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Internal standard response variations during incurred sample analysis by LC–MS/MS: Case by case trouble-shooting

Aimin Tan [a,b,*], Saleh Hussain [a], Adrien Musuku [b], Robert Massé [b]

[a] Anapharm, Richmond Hill, Ontario, Canada
[b] Anapharm, Ste-Foy, Québec, Canada

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ABSTRACT

Internal standard (IS) responses can directly impact the accuracy of reported concentrations in bioanalysis as the majority of LC–MS/MS methods are based on analyte/IS response ratios for quantitation. Due to the complexity of incurred sample matrices and drug formulation, variable IS responses are quite common upon applying a validated method to the analysis of incurred samples. To maintain the integrity of a study and to avoid economic losses, it is therefore extremely important to monitor IS response variations during bioanalysis and to quickly identify the root causes if variations are observed. Presented in this article are twelve trouble-shooting examples from the analyses of incurred samples by a wide variety of bioanalytical methods, including human error, malfunctioning equipment/instruments, wrong material, matrix effect and inherent issues with a bioanalytical method. Insightful ideas for how to trouble-shoot and how to develop more reliable bioanalytical methods can be drawn from these practical examples.

1. Introduction

Internal standards are commonly used in quantitative bioanalysis, particularly in LC–MS/MS based bioanalysis [1,2]. The main purpose of utilizing internal standards is to correct any variation other than that related to the amount of analyte present in a sample, such as the variability in dilutions, evaporation, degradation, recovery, adsorption, derivatization, injection, and detection. Hence, internal standards should be added in sample processing procedure as early as possible, usually added immediately after the aliquoting of samples. In addition, their chemical and physical properties should be as close to those of the analytes as possible. By finding a good internal standard and using analyte/IS response ratios for quantitation, variations in absolute responses other than those related to analyte concentration could be corrected, which help maintain the accuracy of quantitative results.

There are mixed expectations regarding internal standard response variations. In one hand, internal standard response variations in bioanalysis by LC–MS/MS are somewhat expected if considering the many differences between incurred samples and the pooled control blank plasma, in which calibration standards (CS) and quality control (QC) samples are prepared (Table 1). On the other hand, too much variation in internal standard responses during bioanalysis could trigger doubt in the reliability of the quantitative results obtained. To maintain the integrity of a study and to avoid economic losses, it is therefore extremely important to monitor IS response variations during bioanalysis and to quickly identify the root causes if variations are observed, especially for those causes that affect an analyte and its internal standard differentially.

Despite its importance in bioanalysis of incurred samples, no article (to the best knowledge of the authors) has been published for this topic (i.e. comprehensive coverage on IS response variation during the analysis of incurred samples) except for some individual case studies. For example, Keyhani et al. reported consistently higher IS responses for incurred samples than those of calibration standards and quality control samples [3]. A related topic is matrix effect during mass spectrometric detection, which has been extensively addressed by many [4–10]. However, internal standard response variations in incurred sample analysis could be caused by many factors other than matrix effect, such as addition of internal standard, variation in recovery, variation in injection volume, variation in separation and instrumental conditions. A comprehensive and systematic discussion supported by real cases on internal standard response variations during the analysis of incurred samples would be desirable. In recent years, the authors had the privilege to work with a wide variety of bioanalytical methods, compounds, and studies for world-wide clients. Summarized in this article are several practical trouble-shooting examples for IS response variations during the analysis of incurred samples, which would be interesting to many working in bioanalysis field. Additionally, insightful

* Corresponding author at: Anapharm, 2500 Einstein Road, Québec, QC, Canada G1P 0A2. Tel.: +1 418 527 4000; fax: +1 418 527 3546.
E-mail addresses: aimintan@hotmail.com, atan@anapharm.com (A. Tan).

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Table 1
Matrix differences between CS/QC and incurred samples.

	CS/QC	Incurred sample
Screening criteria for matrix sources	Usually loose	Usually specific and strict dependent on the objectives of a study, such as age 40–50 and non-smoker
No. of lots/sources	Usually more than one source (pooled)	One single source
pH	Averaged due to pooling	More variable
Extra components associated with medication	None	Metabolite(s), co-medication and non-active ingredients in formulation
Amount collected	Usually large, e.g. 200 mL per collection	Usually small, e.g. 7 mL per sampling time
No. of freeze/thaw cycles prior to being extracted	Usually 2 or more	Usually 1
Storage tube and pre-use storage	Usually stored at −20 °C and without special protection until being selected for a specific study	Could be collected under sodium light and stored at -80 °C immediately after collection
Amount of anticoagulant	May be different because of different amounts collected	

Note: CS = calibration standard; QC = quality control.

ideas could be drawn from these trouble-shooting examples to help develop reliable and rugged bioanalytical methods.

2. Experimental

Due to the nature of this article and the number of methods involved, only general experimental information is given in this section. More detailed information relevant to trouble-shooting will be given in the case studies.

2.1. Chemicals and reagents

Acetonitrile and methanol (Omnisolv), acetic acid (glacial, AnalaR), formic acid (AnalaR), ammonium acetate (AnalaR), ammonium formate (AnalaR), hydrochloric acid (Assured), and phosphoric acid (85%, AnalaR) were all purchased from EMD (Toronto, Canada). Ammonium hydroxide and Trizma® base were obtained from Sigma (Oakville, Canada). Human EDTA K_2 and K_3 plasma were obtained from Valley Biomedical (Winchester, Virginia, USA). Water was produced in-house with Milli-Q water system (Millipore, Billerica, MA, USA). High purity liquid nitrogen was supplied by Prodair (Mississauga, Ontario, Canada).

2.2. Calibration standards and quality control samples

Stock solutions were prepared in methanol, acetonitrile, or methanol/Milli-Q type water (50/50, v/v) depending on the properties of the compounds. All intermediate and working solutions were prepared by successive dilution of the respective stock solutions in the same solvent used for stock solutions. Calibration standards and quality control samples were prepared in control (blank) human EDTA K_2 or EDTA K_3 plasma. A typical batch for incurred sample analysis included calibration standards and quality control samples at eight and four concentration levels, respectively (duplicate at each level).

2.3. Sample processing

All the three common extraction methods, e.g. protein precipitation (PP), liquid–liquid extraction (LLE) and solid-phase extraction (SPE), were involved depending on the analyte being quantified. Some were performed manually and others automatically with a MultiPROBE II EX HT robotic liquid handling system (Perkin Elmer, Shelton, Connecticut, USA).

