# Peer Reviewed: Isotopically Labeled Analogues for Drug Quantitation

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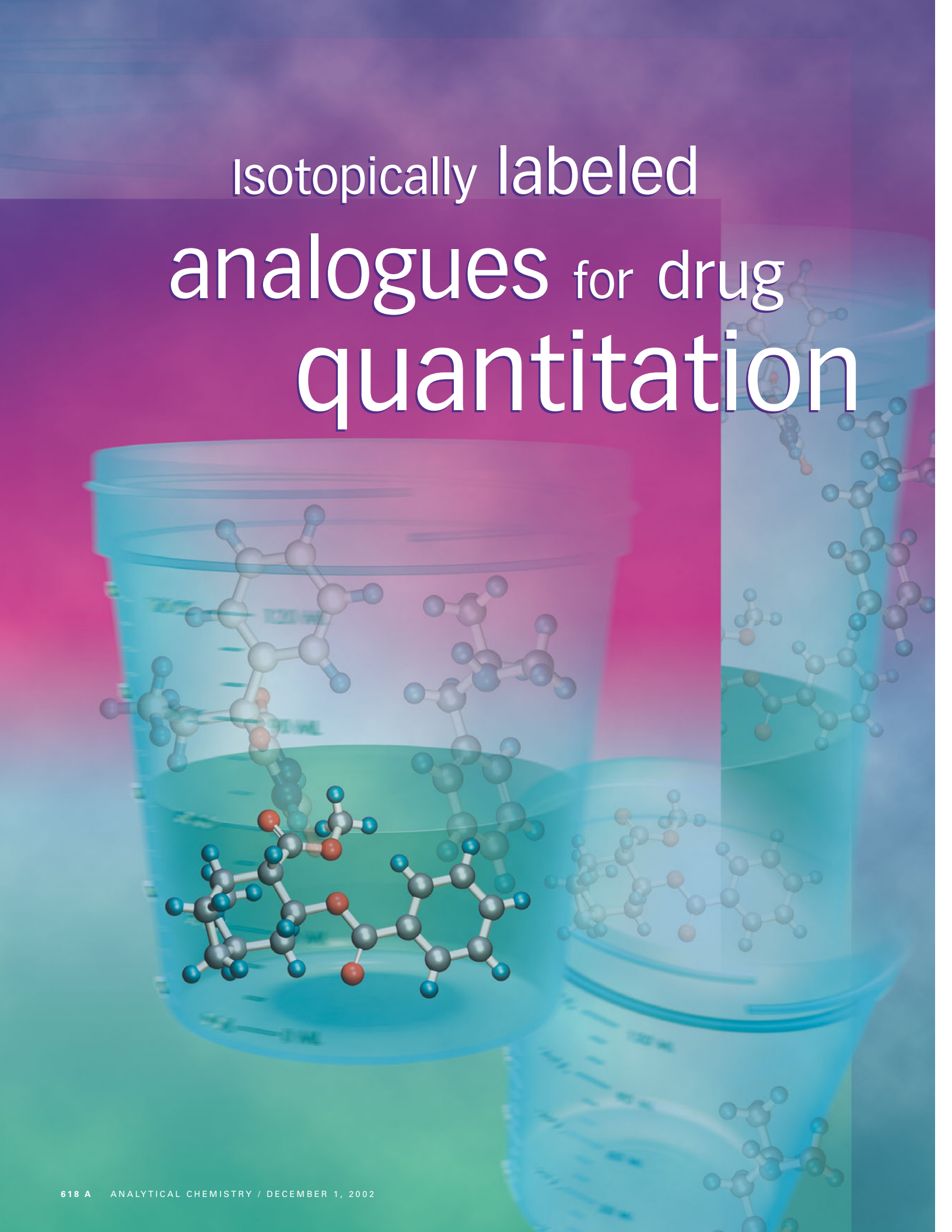
# Isotopically labeled analogues for drug quantitation



# Issues to address when isotopically labeled analogues of analytes are used as internal standards.

The detection of drugs and their metabolites (collectively referred to as drugs in this article) in biological tissues and fluids (media) has always been an important component of clinical diagnosis, forensic testing, pharmacological research, and drug discovery. Thanks to advances in analytical instrumentation and a greater understanding of metabolism, we can now detect drugs at concentrations that were once undetectable (*1, 2*). At the same time, a growing emphasis on monitoring illegal drug use in the workplace has required massive testing of urine specimens, which has stimulated significant advances in specimen pretreatment technologies.

New GC/MS/MS and LC/MS/MS instruments are capable of achieving higher specificity and S/N, which is advantageous for identifying unknown metabolites at very low concentrations. However, robust GC/MS methods still play a major role in identifying higher levels of well-characterized drugs in therapeutic monitoring, emergency room screening, and workplace testing. In most situations, the newer instruments do not generate better quantitative results than GC/MS.

