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Determination of Carryover and Contamination for Mass Spectrometry–Based Chromatographic Assays

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ABSTRACT

The Third American Association of Pharmaceutical Scientists/Food and Drug Administration Bioanalytical Workshop, held in 2006, reviewed and evaluated current practices and proposed that carryover and contamination be assessed not only during the validation of an assay but also during the application of the method in a study. In this article, the potential risks of carryover and contamination in each stage of a bioanalytical method are discussed, to explain to the industry why this recommendation is being made.

KEYWORDS: Carryover, contamination, extraction, chromatography, detection, bioanalysis, accuracy, precision, memory effect

INTRODUCTION

Sample carryover is a major problem that can influence the accuracy and precision of high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalysis, with the consequences being more pronounced at lower concentrations.[1] The continuous increase in sensitivity of new-generation LC-MS/MS instruments, with detection limits in the low pg/mL range and the possibility of using wider calibration ranges (>10^4), has also drastically increased the risk of carryover during bioanalysis.[2] Reduction of carryover during assay development consumes time and resources and can lead to reduced productivity and delays in the drug discovery and development process.[3,4]

Carryover in general is serial in nature and is caused by residual analyte from a sample analyzed earlier in the run. It does not necessarily involve only the next sample in the sequence and can affect several samples in a sequence, if many samples above the calibration ranges are analyzed. Carryover can also be random, where carryover from late-eluting residues on chromatographic columns may affect chromatograms several samples later. Carryover from analyte residues can also occur via dislodgment from a sample's flow path through a chromatographic system and mass spectrometric detection system.

Contamination, conversely, tends to be more random, and precautions should be taken to avoid contamination during sample preparation techniques (extraction) using both manual and automated procedures. The potential for contamination and carryover is highly dependent on the calibration range selected for a given assay.

Carryover and contamination can affect both the accuracy and precision of a method and should be investigated and minimized or eliminated during method development, assessed during method validation, and monitored routinely in study samples analysis. It is critical that unexpected or random carryover and contamination not go unchecked. Unless this random carryover and contamination occurs in samples with known analyte concentrations, such as calibration standards, quality control samples, or placebo/predose samples, the contamination will go undetected and potentially erroneous results will be reported for individual samples, or an entire bioanalytical batch. When blanks or low-concentration samples follow, or are in close proximity to, high-concentration samples, there is a potential risk of contamination and carryover. This article will review the potential risks of carryover and contamination during 3 stages of a bioanalytical method (extraction, chromatography, and detection) and provide some important considerations that should be used to assess and prevent them.

CARRYOVER AND CONTAMINATION: SAMPLE PREPARATION (EXTRACTION)

For a bioanalytical assay, the major sources of cross-contamination during sample preparation (extraction) are spills, aerosols, and drips during the liquid transfer steps.[5] Table 1 lists the steps required to perform 3 common bioanalytical sample preparation techniques for small molecules and the potential risk of carryover and cross-contamination.[5] For solid phase extraction there is a moderate risk of carryover during the sample aliquoting,

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Table 1. Degree of Risk of Carryover and Cross-Contamination During Sample Preparation*

Preparation Steps	Carryover	Cross-Contamination	SPE	LLE	PPT
Aliquot sample, addition of internal standard and reagent, mixing	Medium	Medium	√	√	√
SPE elution	Low	High	√	√	—
Vigorous mixing	High	High	—	√	√
Transfer of supernatant/extract	Medium	High	—	√	√
Evaporation of extract	Medium	High	√	√	√
Dilute extraction and mixing	Medium	Medium	√	—	√

*SPE indicates solid phase extraction; LLE, liquid-liquid extraction; PPT, protein precipitation. Check marks (√) indicate the at-risk steps involved in SPE, LLE, or PPT.[5]

evaporation, and reconstitution steps. However, the chances of cross-contamination are quite high during the elution and evaporation steps. The risk of cross-contamination is also very high during the vigorous mixing of organic solvents, supernatant transfer, and evaporation steps for liquid-liquid extraction (LLE) and protein precipitation (PPT).[5]

Manual Extractions

Since the early 1990s there has been a shift toward the use of automated liquid handlers to carry out extractions.[4] Some bioanalytical laboratories, however, still carry out these extractions manually. Speed and throughput are compromised in extractions done manually, but problems due to carryover and contamination are generally less pronounced. It follows that it is easier to limit, or avoid, these mitigating effects when the sample preparation and extractions are done manually.

