Intensity of the Internal Standard Response as the Basis for Reporting a Test Specimen as Negative or Inconclusive

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Abstract

Under normal circumstances, a test specimen is reported as “negative” when the response of the analyte is absent. However, if the intensity of the internal standard (IS) is low, indicating interference factors, the test could be considered “inconclusive.” A quantitative hypothesis, \( A = (R \times I \times S)/L \), serves as the “cutoff” for the acceptable signal-to-noise (S/N) ratio for the IS in making “negative/inconclusive” decisions, where \( A \) is the acceptable S/N ratio for internal standard; \( R \) is the relative response of the IS and the analyte (same concentration); \( I \) is the concentration of the IS; \( S \) is the (minimal S/N ratio); and \( L \) is the limit of detection. The hypothesis was empirically tested using the 9-carboxy-11-nor-\( \Delta^2 \)-tetrahydrocannabinol (THC-COOH) analyte, THC-COOH-d₄, IS, with ibuprofen and hydrogen peroxide (H₂O₂) as interference factors. Urine specimens containing 0–5 ng/mL of THC-COOH were spiked with various quantities of ibuprofen or H₂O₂, followed by liquid–liquid extraction, derivatization, and gas chromatography–mass spectrometry (GC–MS) analysis under selected-ion-monitoring mode. Among the “adulterated” test specimens evaluated (those with an S/N for the internal standard below the acceptable IS S/N “A”) the quantitative criterion was indeed found to provide a useful guide for making negative/inconclusive decisions. This equation could be programmed into the instrument software to flag results as being inconclusive when they do not meet the criteria described in this paper.

Introduction

Interference factors such as adulteration, substitution, or badly decomposed or contaminated postmortem specimens may result in poor recovery of the analyte and internal standard (IS), causing false-negative or inconclusive results in toxicology tests. Even under normal analytical conditions where the IS is poorly recovered, the substance may escape detection if it is present at or near the limit of detection (LOD). As the most commonly abused substance in the United States, marijuana (in the form of the commonly targeted metabolite, 9-carboxy-11-nor-\( \Delta^2 \)-tetrahydro-cannabinol, or THC-COOH) constitutes a substantial portion of positive specimens reported by forensic urine drug testing laboratories (1). Therefore, specimen adulteration (and substitution) activities have most commonly been associated with urine specimens derived from marijuana users. Approaches proven to be effective for the adulteration of THC-COOH-containing specimens include the addition of oxidants, such as hydrogen peroxide (H₂O₂) and pyridinium chlorochromate (2–4), bleach (hypochlorite) (5), nitrite or ammonia-containing substances (6), and ibuprofen, which may interfere with the derivatization of the analyte in the sample preparation process (7).

The most common analytical approach is to incorporate an isotopically-labeled analogue of the analyte as the IS, which serves well to identify false negative test results when the IS is absent and the analyte’s signal is absent. An unexpected low response from the IS, even in the absence of the analyte’s signal, is indicative of abnormal specimen conditions, and should alert the analyst to investigate further before issuing a “negative” or “inconclusive” report. However, lacking a quantitative tool, the analyst’s decision to issue a negative or inconclusive finding is often subjective in nature.

The objective of this study was to develop a quantitative criterion that would be helpful in making “negative/inconclusive” decisions. Parameters that are included in formulating the proposed acceptable (A) IS signal-to-noise (S/N) “cutoff” are: (i) the relative response (R) of the analyte and the IS when they are present at the same concentration; (ii) the concentration of the IS (I) added into the test specimen; (iii) the minimal signal-to-noise ratio (S) for an acceptable signal; (iv) the limit of detection (LOD) of the adapted analytical protocol. In developing an equation to quantitatively analyze the response of the IS for determining the reliability of the procedure to see the analyte at the LOD, we considered the

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following variables:

(i) (I) The amount of IS added to the specimen. The IS is normally added in much higher concentrations than the LOD of the procedure. Therefore, detection of the IS cannot be used to indicate that the analyte is truly negative at the LOD of the procedure. The response of the IS would be detected well after the analyte response at the LOD of the procedure dropped below detectable limits. (ii) (R) The relative response of the analyte to the IS. If the analyte has a greater response than the IS, the acceptable signal-to-noise ratio of the equation would need to be adjusted lower to account for the lower response of the IS. If the analyte has a lower response than the IS, the acceptable IS S/N should be adjusted higher. (iii) (S) The minimum acceptable S/N for designating a real response. Raising the minimum acceptable S/N for the procedure results in the acceptable IS signal-to-noise being adjusted higher. (iv) (L) The LOD for the procedure takes into account the concentration of the analyte that is detectable. A low LOD results in a higher acceptable IS signal-to-noise.

Laboratory studies were conducted adapting THC-COOH and THC-COOH-d₃ as the analyte and IS (4,8), with ibuprofen and H₂O₂ serving as the interference factors.

**Experimental**

**Chemicals and reagents**

Standard (THC-COOH, 100 µg/mL in ethanol) and deuterated IS (THC-COOH-d₃, 100 µg/mL in methanol) were obtained from Research Triangle Institute (Research Triangle Park, NC) and Radian (Cerilliant) Corporation (Austin, TX), respectively. The chemical derivatization reagent (iodomethane) was purchased from Ferak Berlin GmbH (Berlin, Germany). The sources of the interference reagents and extraction solvents were as follows: ibuprofen, Sigma-Aldrich (Steinern, Germany); H₂O₂, Nichon Shiyaku Industries (Osaka, Japan); tetramethylammonium hydroxide (TMAM), ACROS (Fair Lawn, NJ); n-hexane and ethyl acetate, Mallinkrodt Chemical (Phillipsburg, PA); dimethyl sulfoxide (DMSO) and glacial acetic acid, Panreac Quimica SA (Barcelona, Spain); potassium hydroxide, Katayama Chemical (Osaka, Japan). Other solvents were analytical or HPLC grade.

**Drug-free urine and “adulterated” urine test specimens**

Drug-free urine specimens were collected from laboratory co-workers, who signed an institutional consent form for their urine to be used in this experiment. This urine was confirmed negative for THC-COOH by gas chromatography–mass spectrometry (GC–MS). Urine specimens were filtered with 55-mm filter paper (Toyo Roshi Kaisha, Taito-Ku, Tokyo, Japan) upon collection and refrigerated.

Two categories of adulterated urine specimens were prepared by adding various amount of ibuprofen or H₂O₂ to arrive at desired concentrations of the adulterants (further described in the Result and Discussion section).

**Sample preparation and extraction**

Analytical procedures reported in the literature for the analysis of THC-COOH were slightly revised and adapted in this study. Specific procedures are described as follows. To a clean 16 × 100-mm borosilicate glass tube (Kimble; Vineland, NJ) was added 1 mL of urine sample (calibration standard or test specimen) containing a predetermined concentration of THC-COOH. The IS was added to each tube in the analytical batch using 15 µL working stock solution (1 µg/mL), with a final concentration of 15 ng/mL for the standards and test samples. The mixture was made alkaline with 200 µL of 10-N KOH and incubated for 20 min in a heating block device set at 60°C. The mixture was cooled to room temperature and adjusted to pH 3.5 ± 0.5 by adding 2 mL glacial acetic acid, followed by the addition of 2 mL n-hexane/ethyl acetate (9:1, v/v) mixture. The mixture was vortex-mixed and centrifuged at 2500 rpm (r = 16.5 cm) for 5 min. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 50°C (4,8–12). The extract residue was suspended in 100 µL of TMAH/DMSO (1:20) and vortex-mixed for 2 min, followed by the addition of 100 µL diluted iodomethane (1:50, v/v, in DMSO). The mixture was incubated at room temperature for 5 min, then acidified with 200 µL of 0.1-N HCl, and extracted with 1 mL n-hexane. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 50°C. Finally, the residue was reconstituted with cyclohexane. Typically, the reconstitution volume was 100 µL, while 1 µL was injected for GC–MS analysis (4,13,14).