If analytical instrumentation and specimen pretreatment technologies are the "hardware" aspects of the analytical sciences, then isotopically labeled analogues (ILAs) may be considered the complementary "software" component that is needed for advanced hardware to reach its full potential in quantitation. In this article, we look at how to overcome problems associated with using ILAs as internal standards (ISs) for the quantitation of drugs in media (*3–5*). All of the data and discussion are drawn from GC/MS studies for two reasons: The use of ILAs as ISs was originally developed for GC/MS, although the approach is readily adaptable to GC/MS/MS, LC/MS, and LC/MS/MS applications; and, despite many GC/MS/MS, LC/MS, and LC/MS/MS studies using ILAs as ISs, we know of none that try to better understand the standards themselves.

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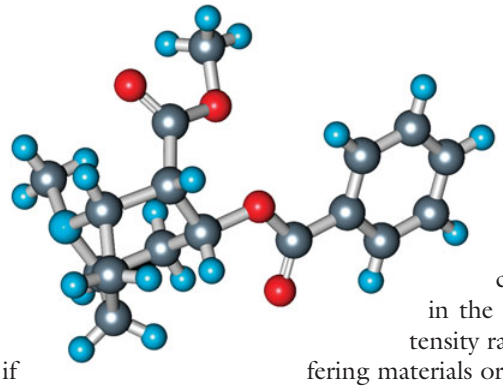


## Significance of accurate quantitation

Recent U.S. regulations for monitoring workplace drug testing include monitoring quantitative data, making it an important aspect of quality control practices (*6*). Furthermore, a specific "cutoff" value was adopted as one of the essential criteria that determines if a specimen is "positive" or "negative" for a targeted drug.

On the other hand, nonroutine drug analysis often requires detecting and interpreting quantitative data for drugs at very low levels with small interspecimen concentration differences. Furthermore, sample preparation often requires a 100- or 1000-fold increase in concentration, thereby resulting in analytical results that must be multiplied by 2 or 3 orders of magnitude, which can significantly magnify any inaccuracies in the raw data. Thus, proper interpretation and use of analytical findings rely heavily on the accuracy of raw analytical data. For example, in studies that determine whether drugs found in hair are from external contamination or were ingested by a subject; assess whether a subject's race affects hair assays (arising from differences in the drug incorporation or recovery in the sample pretreatment step); and link variations in hair susceptibility to incorporating environmental contaminations to differences in race or hair treatment (*7–10*).

Accurate quantitation requires proper calibration (standardization) to fully account for artifacts arising from variations in the matrix, specimen preparation, and instrumental conditions. The most commonly used calibration techniques are the analytical or working curve, standard additions, and IS methods (*3, 4*). The IS method is often preferred because it overcomes the matrix effect and replication issues associated with the other two methods.

Of the various analytical detection methods for drugs, MS has proven to be one of the most selective and sensitive. In particular, the selected ion monitoring (SIM) approach has been used for several decades to achieve better accuracy and precision in ion-intensity measurements and is integral to MS quantitation protocols with ISs. A typical protocol involves monitoring several selected ion pairs derived from the targeted drug and the ILA. Several calibration standards containing known amounts of the drug are processed in parallel with test specimens. All calibration standards and test specimens are spiked with the same amount of the IS, and quantitation is achieved by comparing a selected drug-to-IS ion-pair intensity ratio in the test specimen with the same ratio in the calibration standard(s).

ILAs are the preferred ISs for quantitative analyses because they have practically identical chemical properties and mass spectrometric fragmentation patterns as the drug analyte; thus, they can best compensate for errors due to the incomplete recovery of a drug in the sample preparation process or varying GC or MS conditions. Moreover, the absence of an IS in the final data or altered response and ion-intensity ratios can alert the analyst to look for interfering materials or mechanisms (*11, 12*).

## Basic considerations

This quantitation approach works only if the ILA is isotopically pure (an extrinsic factor) and an adequate number of labeling isotopes are positioned at appropriate locations (an intrinsic factor), so that high-mass ions retaining at least three labeling isotopes are present in the final spectrum with significant intensities. Little or no cross-contribution (which is discussed later) between the ions designated for the drug and the IS occurs.

***Isotopic impurities.*** Using an impure ILA can lead to false positive or systematic errors in a positive specimen. This problem is well illustrated by a benzoylecgonine (BZ; a metabolite of cocaine) study in which a high concentration of an ILA IS (1500 ng/mL $^{2}H_{3}$-BZ) was used (*13*). At that time, high concentrations of ISs were commonly used because the BZ concentrations encountered in positive samples were typically high (>5000 ng/mL). High IS concentrations helped reduce errors caused by naturally abundant $^{13}$C-atoms in BZ, adding to the ion intensity of the signal designated for the IS.