There are several ways to overcome these problems during manual extractions. For instance, to reduce or eliminate carryover, the glassware in which analyte stock solutions are prepared should not be reused for preparing other solutions, such as buffers, working internal standard solutions, and dilute analyte solutions (spiking solutions). Those flasks should be cleaned separately (not with the other glassware) to prevent carryover of analytes. Workbenches, pipettes, vacuum manifolds, evaporation needles, and other items should be cleaned with appropriate reagents before each extraction. Moreover, when performing extractions for HPLC assays, bioanalytical scientists need to be extra vigilant if they share equipment or glassware with others. The poorer selectivity of HPLC detection techniques means that if reagents/solvents or common glassware are contaminated with analytes, albeit from a different assay, they may be detectable by HPLC and influence the selectivity and accuracy of the assay. Cross-contamination between assays may also affect quantitation for MS-based assays, if the cross-contamination analyte co-elutes with the analyte of interest, potentially causing sequential or random ionization suppression/enhancement.

Pipetting using handheld devices should be done slowly to minimize foaming and aerosol formation. Pipettes with aerosol barrier tips are commercially available and may be used. The airflow through these tips reduces the flow of aerosols or liquid into the pipette barrel, which helps to prevent carryover and contamination.[6]

The selection of appropriately sized test tubes is imperative to avoid splashing during the vortexing steps of sample preparation. Contamination from extraction solutions can be avoided by using separate refillable bottles for extraction solvents. These bottles should be emptied and refilled daily. In some cases contamination or interference could arise from impurities in buffers/organic solvents, such as methanol and acetonitrile, and the use of high-purity reagents is recommended.

Automated Extractions

When extractions are performed using automated liquid handlers, the potential of carryover and cross-contamination increases because the samples are clustered together in a 96- or 384-well format. This physical characteristic, with each sample being in close proximity, leads more readily to cross-contamination. Using fixed tips is less expensive than using disposable tips, but fixed tips are more likely to lead to carryover problems. This effect is more pronounced when the analyte is "sticky" and prone to adsorption to the surface of the tip. Appropriate methodology involving washing and rinsing solutions can be used for fixed tips to lower the risk of carryover considerably in most cases, but the requirement for extensive washes between steps will ultimately affect sample throughput.

Currently, there are several automated liquid handlers that can control the dispensing height, dispensing speed, position of tips, and adjustment of air gap to prevent dripping

and thereby limit contamination. Nevertheless, transfer of organic solvents is a potential source of contamination due to dripping. During PPT or LLE, the mixing step may generate aerosols or allow organic solvents to climb over the barriers between wells because of capillary action. For example, the capillary action in polypropylene microtiter plates is highest for heptane > ethyl acetate > 75% methanol or acetonitrile > water > 50% dimethyl sulfoxide. Capillary action thus reduces the usable volume of the wells, thereby affecting accuracy and precision.[7]

To avoid cross-contamination during the mixing steps in a PPT or an LLE, heat-sealing films can be used. Heat-sealing films are also available with pierceable sealing foil, which further limits contamination. Caution should be exercised while removing the films because of the potential for contamination from the droplets on the film. An additional step of centrifugation could be performed to remove the droplets.

Some automated liquid handlers can mix the sample using disposable tips, which helps eliminate the risk of contamination from sealing films. The bioanalytical scientist should consider these factors in designing the analytical method and determining when it is appropriate to use 96-well plates with larger volume, fixed tips or disposable tips, or square well or round well plates, and should also consider displacement of solution from tips when tips are used for sample mixing.[5]

The use of surrogate markers or contamination markers for LC-MS/MS is becoming very popular in tracking the cross-contamination when extraction is performed in a 96- or a 384-well format. A surrogate marker, often an analog of the analyte, is ionizable at the MS interface, extracted with the analyte, and eluted in the HPLC method but not co-eluted with the analyte or the internal standard. The method is developed for the analyte and the extraction recoveries, and chromatography is determined for the surrogate marker. To monitor the cross-contamination, high concentrations of markers are spiked in a checkerboard pattern as shown in Figure 1. The markers are added to a clean 96-well plate and evaporated (if required); then the spiked plate is used for sample preparation.[5] The presence of both markers in any well indicates cross-contamination has occurred. The response of an unspiked marker in the well is subsequently measured, with the result indicating the degree of contamination.[4] Routine application of this technique does add to the time and expenses required to develop a bioanalytical method, as extraction and chromatographic conditions for the surrogate as well as the analyte of interest will need to be developed. Despite this limitation, the most notable advantage of the application of this technique is that cross-contamination can be assessed for all samples individually. If significant cross-contamination is observed, only those affected samples, rather than the whole batch, would be failed (deactivated).