2.4. LC–MS/MS analysis

The LC system consisted of a solvent delivery module (Hewlett Packard series 1100 from Agilent, Santa Clara, California, USA) and an autosampler (PE series 200 of Perkin Elmer, Toronto, Canada). Various columns and mobile phases were used in isocratic or gradient modes.

Mass spectrometric detection was carried out with a Sciex API 4000 or API 5000 equipped with a TurboIonSpray interface (MDS Sciex, Toronto, Canada). The Analyst™ software (version 1.4.1, MDS Sciex, Toronto, Canada) was used for data acquisition and processing. Calibration curves were constructed using the respective analyte/IS peak area ratios against analyte concentrations with a weighted ($1/C^2$) least-squares linear regression.

2.5. Log D calculation

The log *D* (hydrophobicity) values used in this article were determined by Pallas software, version 3.1 (CompuDrug International, Inc., Sedona, Arizona, USA).

3. Case studies

3.1. Case 1—variation or error in the addition of internal standard

As quantitation is based on analyte/IS response ratios, a prerequisite for good accuracy is that the same amount of internal standard is added to all the samples including calibration standards, quality controls, and unknown (incurred) samples. Any difference in the amount of internal standard added to a sample will directly affect its ratios, and therefore its reported concentration. Hence, this type of variation or error should be minimized or avoided whenever possible.

It is relatively easy to identify variation or error in the addition of IS for CS and QC samples. As their concentrations are known, large variation in internal standard addition for CS and QC samples would result in high bias from nominal concentration, the rejection of CS and QC samples, or even the rejection of the whole run. However, the variation or error in the addition of IS for unknown incurred samples would be difficult to be ascertained unless the addition of internal standard is doubled or missed as shown in Fig. 1. Even with doubled or near zero IS responses observed, it could be other reasons than the addition of IS. For example, missed addition or incorrect amount of derivatization reagent can produce similar IS response as well.

Though human error is usually the cause in this case, proper method development can make large difference in reducing this type of error or variations. Firstly, large volume of IS, such as 200 μL or more should be used if possible. Considering that an internal standard is usually added by a repeater pipet, small volumes (such as 50 μL or less) are more prone to imprecision than large ones. In addition, it would be extremely difficult to visually spot missed or doubled addition for an internal standard when the volume of the internal standard is much smaller than the volume of sample and/or other reagents (e.g. buffer). Secondly, it would be helpful to

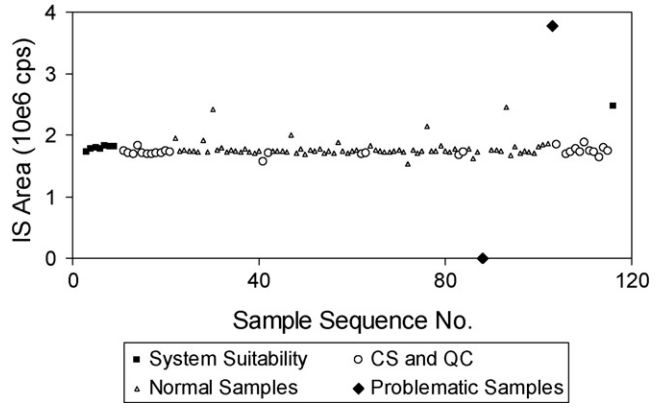

Fig. 1. Example of missed and double addition of internal standard. Analyte: clopidogrel carboxylic acid; Extraction: evaporation-free protein precipitation; Sample volume: 50 μL; IS volume: 150 μL.

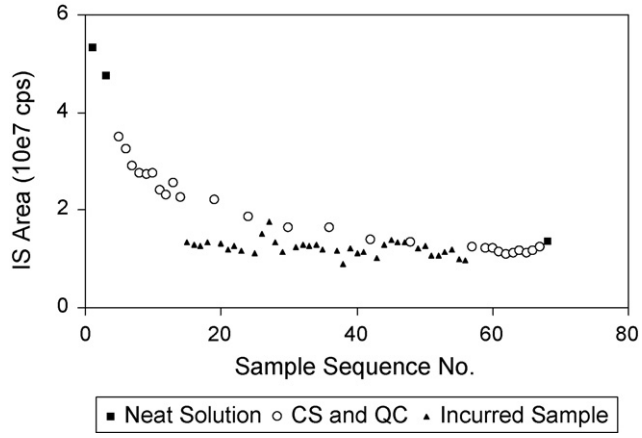

Fig. 3. Gradually decreased internal standard responses caused by the charging of mass spectrometer. Analyte: escitalopram.

reduce errors by adding the IS solution (usually colorless) first and then incurred samples, which are usually colored, such as slightly yellowish for plasma samples or dark red for whole blood samples.

3.2. Case 2—random and sharp drop in IS response

As shown in Fig. 2, internal standard responses dropped suddenly during the course of a run and indiscriminately for CS, QC, or incurred samples. For the CS or QC samples, their accuracy was not affected. Considering otherwise stable IS response for the partially injected run, a problem with the mass spectrometer or sample processing was not likely. Instead, the root cause was identified with the autosampler.

In bioanalysis, extracted samples are usually stored in either autosampler vials or wells in a plate (such as 96-well plate) sealed with pierceable caps or cover. During injection, the autosampler needle has to pierce the caps or cover to load samples. The debris produced might completely or partially block the needle of an autosampler, which would result in no sample or randomly low volumes of sample injected. Accordingly, no response or low IS responses will be observed. As most autosamplers have a built-in needle flushing mechanism, the debris in the needle might be flushed out later partially or completely. Therefore, the injected volume can be back to normal at a later time without any intervention of operators. Apparently, when a needle will be blocked and when the blocked needle will be cleared by flushing, as well as how it will be blocked (completely or partially) are difficult to be predicted. Hence, there will be no definite pattern for this type of IS variations. The affected injections always have lower IS responses than those of other samples. Despite their lowered IS responses, the accuracy of quantitation can usually be maintained except for situations where none or extremely low volume of samples are injected, resulting in responses outside the limits of linear range.

To solve the problem depicted in this example, the affected run can be re-injected in a different LC–MS/MS system or on the same system after the needle of the autosampler is cleared.