This study examined two lots of commercially available 0.1 mg/mL $^{2}H_{3}$-BZ in methanol (*13*). After the addition of 4.5 μg of $^{2}H_{3}$-BZ IS into a 3-mL urine sample (corresponding to 1500 ng/mL $^{2}H_{3}$-BZ), solid-phase extraction, derivatization, and evaporation, the resulting residue was reconstituted to 100 μL for GC/MS analysis. However, BZ ions (*m/z* 331, 272, and 210) were observed in samples without the drug. These two lots of the ILA were found to contain 0.472% and 1.87% impurities of BZ.

Isotopically impure ISs also introduce systematic errors into the data for positive specimens. Data in Table 1 in Supporting Information demonstrate systematic errors, such as higher reported results if the concentration of BZ is lower in the test specimen than the calibration standard, and vice versa. They also demonstrate that the deviations increase as the isotopic impurity in the IS increases. No error will be introduced if the analyte concentrations and the calibration standard are the same.

***Cross-contribution.*** Typically, the drug and IS are not resolved adequately by chromatography. Thus, the ILA must generate at least one ion—preferably two or three—free from cross-contributions by the ions designated for the drug. (In fact, current practice for drug confirmation requires at least three

"interference-free" ions, which provides two ion-intensity ratios for monitoring.)

Therefore, the isotopic labels in the ILA must be positioned at appropriate locations in the molecular framework so that the fragmentation process yields a sufficient number of intense, high-mass ions that retain $n$ (the nominal mass difference between the analyte and the IS) labeling isotopes and do not interfere with the ion intensities attributed to the drug. In other words, a drug could generate [M + $n$] ions that would then overlap with the ILA ion corresponding to the drug's [M] ion. Thus, if deuterium is the labeling isotope, a value of $n \geq 3$ is required under normal circumstances. Otherwise, naturally occurring isotopic abundances in the drug will yield ions designated for the ILA and add to its ion intensity.

Secobarbital/$^{13}C_4$-secobarbital (SB/$^{13}C_4$-SB) data in Figure 1 illustrate how ion-intensity cross-contributions affect the accuracy of analyte quantitation (*14*). In this example, cross-contributions for the first pair of ions (red curve) are so limited that the linearity of the SB/IS ion-pair intensity ratio extends through a wide analyte concentration range. On the other hand, cross-contributions between the ion pair for the blue curve are more significant and would lead to an error if they were used to determine the analyte concentration. The error becomes more serious if the drug concentration in the test specimen is significantly higher or lower than the concentrations in the calibration standard.

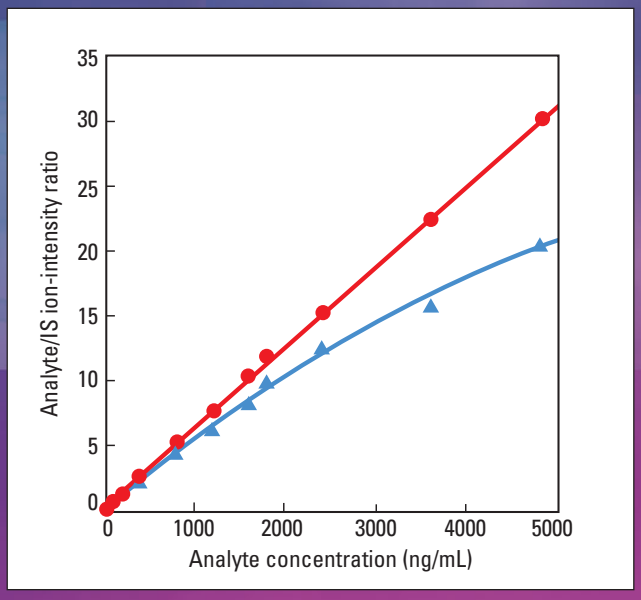



**FIGURE 1.** IS calibration using data from ion pairs with different degrees of cross-contribution between SB and $^{13}C_4$-SB.

The red line is based on the ratio of intensities for *m/z* 196/200; the blue hyperbolic result uses *m/z* 181/185. 0.23% of the measured intensity of *m/z* 196 (SB) is contributed by $^{13}C_4$-SB, whereas 0.017% of *m/z* 200 ($^{13}C_4$-SB) is contributed by SB. On the other hand, 1.6% of the measured intensity of *m/z* 181 (SB) is contributed by $^{13}C_4$-SB, whereas 0.29% of *m/z* 185 ($^{13}C_4$-SB) is contributed by SB. Data adapted from Ref. 14.

## Different approaches to IS selection

Drug ILAs with high isotopic purity are now widely available from commercial sources. On the other hand, the compound's fragmentation mechanism controls whether a specific drug/ILA IS system is indeed free of intrinsic factor interference. By appropriate positioning of isotopes in the molecule, experimenting with various chemical derivatizations, and selecting alternative ionizations, drug/ILA IS ion pairs that are free of or that have minimal cross-contribution can be obtained.

