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Isotopic Analogue as the Internal Standard for Quantitative Determination of Benzoylecgonine: Concerns with Isotopic Purity and Concentration Level

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ABSTRACT: Empirical data is used to demonstrate the observation and quantification of benzoylecgonine in negative test samples when high concentrations of deuterated benzoylecgonine are used as the internal standard in the assay process. On the quantitative determination of true positive samples, inaccuracy introduced by the isotopic impurity of the internal standard is calculated as a function of the impurity and the concentration levels of the internal standard used.

KEYWORDS: toxicology, benzoylecgonine, cocaine, internal standard, isotope dilution, isotopic purity, mass spectrometry, selected ion monitoring, stable isotope

The isotopic analogue of an analyte is often considered the most suitable internal standard for a quantitative analysis process and is widely used in the determination of drugs and drug metabolites in biological matrices. The similarities of the isotopic analogue and the analyte in their chemical properties and the mass spectrometric fragmentation process compensate for possible errors that may derive from the loss of the analytes in the sample preparation process and variations of gas chromatographic (GC) and mass spectrometric (MS) conditions. However, false identification or inaccurate quantification may result if the isotopic purity of the internal standard used is not at an acceptable level and data are not interpreted properly. This work illustrates an example of error of this source and the determination of the isotopic purity of d_3-benzoylecgonine used in urinalysis of the cocaine metabolite.

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Materials and Methods

The standard operating procedure of the U.S. Navy's Drug Screening Laboratories [1] is adopted for the extraction/derivatization of benzoylecgonine from urine samples and for the gas chromatographic and selected ion monitoring mass spectrometric analysis. Briefly, 3 mL of urine sample is buffered with ammonium hydroxide. Solid phase extraction is performed with the DuPont PREP Type W Extraction Cartridge (Wilmington, Delaware). The acetone/ethyl acetate eluate is then alkylated with tetramethylammonium hydroxide/trimethylphenylammonium hydroxide/dimethyl sulfoxide (TMAH/TMPAH/DMSO) and iodopropane. A Hewlett-Packard 5970B mass selective detector coupled to a 5890 series Hewlett-Packard gas chromatograph equipped with a 15-m by 0.251-mm inside diameter (0.25-μm film thickness) J & W DB-5 column (Folsom, California) is used for analysis. The injector temperature is maintained at 270°C and the collision energy is 70 eV. Ions at *m/z* 331, 210, and 272 (analyte) and at 334 and 213 (internal standard) are monitored. Ions at *m/z* 210 and 213 are used for quantification.

The amount of internal standard added to each sample is equivalent to 1500 ng/mL of d_3-benzoylecgonine. The concentration of the analyte in a specific sample is quantitated against a calibration standard which has a concentration of 150 ng/mL of benzoylecgonine.

Results and Discussion

Mass spectrometric and theoretical aspects pertaining to the use of stable isotope methodologies have been discussed in the literature [2-6]. On the practical aspect, one of the factors that is often considered in deciding the amount of the internal standard used in a specific assay process is the anticipated concentration level of the analyte in the test sample. If the concentration of the analyte in the sample is very high, the use of an insufficient amount of the internal standard may complicate the analytical process in at least two ways: (1) before GC/MS analysis, the extract may have to be reconstituted at such a volume that the concentration level of the internal standard is too low to generate significant ion intensity and (2) the contribution of the isotopic ions derived from the analyte may become significant enough to interfere with these ions derived from the internal standard. Since the concentration of benzoylecgonine in urine samples is often found at the 10 000-ng/mL level or higher, it is logical to use the internal standard at the 1 500-ng/mL level.

False Identification

The mass spectra of propyl derivatives of benzoylecgonine and its deuterated analogue are shown in Fig. 1. Because of the high concentration level of the internal standard used, *m/z* 331, 210, and 272 ions are observed in all negative samples. The source of these ions is attributed to the added internal standard and confirmed by the absence of these ions in samples in which the deuterated internal standard is not used. The concentrations of benzoylecgonine thus introduced by the addition of the internal standard are calculated as follows:

$$\frac{W}{(1500 = W)} = \frac{(\text{Ion int. of } m/z\ 210)}{(\text{Ion int. of } m/z\ 213)}$$

where W is the concentration of benzoylecgonine introduced by the addition of the internal standard.

Two lots of the deuterated internal standard are used in this study. The concentrations of benzoylecgonine thus calculated are 7.90, 7.07, 7.18, 6.73, 6.78, and 6.83 ng/mL in Lot A and 27.92, 28.47, 26.75, 27.94, and 28.86 ng/mL in Lot B. The respective averages derived

