1000	0.07027	0.007174	0.9981	1.02	26.8	2.4
	Mean	0.006863				
	CV (%)	5.49				

Notes: IS, internal standard; LLOQ, lower limit of quantitation; LLQC, lower limit quality control.

Table 5
Comparison of between-run accuracy and precision with or without cross contribution from the internal standard to the analyte (the IS in the analyte solutions are equivalent to 5% of the analyte concentrations).

Sample type	Nominal conc. (ng/mL)	No analyte in IS			IS contributed to 1-fold LLOQ		
		Mean measured conc. (ng/mL)	CV (%)	Bias (%)	Mean measured conc. (ng/mL)	CV (%)	Bias (%)
LLQC	1.00	1.012	8.3	1.2	1.018	10.5	1.8
QC1	3.01	3.129	8.4	4.0	3.171	3.6	5.4
QC2	301.11	303.281	2.7	0.7	303.807	2.1	0.9
QC3	702.59	687.174	3.3	−2.2	683.607	2.1	−2.7

Notes: LLQC, lower limit quality control; QC, quality control; Conc., concentration; IS, internal standard; LLOQ, lower limit of quantitation; n = 18 (3 runs, 6 replicates/run) for all samples.

added internal standard to the analyte and other unknown and variable sources, such as matrix selectivity, carry-over, and contamination. For those sources, the criterion of 20% must be used to ensure that the bias at the LLOQ level is within ±20%. While for the cross signal contribution from an internal standard to the analyte, the criterion of 20% is not an absolute necessity and should be method specific because the amount of extra analyte introduced by the added IS solution is known and constant as well as each method has different signal to noise (S/N) ratio and variability at the LLOQ level.

3.5. Reference standard impurity vs. isotopic interference

So far, the two types of cross signal contribution, i.e. reference standard impurity and isotopic interference, have been treated equally. Indeed, the distinction is not necessary for the cross signal contribution from an internal standard to the analyte because the same amount of internal standard is added to all the samples. For an analyte, such distinction is also not necessary between calibration standards and quality controls because they are prepared the same way. However, if the impurity in the analyte reference standard is a factor in cross signal contribution, then systematic errors in the quantitation of unknown study samples may exist because study samples are not prepared with the same reference standard as used for the calibration standards and quality controls. Instead, the analyte in study samples originates from the medication used in a study.

As demonstrated by the results from theoretical simulations (Table 6), the systematic errors vary with the IS concentration and the weighting factor used in regression, and they are also concentration-dependant. For example, by increasing the IS concentration from 20% to 100% of the ULOQ, the systematic errors for most of the high concentration samples are reduced from about 10% to 2%. Using $1/C$ weighted regression, instead of $1/C^2$ weighted regression, the systematic errors increase for all concentrations, e.g. from 4% to 7% for high concentration samples. The largest change occurs at the lower limit of quantitation, from −1.0% to −13.9%, which highlights the importance of stronger weighting factor for lower concentrations. Similar simulation results were also obtained for a concentration range of 100-fold (data not shown).

To check the validity of the simulation results, i.e. those in Table 6, a run (batch) including two series of calibration standards was extracted, one equivalent to using pure analyte reference standard (pure CS) while the other equivalent to using impure analyte reference standard (non-pure CS, containing 5% of the internal standard). By using non-pure CS series as calibration standards in the

Table 6
Comparison of biases between calibration standards and real samples when analyte reference standard contains 5% of internal standard (simulation).

CS no.	Conc.	IS = 20% ULOQ		IS = 50% ULOQ		IS = 100% ULOQ	
		Bias (%) for CS	Bias (%) for real sample	Bias (%) for CS	Bias (%) for real sample	Bias (%) for CS	Bias (%) for real sample
1	1	−2.3	−2.3	−1.0 (−13.9)	−1.0 (−13.9)	−0.5	−0.5
2	2	3.7	3.7	1.5 (−3.3)	1.5 (−3.3)	0.8	0.8
3	20	8.6	9.1	3.6 (6.0)	3.8 (6.3)	1.8	1.9
4	100	6.9	9.6	3.0 (6.1)	4.0 (7.1)	1.5	2.0
5	200	4.4	9.6	2.0 (5.1)	4.0 (7.2)	1.0	2.0
6	400	−0.3	9.7	0.0 (3.1)	4.0 (7.3)	0.0	2.0
7	800	−8.6	9.7	−3.7 (−0.7)	4.0 (7.3)	−1.9	2.1
8	1000	−12.3	9.7	−5.4 (−2.5)	4.0 (7.3)	−2.8	2.1

Notes: CS, calibration standard; Conc., concentration in arbitrary unit; IS, internal standard; ULOQ, upper limit of quantitation.
The % bias values in brackets for IS = 50% ULOQ are associated with $1/C$ regression while others are from $1/C^2$ regression.