However, all of these approaches have certain limitations. The number and positions of the isotopic atoms are entirely determined during the manufacturing process by the practical synthesis routes. Available functional groups and reagents limit chemical derivatization choices. Chemical ionization procedures are the only practical alternatives, but they have been criticized for their inferior discriminating power for definite identification in a routine high-throughput environment (*15*). In the following discussion, we examine the first two approaches.

***Labeling various positions or using different isotopes.*** Various $^2$H-labeled analogues of amphetamine and methamphetamine with different number of deuteriums and positions were evaluated as ISs for the analyses of these two drugs (*16*). Suitability was judged primarily by the availability of three ions that are interference-free, retain some of the drug's structural features, and have relatively high mass and sufficient intensity. Another study focused on evaluating ions *m/z* 91 and 118 from the heptafluorobutyryl derivative of $d_5$-, $d_8$-, and $d_{11}$-methamphetamine for contributions to ions designated for unlabeled methamphetamine (*17*).

These and other studies indicated that MS fragmentation patterns can include [M – $H_n$] processes that generate a series of "cluster ions", where $n$ is the number of hydrogens involved in the process. When this occurs, [M – $^2H_n$] processes in a $^2$H-analogue of the drug are likely to generate ions interfering with the intensities of ions attributed to the unlabeled drug. Thus, $^{13}$C-labeled ILAs may be a better solution.

A very limited number of $^{13}$C-labeled drug analogues are now commercially available, and two of them have been thoroughly studied along with their $^2$H-analogues (*18–20*). The resulting cross-contribution data determined by various procedures for the secobarbital/$^2H_5$-secobarbital (SB/$^2H_5$-SB), and the secobarbital/$^{13}C_4$-secobarbital (SB/$^{13}C_4$-SB) systems indeed demonstrated the merits of $^{13}$C-labeled ILAs.

***Derivatization.*** Because fragmentation characteristics and

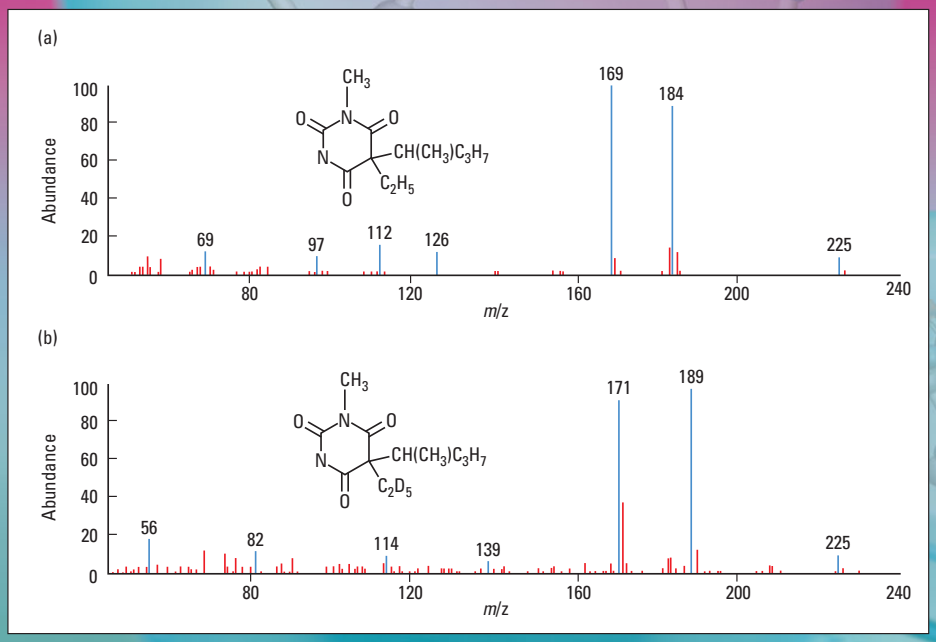



**FIGURE 2.** Mass spectra of methyl derivatives of (a) PB and (b) $^2H_5$-PB.

Adapted with permission from Ref. 13.

the resulting drug ions may be altered by attaching a derivative, these agents can play an important role in generating more favorable ion pairs with minimal or no cross-contribution. A study to evaluate the effectiveness of this approach using methamphetamine and several $^2H$-analogues concluded that the degree of cross-contribution varied with the ILA IS and the derivatization route selected for the assay protocol (*21*). Thus, if a specific derivatization procedure is preferred, a specific ILA IS that may generate the most favorable ion pairs should be selected.

## Protocols for ion pair selection

Even when following the previous "rules", an ILA with adequate isotopic purity and mass difference may still cross-contribute to the drug ions as a result of intrinsic ion fragmentation mechanisms. Understanding the fragmentation pathways for a compound helps researchers select appropriate positions for labeling isotopes, but it does not help them identify the sources of the cross-contributing ions. Such ions have low intensities and pathways that are not well understood. Cross-contribution data for a specific drug/ILA IS system requires empirical evaluation. Various evaluation protocols have been explored and briefly described as follows (*20*).

