An empirical study on the selection of analytes and corresponding cutoffs for immunoassay and GC–MS in a two-step test strategy—buprenorphine example

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(i) Standard solutions of buprenorphine (B) and three metabolites; (ii) immunoassay (IA) reagents designed for the analysis of B and/or its metabolites; and (iii) clinical urine specimens collected from patients (under B-treatment), constitute the B-System for fundamental study of parameters critical to the two-step test strategy, an analytical approach designed for a high-volume testing environment. The cross-reacting characteristics of IA reagents were examined using standard solutions of B and its metabolites. Resulting data were used as the basis for selecting target analytes suitable for the preliminary and the confirmatory test steps. Test data derived from IA and GC–MS analysis of clinical urine specimens (with natural distribution of B and its metabolites) were quantitatively correlated. Correlation parameters were examined: (i) to verify whether the analyte-pair targeted by the IA and GC–MS test steps has been properly selected; and (ii) to decide on appropriate cutoffs for the two test steps. In conclusion, this study has demonstrated that the most effective analyte(s) that should be targeted in the GC–MS determination step vary with the IA selected in the preliminary test step. All analytes that generate significant responses to the IA reagent should be targeted in the GC–MS test step.

Introduction

The need for, and prevalence of, workplace drug-testing programs mandates the development of an effective two-step test strategy, using different methodologies, such as immunoassay (IA) and gas chromatography–mass spectrometry (GC–MS), to cope with the high-volume test environment and to minimize the risk of test error. Successful implementation of this approach relies on the establishment of a reasonable correlation between the preliminary and the confirmatory test data and the selection of an appropriate cutoff for each test step. Correlation studies on test data derived from IA and GC–MS were mostly qualitative and reported by IA reagent manufacturers in their product package inserts. These studies place emphasis on the consistency of the positive/negative test results derived from these two test steps. Nevertheless, quantitatively correlating the IA’s apparent analyte concentration to the analyte concentration as determined by GC–MS can be more informative and valuable. Specifically, parameters derived from the latter correlation study can help select: (i) the most suitable analyte(s) to be targeted in the two test steps; and (ii) the most effective IA cutoff for a specific GC–MS cutoff adopted by the test protocol. (In addition to technical considerations, test objective, drug metabolism, and potential interpretations of positive/negative test results are also important issues to be addressed while selecting the targeted analyte(s) and the cutoff for the GC–MS test step.)

Fundamental parameters shaping quantitative correlation of the IA and GC–MS test data are: (i) cross-reacting characteristics of the selected IA toward metabolites that are not targeted in the GC–MS determination step; and (ii) the distributions of these metabolites in the test specimen population. Only with a reasonable data correlation and a cutoff corresponding well to the cutoff deemed appropriate for the GC–MS methodology, can the IA serve as an effective preliminary test method in a two-step test protocol. Buprenorphine (B), its metabolites, IA reagents from various sources, and urine specimens collected from patients under B-treatment constitute a unique system suited for this study as further described below.

Buprenorphine, a semi-synthetic opioid derived from the baine, is a powerful analgesic exhibiting both partial agonist activity at the μ-opioid receptor and antagonist activity at the κ-opioid receptor. Having long been prescribed for pain relief and anaesthetic induction, B has been adopted as a substitution agent for managing opiate-dependent individuals in France (1966), the US (2002), and Taiwan (2006). Yet, significant numbers of B-related fatalities have been reported in France and elsewhere. Since the use of B as a substitution drug is a very recent event (especially in non-European countries), only a few IA reagents designed for the analysis of B and/or its metabolites...
are available. Literature reports addressing these IAs are very limited in number, scope, and depth. Specifically, we could find only six articles on this subject matter. Three of these articles limited their studies to one single microplate reagent each.6-8 Two articles studied an analyzer-based reagent without,9 and with,10 comparison to a microplate reagent. The sixth article was on a newly developed product.11 On the contrary, IA study on methadone (a drug with a much longer history for treating heroin addiction) has been much more thorough.12-17 It has also been noted that GC–MS analysis of B-related compounds18-22 is not as well studied as the application of the more advanced liquid chromatography–tandem mass spectrometry (GC–MSMS) instrumentation to B-associated pharmacokinetic studies.23-34

With this background in mind, we have selected the B-System (B and metabolites, IA reagents, and clinical urine specimens) to study parameters (such as cross-reacting characteristics, target analyte selection, and correlation of IA/GC–MS test data) that are critical to the development of a successful two-step test strategy. Fully understanding the parameters’ role in a two-step test strategy is enlightening on its own; specific information derived from this exemplar system will also facilitate the development and implementation of testing programs helpful to monitoring compliance, assessing treatment effectiveness and deterrence function, important components of the B-treatment program.