**GC–MS analysis**

GC–MS analysis was performed on an Agilent 6980 GC interfaced to an Agilent 5975 MS (Agilent, Palo Alto, CA). A 12 m HP-5 crosslinked 5% phenyl methyl siloxane capillary column (200 µm i.d., 0.33 µm film thickness) from Agilent was used for this study. Helium carrier gas flow rate was set at 1.0 mL/min. The injector and GC–MS interface temperatures were maintained at 260°C and 250°C, respectively. The sample was introduced into the gas chromatograph in the splitless mode. The initial oven temperature was held at 150°C for 1 min, then raised to 270°C at 30°C/min, and held for 5 min. The final temperature was set at 300°C and held for 4 min to clean up the system before recycling back to the initial temperature for next injection. The following ions were selected to monitor methyl-derivatives of the analyte and the IS: m/z 313, 357, and 372 for THC-COOH; and 316, 360, and 375 for THC-COOH-d₃.
Table I. Ibuprofen Interference on GC–MS Analysis of THC-COOH

<table>
<thead>
<tr>
<th>Exp. set#</th>
<th>THC-COOH conc. (ng/mL)</th>
<th>Ibuprofen conc. (pg/mL)</th>
<th>Responses of the IS &amp; its background noise</th>
</tr>
</thead>
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<tr>
<td></td>
<td>IS*</td>
<td>max</td>
<td>min</td>
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<tr>
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<tr>
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</tr>
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<tr>
<td>III-3</td>
<td>1327</td>
<td>70</td>
<td>63</td>
</tr>
</tbody>
</table>

* Signal level (peak height).
1 S/N = signal-to-noise ratio
2 N: negative; I: inconclusive; P: positive.
3 Intensities of the ions designating the analyte and the IS were abundant and meet the ion intensity ratio required for “positive” designations.

**Calculation of S/N**

The IS peak height and average noise peak height were used to calculate the S/N (Figure 1). The average noise peak height is the average of the max noise + minimum noise peak height divided by 2, measured in the region adjacent to the IS peak. The noise peak height is affected by many variables, including dirty detector, column degradation, specimen matrix, carrier gas purity, and putrefaction of the specimen.

**Result and Discussion**

Under normal circumstances, a test specimen will be reported as “positive” when the analyte’s signal is above the detection limit, and “negative” when the analyte’s response is absent or at the noise level. However, if the intensity of the IS is critically low, indicating the presence of interference factors, the test should be considered “inconclusive,” even in the absence of the analyte’s signal. A quantitative criterion is developed to serve as the “cutoff” for making “negative/inconclusive” decisions when the response of the IS falls in the critical range. Specifically, the hypothesis states that when

\[
\text{S/N IS} > (R \times I \times S)/L\quad \text{Eq. 1}
\]

the specimen can be reported as “negative”; otherwise, the test should be considered “inconclusive,” where R is the relative response of the IS and the analyte (when present at the same concentration); I is the concentration of the IS; S is the 3 (the minimal S/N for an acceptable signal); and L is the limit of detection of the analytical protocol.

In this empirical study, ibuprofen and H₂O₂ were adapted as the interference factors and added into THC-COOH-containing urine specimens to create circumstances under which the signal levels of the analyte (THC-COOH) and the IS (THC-COOH-d₃) are at the critical levels, requiring a quantitative guide for making negative/inconclusive decisions. Ibuprofen and H₂O₂ interfere with the GC-MS assay of THC-COOH with different mechanisms. Specifically, ibuprofen competes with the analyte (and the IS) at the derivatization step, while H₂O₂ destroys the analyte (and the IS) through an oxidation reaction.
Table I. (continued) Ibuprofen Interference on GC–MS Analysis of THC-COOH

<table>
<thead>
<tr>
<th>Exp. set#</th>
<th>THC-COOH conc. (ng/mL)</th>
<th>Ibuprofen conc. (µg/mL)</th>
<th>Responses of the IS &amp; its background noise</th>
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<tr>
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</table>

* Signal level (peak height).†

S/N = signal-to-noise ratio.

N: negative; I: inconclusive; P: positive.