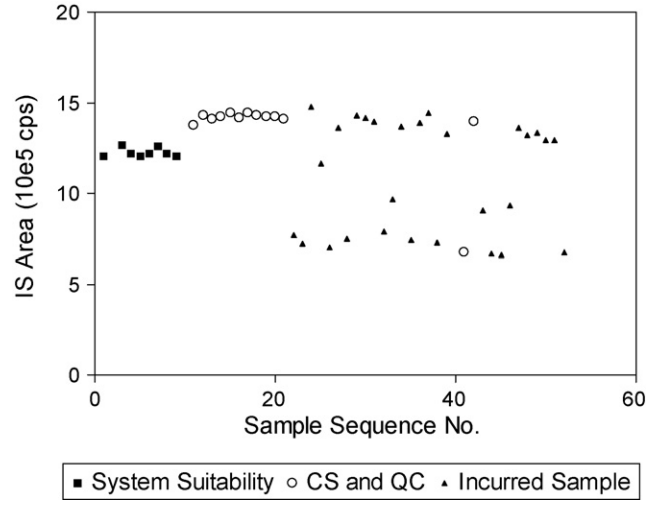

Fig. 2. Low and variable internal standard responses caused by autosampler problem occurred during the middle of run injection. The analyte and method were the same as in Fig. 1.

3.3. Case 3—gradual decrease of IS responses

This was observed in the chiral separation of escitalopram with a long chromatographic run time (14 min/injection). At the beginning of the run, there was a steep decrease in IS response and then followed by slower and continuous decrease in IS signal (Fig. 3), which is quite similar to the current *vs.* time curve during the charging of a capacitor.

For those with good knowledge of mass spectrometer instrumentation, it would be relatively easy to identify the root cause for this case, i.e. the "charging" of a mass spectrometer. Specifically, the components inside mass spectrometers, such as the rods of quadrupoles, get dirty and contaminated during routine use in bioanalysis, particularly when high flow rate is used without split. When this happens, the number of ions transmitted will decrease continuously in a similar way as the charging of a capacitor.

Whether this had an impact on the accuracy of quantitation or not depends on how well the IS followed the analyte in MS/MS detection and if there was still enough signal/noise (S/N) ratios, especially for the samples injected at the end of the run. In some instances the accuracy would be impacted and in others not. In the case shown in Fig. 3, the IS was able to compensate the variation well, therefore, the accuracy of quantitation was maintained. To solve this type of problem, the contaminated components in mass spectrometer should be cleaned.

3.4. Case 4—low IS responses caused by autosampler problem plus the charging of mass spectrometer

Sometimes two different problems can emerge at the same time as shown in Fig. 4. In this case, the mass spectrometer was clearly charging as demonstrated by the gradual decrease of IS response. At the same time, there were randomly scattered low IS responses,

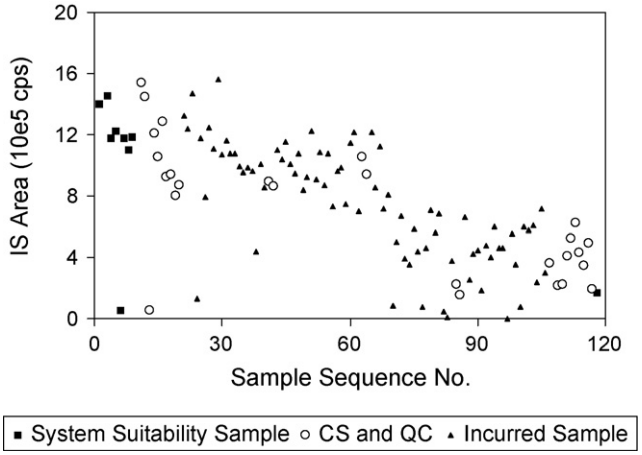

Fig. 4. Significant internal standard response variation caused by autosampler problem and the charging of mass spectrometer. Analyte: esomeprazole.

which is characteristic of autosampler needle blockage. When this batch was re-injected in a different LC–MS/MS system, stable IS responses were observed for all the samples.

3.5. Case 5—low IS responses for most of the extracted samples

After an LLE-based method for tamsulosin had been successfully applied to several studies, an unexpected issue emerged during the early stage of a new study (Fig. 5). In this case, the system suitability samples (extracted blanks reconstituted with analyte and IS in neat solution) were normal, i.e. with similar responses as those in previous studies. However, the internal standard responses for almost all the CS, QC, and incurred samples were much lower than expected. Only a few samples, randomly scattered in the run, had normal IS responses. Based on the above, issues with the method, lab personnel, reagents, or the LC–MS/MS system used could be ruled out. Furthermore, it was observed that the accuracy of CS and QC were not affected, despite lowered IS responses, which means that the IS had followed the analyte well and the abnormality occurred after sample aliquoting step.

By interviewing the lab persons involved, it was found that a new type of caps, i.e. rubber-lined caps, had been ordered and put into use in the lab just after the start of this new study to reduce the chance of leakage associated with the PTFE (polytetrafluoroethylene)-lined caps during the liquid–liquid

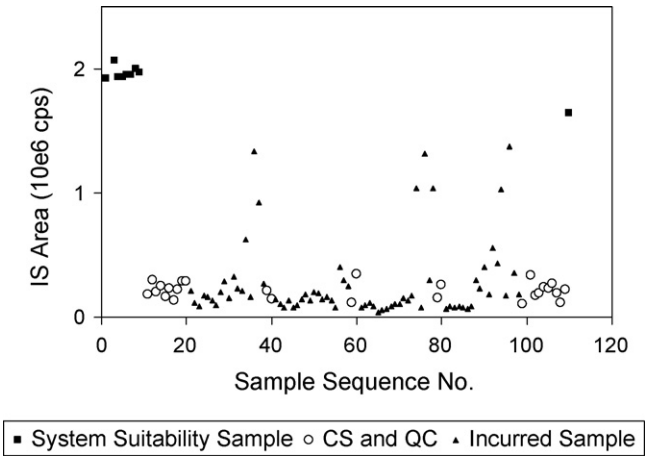

Fig. 5. Low internal standard responses caused by using rubber-line caps, instead of PTFE-lined caps during liquid–liquid extraction. Analyte: tamsulosin; Internal standard: tamsulosin-d_4.

Table 2
Comparison of IS responses for an LLOQ sample by using rubber-lined and PTFE-lined caps during LLE.