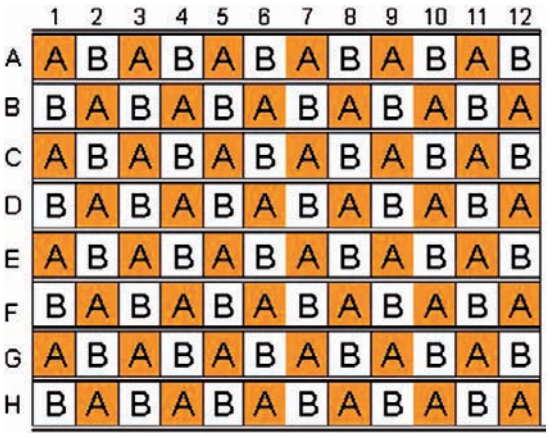

Figure 1. Use of 2 additional analytes (A and B) as surrogate markers[5]

In cases when extraction contamination and carryover are not observed in control samples but are suspected (eg, upon random sample repeat), additional investigational analysis may be required. This will allow the cause to be identified and appropriate and corrective action to be performed, to ensure the integrity of the results of other samples in the batch, and subsequent analysis of batches.

Carryover and Contamination: Chromatography

Carryover and contamination from a chromatographic system can be caused by residues of a previously injected sample that are absorbed on, or trapped within, the autosampler. Carryover can also be caused by residues on columns that may randomly affect chromatograms several samples later. There are many publications that describe measures to deal with autosampler carryover, but only a few discuss column carryover. This section discusses autosampler carryover, the origins of carryover, and the means to overcome issues associated with column carryover.

Types and Features of HPLC Carryover

The primary causes of HPLC carryover can be divided into 2 categories: autosampler carryover and column carryover. Autosampler carryover results from the residue of a previously injected sample absorbed on and/or trapped in the autosampler needle, injection port, transfer tube, sample loop, or injector valve. Typical autosampler carryover has a similar retention time to that of the analyte. This often introduces a positive bias (% relative error) and consequently has a major impact on the accuracy of quantitation, most significantly at lower analyte concentrations. Column carryover, however, can be caused by the residue of a previously injected sample on the column, both in its original form and occasionally in different forms of the analyte (eg, analyte:reagent adducts and analyte dimers)[8] that can

decompose in the ion source back to the original form of the analyte. Typical column carryover has uncertain analyte retention times and often generates random error that affects mainly the method precision.

Interaction Mechanisms and Solutions Used to Reduce HPLC Carryover

Autosampler carryover is largely associated with the interaction of an analyte with the flow path components of the system; it has a close relationship with the chemical/physical characteristics of both the analyte and the analysis system. Analysis of extremely basic and hydrophobic compounds can be particularly problematic, because of their tendency to be present in a charged form and to adsorb to the sample path of an autosampler through ionic interaction with metallic surfaces and through hydrophobic interaction with plastic materials.[9] Great efforts have been made by scientists and engineers to reduce carryover in 2 ways: by removing it by rinsing, and by preventing it in the first place.[9-14] Rinsing can be effective, but selection of the most effective rinsing solution, optimized for time, is no trivial matter. Rinse solution chemistry can have a huge impact and should be carefully considered to best counteract carryover. "Like dissolves like" is the primary rule to follow. Generally speaking, acetonitrile or 90% acetonitrile is an acceptable choice for rinsing/removing analytes adsorbed by hydrophobic interaction (eg, lipophilic compounds). A more protic solvent, such as methanol or 90% methanol, is an alternative for more polar lipophilic compounds. Acidified acetonitrile, alkalized acetonitrile, or methanol/isopropanol/water solution is quite efficient and universally used to dissociate analyte adsorption caused by dipole-dipole and ionic interaction (hydrophilic compounds).

Matching the pH to the organic/water or buffer ratio of the rinsing solution can dramatically reduce carryover since the pH of the rinsing solution influences the analyte charge state. For example, a basic compound exists in a positively charged state under acidic and neutral conditions and is uncharged in alkaline conditions. An acidified organic/water or an alkalized organic needle/valve wash solution is useful in removing it, but selection of an acidified organic or alkalized organic/water solution will greatly compromise the rinsing effectiveness. This effect occurs because when charged (ionized), a basic compound easily dissolves in organic/water or acidified organic/water solutions. However, in an uncharged state, it has more affinity toward pure organic or alkalized organic needle/valve wash solutions.

