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Selecting an Appropriate Isotopic Internal Standard for Gas Chromatography/Mass Spectrometry Analysis of Drugs of Abuse—Pentobarbital Example

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ABSTRACT: Internal standards are commonly used for the quantitative determination of drugs of abuse and their metabolites (**drug/metabolite**) in biological fluids and tissues by the selective ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS) procedure. Analogs of drugs/metabolites that are labeled with three or more deuterium atoms (**isotopic analog**) at appropriate positions are considered to be the most effective internal standards for these applications. Before a specific deuterated analog can be adopted as an internal standard in a GC/MS assay, the mass spectrum of the compound or its derivative must be evaluated along with the corresponding spectrum from the parent drug/metabolite. There should be an adequate number of sufficiently high-mass ions (typically three for the drug/metabolite and two for the isotopic analog) that can be attributed to each analyte, and these ions should be sufficiently free of interference from the other analyte of the pair (**cross-contribution**). Interferences may be caused by the presence of an isotopic impurity in the deuterated analog (extrinsic factor) or may be due to the ion fragmentation characteristics of the compound (intrinsic factor). The extrinsic factor may be corrected by the manufacturer with different synthetic methods and purification procedures, while the intrinsic factor may be partially or wholly corrected through the use of different chemical derivatives (sample preparation stage) or different ionization (GC/MS assay stage) procedures.

In this study, pentobarbital/d_5-pentobarbital is used as the exemplar analyte/deuterated analog pair to illustrate the ion selection and evaluation procedures. Full-scan mass spectra were employed for preliminary ion selection. SIM data were then used to calculate the extent, if any, of cross-contributions. SIM ion chromatograms obtained under a lower GC oven temperature were used to differentiate sources (ion fragmentation mechanism versus isotopic impurity) of cross-contributions.

KEYWORDS: toxicology, barbiturates, GC/MS, internal standard, pentobarbital

Internal standard method is currently the most commonly adopted approach for the quantitative analysis of abused drugs in biological matrices. When a mass spectrometer is used as the detection device, for example, in gas chromatography (GC)/mass spectrometry (MS) and high performance liquid chromatography (HPLC)/MS applications, an *isotopic analog* of the *analyte* with an adequate number of deuterium atoms at appropriate positions is often the preferred internal standard. With its practically identical chemical properties and mass spectrometric fragmentation mechanisms, the use of an isotopic analog (of the analyte) as the internal standard offers the following advantages:

1. Errors derived from (a) incomplete recovery of the analyte in the sample preparation process or (b) varying gas chromatographic and mass spectrometric conditions are compensated.
2. The presence of interfering materials (or mechanisms) that prevents the detection of the analyte will also cause the absence of the internal standard in the final chromatogram, thus alert the analyst to conduct further investigation [*1*].

A typical quantitative GC/MS protocol usually involves monitoring several selected ions (SIM) from the analyte and from the isotopic analog. Quantitation is achieved by comparing a selected analyte-to-isotopic analog ion intensity ratio observed from the *test sample* and the same ratio observed from the *calibration standard.* The calibration standard contains the same amount of the internal standard and a known amount of the analyte and is processed in parallel with the test sample. The analyte concentration in the test sample can be calculated using a one-point calibration approach as shown in Fig. 1.

Not all deuterium-labeled isotopic analogs (of the analyte) are suitable internal standards. Since the analyte and the internal standard are rarely separated adequately, the proposed isotopic analog must generate at least one (preferably two or three) ion that is relatively free from cross-contribution by the analyte. There must also be at least three ions from the analyte that are relatively free from cross-contribution by the proposed isotopic internal standard. If there are significant cross-contributions, then the quantitative result and ion intensity ratio data (that are commonly used as important parameters for analyte identification) may become unreliable. Under these circumstances, elaborated deconvoluting procedure [*2*] may be required to resolve the peak intensity data resulting from the overlapping of the analyte and the internal standard. Furthermore, non-linear calibration models may have to be adopted for obtaining accurate quantitative information [*3–5*].