Table 7
Comparison of biases between calibration standards and real samples when the analyte reference standard contained 5% of the internal standard (experiment).

CS no.	Conc. (ng/mL)	$1/C^2$ Weighing		$1/C$ Weighing	
		Bias (%) for CS	Bias (%) for real sample	Bias (%) for CS	Bias (%) for real sample
1	1.00	−0.8	−10.4	−11.5	−21.4
2	2.01	1.3	−5.6	−2.7	−9.8
3	20.07	2.9	3.3	4.9	5.3
4	100.37	1.0	4.5	3.5	7.1
5	200.74	3.3	2.9	6.0	5.5
6	401.48	−0.1	4.6	2.5	7.3
7	802.96	−2.6	4.4	−0.1	7.1
8	1003.70	−5.0	2.9	−2.6	5.5

Notes: CS, calibration standard; Conc., concentration, internal standard concentration equivalent to 50% of the upper limit of quantitation; *n*, 6 for each CS and real sample.

regression of the calibration curve and the pure-CS series as quality controls, the systematic errors for future study samples can be estimated from the % bias values of the pure-CS samples. Moreover, six replicates were processed for each calibration standard in order to improve the precision of this evaluation [11]. Overall, these experimental results (Table 7) are comparable with those of simulations, except for large difference at the very low concentrations, i.e. CS1 and CS2. Specifically, the systematic errors from the experiment at the LLOQ level are −10.4% and −21.4% for $1/C^2$ and $1/C$ weighted regressions, respectively. The latter is even outside the acceptance criterion (±20%). The difference between the simulation and experimental results might be due to unavoidable experimental variations and the inadequate strength of the weighting factors used.

On the other hand, when the pure-CS samples were used as standards in regression, very good accuracy and precision for back-calculated concentrations were obtained for pure-CS samples and no significant differences were observed between $1/C^2$ and $1/C$ weighing. Specifically, for $1/C^2$ regression, the % bias ranged from −1.78% to 0.87% and CV% were between 1.02 and 5.63%. For $1/C$ regression, the % bias ranged from −1.85% to 0.91% and CV% were between 1.02 and 5.64%. These values, particularly the % bias, are much better than the corresponding % bias for the back-calculated concentrations of non-pure CS samples (Table 7), which demonstrates that the systematic errors presented in Table 7 are not due to any experimental errors.

3.6. Implications of systematic errors and change of reference standard lot or supplier on stability evaluation, method transfer, cross-validation, and the validity of a validated method

As mentioned in the previous section, appreciable systematic errors could exist for unknown study samples while being quantified by a calibration curve prepared from a reference standard that contains traces of the internal standard. In other words, the systematic bias may be significant enough to cause failures or bias the result interpretation in stability evaluation, cross-validation, and method transfer when reference standards with different amounts of IS impurity are used in two different occasions, e.g. time 0 analysis run and stability evaluation run or analyses in lab A and lab B. For example, when stability samples were prepared and quantified to obtain comparison values, a pure analyte reference standard was used. Several months later, a new analyte reference standard that contains 5% of the internal standard is used and the IS concentration used in the bioanalytical method is equivalent to 20% of the ULOQ. Based on the results shown in Section 3.5, around 10% of bias is added to the real percentage of change, which could fail an otherwise acceptable stability evaluation or worse accept an otherwise unacceptable one. The same can be said about cross-validation and method transfer. Given the trend of increasing cross-validations and method transfers either nationally or internationally [12], this finding is of great significance and its impact can be far reaching. The potential significant systematic error associated with impurities in reference standards should be taken into consideration during experimental design and/or trouble-shooting.