Experimental

Immunoassay reagents, standard, and specimens

Four microplate B ELISA (enzyme-linked immunosorbent assay) kits and an instrument-based enzyme immunoassay (EIA) kit included in this study were from Diagnostix Corp. (Mississauga, ON, Canada); Immunalysis Corp. (IMS: San Dimas, CA, USA); International Diagnostic Systems Corp. (IDS: St. Joseph, MI, USA); Neogen Corp. (Lexington, KY, USA); and Microgenics’s analyzer-based CEDIA (cloned enzyme donor immunoassay) for B (Fremont, CA, USA).

B, norbuprenorphine (NB), buprenorphine-d1 (B-d1), norbuprenorphine-d3 (NB-d3), B-3-β-δ-glucuronide (BG) and NB-3-β-δ-glucuronide (NBG) (in 1 or 0.1 mg ml−1 methanol solutions) were from Cerilliant Corp. (Austin, TX, USA). The derivatization reagent, acetic anhydride (AA), was from Finechem (Wellington, Auckland, New Zealand). Liquid–liquid extraction kits (Toxi-Tubes A) and solid-phase extraction cartridges (Bond Elut C18 SPE, 200 mg, 40 Å) were obtained from Varian (Walnut Creek, CA, USA). β-Glucuronidases (Helix pomatia) are obtained from Sigma Aldrich Fine Chemicals (Saint Louis, MO, USA).

Clinical urine specimens came from the B-treatment program, which has been instituted following the IRB protocol of Taipei City Hospital Songde Branch (Taipei, Taiwan). All specimens were kept frozen until analysis.

Microplate ELISA procedure

Assay procedures provided by respective ELISA kit manufacturers were followed and briefly outlined in Table 1. Dose–response calibrations were established using a series of standard solutions with predetermined concentrations. These standards were prepared by diluting the standards provided by the ELISA reagent manufacturers or standard compounds from Cerilliant. Buffer (provided by ELISA reagent manufacturers) or drug-free urine were used as the matrix for the preparation of standard solutions. For cross-reactivity studies, standards were diluted to preset concentrations and tested. The observed concentrations were then divided by the targeted concentrations to derive the test compounds’ cross-reacting characteristics toward the ELISA kits examined. For the assay of clinical specimens, all specimens were diluted with drug-free urine by predetermined factors.

Table 1 Testing procedures and parameters for microplate ELISA from different manufacturers

<table>
<thead>
<tr>
<th>Step</th>
<th>Neogen</th>
<th>IDS</th>
<th>IMS</th>
<th>Diagnostix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load sample/µl</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Add drug-enzyme/µl</td>
<td>180</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Incubation/min</td>
<td>45</td>
<td>30</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Add wash buffer/µl</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Add substrate/µl</td>
<td>150</td>
<td>150</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>Incubation/min</td>
<td>30</td>
<td>15</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Add stop solution/µl</td>
<td>50</td>
<td>150</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>Read wavelength/nm</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
</tbody>
</table>

Instrument-based EIA procedure

The CEDIA B assay is intended for use on automated clinical analyzers. The analyzer should be capable of maintaining a constant temperature, pipetting, mixing reagent, measuring enzymatic rates at an absorbance of 660 nm, and timing the reaction. The test was performed on a Hitachi 7180 analyzer at Fooyin Hospital (Tong Kong, Pingtong Hsien, Taiwan), using the test protocol supplied by Microgenics. The concentrations of calibration standards were 0, 5, 20, 50, and 75 µg l−1.

Extraction and derivatization procedure for GC–MS analysis35,36

For liquid–liquid extraction a 1 ml urine sample was added to a Toxi-Tubes A and brought up to 5 ml with double-distilled water. After 10 min mechanical shaking, the mixture was centrifuged at 2500 rpm for 10 min. The organic layer was transferred into a clean 16 × 100 mm glass tube and dried under a slow stream of nitrogen at 50 °C.

The dried residue was re-dissolved in 100 µl AA, vortex mixed, capped, and incubated at 80 °C for 20 min. The reaction mixture was dried at 65 °C in a heating block under a slow stream of nitrogen. The residue was re-dissolved in 100 µl ethyl acetate and 1 µl was used for GC–MS analysis.

For the collection of standard spectra and the evaluation of ion intensity cross-contribution data, 10 µl of the analyte and internal standards were individually placed into 16 × 100 mm glass tubes and dried under a stream of nitrogen at 50 °C. The same derivatization procedure described above was then followed.