Realizing the significances of the cross-contributing phenomenon, we have conducted this study in which an empirical approach is used for evaluating, and thereafter selecting, ions for the analyte

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$$\frac{\left[\frac{(\text{Selected Ion Intensity})_{\text{Analyte}}}{(\text{Selected Ion Intensity})_{\text{Internal Std}}}\right]_{\text{Test Sample}}}{\left[\frac{(\text{Selected Ion Intensity})_{\text{Analyte}}}{(\text{Selected Ion Intensity})_{\text{Internal Std}}}\right]_{\text{Calibration Standard}}} \times \left[\text{Standard Conc.}\right]_{\text{Calibration Standard}} = \left[\text{Analyte Conc.}\right]_{\text{Test Sample}}$$

FIG. 1—*Formula for the calculation of the analyte concentration using an one-point calibration protocol.*

and the internal standard for SIM applications. Pentobarbital/d_5-pentobarbital are used as the exemplar analyte/isotopic analog pair to illustrate the ion selection and evaluation process.

Experimental

Materials

Pentobarbital (analyte) and 0.1 mg/mL methanol solution of d_5-pentobarbital (internal standard) were purchased from Sigma (St. Louis, MO) and Radian (Austin, TX), respectively. The derivatization reagents and solvent—tetramethylammonium hydroxide (TMAH) (24% in methanol), iodomethane, and dimethylsulfoxide (DMSO)—were purchased from Eastman Kodak (Rochester, NY), Mallinckrodt (Paris, KY), and Aldrich (Milwaukee, WI), respectively.

Derivatization

Pentobarbital and d_5-pentobarbital were analyzed as their methyl derivatives obtained by the procedure described below. Pentobarbital (or d_5-pentobarbital) was dissolved in freshly prepared TMAH/DMSO (1:20) solution, followed by the addition of iodomethane. The mixture was vortex-mixed briefly and 0.1 N HCl was added five minutes later. Isooctane was added to the mixture to extract the derivatization product by thorough mixing and layer separation with centrifugation. The organic phase was isolated by pasteur pipetting or decanting (after freezing the lower aqueous layer in dry ice/isopropanol bath). The solvent was evaporated under a stream of air (or nitrogen) at room temperature. The residue was reconstituted with appropriate volume of ethyl acetate prior to GC/MS analysis.

GC/MS Analysis

Full-scan and SIM mass spectrometric data were obtained using a Hewlett-Packard (Palo Alto, CA) HP 5890 Gas chromatograph interfaced to a Hewlett-Packard HP 5970 mass selective detector (MSD). The gas chromatograph was equipped with a 13-m J & W (Folsom, CA) DB-5 (5% phenyl polysiloxane phase) fused silica capillary column (0.25 mm ID; 0.25 μm film thickness). The injection port was equipped with a split silanized glass insert packed with OV-101 (80/100 mesh). Helium was used as the carrier gas with a flow rate of 1.0 mL/min and a split ratio of 10:1. For routine analysis, the injector, oven, and interface temperatures were maintained at 270, 160, and 270 °C, respectively. Oven temperature was maintained at 130 °C for partial resolution of pentobarbital and d_5-pentobarbital (as methyl derivatives) as discussed later.

Full-scan spectra were obtained with the MSD operated in full-scan mode scanning from m/z 45 to 320 amμ. The MSD was used under SIM mode (dwell time 50 ms) when monitoring m/z 169, 184, 185, 170 and 189, 171, designated for pentobarbital and d_5-pentobarbital (as methyl derivatives), respectively, for evaluation.

A solution containing the same concentration of pentobarbital and d_5-pentobarbital was prepared and appropriate volumes of this mixture containing 70 ng of each compound were injected into the MSD for assessing relative cross contribution information as described later.

Results and Discussion

Criteria for Selecting an Isotopic Analog As Internal Standard

A specific isotopic analog can be adopted as an internal standard in a GC/MS application only if there are an adequate number (typically three for the analyte and two for the isotopic analog) of ions that are sufficiently free of cross-contribution. This requirement is satisfied if the following conditions can be met:

1. The isotopic analog is labeled with a *sufficient number of atoms of a selected isotope* (typically deuterium) so that the corresponding ions from the internal standard and from the analyte will have a significant difference in their masses. If the difference is not sufficient, the [M + n] ion (designated for the analyte) due to the naturally occurring isotope abundance may make a significant contribution to the intensity of the ion (designated for the isotopic analog) that corresponds to the [M] ion of the analyte. (M is the mass of the ion derived from the analyte and selected for monitoring; n is the nominal mass difference of the ions designated for the analyte and the isotopic analog.) If deuterium, as in most realistic applications, is used as the labeling isotope for the internal standard, a difference in three mass units between the analyte and the isotopic analog is sufficient under normal circumstances. (It should be noted, however, that if the concentration of the analyte is unproportionally higher than the concentration of the internal standard included in the assay process, the intensity of the [M + 3] ion originated from the analyte may become significant enough to require an additional analysis of a diluted aliquot.)
2. The analyte and the isotopic analog undergo an appropriate fragmentation process to generate several high intensity ions that include the labeling isotopes with insignificant intensity of [M − nH] ions. To meet this requirement, the *labeling isotopes must be positioned at appropriate locations* in the molecular structure so that, after the fragmentation process, a sufficient number of high-mass ions (with significant intensities) that retain the labeling isotopes are present and will not interfere with the intensity measurement of the corresponding ions derived from the analyte. These ions (from the isotopic analog) and their counterparts in the analyte may then be monitored for ion ratio

evaluation and further used for qualitative compound identification and quantitative determination.