Caps used	Analyte area (cps)	IS area (cps)	Analyte/IS area ratio
Rubber-lined	1322	130,047	0.01017
	1204	115,882	0.01039
PTFE-lined	15,480	1,432,742	0.01080
	15,340	1,427,532	0.01075

Note: IS = internal standard; PTFE = polytetrafluoroethylene; LLOQ = lower limit of quantitation.

extraction shaking (mixing) step. Accordingly, it was suspected that the mixed usage of both rubber-lined and PTFE-lined caps during the liquid–liquid extraction was the root cause. Since rubber is not resistant to the organic solvent, some analyte and IS in organic solvent might have been soaked into the rubber and were retained, which reduced recovery. To further prove this, the lower limit of quantitation sample was extracted in duplicate with both rubber-lined and PTFE-lined caps. The results (Table 2) clearly supported the speculation. In addition, they also proved the analyte/IS area ratio, i.e. quantitation, were not otherwise affected due to the use of a deuterated internal standard.

3.6. Case 6—High IS responses observed for incurred samples only

During the analysis of repaglinide in a study, consistently high IS responses (higher than those of CS and QC samples) were observed for incurred samples from a few subjects (Fig. 6a). Since the IS

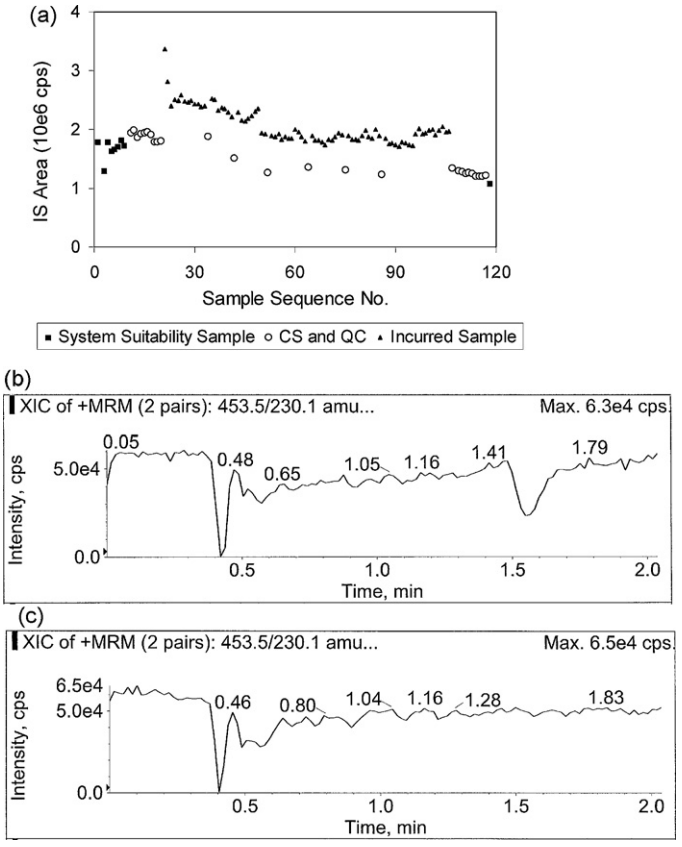

Fig. 6. (a, top) High internal standard responses were observed for incurred samples only. Analyte: repaglinide; Extraction: automatic liquid–liquid extraction. (b, middle) Post-elution infusion results show that ion suppression existed near the retention time of the analyte (1.57 min) from the pooled control blank used for the preparation of CS and QC samples. (c, bottom) Absence of ion suppression near the retention time of the analyte in subject pre-dose sample.

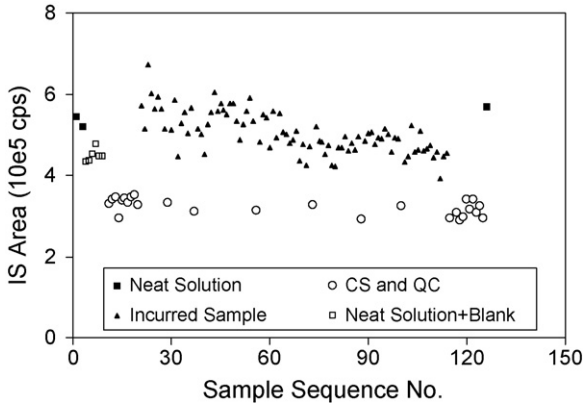

Fig. 7. High internal standard responses were observed for incurred samples only. Analyte: p-hydroxy-atorvastatin; Extraction: liquid–liquid extraction.

responses of the pre-dose samples also showed this anomaly, the issue was unlikely related to drug formulation or metabolite(s). Instead, it must be related to the difference between individual subject matrix and the matrix used for the preparation of CS and QC samples.

Initially, it was thought that ion enhancement was the cause of high IS responses in the subject that had high IS responses. Accordingly, post-column (post-elution) infusion tests [7] were performed by injecting a pooled control blank (which was used for the preparation of CS and QC samples, Fig. 6b) and the pre-dose sample from the subject that had high IS responses (Fig. 6c). Contrary to the initial speculation, the results indicate that ion suppression, instead of ion enhancement, was the root cause. Specifically, ion suppression was found near the retention time of analyte (1.57 min) in control blank, but not in the pre-dose sample. Since the consistently high IS responses were observed only in a few subjects, i.e. most subjects had "normal" internal standard responses (similar to those of CS and QC samples), it is therefore speculated that the component or components that caused the ion suppression are present in most of the control blank sources. Whenever several lots are pooled, i.e. during the preparation of CS and QC samples, the chance of ion suppression for CS and QC samples is very high. While for incurred samples, since the matrix is from individual sources, high IS responses in a few subjects could be observed when there is less or no ion suppression. In short, it was still an issue of ion suppression in this case.

3.7. Case 7—high IS responses observed for incurred samples only

In this case, consistently high IS responses were also observed as in case 6 (Fig. 7). However, the high IS responses in incurred samples can not be fully explained by the absence of ion suppression as in case 6. Specifically, the ion suppression for internal standard in the extracted control blank used for the preparation of CS and QC was estimated at −18% (by comparing the IS responses of neat solutions with those of extracted control blanks reconstituted with neat solutions). Hence, the IS responses in incurred samples of a subject without ion suppression should be approximately +18% more than those of the CS and QC samples. Apparently, the difference in IS responses between incurred samples and the CS and QC samples was much larger than +18% (around 50%). Therefore, there must be an additional cause for the high IS responses in incurred samples in this case.