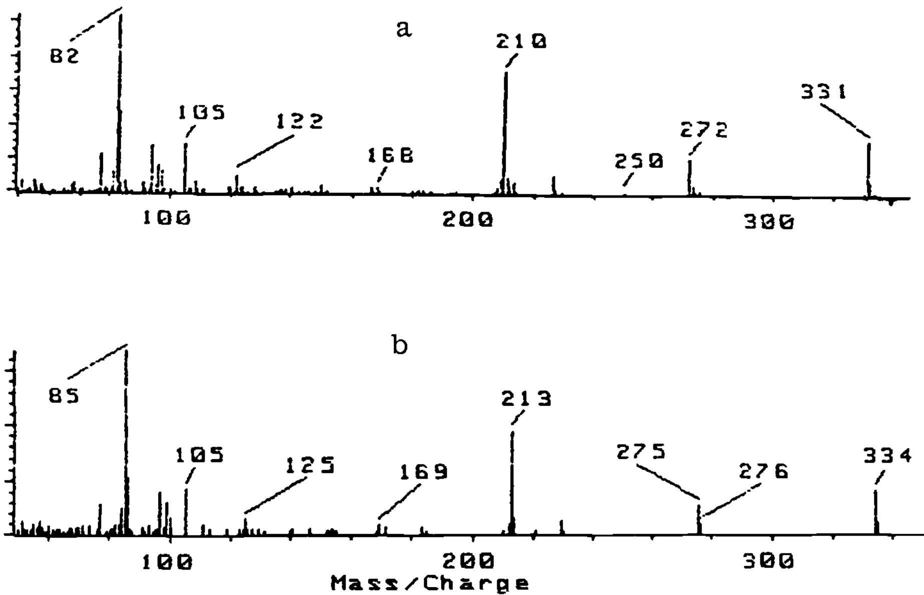

FIG. 1—*Mass spectra of propyl derivatives of benzoylecgonine* (a) *and* d_3*-benzoylecgonine* (b).

from these two lots are 7.08 and 27.99 ng/mL in the 3 mL of urine used for analysis. These values correspond to isotopic impurity of 0.472 and 1.87% in these deuterated internal standards.

Inaccurate Quantification

The concentrations of benzoylecgonine in positive samples are calculated, with and without the consideration of the levels of benzoylecgonine introduced to the samples, as follows:

Without the consideration of the isotopic impurity introduced:

$$\frac{(150 \text{ ng/mL})/(1500 \text{ ng/mL})_{\text{cal.std.}}}{(X \text{ ng/mL})/(1500 \text{ ng/mL})_{\text{sample}}} = \frac{(\text{Ion int. of } m/z \text{ } 210)/(\text{Ion int. of } m/z \text{ } 213)_{\text{cal.std.}}}{(\text{Ion int. of } m/z \text{ } 210)/(\text{Ion int. of } m/z \text{ } 213)_{\text{sample}}}$$

With the consideration of the isotopic impurity introduced:

$$\frac{(150 + W \text{ ng/mL})/(1500 - W)_{\text{cal.std.}}}{(X + W \text{ ng/mL})/(1500 - W)_{\text{sample}}} = \frac{(\text{Ion int. of } m/z \text{ } 210)/(\text{Ion int. of } m/z \text{ } 213)_{\text{cal.std.}}}{(\text{Ion int. of } m/z \text{ } 210)/(\text{Ion int. of } m/z \text{ } 213)_{\text{sample}}}$$

where X is the concentration of benzoylecgonine in the sample and W is the concentration of benzoylecgonine in the urine sample that was contributed as a result of the isotopic impurity of the internal standard.

Results thus calculated are tabulated in Table 1. These calculations experimentally evince the concern in using high concentration levels of isotopic analogues as internal standards. Quantitative evaluation of the results shown in Table 1 indicates that (1) no error will be introduced if the analyte concentration is at the exact level of the calibration standard; (2) a higher apparant result will be reported if the analyte concentration is below the concentra-

TABLE 1—*Quantitation error as a function of the isotopic impurity level in the internal standard and the relative analyte concentration.*[a]

Isotopic Impurity[b]	Apparant Concentration[b]	True Concentration[b]	% Error
	83.82	80.70	+3.87
	141.1	140.7	+0.284
	146.0	145.7	+0.206
7.080	147.1	147.0	+0.0680
	147.3	147.2	+0.0679
	148.1	148.0	+0.0676
	252.1	256.9	−1.87
	84.19	72.28	+16.5
	154.1	154.8	−0.453
27.99	155.5	156.6	−0.702
	156.8	158.0	−0.759
	161.7	163.9	−1.34
	273.5	296.6	−7.79
	288.9	314.6	−8.17
	292.5	319.1	−8.34
	293.9	320.7	−8.36
	298.3	326.0	−8.50

[a]Results were obtained based on a calibration standard of 150 ng/mL.
[b]In ng/mL.

tion of the calibration standard, while the opposite will result if the analyte concentration is above that of the calibration standard; and (3) the degree of the deviation of the apparent concentrations from that of the true concentrations depends on the level of the isotopic impurity of the internal standard used.

Conclusion

Empirical results presented in this study demonstrate that the observation of the analyte at a low level should be interpreted with caution. It is likely that a small amount of the analyte will be observed when a high concentration of the isotopic analogue is used as the internal standard. On the quantitative aspect, to minimize errors that are caused by the isotopic impurity of the internal standard, the calibration standard concentration should be as close to the analyte concentration as possible. The level of the isotopic impurity of the internal standard that may be used for a particular assay, without correction, depends on the acceptable percent error and the difference of the analyte concentrations in the sample and that in the calibration standard.

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