3. The isotopic analog is manufactured with sufficient *isotopic purity.* Otherwise, the addition of the internal standard may result in the observation of a significant amount of the analyte in a true negative sample and may also introduce errors in quantitation. This will become a problem, especially when a high concentration of the internal standard is used [6].

Preliminary Ion Selection—Based on Full-Scan Mass Spectra Data

Full-scan mass spectra of the exemplar *pentobarbital/d_5-pentobarbital* (analyte/isotopic analog) pair are graphically presented together with the same mass axis scale in Fig. 2. Relative ion intensity data are shown in Table 1. The mass spectrum of d_5-pentobarbital (Part *b*) shows 100% and 84% relative intensities for m/z 189 and 171 ions (Table 1). Since the full-scan mass spectrum (Part *a*) of pentobarbital show 0% for these two ions (Table 1), they *appear* to be free of interference and can be designated for d_5-pentobarbital in SIM data acquisition.

TABLE 1—*Full-scan and SIM mass spectral data of pentobarbital and d_5-pentobarbital—selecting ions for internal standard application.*

Ion (m/z)	d_0-Pentobarbital Full-scan (Relative intensity)	d_0-Pentobarbital SIM Intensity and % Contribution by Analog	d_5-Pentobarbital Full-scan (Relative intensity)	d_5-Pentobarbital SIM Intensity and % Contribution by Analog
Ions Designated for Pentobarbital				
169	100%	1,525,511 (2.70%)	3%	41,132
184	84%	1,200,754 (1.90%)	2%	22,867
185	8%	143,391 (2.75%)	1%	3,939
170	11%	123,205 (23.2%)	0%	28,534
Ions Designated for d_5-Pentobarbital				
189	0%	1,996	100%	1,004,286 (0.20%)
171	0%	16,769	84%	946,005 (1.77%)

The full-scan mass spectrum of pentobarbital shows 84%, 8%, 100%, and 11% for m/z 184, 185, 169, and 170, respectively (Table 1). The relative intensities of these four ions observed in the full-scan mass spectrum of d_5-pentobarbital are 2%, 1%, 3%, and 0%, respectively (Table 1), indicating no or low cross-contribution and *may be* suitable for SIM data acquisition for pentobarbital.

It should be noted that ion intensity data from full-scan mass spectra are generally not very accurate, and are often affected by the threshold setting used in the data acquisition process. For example, if the threshold is set too high, ions with low intensities may not even be observed or observed with inaccurate low intensities. Thus, the *apparent* absence of cross-contribution for some ions (m/z 189 and 171 designated for d_5-pentobarbital and m/z 170 designated for pentobarbital) *may* have resulted from the use of an inappropriate threshold setting (too high). On the other hand, the *apparent* cross-contribution for other ions may have been caused by extrinsic (presence of isotopic impurity) or intrinsic (ion fragmentation process) factors. Further evaluations of the above mentioned interference (and non-interference) are essential.

Further Evaluation of Selected Ions—Based on SIM Data

While full-scan mass spectra are valuable for preliminary ion selections for SIM data acquisition, they do not provide adequate quantitative information, especially for those ions showing low intensities. SIM data are needed to determine whether it is indeed