By examining the properties of the analyte and the analytical method used, it was suspected that there were differences in recovery among incurred samples and CS and QC samples. Accordingly, recoveries were evaluated for the analyte and its internal standard during extraction from pooled control blank or individual subjects (Table 3). As the results show, internal standard recovery could vary from 67.19 to 89.99% for the four subjects tested despite the fact that the ratios of analyte to IS were relatively independent of subject sources, i.e. no impact on quantitation.

3.8. Case 8—low IS responses for incurred samples only

In this case, rosuvastatin in plasma samples was extracted by LLE with automatic transfer of organic layer [11]. During the analysis of incurred samples, consistently low IS responses were observed for a few subjects in the study (Fig. 8a). As the same anomaly occurred to the pre-dose samples, the problem was unlikely related to drug formulation and metabolite(s). In addition, since the IS responses of all the system suitability, CS and QC samples were normal, there should be no issue with the reagents or the LC–MS/MS system used.

To find out the root cause, sample processing procedures including the organic transfer step performed by the robotic liquid handling system were carefully examined. It was found that the thickness of the intermediate layer between the organic and aqueous phases varied among different matrix samples. Specifically, the intermediate layers of incurred samples for some subjects were thicker than those of the CS and QC samples (prepared in pooled plasma). As the aspirating height of the robotic liquid handling system, i.e. how deep a disposable tip should go into a sample tube during organic transfer, was set based on CS and QC samples during the method validation, this height was not adequate for incurred samples of some subjects (Fig. 8b, it should be noted that the automatic liquid level sensing was disabled in this case due to low conductance of organic layer). As a result, a small amount of intermediate layer, which contained salts, was transferred together with the organic layer. The transferred salts caused the ion suppression, i.e. lowered internal standard responses of incurred samples.

To solve the problem, the aspirating height was re-adjusted. When the affected samples were re-analyzed, their IS responses fell in the normal range, i.e. within ±50% of the mean IS response of the accepted CS and QC samples. The reassay concentrations matched those of the original analyses despite the lower IS responses, which indicates that the lowered IS responses did not affect the accuracy of quantitation and the deuterated IS was able to compensate for this variation. It should be noted that this type of IS response variations can occur to other types of manual LLE methods as well when organic phases are decanted, such as flash-freeze LLE.

Table 3
Recovery difference among different subjects and pooled control blank used for the preparation of CS and QC samples.

Matrix	Analyte recovery (%)	IS recovery (%)	Analyte/IS response ratio	IS responses (6 replicates)	
				Mean	CV (%)
Blank for CS&QC	64.21	73.41	0.2143	163872.7	11.58
Subject 31	82.45	89.99	0.2060	268513.0	1.38
Subject 47	66.19	75.58	0.1978	237824.8	3.06
Subject 49	61.06	69.82	0.2000	226461.3	3.69
Subject 54	61.30	67.19	0.2088	168789.7	5.17

Note: IS = internal standard.

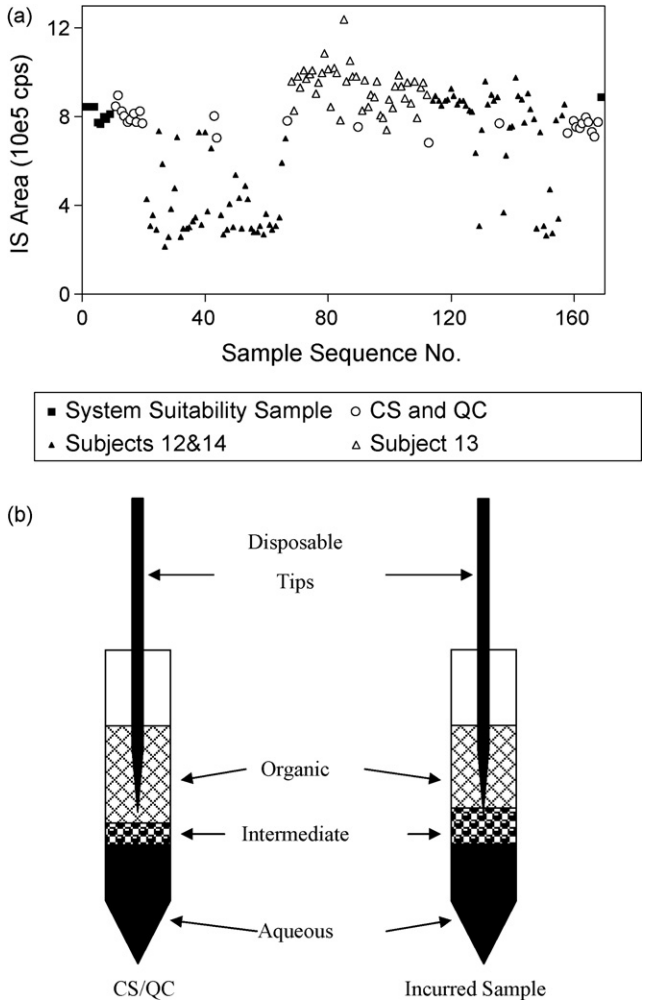

Fig. 8. (a, top) Low internal standard responses were observed for incurred samples of some subjects. (b, bottom) Due to variable thickness of intermediate layer between aqueous and organic phases in liquid–liquid extraction, inappropriately set aspirating height could result in partial transfer of salt-containing intermediate layer, which caused ion suppression.