Hydrolysis procedure for the analysis of total B and NB35

Each urine specimen (or BG and NBG standards) was aliquoted into a 15 ml centrifuge tube, followed by the addition of 1 ml 1.0 mol l−1 acetate buffer and 1000 fishman units of β-glucuronidase (100 µl 10 000 fishman units ml−1). The samples were...
capped and incubated at 60 °C for 4 h. This condition has been found effective for complete hydrolysis of BG and NBG.  

**GC–MS analysis**

An Agilent 6890N gas chromatograph/5975 mass selective detector system operating at 70 eV with ion source temperature set at 230 °C was used for this study. The gas chromatograph was equipped with a 12 m HP-5 (Wilmington, DE, USA) capillary column cross-linked 5% phenyl methyl siloxane with 200 μm I.D. and 0.33 μm film thicknesses. The injector temperature and GC–MS interface temperature were maintained at 280 °C. The sample was introduced into the gas chromatograph in splitless mode and the helium carrier gas flow rate was set at 1.2 or 1.0 ml min⁻¹.

Our earlier studies have concluded that derivatization with AA produced the best results for the analysis of B and NB. For the analysis of the acetyl-derivatives, the initial oven temperature was held at 200 °C for 1 min, then raised to 300 °C at 30 °C min⁻¹, and held for 5 min. Full-scan mass spectra of B and NB and their deuterated analogs (all as acetyl-derivatives) are shown in Fig. 1. Data derived from these full-scan mass spectra were used to select the following ions for designating B/d₄ and NB/d₃: m/z 420, 408, 452/424, 412, 456 and m/z 440, 441, 422/443, 444, 425. The ions in bold were used for quantitation.

**Results and discussion**

Immunoassays for the analysis of B (and/or its metabolites), designed in the ELISA (microplate) format, are available from Diagnostix, IMS, IDS, and Neogen. The chemical analyzer-based reagent is from Microgenic. While IAs in ELISA format require minimal instrumentation, the analyzer-based format is more suited to automation and can become more cost effective under a high-volume test environment. Both formats have their merits under different testing environments.

In this study, standard solutions of B and related compounds were prepared to first examine the fundamental characteristics of these IAs. Clinical urine specimens were then used to evaluate how the responses derived from these IAs could be best related to the concentrations of specific B and/or its metabolites as determined by GC–MS. Analytical findings are interpreted and applied to selecting the most suitable targeted analytes and corresponding cutoffs for the IA and the GC–MS test steps in the intended application.

**Cross-reacting characteristics toward B metabolites and common opioids**

Reagent package inserts from the manufacturers include comprehensive lists of compounds toward which no

![Fig. 1 Mass spectra of acetyl-derivative of B (a)-1 and B-d₄ (a)-2; and di-acetyl-derivative of NB (b)-1 and NB-d₃ (b)-2.](image-url)
cross-reactivity was observed. However, these inserts provide minimal and, in some cases, inconsistent cross-reactivity data toward B and its metabolites. Thus, we have conducted a series of studies using a common set of standards at several concentration levels. Data derived from our study are included in the upper part of Table 2, while those provided by the manufacturers (wherever available) are included in the footnotes. The three known B metabolites and opioids, that are most likely to present in the specimens collected from patients under B-treatment, are included in the cross-reactivity study.

All reagents adopt B as the target analyte (100% cross-reactivity). Cross-reacting data shown in Table 2 were calculated the same way as reported by Zeck et al., except that immunological HPLC was used in their study to separate the analytes. Thus, chromatogram peak areas resulting from UV detection were used to derive the cross-reactivity data. While in this current study, individual solutions for compounds of interest were prepared, the absorbance data resulting from the IA protocol for each solution were used to derive the observed concentration that is equivalent to B.

Similar to data compiled for various drugs and IA reagents from various manufacturers, the cross-reactivity data summarized in Table 2 also reveal the concentration-dependent characteristic. Thus, it is critical to use the same set of standards when evaluating the cross-reactivity characteristics of IA reagents from different sources or lots. Therefore, cross-reactivity data should be presented along with the concentration under which these data were observed. Since only one measurement was conducted at each concentration, no precision data can be derived. However, these data clearly indicate that the reagent from IMS shows significant response toward NB, while all other reagents show significant responses toward BG. This observation implies that the most effective analyte(s) that should be targeted in the GC–MS determination step vary with the IA reagent selected in the preliminary test step. Specifically, B and NB should be targeted in the GC–MS test step if ELISA from IMS is selected; on the other hand, total B (B plus BG) should be the targeted analyte when IAs from the other sources are selected. This assertion is supported by data derived from clinical urine specimens as further discussed in a later section.