† Intensities of the ions designating the analyte and the IS were abundant and meet the ion intensity ratio required for “positive” designations.

Analytical parameters

For the purpose of this study, the concentration of the IS (I) was set at 15 ng/mL, as commonly adapted by urine drug testing laboratories, while the S/N for an acceptable signal is set at 3. A series of preliminary experiments (data not shown) were performed to establish the limit of detection of the adapted analytical protocol as 3 ng/mL and the relative response of the analyte (m/z 313) and the IS (m/z 316), when they are present at the same concentration, as 0.85. Additional series of preliminary studies indicated the best analyte (THC-COOH) concentration suitable for this study is at the 0–5 ng/mL range, while the amounts of the interference factors, ibuprofen and H2O2 (35% solution), are 350–450 µg/mL and 75–200 µL, respectively.

When the amounts of the interference factors are below the lower limits, the signals of the IS and the analyte are not significantly interfered; thus, a specimen containing the analyte above the LOD can be properly reported as being positive or negative, using the normal ion intensity ratio criteria. On the other hand, when the amounts of the interference factors are above the higher limits, the signal for the IS becomes absent, resulting in the test being listed as inconclusive, again without requiring any additional criterion in the decision-making process.

When the amounts of the interference factors are in the critical range (75–200 µL for H2O2 and 350–450 µg/mL for ibuprofen), the signals of the IS and the analyte fall in the critical range, requiring a quantitative criterion to make a negative/inconclusive interpretation of the test results. Specifically, the analytical parameters discussed previously allow for the calculation of the acceptable S/N for the IS.

\[ A = (R \times I \times S)/L = (0.85 \times 15 \times 3)/3 = 13. \]

Thus, when the S/N of the IS is greater than 13, the test result can be interpreted as N (negative), otherwise, as I (inconclusive). The following two sections will focus on the discussion of data collected when the amounts of the interference factors are in the critical range.

Ibuprofen study

Data from Table I clearly demonstrates the concentration of ibuprofen that may cause interference to the analytical protocol to fall between 350–450 ng/mL. With this information in mind, four sets of a five-concentration series of standards containing 0, 1, 2, 3, and 5 ng/mL THC-COOH (each with 15 ng/mL IS) were spiked with 0, 350, 400, and 450 ng/mL of ibuprofen. Data derived from this batch of the experiment are shown in Sections I-1 to I-4 in Table I. Intensity data shown in the table are the peak heights for the IS (m/z 316).

The S/N of the 0, 1, and 2 ng/mL standards in Sets 1 and 2 are larger than the critical value, \((R \times I \times S)/L = 13\), thus, they are correctly designated as “N.” For the 3 and 5 ng/mL standards, the three ions monitored for the analyte are at higher intensity level. The two ion intensity ratios are within ±20% of corresponding ratios observed in the calibration standard; thus, they are designated as “P.” Set I-3 concentrations all fell below the value calculated with the proposed equation and are designated as inconclusive. For the set of standards (Set I-4) containing 450 ng/mL ibuprofen, the ion intensities of the analyte and the IS are all at the noise level; thus, they were designated as “I.” These designations are based on the intensity of the IS; however, the value calculated by the equation would have come to the same conclusion.

Data derived from Sets I-3 and I-4 clearly indicate that the truly critical concentration of ibuprofen falls within the 400–450 ng/mL range. Thus, a second batch of standards containing 420 and 440 ng/mL of ibuprofen was prepared, and the experiment was conducted on a different day. The resulting data shown in Sets II-1 and II-2 (Table I) demonstrate the expected trend (i.e., as the concentration of ibuprofen increases, the standards with higher analyte concentrations are designated as “I”).

To further confirm the observed phenomenon, a third set of standards with ibuprofen concentrations at 300, 340, 360, 380, and 400 ng/mL was studied on yet another day. Data shown in Sets III-1, III-2, III-3, and III-4 (Table I) nicely agree with the data observed in Set I, except for the data from Set III-1 at 0 ng/mL THC. It appears that, in that specimen, unknown factors caused a significant decrease in the IS response relative to all of the other specimens in Set III-1. The equation did, however, correctly identify this specimen as inconclusive because the IS was not significantly recovered to ensure that the analyte of interest would have been detected if it had been present at the LOD.