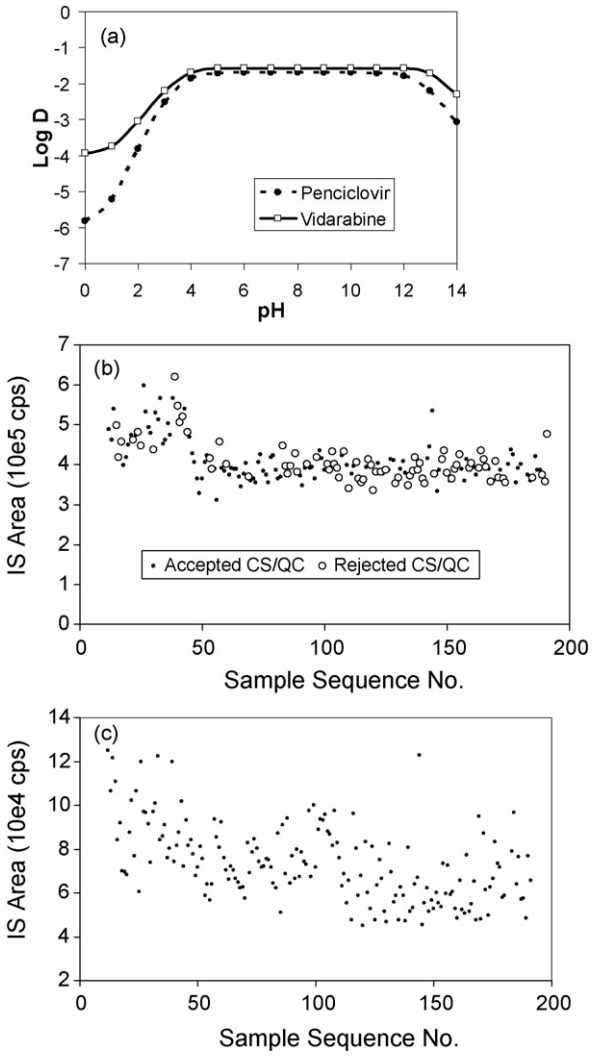

Fig. 9. (a, top) Hydrophobicity (log *D*) vs. pH curves for penciclovir and vidarabine. (b, middle) Less internal standard response variation observed while using vidarabine as the internal standard (CV = 13.02%) but 43% of the CS and QC samples were rejected. Extraction: MCX (mixed-mode strong cation exchange)-based solid-phase extraction. (c, bottom) More IS response variation observed while using deuterated internal standard, penciclovir-d_4 (CV = 23.81%) but 100% of the CS and QC samples were accepted.

3.9. Case 9—less IS response variation does not necessarily translate to good accuracy

Initially, a method based on mixed cation exchange (MCX) solid-phase extraction was validated for penciclovir by using vidarabine as its internal standard due to their similar properties, particularly hydrophobicity (Fig. 9a). However, some discrepancies were observed during the re-analysis of a few samples with dilution, specifically the concentrations obtained after dilution (with dilution factor considered) were significantly lower than the corresponding extrapolated values in the first analysis without dilution.

Through careful examinations of all the results obtained and the bioanalytical method employed, it was suspected that the discrepancies were most likely due to recovery variation between original and re-analyses. The assumption was as the following. Despite the overall similarity in hydrophobicity *vs.* pH characteristics, there is a relatively large difference in hydrophobicity between vidarabine and penciclovir in acidic conditions (pH 1–2), in which the mixture of sample and IS was loaded onto an MCX plate and the loaded MCX plate was washed. For vidarabine, its hydrophobicity is relatively less variable than that of penciclovir in this pH range. Because of this difference, minor change in pH during the analysis of incurred samples, differential recovery variation between the analyte and its internal standard would be caused, i.e. potential impact on the accuracy of quantitation.

To prove this assumption, 192 samples (mainly composed of CS and QC samples) were extracted by adding two different internal standards simultaneously, i.e. vidarabine and penciclovir-d_4. After extraction, the analyte (penciclovir) and the two internal standards were all monitored during LC–MS/MS analysis. When vidarabine was employed as the internal standard for quantitation, the accuracy was not satisfactory despite the relative stable IS responses (CV in IS responses was 13.02%, Fig. 9b). While penciclovir-d_4 was used as the internal standard, all the CS and QC samples met the acceptance criteria in accuracy though there were more variations in responses (CV in IS responses was 23.81%, Fig. 9c). These results demonstrate that stable IS responses can not be automatically translated to good accuracy unless they reflected the variations the analyte experienced.

3.10. Case 10—gradual increase of IS responses

In this case, internal standard responses increased over time almost linearly for all the samples, CS, QC, and incurred samples

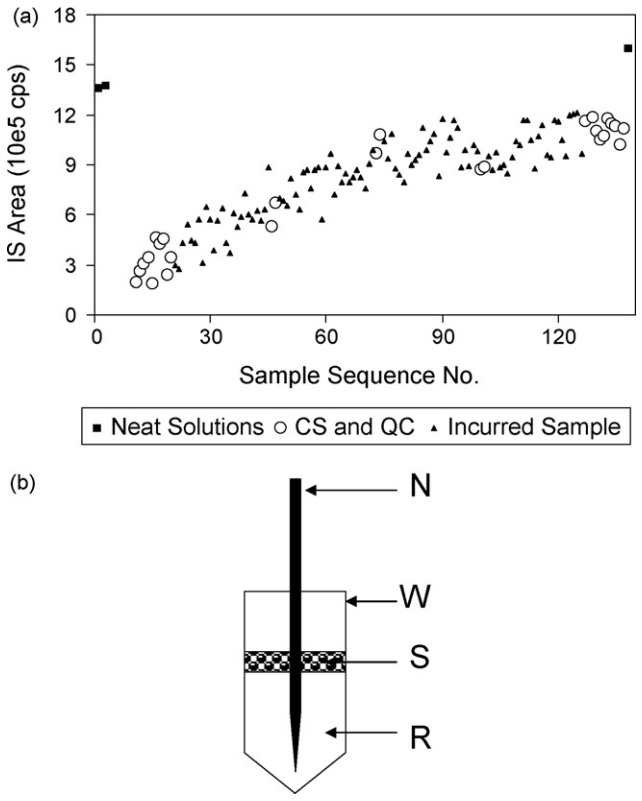

Fig. 10. (a, top) Gradually increasing IS responses due to increased mixing of the transferred supernatant (100 μL) with reconstitution solution (400 μL). (b, bottom) Schematic diagram showing the autosampler needle loaded sample near the bottom of a well (96-well plate), where was mainly reconstitution solution (deficient of the analyte and its internal standard) when homogeneous mixing had not been reached. N: autosampler needle; W: a well in a 96-well plate; S: supernatant; R: reconstitution solution.

alike (Fig. 10a). The accuracy of all the CS and QC samples were not affected. Considering this and the similar IS responses of the system suitability samples injected at the very beginning and the end of the run, it was unlikely that this almost linear increase of IS response was caused by malfunctioning of the LC–MS/MS system.