***Direct measurement protocol.*** The methyl derivatives of pentobarbital (PB) and its $^2H_5$-analogue ($^2H_5$-PB) were used to illustrate the evaluation procedure (*22*). First, full-scan mass spectra of PB and $^2H_5$-PB were obtained (Figure 2 and Table 2 in Supporting Information). Relative ion-intensity data with $^2H_5$-PB show 100% and 84% for ions at *m/z* 189 and 171, respectively. Because the full-scan mass spectrum of PB indicates 0% intensity for these two ions, they appear to be free of cross-contribution and can be used to designate $^2H_5$-PB and for SIM data acquisition. The full-scan mass spectrum of PB shows 84%, 8%, 100%, and 11% for ions at *m/z* 184, 185, 169, and 170, respectively. The corresponding intensities of these ions observed in the full-scan mass spectrum of $^2H_5$-PB are 2%, 1%, 3%, and 0%, respectively, indicating no or low cross-contribution, and therefore these peaks may be suitable for the designation of PB.

However, ion-intensity data derived from full-scan mass spectra are statistically inaccurate. Data for ions with low intensities are especially sensitive to the threshold settings used for data acquisition. For example, if the threshold is set too high, ions with low intensities may be observed with inaccurately low intensities or not seen at all. Thus, the apparent absence of cross-contribution for some ions may have resulted from an inappropriately high threshold setting. Further evaluation is essential.

In the second step, the ions selected from the full-scan data are studied by SIM to determine if these ions are suitable to designate PB and the extent of cross-contribution. Under typical chromatographic conditions, $^2H_5$-PB has a slightly shorter GC retention time than PB, but the difference is insufficient to separate the peaks. When run at a lower temperature, significant resolution of the PB and $^2H_5$-PB peaks can result and allow for determining the source of cross-contribution.

SIM single-ion chromatograms representing some of the PB and $^2H_5$-PB ions are shown in Figure 3. Single-ion chromatograms in Figures 3g–i reveal that the *m/z* 189 ion designated for $^2H_5$-PB is ideal because no PB ion contributes to the intensity of this peak. Alternatively, Figures 3d–f and SIM peak area integration data indicate the *m/z* 170 ion cannot be used to designate PB because it has an unacceptably high intensity (15.8%) contributed by $^2H_5$-PB. SIM peak area integration data also indicate that the cross-contribution to *m/z* 185 (Fig-

Quantitation works only if the ILA is pure and enough labeling isotopes are properly positioned.

ures 3a–c; designated PB) and 171 (Figures 3j–l; designated $^2H_5$-PB) are 1.35% and 1.71%, respectively.

***IS and standard addition protocols.*** Cross-contribution data from the direct measurement protocol are based on ion-intensity data collected from separate analytical runs for the drug and the ILA. Variations in stock solution concentrations, chemical derivatization yields, and instrument conditions, along with losses in the sample preparation step arising from adsorption and resolubilization, can also severely limit data accuracy.

IS and standard addition are two alternative approaches that imitate calibration methods routinely used in quantitative analysis protocols (*3, 4*). These protocols have recently been used to evaluate cross-contribution data (*20*). SB and its two ILAs, $^2H_5$-SB and $^{13}C_4$-SB, were adapted as the exemplar system for this study.

For the IS method, a set amount of a chromatographically resolved third compound is used. In this example, PB was used as the IS and incorporated into separate experiments for evaluating the SB/$^2H_5$-SB and SB/$^{13}C_4$-SB systems. Normalizing the MS intensities of the ions to a selected ion compensates for variations in the GC/MS conditions and the yield of the derivatization step. In this study, the ions designated for SB, $^2H_5$-SB, and $^{13}C_4$-SB were normalized to the intensity of the *m/z* 169 ion, which was derived from the PB IS.

The standard addition approach includes two sets of experiments for evaluating each SB/ILA system. For example, to evaluate the cross-contribution between SB and $^2H_5$-SB, experiments compared the contribution of SB (interfering analogue) to the intensities of ions designated for $^2H_5$-SB (analogue suffering interference) and the contribution of $^2H_5$-SB (interfering analogue) to the intensities of ions designated for SB (analogue suffering interference). Similarly, two additional sets of experiments were carried out to evaluate the cross-contribution data for the SB/$^{13}C_4$-SB pair. Thus, a total of four sets of experiments were conducted in this study (*20*).

The first set of standard addition experiments found the intensities of the *m/z* 201, 200, 143, and 116 ions—those designated for $^2H_5$-SB (the analogue suffering interference)—which were contributed by a set amount (5 µg) of SB. Because the four ions were designated for $^2H_5$-SB, their intensities derived from the presence of $^2H_5$-SB were much higher than from the same amount of SB. Furthermore, the relative intensities of these ions, resulting from the presence of a set amount of $^2H_5$-SB (or SB), was not the same. Thus, the amount of each $^2H_5$-SB standard addition that was needed to make the optimal increase in the signal (0.5–2 times the original signal derived from 5 µg of SB) also varied with the ion to be evaluated. Thus, the presence of the four ions in SB was estimated by additions of different amounts of the standard $^2H_5$-SB.