The pK_a of an analyte is a good indicator that should be considered when making pH adjustments to the needle/valve wash solutions. For an analyte that is hard to dissolve in common solvents (methanol, acetonitrile, or aqueous mixtures thereof), strong solvents such as tetrahydrofuran, dimethylsulfoxide, or a halohydrocarbon (eg, methylene chloride) can be used. Use of such strong solvents can, however, cause nonmetallic tubing to swell, which greatly reduces the rupture pressure of the tubing and should be avoided under ultra performance chromatography (UPLC) conditions. An ion pair reagent such as perchloric acid can be used as a rinsing solution, to reduce sample adsorption caused by ionic or coordination interactions, but the possible effect of the counterion should be considered in MS-based assays, as it may suppress ionization. Also, the introduction of any nonvolatile ion pair reagents into the MS system must be avoided.

Most modern autosamplers are equipped with 2 or more needle- and valve-wash lines, allowing multiple rinses to be performed. The first rinsing solution removes analyte residues and involves a weaker solution or mobile phase. The last rinsing solution has better compatibility with the detection system. If only 1 needle- or valve-wash for the autosampler is available, the options for selecting suitable rinsing solutions are more limited, and the compatibility of the rinsing solution with the mobile phase must be considered.

Autosampler Design

Many improvements have been made in autosampler design, materials, and techniques to prevent or limit carryover. The first is the "push-to-fill" design, which is an automated version of a manual injection. In this design, a needle attached to a motor-driven syringe is moved to the sample vial, is filled, and then transfers the sample to the injection loop. The valve rotor is moved, and the sample is injected. Any sample residue left inside the needle, the syringe, or the connecting tubing can be flushed out with a wash solvent or rinsing solution. Another setup involves the "needle-in-loop" design, which combines the needle and loop as 1 component, so that both the needle and the loop are flushed with the mobile phase during the sample elution and no additional internal rinsing of the needle is required. As rinsing takes place during the chromatographic run, it is best to leave the loop in the inject position during the entire run for maximum flushing, especially during gradient elution chromatographic methods. An alternative design is the "load-ahead" autosampler, in which the loop is removed from the inject position before the run is complete. This may have the potential for less thorough flushing of the inside of the loop.

Carryover can also result from sample residue left on the outside of the sample needle. The vial septum is the first line of defense to remove any residue on the outside of the needle. A well-chosen septum will act as a "squeegee" and wipe the outside of the needle. Polymeric septa, such as silicone or polytetrafluoroethylene-faced silicone, work well

in this regard. In the "needle-in-loop" design, there is normally no valve wash but there is an external needle wash to avoid injection seal contamination. There are 2 common techniques for external needle wash. The first technique is "dip only," which is a static dip approach used to wash the external needle by dipping it into a vial of wash solvent. The second technique is the "active rinse," in which the needle is dipped into a wash station with wash solvent flowing on the outside of the needle. This approach is slower but more effective than a static dip technique, but the static dip is better than no rinsing at all.

Over the years, injection needle coatings have been developed to prevent carryover caused by basic or ionic compounds adsorbed to metallic needle surfaces (eg, stainless steel alloy) by ionic or coordination interaction. Three kinds of common needle coatings are commercially available: Teflon, polyetheretherketones (PEEK), and platinum. Teflon coating is mechanically weak (coating layers can peel off after ~300 injections). PEEK is a thin-layer coating (of a few dozen micrometers) that is technically complex, is chemically stable, and has utility across a broad pH range. Platinum coating is also a thin layer (of a few micrometers) and due to a special coating process is very durable and can last more than 20 000 injections.

Adsorption of lipophilic analytes, via hydrophobic interaction, with resinous materials on rotor seals can be a significant cause of carryover. Vespel is common material employed in rotor seals with excellent durability, but unfortunately it has a strong affinity for lipophilic molecules. Delrin is another common material that can be used with an alkaline mobile phase with little adsorption of hydrophobic compounds. PEEK seals are also available and can be used with the mobile phase across the entire pH range with little adsorption of lipophilic compounds.