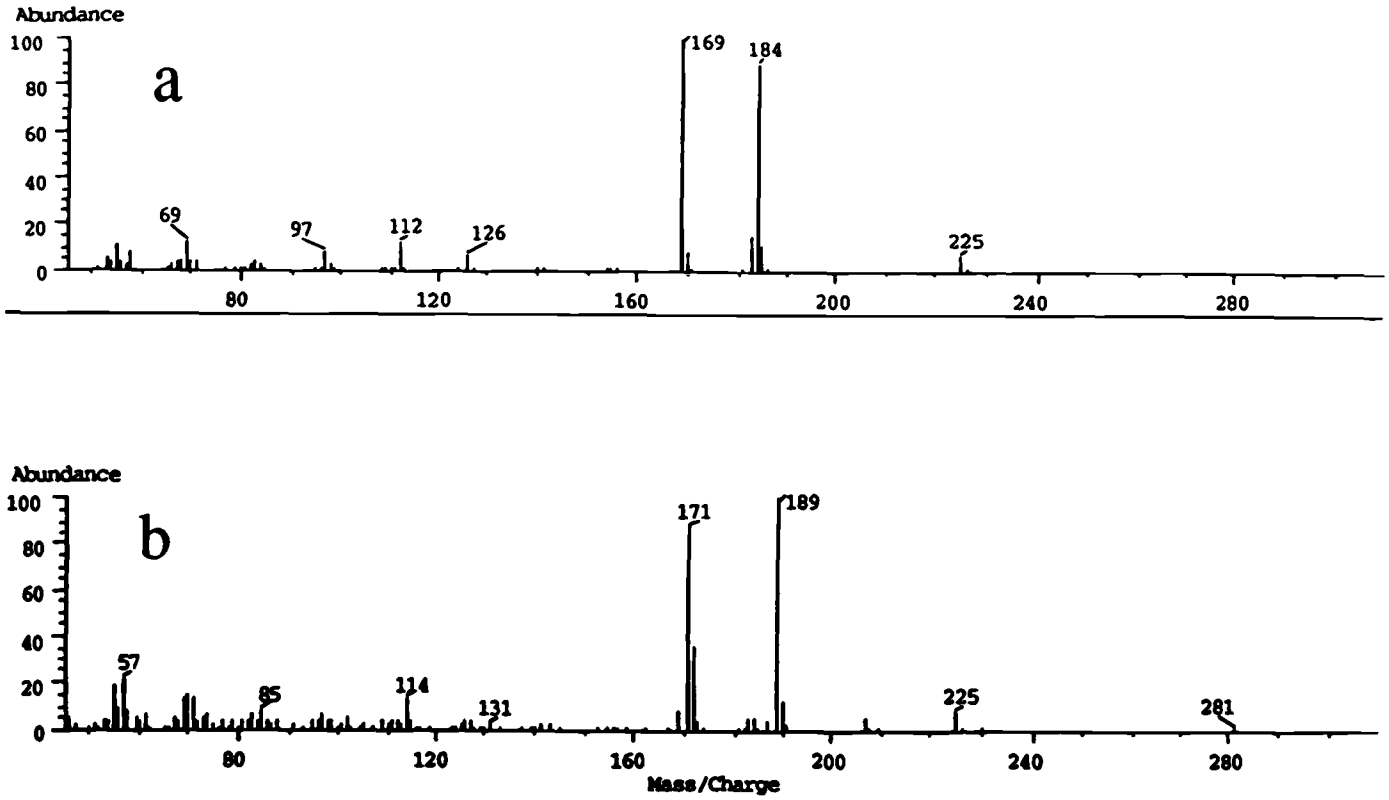

FIG. 2—*Mass spectra of methyl derivatives of pentobarbital* (a) *and d_5-pentobarbital* (b).