To find out the root causes, the analytical method was examined. In this method, an evaporation-free protein precipitation procedure was used [12]. Briefly, 100 μL of plasma sample was first protein-precipitated with 900 μL of methanol (with IS in it) and then 100 μL of the supernatant was automatically transferred to a 96-well plate, where the wells had been pre-filled with 400 μL of reconstitution solution for later dilution of the transferred supernatant. As Fig. 10b shows, the pre-filled reconstitution solution was located at the bottom of the square-shaped well in a 96-well plate while the transferred supernatant was at the top. During injection, the needle of the autosampler loaded samples near the bottom of each well. When the supernatant and the reconstitution solution are homogeneously mixed, there should be no problem. However, due to the relative large volume (500 μL) in each well and its square shape, homogeneous mixing takes time. Owing to insufficient mixing time used by the lab technician, homogeneous mixing had not been reached in this case prior to the injection of the run. Accordingly, when the autosampler loaded a sample, the part of the solution near the bottom of the well was loaded, where there were less analyte and internal standard, i.e. less supernatant. However, since both the analyte and its IS were originally transferred from the same portion of supernatant and they had encountered

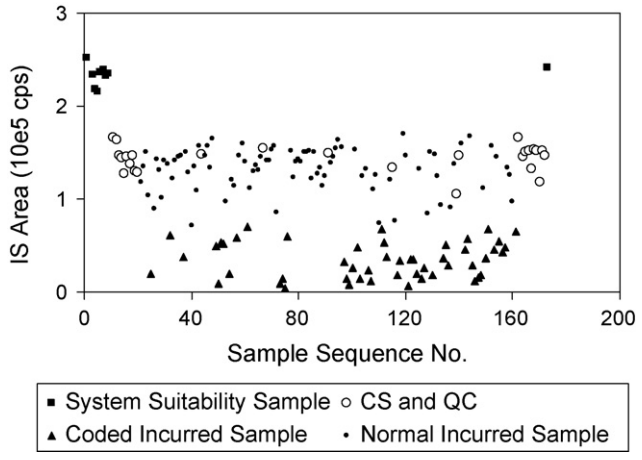

Fig. 11. Randomly scattered low internal standard (IS) responses observed for incurred samples only, whose IS responses were within normal range during repeat analyses. Analyte: olanzapine; IS: olanzapine-d_3; Sample pretreatment at clinic: 25% (w/v) L-ascorbic acid added to plasma in a ratio of 1.25:100 (v/v); Extraction: MCX (mixed-mode strong cation exchange)-based solid-phase extraction.

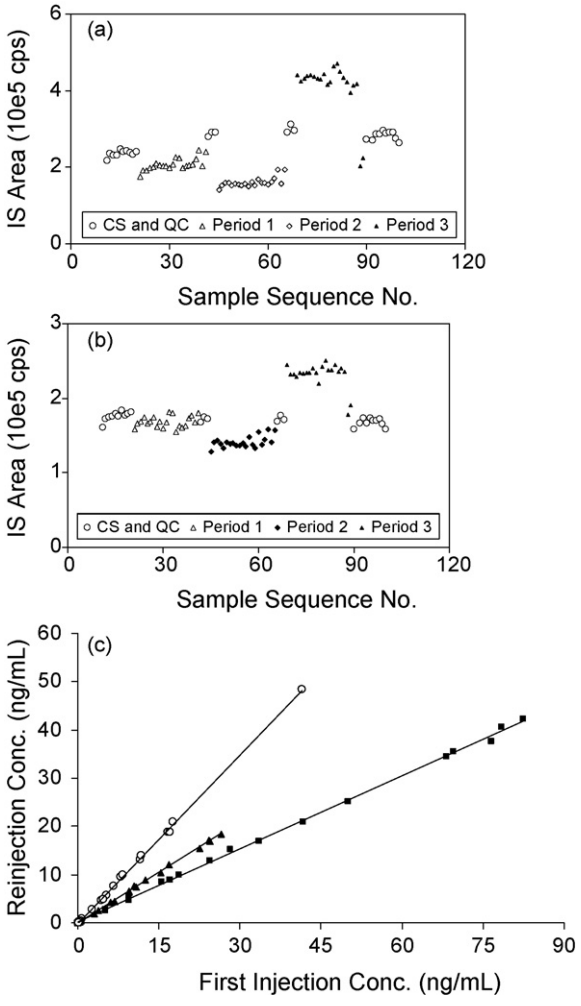

Fig. 12. (a, top) Most incurred samples from period 3 had unacceptable high internal standard responses during the original injection on system LC–MS/MS (A). Analyte: atorvastatin; IS: atorvastatin-d_5; Extraction: liquid–liquid extraction. (b, middle) Previously coded incurred samples of period 3 were not coded any more during the reinjection on a different system, LC–MS/MS (B). (c, bottom) Linear correlations between the concentrations obtained from original injection and those of reinjection but with different slopes for different periods. The slopes are 0.6972, 0.5054, and 1.1647 for period 1 (solid triangle), period 2 (solid square), and period 3 (empty dot), respectively.

similar dilution (albeit not homogeneously), the accuracy was not compromised due to the usage of analyte/IS area ratios for quantitation. Apparently, the mixing continued over the course of the injection through diffusion, which resulted in gradual increase of IS response like in this case. When this run was reinjected after proper mixing, high and stable IS responses were observed for the whole run.

3.11. Case 11—randomly scattered low IS responses for incurred samples only and not repeated during re-analysis (root causes to be identified)

In this case, variable and randomly low IS responses were observed for incurred samples only (Fig. 11). Despite similar pattern as in case 2, the root cause was unlikely related to autosampler issue because no CS or QC samples were affected in this case. In addition, since all the CS and QC were accepted, there should be no issue with the LC–MS/MS system or reagents used. Furthermore, when those incurred samples with unaccepted low IS responses were re-analyzed, their internal standard responses were back to normal. The reassay results matched those of the first analyses if low internal standard responses were not considered.

Based on the above, it was deemed necessary to use the incurred samples for further trouble-shooting. Unfortunately, this was not granted by the client, which left the root cause unidentified. The speculation is that the randomly low IS responses were related to the ascorbic acid added to the incurred samples at the clinic for the stabilization of the analyte. Although ascorbic acid was also added in the pooled control plasma used for the preparation of the CS and QC samples, there exist differences as outlined in Table 1. For example, the incurred samples went through only one thawing during the first analysis while the plasma matrix for CS and QC samples went through thawing twice when they were extracted.