Sets I-3 and III-5 had the same concentration of interfering substance (400 µg/mL ibuprofen); however, Set III-5 had a much lower S/N. This demonstrates the importance of taking into consideration the S/N when calculating the acceptable recovery of the IS for the identification of the analyte of interest at the LOD of the procedure.

Hydrogen peroxide study

Several sets of standards containing 0, 1, 3, and 5 ng/mL of
THC-COOH included in this study were spiked with 0, 75, 100, 150, 200, and 300 μL of H2O2 solution (35%). During the preliminary phase of this study, H2O2 was added into these standard solutions immediately following their preparation. These “adulterated” standards were then stored for later analysis. The IS was added when the analytical batches were assembled as practiced in a normal analytical protocol. Data derived from this standard protocol (in terms of the timing in adding the IS) were found sporadic. It was concluded that the reaction between the analyte and H2O2 had occurred prior to the addition of the IS, and thus was not suitable for the intended study.

An alternate approach was then adapted in which H2O2 was added into the standard solution after the addition of the IS, allowing the analyte and the IS to react competitively with the adulterant. Data shown in Table II demonstrates that, as the amount of the H2O2 solution increases from 75 μL (Set 2) to 200 μL (Set 5), the standards containing increased concentrations of the analyte are designated as “I.” These designations are based on the quantitative criterion proposed in equation 1.

**Conclusions**

The validity of the proposed quantitative criterion for designating a test result as “negative” or “inconclusive” in the event of poor recovery of the IS has been demonstrated by the ibuprofen and H2O2 studies. In the absence of an interfering substance, the equation correctly identified all true negatives and positives. The equation did properly identify several false negative results for concentrations at or above the 3 ng/mL LOD of the procedure when the interfering substance was in the critical range. It is interesting to note in data set III (concentrations 0 to 1 ng/mL), where the equation showed true negative results, that an analyst using subjective judgment may have incorrectly designated these true negative results as inconclusive due to the poor recovery of the IS. It could be incorrectly argued in some of the data presented that some of the 0 ng/mL–2 ng/mL concentrations were incorrectly identified as inconclusive when they were, in fact, negative. However, it is important to realize that the analyst does not know the starting concentration and can only rely on the IS response in making a decision as to whether the test would have detected the analyte at the LOD of the procedure. The equation provided has proven very reliable in quantitatively establishing whether the recovery was sufficient to call a specimen a true negative.

This same equation would prove extremely valuable in the analysis of postmortem specimens where the specimens are contaminated or putrefied, leading to large backgrounds and poor recovery of the IS.

The equation was developed to take into consideration the use of isotopic IS or non-isotopic IS. However, the authors’ experiments were limited to isotopic IS and further research will be when needed to prove how effective the proposed equation would be using a non-isotopic IS. If a non-isotopic IS is used, this equation should still be applicable for evaluating the probability of seeing the analyte of interest at the LOD of the procedure given a known R for the IS and analyte. It is very important, when using a non-isotopic IS, that R be calculated after the specimen has been extracted to take into consideration differences in extraction efficiency for the IS and analyte.

**Table II. Interference of H2O2* on GC-MS Analysis of THC-COOH**

<table>
<thead>
<tr>
<th>Exp. set #</th>
<th>THC-COOH conc. (ng/mL)</th>
<th>H2O2 μL</th>
<th>Responses of the IS &amp; its background noise</th>
<th>Result (N/I/P)</th>
</tr>
</thead>
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<td></td>
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<td>S/N</td>
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<tr>
<td>5</td>
<td>0</td>
<td>300</td>
<td>225</td>
<td>402</td>
</tr>
</tbody>
</table>

* H2O2: concentration 35%.
* Signal level (peak height).
* S/N = signal-to-noise ratio.
* N: negative; I: inconclusive; P: positive.
* Intensities of the ions designating the analyte and the IS were abundant and meet the ion intensity ratio required for “P”.

**Acknowledgments**

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**References**

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