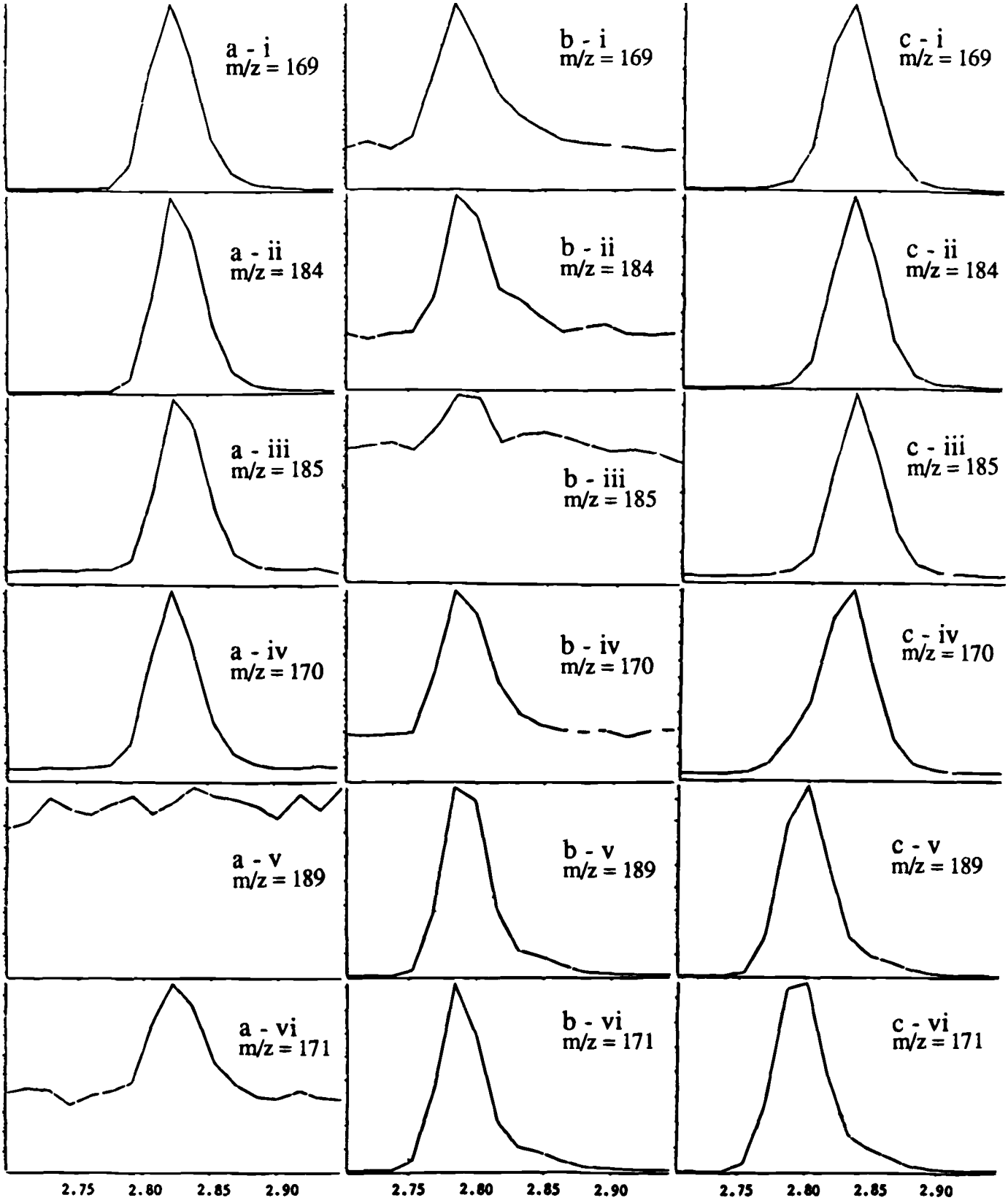

FIG. 3—*Single ion chromatograms of pentobarbital* (a), *d_5-pentobarbital* (b), *and pentobarbital/d_5-pentobarbital mixture* (c) *(obtained with chromatographic oven temperature at 160°C).*