Column Carryover

Column carryover is very compound-dependent and is related mainly to analyte:reagent interaction. The so-called sticky analytes often have unique chemical and physical characteristics. Compounds having active positive carbon atoms in the molecule, or strong electron withdrawing groups (eg, fluoride ions), have a strong tendency to form adducts with common organic, acid, salt, and solvent ions. Compounds that contain dipolar ions or are rich in hydroxy groups can form low-molecular-weight polymers (typically dimers) at high concentrations. The different adduct or polymer forms of an analyte can then decompose in the ion source (by in source collision-induced dissociation) back to the original analyte form and cause random carryover- and contamination-like effects that can affect the quantitation of the assay. These 2 cases may be thought of as late-eluting interference effects but should also be considered as a special case of carryover due to analyte interaction with the mobile phase, extending analyte retention on the column. This type of carryover can be observed as a highly variable analyte response, particularly at low analyte concentrations. The potential for this type of analyte-adduct formation or polymerization should be taken into account during the method development process. Precautions should be taken to avoid adduct formation or analyte polymerization during extraction, chromatography, and detection. Gradient elution could be considered an option for removing such effects when adduction or polymerization cannot be minimized effectively. The extended interaction of basic compounds, caused by ionic interaction with active acidic sites on silicone-based stationary phases, is well known. Careful selection of column chemistry will provide many good options to overcome this kind of problem.

Assessment and Accepted Criterion for Autosampler Carryover

Carryover can be assessed by injecting 1 or more blank samples after a high-concentration sample or standard.[1] The commonly accepted criterion for carryover is that the peak area of the analyte in a blank sample that follows a standard prepared at the upper limit of quantitation (ULOQ) must be less than 20% of the peak area of the lower limit of quantitation (LLOQ) sample. This criterion is closely correlated to the dynamic range of a bioanalytical assay. Considering that carryover is proportional to the concentration of analyte in the preceding sample, the higher the concentration of the preceding sample, the higher the peak area will be in the sample that follows. Therefore, the selection of the LLOQ of an assay is directly related to the ULOQ and any subsequent carryover. In addition, because the peak response from carryover in the blank sample is also directly related to the sensitivity of the detector, the absolute peak response may vary from day to day or from system to system for the same analyte. Therefore, autosampler carryover evaluation should be performed for each analytical run to ensure that it does not affect the accuracy of quantitation. An assessment of autosampler carryover may be challenging when the response of an analyte at the LOQ is close to the limit of detection, where it may be difficult to accurately differentiate carryover from background noise. In such cases, additional experiments may be required when considering the impact of any carryover on the integrity of the data.

Carryover and Contamination: Mass Spectrometry Detection

Artifactual Contamination Caused by Cross-Talk

"Cross-talk" is caused by the slow removal of ions from the collision cell.[15] This can become a problem if different

analytes of interest have the same monitored fragment ions. For example, cross-talk occurs when fragment ions from the first mass transition scan event of an analyte have not cleared the collision cell before a second mass transition scan event of another analyte takes place. The impact of this cross-talk leads to signal/response artifacts in the next mass transition, so it has an impact on the quantitation of the analytes of interest. Modern triple-quadrupole mass spectrometers have been redesigned so that collision cells evacuate the ions quickly before the next mass transition scan event takes place.[16] For the old mass spectrometers, this problem still remains, but it can be resolved by adding a "dummy ion transition" scan event between the 2 analytes of interest, and thereby allowing time for the collision cell to empty of the common fragment ion, which eliminates the "artifactual contamination" caused by cross-talk.

Intersprayer cross-talk[17,18] has also been reported using multiplexed electrospray technology. An evaluation of the cross-talk effect using this type of mass spectrometer platform should be considered in the development and application of methods that use this technique.

Memory Effect I: Column Carryover

Memory effect I is observed as an elevated, downward-drifting baseline in a blank sample analyzed after a high-concentration sample.[19] This suggests that the analyte from the previous injection was still eluting off the column at the time when the blank injection was made. The elevated baseline is in fact the tail of the peak from the previous injection (Figure 2). This is common for analytes that exhibit strong interactions with silanol groups on the chromatographic column, and that have a very short run time, such that the analyte peak has had insufficient time to fully elute from the column. This raised baseline in the subsequent samples may affect the analyte if present at low concentrations—that is, the peak becomes hard to accurately differentiate from the background noise. This problem can be improved by selection of end-capped columns to minimize the residual silanol effects, careful selection of the mobile phase pH, and adjustment of the chromatographic run time.