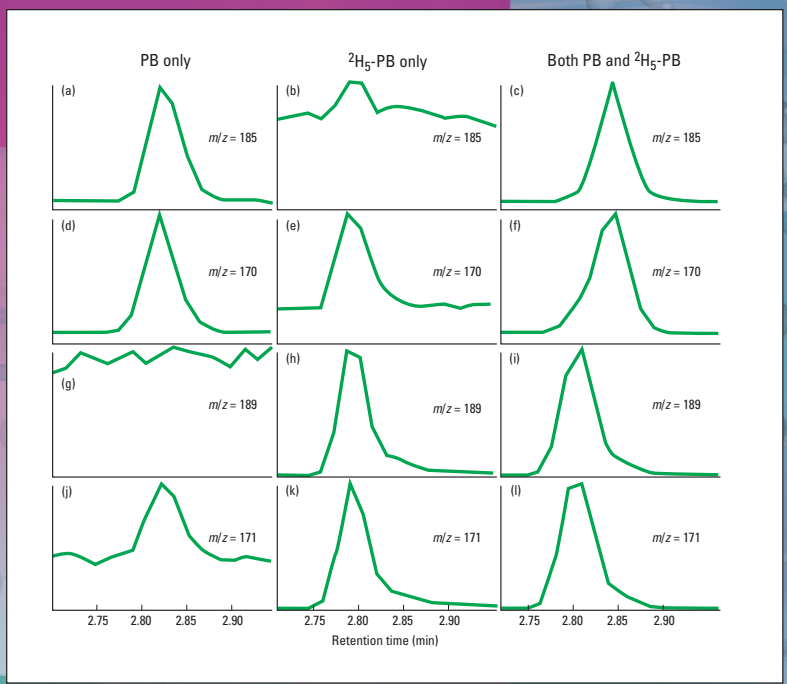



**FIGURE 3.** Single-ion chromatograms of PB, $^2H_5$-PB, and PB/$^2H_5$-PB mixtures.

(a–c) *m/z* 169; (d–f) *m/z* 170; (g–i) *m/z* 189; (j–l) *m/z* 171. Adapted with permission from Ref. 13.

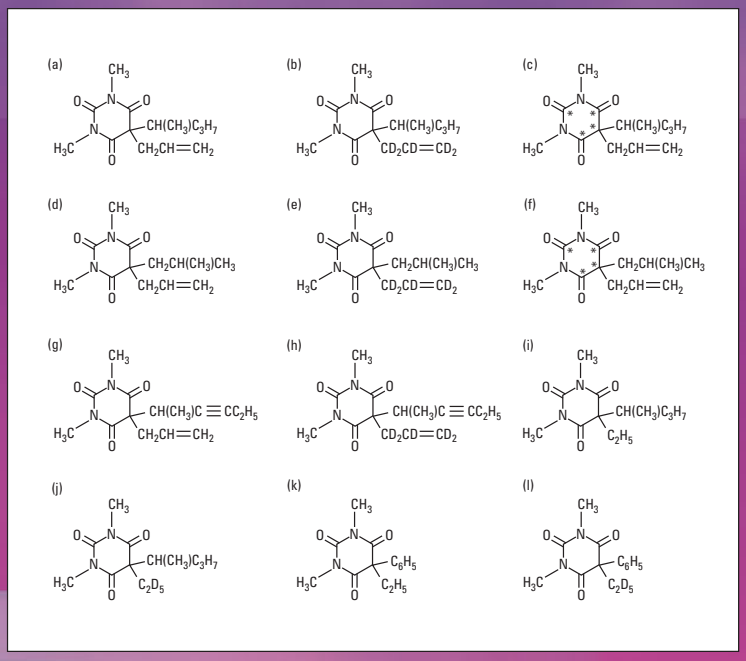


**FIGURE 4.** Structures of analytes/isotope-labeled analogues (all as methyl derivatives): (a–c) SB/$^{2}H_5$-SB/$^{13}C_4$-SB; (d–f) BB/$^{2}H_5$-BB/$^{13}C_4$-BB; (g, h) methohexital/$^{2}H_5$-methohexital; (i, j) PB/$^{2}H_5$-PB; (k, l) phenobarbital/$^{2}H_5$-phenobarbital.

Data resulting from these addition experiments are used in three different ways to estimate the intensities of these ions before the addition. In method A, the ion intensities observed from each series of additions, which include the addition of none and four levels of $^{2}H_5$-SB, are used directly. In methods B and C, ion intensities are normalized to the base ion intensity of the interfering analogue (*m/z* 196, resulting from the presence of 5 µg of SB) and a set amount of a third compound (PB, *m/z* 169), respectively. Normalized ion intensities are then used for cross-contribution data evaluation.