In addition, whether a validated method needs partial re-validation should be evaluated when a reference standard of different lot or supplier from that used in the original validation is to be employed, despite the fact that the analyte compound and its purity may be comparable. For example, the analyte reference standard used in method development and original validation contains much less impurity of the internal standard and a low IS concentration has been selected. Apparently, there should not be any issue with the original validation. However, when the method is later used for study sample analysis utilizing a new lot or a new supplier for analyte reference standard and it happens that the new analyte reference standard contains more IS as impurity, the original linear calibration curve may become intrinsically non-linear, which could cause various issues in addition to the systematic errors for unknown study samples mentioned above, such as high run failure and reassay rates as well as compromised quality of the reported results.

On the other hand, issues may also arise even the other way around, e.g. less pure analyte reference standard used in the original method validation while purer analyte reference standard is to be used in the sample analysis (the application of the validated method). It is possible that a non-linear calibration has been employed due to the reasons mentioned previously. Now, the calibration curve becomes linear with the purer reference standard. Variable and unreliable results may be obtained while an intrinsically linear curve is forced to be regressed non-linearly. However, there should be no issues in cases where linear calibration and appropriate IS concentration have been used in the original validation.

Regarding a change of reference standard for an internal standard, re-validation of accuracy and precision should be performed if the amount of analyte in the new internal standard is significantly increased, e.g. from less than 20% of the LLOQ to 50% or 100% of the LLOQ.

Based on the above, it is recommended that such information as how much cross contributions are tolerable in a specific method should be written in the method standard operating procedure (SOP) to guide the re-purchasing or re-synthesis of reference standards.

3.7. Methods for more than one analytes

The situation associated with a multiple analyte method could be quite complicated depending on how many analytes and how many internal standards are included in the method as well as the analytical ranges, particularly when the concentration ratios between or among the analytes are not constant across different

concentration levels. Nevertheless, in principle, the findings presented in the above are still applicable to bioanalytical methods of multiple analytes. For example, cross signal contribution to an IS could cause issues of linearity, accuracy, and systematic error whether it comes from the associated analyte (the analyte for which the internal standard is intended) or a co-analyte. The cross signal contribution to an analyte either from its internal standard or a co-internal standard would cause the shifting of calibration curves while accuracy may be maintainable. However, it should be emphasized that any cross signal contribution to an analyte from a co-analyte due to impurities in its reference standard could cause additional systematic errors. As to the details of how cross signal contributions affect the accuracy, linearity, and systematic error in a multi-analyte method, further research is necessary.

4. Conclusions

Cross contributions in mass spectrometric response between an analyte and its internal standard are quite common due to isotopic interference and/or impurity in the reference standards used. When an analyte contributes to the response of the internal standard, high IS concentration should be used during sample processing to improve linearity and accuracy unless there exists mutual ion suppression between an analyte and its stable isotope labelled IS [7,13]. Moreover, while the analyte reference standard contains its internal standard, systematic errors could exist for real samples, which cannot be revealed by the results of quality controls and calibration standards. To reduce the magnitude of such systematic errors, in addition to high IS concentration, $1/C^2$ weighted regression is also preferred regardless of the concentration range.

The cross response contribution from an internal standard to the analyte does not affect linearity, only the intercept because the same amount of internal standard is added to all samples in a batch. The contribution may not need to be controlled to be within 20% of that of the lower limit of quantitation sample. Acceptable accuracy and precision could be achieved with contributions higher than 20% of that of the LLOQ, e.g. 200%, though the exact percentage is method specific.

When a reference standard from different lot or supplier is used for a validated method, a careful evaluation should be performed to see if a partial re-validation is necessary even when the purity of the new reference standard is higher than that of the original reference standard used in the method validation.

Since the aforementioned systematic errors are related to the impurity in analyte reference standard, they could bias the interpretation and/or cause issues in the evaluation of long-term stability in matrix, cross-validation and the transfer of a method when reference standards of different lot or supplier are used in the two different occasions.

Although the examples given in this paper are for a small molecule compound quantified by LC–MS, the principle and findings are also applicable to the quantitation of other types of molecules (e.g. peptide, protein, carbohydrate) and other mass spectrometric technologies, such as GC–MS, ICP–MS.

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