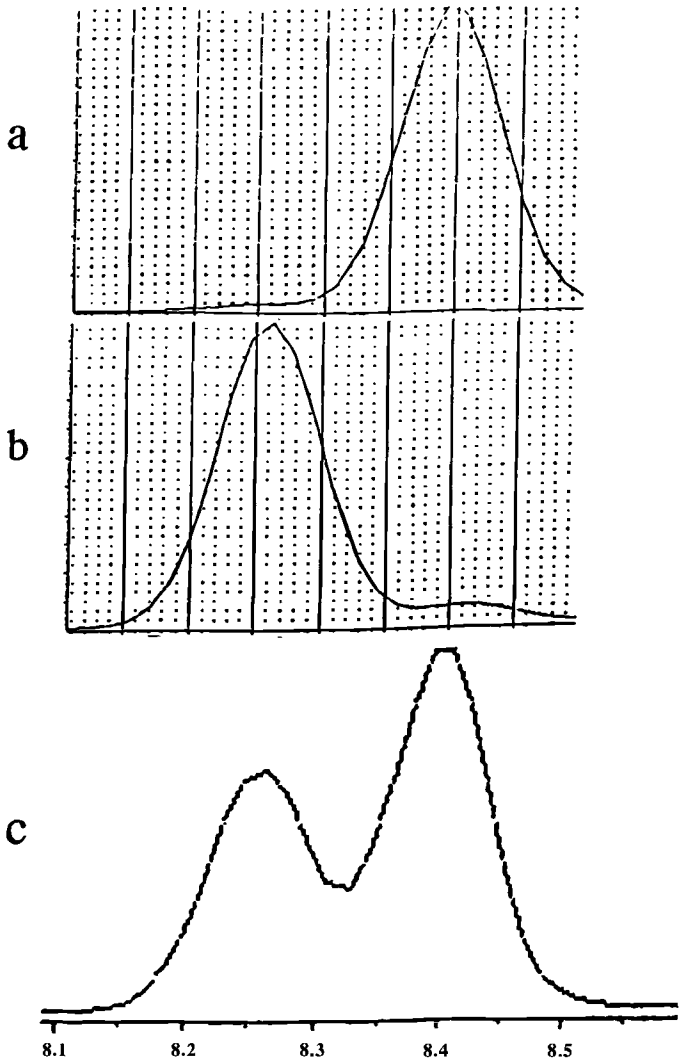

FIG. 4—*Single ion chromatograms for m/z = 169* (a), *189* (b) *and composite ion chromatogram* (c) *of a pentobarbital/d_5-pentobarbital mixture (obtained with chromatographic oven temperature at 130°C).*

without cross-contribution and, if present, the extent and source of the cross-contribution.

SIM single ion chromatograms of the six ions (m/z 169, 184, 185, 170, 189, and 171 selected from the mentioned full-scan mass spectra data) collected from pentobarbital, d_5-pentobarbital, and pentobarbital/d_5-pentobarbital mixture are presented in Fig. 3-*a*, 3-*b*, and 3-*c*, respectively. These ion chromatograms were obtained using the same amount (70 ng) of pentobarbital and d_5-pentobarbital.

Single ion chromatograms shown as Fig. 3-a-vi and 3-b-vi reveal that the m/z 189 ion designated for d_5-pentobarbital is ideal—pentobarbital will not make observable cross-contribution. On the contrary, Fig. 3-a-v and 3-b-v and SIM peak area integration data shown in Table 1 indicate unacceptable high intensity of the m/z 170 ion contributed by d_5-pentobarbital, thus disqualifying the use of this ion for SIM data acquisition for pentobarbital.

SIM peak area integration data shown in Table 1 also indicate that cross-contribution to m/z 169, 184, 185 (designated for pentobarbital), 189, and 171 (designated for d_5-pentobarbital) are 2.70%, 1.90%, 2.75%, 0.20%, and 1.77%, respectively. These five ions are tentatively considered acceptable for SIM data acquisitions, and further evaluated in the following section.

Differentiation on Sources of Cross-Contribution—Intrinsic Fragmentation Mechanism vs. Isotopic Impurity

A deuterated analog of the analyte show slightly shorter retention time. The difference, however, are not significant enough for peaks separation under normal operation chromatographic conditions—unless the deuterium atoms are as many as, for example, 9. In order to differentiate whether the observed cross-contribution derives from ion fragmentation mechanism or is caused by the presence of an isotopic impurity component, SIM data are obtained at a chromatographic oven temperature that was low enough to show partial resolution of analyte/isotopic analog pairs.

Single ion chromatograms (Fig. 4-*a* and 4-*b*), obtained from a mixture of 70 ng of pentobarbital and 70 ng of d_5-pentobarbital, show observable separation of the m/z 169 (designated for pentobarbital) and the m/z 189 ions. The composite[4] ion chromatogram (Fig. 4*c*) also shows partial resolution of the pentobarbital/d_5-

[4] The term "composite ion chromatogram" is used here to indicate the sum of intensity of the SIM ions (m/z = 169, 185, 170, 184, 189, and 171, in this case). The term, "total ion chromatogram," is reserved for use for full-scan data.

pentobarbital pair. Identification of the source of cross-contribution are shown below.

Interference through Fragmentation Mechanism—Ion chromatogram peaks and peak integration data shown in Fig. 5 and Table 2 indicate that the cross-contributions of ions m/z 169, 184, 185, and 170 ions by d_5-pentobarbital fragmentation mechanisms appear at a slightly shorter retention time and are 1.94%, 1.67%, 1.35%, and 15.8%, respectively. Similarly, pentobarbital contributes (through fragmentation mechanism appearing at a slightly longer retention time) 0.0% and 1.71% to the ions m/z 189 and 171 that are designated for d_5-pentobarbital.