With the standard addition approach, the intensity of a designated signal (*y*) detected in the test sample is plotted against the quantity (*x*) of the standard added. Least-square fit equation and correlation coefficient data are calculated. The equivalent quantities of the standard ($^{2}H_5$-SB) that will generate the same ion intensities derived from 5 µg of SB is the calculated *x* value when $y = 0$ (*3*, *4*). Resulting equivalent quantities of the standard are then divided by the quantity of the interfering compound (5 µg of SB) to derive the percentage of cross-contribution data for the ions studied. Correlation coefficient data indicate that method B provided the best, linear results.

Cross-contribution data for the ion pairs for both systems (SB/$^{2}H_5$-SB and SB/$^{13}C_4$-SB) are found in Table 3 in the Supporting Information. These data indicate that all methods produce practically the same order in the degree of cross-contribution in all ion pairs studied. Thus, all methods can be used to select the best ion pair within a selected drug/ILA IS pair for the intended quantitative analysis protocol.

Numerical values derived from method B are believed to be the most reliable ones because data are derived from multiple data points, and linearity provides assessment of the quality of the resulting cross-contribution data. In addition, the data normalization procedure adapted by the IS method is similar to that adapted by method C of the standard addition approach, which was proven less effective than the normalization procedure adapted by method B.

Accuracy and precision in measuring the low intensity of the cross-contributing ion are limiting factors in determining the extent of cross-contribution, whether by direct measurement, IS, or standard addition protocols. Additional studies address improvements in these measurements (*23*, *24*).

## Linear versus nonlinear calibration models

As shown in Table 1 in Supporting Information and the blue line in Figure 1, drug concentrations derived from one-point calibration often include systematic errors because this method is built on assumptions (absence of cross-contamination and isotopic impurity) that are impractical (*13*, *14*). The error is absent when the drug concentration in the test specimen is the same as that in the calibration standard, but it systematically increases as the drug concentration in the test specimen increasingly differs from that of the calibration standard.

Multiple-point linear calibration approaches, in which the observed drug-to-ILA IS ion-pair intensity ratios are plotted against the drug concentration, are commonly used to extend quantitation to a wider concentration range. However, systematic errors still occur as a result of the inherently nonlinear nature of the calibration curve. Basically, one-point and multiple-point approaches previously described are based on linear models; thus, similar errors will be observed as long as the assumptions for the linear model are violated.

For calibration purposes, a linear relationship with a zero-intercept between the measured response and the drug concentration is preferred. Thus, logarithmic-transformed ion-pair in-

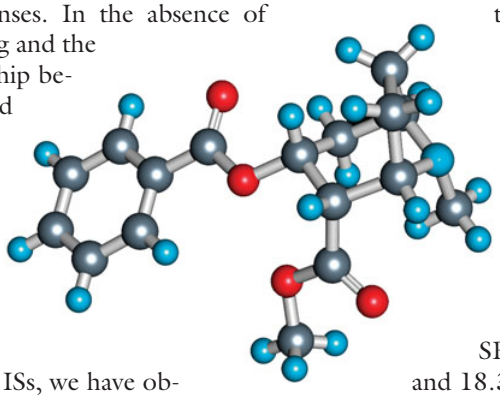
tensity ratios were proposed to establish the standard curve, which was reportedly linear with an upper concentration of 500,000 ng/mL of BZ (*25*). Our studies have demonstrated that the hyperbolic model works well for cases in which calibration data have been derived from ion pairs with significant cross-contributions, as demonstrated by the blue line in Figure 1 (*14*). The hyperbolic model is effective because it accounts for the cross-contribution phenomenon shown in Equation 1 (*26*)

$$y = (xX + aA') / (xX' + aA);$$
$$(x + aA'/X) / (xX'/X + aA/X) = (x + C_1) / (C_2 x + C_3) \quad (1)$$

where $y$ is the observed ion-pair intensity ratio; $x$ is moles of the drug in each standard; $X$ is intensity of the ion designated for the drug; $a$ is moles of the IS in all standards; $A'$ is intensity of the IS cross-contributed to the ion designated for the drug; $X'$ is intensity of the drug cross-contributed to the ion designated for the IS; and $A$ is intensity of the IS for the ion designated for the IS.

After reducing the equation, constant $C_1$ expresses the cross-contribution of the IS to the intensity of the ion designated for the drug; constant $C_2$ expresses the cross-contribution of the drug to the intensity of the ion designated for the IS, whereas constant $C_3$ reflects the moles of the IS used, the relative purity of the drug and the IS used in preparing the standard solutions, and the relative intensities of the ions designated for the same amount of the drug and the IS.