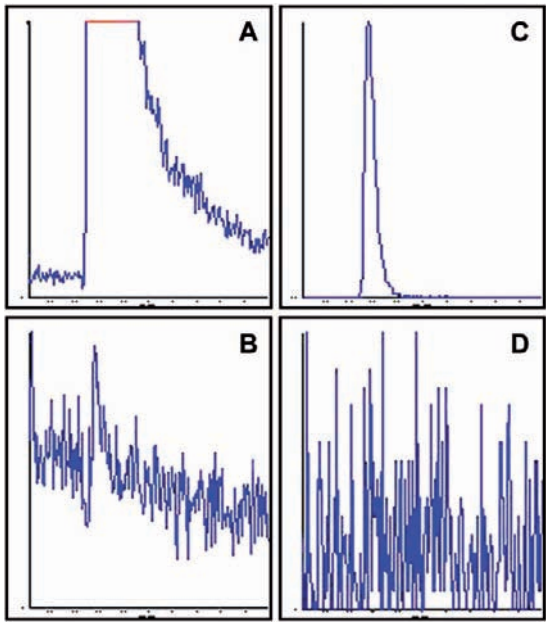

Figure 2. Memory effect due to chromatographic peak tailing: (a) ULOQ sample magnified to show peak tailing; (b) peak tailing from ULOQ (a) causes memory effect in blank sample that follows; (c) modification of chromatographic conditions for ULOQ; (d) no memory effect in blank that follows ULOQ (c). ULOQ indicates upper limit of quantitation.

Memory Effect II: Additives Such As Triethylamine

If triethylamine (TEA) has been used in the mobile phase for 1 assay, any residual TEA that remains in the system may carry over and have a negative impact on the quantitation of an analyte of interest for subsequent analysis.[20] TEA strongly adsorbs on the surfaces of the mass spectrometer and can produce ion suppression of other analytes, particularly for those present in low concentrations with low detection limits. Hence an evaluation of the impact on the quantitation of these analytes is required and if necessary a thorough cleaning of the system may be indicated to remove or reduce the impact of carryover and contamination from residual TEA.

Chip-Based Technology

In chip-based technology, electrospray ionization (ESI) is integrated into a chip format to form an array of ESI nozzles.[21] This technology is similar, in principle, to flow injection analysis in that each sample has its own unique spray (ESI nozzle) and no chromatography. This MS-based approach has the advantage of directly introducing each sample into the mass spectrometer without the mobile phase or any common sample flow path. The possibility of injection and chromatographic carryover is therefore completely eliminated, and extended calibration ranges can be used. The major disadvantage of this chip-based analysis format is that the analyte may co-elute with its metabolites or there may be endogenous matrix interferences, because of the absence of chromatographic separation. Ion suppression can be significant, and if it is not consistent from matrix to matrix, quantitation of the analyte can be affected. This type of approach is also not suitable for the differential quantitation of isomers (structural or enantiomers), because without chromatographic separation, the isomers cannot be differentiated by the mass spectrometer alone. This approach has significant limitations for quantitative application of bioanalytical methods to support human clinical trials. Conversely, the lack of carryover, and hence the time required to

minimize it, is particularly advantageous during drug development. Using this technique, high-throughput screening of a large number of samples over wide calibration ranges is achievable, with no risk of an impact from carryover and contamination.

Purity of Stable Isotopic-Labeled Internal Standards

The purity of stable isotopic-labeled internal standards, which are commonly used in bioanalytical assays, is an important consideration. For example, if the D_0 of a deuterated internal standard is present in a significant amount, "apparent contamination" from the internal standard can affect the quantitation of an analyte, and in such cases the concentration of the internal standard used needs to be carefully selected relative to the LOQ of a given assay.

Conclusion

It is clear that each stage of bioanalysis (extraction, chromatography, and detection) is susceptible to risk from carryover and contamination. These effects can be both serial and random. During method development and validation, these risks should be understood, and steps need be taken to ensure they are eliminated or minimized. While there is no standard acceptable magnitude of carryover and contamination for a passing bioanalytical run, it is most typically assessed in blanks analyzed after the highest calibration standard. During the routine application of bioanalytical methods in support of preclinical and clinical trials, this type of assessment must be performed for each batch of analysis. It is imperative to ensure that carryover and contamination do not affect the in-process accuracy and precision of the method and thereby guarantee the integrity of the results generated for all samples analyzed. When unexpected/unplanned occurrences of carryover and contamination do occur, the bioanalytical scientist must interpret the impact on the results and carry out the appropriate corrective action to eliminate further occurrences.

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