3.12. Case 12—deuterated IS not following the analyte and reinjection results not matching those of 1st injection (root cause to be identified)

In this case, a run was first injected on LC–MS/MS system A and all the CS and QC met the acceptance criteria except that most of the incurred samples from period 3 (formulation 3) had elevated IS responses (Fig. 12a). Then, the same run was re-injected on a different system, LC–MS/MS system B, within the validated autosampler stability for trouble-shooting purposes. All the CS and QC samples in the reinjected run also met the acceptance criteria. In addition, those coded incurred samples from period 3 were not coded in the reinjection run, i.e. their IS responses were within ±50% of the mean IS responses of the CS and QC samples (Fig. 12b).

Since all the CS and QC samples in both the original and the reinjected runs met the acceptance criteria, a comparison was made between the concentrations of the incurred samples obtained from both runs (Fig. 12c). Though both results, i.e. original injection concentrations and reinjection concentrations, are linearly correlated ($r \geq 0.9995$ for all the three periods), there exist large differences between the two concentrations and these differences varied among the three periods. For example, the concentrations from the reinjected run are around 50% of the corresponding original concentrations for the samples of period 2, i.e. reinjection concentrations < original concentrations. On the contrary, the concentrations from the reinjected run are around 120% of the original concentrations for the samples of period 3, i.e. reinjection concentrations > original concentrations.

To rule out the possibility of different autosampler stabilities between CS/QC and incurred samples (if any), a few selected incurred samples together with some calibration standards were re-injected on LC–MS/MS A one more time after the reinjection on LC–MS/MS B within the validated autosampler stability. Their analyte/IS area ratios were still comparable with those of the original injections on LC–MS/MS A. Unfortunately, further investigations were not performed due to unavailability of incurred samples, which leaves the root cause unidentified. However, considering the good correlation between the original and reinjection results, it was speculated that the differences in concentration were related to differential matrix effects between the analyte and its internal standard from the co-extracted matrix components in the two different LC–MS/MS systems, particularly when the peaks of an analyte and its deuterated IS are partially separated [13–15].

4. IS response variation and its impact on quantitation

The main purpose of trouble-shooting IS response variation is to make sure that the quantitation of unknown samples has not been

Table 4
Summary of trouble-shooting cases and their impact on quantitation.

Case	Observations	Root cause identified	Effect on quantitation or comments
1	Zero or nearly doubled IS response	Missed or double addition of IS	Yes
2	Random and sharp drop in IS response	Autosampler needle blockage	Usually no, unless S/N is too low
3	Gradual decrease of IS responses	Charging of mass spectrometer	Not in this case, but it usually depends on how well an IS follows an analyte
4	Random, sharp drop, and overall downward trend in IS response	Autosampler needle blockage plus charging of mass spectrometer	It depends, but batch should be reinjected.
5	Low IS responses for most of the extracted samples	Mixed usage of right and wrong caps in LLE	It depends, but samples should be reassayed by using correct materials.
6	High IS responses observed for incurred samples only (usually a whole subject)	Relatively less ion suppression in subject samples than in CS/QC	It depends on how well an IS follows an analyte
7	High IS responses observed for incurred samples only (usually a whole subject)	Recovery variation plus relatively less ion suppression in subject samples than in CS/QC	It depends on how well an IS follows an analyte.
8	Low IS responses for incurred samples only (usually a whole subject)	Transfer of salt-containing intermediate layer in LLE	It depends, but samples should be reassayed.
9	Less IS response variation with analogue IS than with deuterated IS	Analogue IS did not follow analyte well	Quantitation affected with analogue IS and it should be changed
10	Gradual increase of IS responses	Insufficient mixing	Not in this case, but should be evaluated case by case
11	Randomly scattered low IS responses for incurred samples only and not repeated during re-analysis	Not conclusive, but speculated as due to ascorbic acid and different cycles of F/T	Not in this case, but should be evaluated case by case
12	Deuterated IS not following the analyte and reinjection results not matching those of 1st injection	Not conclusive, but speculated as due to differential matrix effect between analyte and its deuterated IS	Yes in this case, but should be evaluated case by case

Note: IS = internal standard; CS = calibration standard; QC = quality control; LLE = liquid–liquid extraction; F/T = freeze and thaw.

impacted despite the variable or abnormal IS responses observed. As summarized in Table 4, there is no clear-cut "yes or no" answer to this question for most of the cases studied in this article. The best approach would be to monitor IS response variation during the analysis of incurred samples by using some pre-defined acceptance criteria, such as a range defined by the lowest and the highest IS responses of the CS/QC samples in the batch that meet the acceptance criterion of accuracy. Once variable or abnormal IS responses are observed, each case should be investigated for root causes and the impact on quantitation should be evaluated. Based on the outcome of the investigation or evaluation, the affected samples may be reinjected, re-analyzed or their results may be accepted together with some scientific proof. The last approach not only would be preferable, i.e. saving time and cost, but it may also be the only option in some cases. For examples, in cases like 6 and 7 where all the samples from a single subject have consistently higher or lower IS responses than those of CS and QC samples, the same or similar IS responses would be repeated during the reassays. Without proper investigation or evaluation, either there would be no reportable values for a whole subject (due to abnormal IS responses) or there would be uncertainty on the accuracy of the results obtained if they are to be reported. Both should be avoided during the analysis of incurred samples.

5. Conclusions

As shown above, many factors other than matrix effect could cause variations in internal standard response during the analysis of incurred samples. Accordingly, a variety of factors should be taken into consideration, such as the properties of an analyte and its internal standard, sample processing procedure, analyst and his/her experience, materials, reagents, solutions, equipment and instruments used. Post-elution infusion test and/or reinjection using different sequences or on different instruments are often necessary for trouble-shooting. Though not always practical, it is very much desirable to use incurred samples to perform trouble-shooting experiments.

Since the same phenomenon can be caused by different factors, each case should be dealt individually with an open mind. Higher internal standard responses for incurred samples may not be due to ion enhancement. On the contrary, in some cases it might be due to the lack of ion suppression or relatively less ion suppression.

Stable isotope labeled internal standards may be the best, but they cannot always follow an analyte to compensate variations of experimental condition, particularly when deuterated internal standards are used and when there is partial separation between an analyte and its internal standard. In addition, less variation in internal standard response may not always be interpreted as good results though they are favored. Stable internal standard response is good only when the internal standard behaves the same way as the analyte does.

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