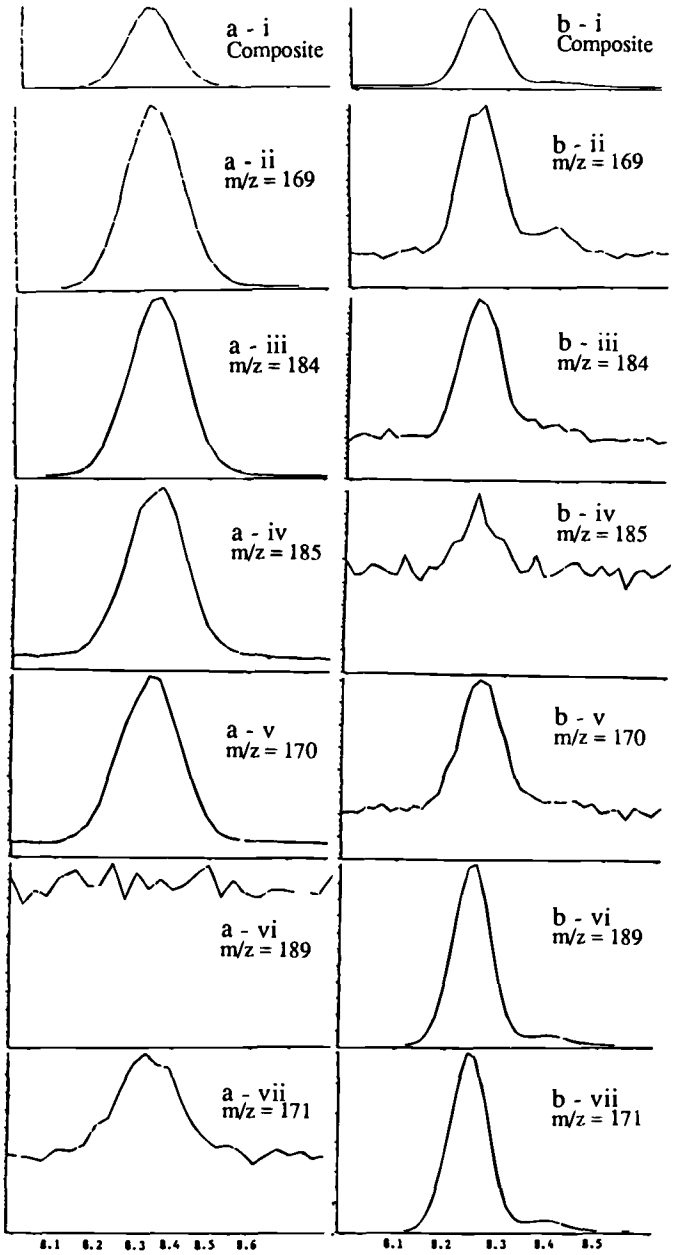

FIG. 5—*Composite ion chromatograms* (a-i) *and* (b-i) *and single ion chromatograms of pentobarbital* (a) *and* d_5*-pentobarbital* (b) *(obtained with chromatographic oven temperature at 130°C).*

Interference Due to Isotopic Impurity—Fig. 5*a* and the left-hand section of Table 2 clearly show that there is no d_5-pentobarbital present as an impurity in pentobarbital. Figure 5*b* and Table 2 show that there are 0.11%, 0.038%, 0%, and 0% of pentobarbital impurity present in d_5-pentobarbital based on data from ions m/z 169, 184, 185, and 170, respectively. Since the intensities of ions m/z 185 and 170 are very low, data from ions m/z 169 and 184 should be considered more reliable. It is nevertheless clear that this lot of d_5-pentobarbital is sufficiently pure isotopically for internal standard applications.

It is not clear why significant intensities of ions m/z 189 (4.61%) and 171 (4.70%) are observed at the retention time corresponding to pentobarbital in the respective single ion chromatograms of d_5-pentobarbital. These could not be attributed to the presence of pentobarbital impurity in d_5-pentobarbital as pentobarbital ion chromatograms shows only 0% and 1.71% (Table 2) for these two ions. One possible speculation is the presence of other deuterated pentobarbitals with less than five deuterium atoms. Since these intensities do not interfere with the SIM data collection for the analyte, they should not cause errors in the quantitation of pentobarbital.

Effect of Cross-Contribution

It is not common to find a deuterated analog that would allow for the selection of five ions (three from the analyte and two from the deuterated internal standard) that are completely free of cross-contributions. We have demonstrated that a significant amount of the analyte may be reported if the deuterated internal standard contains too much analyte (non-deuterated impurity) and the concentration of the internal standard is too high [*6*]. This should not be a serious problem for an experienced analyst who monitors the isotopic purity of the deuterated internal standard and adopts a protocol using a reasonable concentration of the internal standard. What are commonly observed include deviated qualifier ions ratios and significant quantitation errors when the concentration of the analyte in the test sample is significantly different from that in the calibration standard(s). We have conducted a limited evaluation on the extent of this effect and potential correction models [*4*]. Interested readers are referred to this articles for more details.