Constant $C_3$ equals the concentration of the IS when both the drug and the IS are 100% chemically and isotopically pure with identical mass spectral responses. In the absence of cross-contribution between the drug and the IS, $C_1 = C_2 = 0$; thus, the relationship between the drug/IS ion-intensity and concentration ratios reduces to a linear function, $y = Cx$, where $C = 1/C_3$.



## Are $^2$H-analogues the best choice?

While evaluating the effectiveness of the $^{13}C_4$- and $^2H_5$-analogues of SB (*18*) and butalbital (BB) (*19*) as ISs, we have observed an interference phenomenon in cases where $^2H_5$-analogues of these two barbiturates are adapted. Specifically, the intensity ratios of the ion pairs designated for these two drugs and their respective $^2H_5$-analogues increase with the volume of the ethyl acetate solvent used to reconstitute the extraction/derivatization residue. This phenomenon was not observed when the respective $^{13}C_4$-analogues were used as the ISs in parallel experiments.

Because this interference phenomenon was observed with the $^2$H-labeled systems and not with the $^{13}$C-labeled systems, we do not believe the reported self-chemical ionization phenomenon is the underlying cause (*27, 28*). Because the $^2$H-atoms in the $^2H_5$-SB and $^2H_5$-BB are at allylic positions, it was hypothesized that hydrogen/deuterium exchange took place at the ion source. However, the observation of the same phenomenon for drug/$^2$H-analogue pairs both with (SB, BB, methohexital) and without (PB, phenobarbital) this structural feature disproved the hypothesis (*29*) (Figure 4 and Table 4 in the Supporting Information).

Drug/$^{13}$C-analogue systems differ from the corresponding drug/$^2$H-analogue systems by displaying identical retention times for the drugs and the ILA ISs. Thus, retention time difference between the drug and the $^2$H-analogue IS was hypothesized as the underlying factor causing the increase in the ion-pair intensity ratio observed for the drug/$^2$H-analogue systems. To test this hypothesis, several series of experiments were performed in which GC column temperature-programming conditions were varied to modify the separation between the drug and the $^2$H-analogue IS (*29*). The resulting drug/IS ion-pair intensity ratio changes were characterized and evaluated.

The SB/$^{13}C_4$-SB system was again used as the control, and the monitored ion-pair intensity ratio for this system remained constant as the reconstitution volume was increased and the temperature-programming rate changed from 30 to 15 to 5 $^{o}$C/min. This is consistent with the hypothesis because the retention times of the drug and the $^{13}$C-analogue IS demonstrate no separation, regardless of the programming rate.

Data from a series of parallel experiments for the SB/$^2H_5$-SB system are found in Table 5 in Supporting Information. As the programming rate was reduced from 30 to 15 to 5 $^{o}$C/min, the separation between the drug and the $^2$H-analogue IS increased, and the overlap of *m/z* 196 by *m/z* 201 decreased from 89.5 to 77.7, and then to 70.2%. With these three temperature-programming conditions and a reconstitution volume that changed from 20 to 200 µL, the monitored ion-pair intensity ratio for the SB/$^2H_5$-SB system changed 11.92%, 15.71%, and 18.35%, respectively.

Another series of experiments for the SB/$^2H_5$-SB system were performed in which the $^2$H-analogue IS, rather than the drug, was the major component. In this case, with the same programming rate, the separation between the drug and the IS similarly increases, but the overlap is reduced from 100 to 94.3, and then to 62.4%. Again, under these three temperature-programming conditions and a reconstitution volume that changed from 20 to 200 µL, the monitored ion-pair intensity ratio for the SB/$^2$H-SB system changed –7.65%, –14.2%, and –23.2%, respectively.

These phenomena are rationalized as follows. First, when two chromatographically closely eluted compounds have their overlapping portions appear simultaneously at the ion source, the nonoverlapping portions will have a higher ionization efficiency; thus, the overall ionization efficiency of the major component will be lower than the minor one. Second, this difference in ionization efficiency between the major and the minor compounds becomes more significant when the total molecular population at the ion source is higher, resulting from a smaller reconstitution volume. This explains why, as the reconstitution volume is increased from 20 to 200 μL, the monitored ion-pair intensity ratios increases when SB is the major component, but decreases when $^2H_5$-SB is the major component (Table 5 in Supporting Information). Third, as the drug and the $^2$H-analogue IS more closely elute, larger portions of these two compounds will appear at the ion source at the same time. Therefore, the rate of the changes in the monitored ion-pair intensity ratio is much higher when the temperature-programming rate is decreased.

These reasons are consistent with the observed peak overlapping data and ion-pair intensity ratio change characteristics shown in Table 5 and may account for the reported interference on the quantitation of BZ caused by the coelution of fluconazole (*14*). The authors attributed the observed "coeluting interference" to "saturation of the ionization chamber," but did not mention nonproportional variations in BZ/$^2H_3$-BZ ionization efficiencies.

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Note: Tables 1–5 are available as Supporting Information on the Web at http://pubs.acs.org/ac.