Summary

With its practically identical chemical properties and mass spectrometric fragmentation characteristics, a deuterated analog of the analyte (with at least three deuterium atoms) is an excellent candidate for use as an internal standard in GC/MS analysis. However, careful evaluation should be conducted prior to its adoption for routine use. Full-scan mass spectra of the analyte and the deuterated analog are compared for the preliminary selection of ions for SIM data collection—ions (of relatively high masses and intensities) that *appear* to be contributed by only one member of the analyte/deuterated analog pair. SIM data of these ions collected from *each* and the *mixture* of this pair are then evaluated to confirm that there is indeed no contribution (from the other member of the pair) to the ions designated for monitoring each member of the pair, and to determine the extent of the contribution if present. For those ions with cross contributions, SIM data are collected again (under a reduced chromatographic temperature) from the compound (analyte or deuterated analog) that makes the undesired contributions. The exact retention times are carefully examined to determine whether the cross contribution is caused by the presence of an isotopically impure component or due to the compound's

TABLE 2—*Interferences of intensity measurements for ions selected for SIM data acquisitions for pentobarbital and d_5-pentobarbital.*

Ion	Ion Chromatogram of d_0-Pentobarbital Area at Retention Time of		Ion Chromatogram of d_5-Pentobarbital Area at Retention Time of	
	d_0-Pentobarbital	d_5-Pentobarbital	d_0-Pentobarbital	d_5-Pentobarbital
Ions Designated for Pentobarbital				
169	3,452,749 (100%)	0 (0%)	3,739 (0.11%)[a]	66,935 (1.94%)[b]
184	2,556,427 (100%)	0 (0%)	991 (0.038%)[a]	42,698 (1.67%)[b]
185	304,178 (100%)	0 (0%)	0 (0%)	4,107 (1.35%)[b]
170	285,108 (100%)	0 (0%)	0 (0%)	44,941 (15.8%)[b]
Ions Designated for d_5-Pentobarbital				
189	0 (0%)[c]	0 (0%)	84,566 (4.61%)[d]	1,834,203 (100%)
171	33,321 (1.71%)[c]	0 (0%)	91,501 (4.70%)[d]	1,946,276 (100%)

[a]Quantities in this section represent the contribution of deuterated internal standard to the ion intensities measured for the analyte, caused by the presence of the analyte (impurity) in the deuterated internal standard. Percentages inside parentheses represent the degrees of interference, calculated by dividing the intensities contributed by the deuterated internal standard to that by the analyte. For example, 0.11% was obtained from dividing 3,739 by 3,452,749.

[b]Quantities in this section represent the contribution of deuterated internal standard to the ion intensities measured for the analyte, caused by fragmentation mechanism of the deuterated internal standard. This interference will be observed only when the analyte and the deuterated analog are not adequately resolved—which is normally the case. Percentages inside parentheses represent the degrees of interference, calculated by dividing the intensities contributed by the deuterated internal standard to that by the analyte. For example, 1.94% was obtained from dividing 66,935 by 3,452,749.

[c]Quantities in this section represent the contribution of the analyte to the ion intensities measured for the deuterated internal standard, caused by fragmentation mechanism of the analyte. This interference will be observed only when the analyte and the deuterated analog are not adequately resolved—which is normally the case. Percentages inside parentheses represent the degrees of interference, calculated by dividing the intensities contributed by the analyte to that by the deuterated internal standard. For example, 1.71% was obtained from dividing 33,321 by 1,946,276.

[d]Quantities in this section represent the interference caused by the presence of analyte (impurity) in deuterated internal standard. This interference will be observed only when the analyte and the deuterated internal standard are not adequately resolved—which is normally the case. Percentages inside parentheses represent the degrees of interference, calculated by dividing the intensities contributed by the analyte (impurity) to that by the deuterated internal standard. For example, 4.70% was obtained from dividing 91,501 by 1,946,276. See text for the unusually high percentage observed.

ion fragmentation characteristics. These procedures allow for the evaluations of the isotopic purity and the ion fragmentation characteristics of the deuterated analog to determine whether it can be used as the internal standard. Since *small* cross contributions normally do *exist* and the deuterated analog is used as the internal standard under this non-ideal situation, this approach is extremely useful in helping understand the limits of the quantitation methodology and why difficulties are observed under certain circumstances, for example, when the concentrations of the analog and the deuterated analog (internal standard) are extremely different [*6*].

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