All microplate ELISA reagents show minimal cross-reactivity toward common opioids, allowing them to be used for compliance monitoring associated with the B-treatment program. It should be noted, however, that the analyzer-based Microgenec product reportedly exhibited some levels of cross-reactivity toward methadone, codeine, dihydrocodeine, morphine, and

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**Table 2** Cross-reactivities\(a\) (in %) of immunoassays to major B metabolites and selected opioids at various concentration levels (\(\mu g l^{-1}\))

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Conc./(\mu g l^{-1})</th>
<th>CEDIA(^b)</th>
<th>IDS-B</th>
<th>Neogen(^c)</th>
<th>Diagnostix</th>
<th>IMS(^d)</th>
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<tbody>
<tr>
<td>BG</td>
<td>3</td>
<td>133</td>
<td>—</td>
<td>161</td>
<td>71.2</td>
<td>15.7</td>
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<tr>
<td></td>
<td>5</td>
<td>140</td>
<td>70.2</td>
<td>127</td>
<td>32.8</td>
<td>—</td>
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<td></td>
<td>10</td>
<td>134</td>
<td>44.8</td>
<td>72.9</td>
<td>26.1</td>
<td>2.3</td>
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<td></td>
<td>15</td>
<td>127</td>
<td>34.2</td>
<td>53.4</td>
<td>32.6</td>
<td>—</td>
</tr>
<tr>
<td>NB</td>
<td>3</td>
<td>3.3</td>
<td>16.3</td>
<td>2.4</td>
<td>14.3</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.0</td>
<td>14.9</td>
<td>1.5</td>
<td>12.5</td>
<td>157</td>
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<tr>
<td></td>
<td>10</td>
<td>5.0</td>
<td>10.2</td>
<td>1.2</td>
<td>7.7</td>
<td>147</td>
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<tr>
<td></td>
<td>15</td>
<td>4.0</td>
<td>8.5</td>
<td>0.8</td>
<td>5.8</td>
<td>—</td>
</tr>
<tr>
<td>NBG</td>
<td>3</td>
<td>13.3</td>
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<td>14.3</td>
<td>9.09</td>
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<td></td>
<td>5</td>
<td>10.0</td>
<td>10.1</td>
<td>1.30</td>
<td>9.88</td>
<td>5.98</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.0</td>
<td>5.88</td>
<td>1.00</td>
<td>4.75</td>
<td>4.06</td>
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<tr>
<td></td>
<td>15</td>
<td>2.0</td>
<td>5.56</td>
<td>0.80</td>
<td>3.82</td>
<td>—</td>
</tr>
<tr>
<td>Morphine</td>
<td>5</td>
<td>—</td>
<td>1.73</td>
<td>0.19</td>
<td>0.29</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>—</td>
<td>0.99</td>
<td>0.15</td>
<td>0.13</td>
<td>1.11</td>
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<tr>
<td></td>
<td>1000</td>
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<td>0.01</td>
<td>0.00</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>Codeine</td>
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<td>0.15</td>
<td>0.35</td>
<td>2.29</td>
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<tr>
<td></td>
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<td>—</td>
<td>1.06</td>
<td>0.21</td>
<td>0.19</td>
<td>0.91</td>
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<tr>
<td></td>
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<td>—</td>
<td>0.02</td>
<td>0.00</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>Hydrocodone</td>
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<td>0.91</td>
<td>0.14</td>
<td>0.39</td>
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<tr>
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<td>—</td>
<td>0.01</td>
<td>0.00</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>5</td>
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<td>1.72</td>
<td>0.52</td>
<td>0.32</td>
<td>2.97</td>
</tr>
<tr>
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<td>10</td>
<td>—</td>
<td>0.88</td>
<td>0.15</td>
<td>0.20</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>—</td>
<td>0.01</td>
<td>0.00</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>Naloxone</td>
<td>5</td>
<td>—</td>
<td>1.56</td>
<td>0.17</td>
<td>0.33</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>—</td>
<td>0.46</td>
<td>0.10</td>
<td>0.12</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>—</td>
<td>0.01</td>
<td>0.00</td>
<td>—</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^a\) B is used as the target compound (100% cross-reactivity) for all reagents. \(^b\) Reagent insert provides the following cross-reactivities toward buprenorphine glucuronide: 98.0 and 97.0% at 5 and 20 \(\mu g l^{-1}\), respectively. \(^c\) Reagent insert provides 0.82% cross-reactivity toward NB at 59.8 \(\mu g l^{-1}\). \(^d\) Reagent insert provides the following cross-reactivities toward NB: 80, 120 and 120% at 1, 5 and 10 \(\mu g l^{-1}\), respectively.
morphine-3-glucuronide,\textsuperscript{9,10} and other opioids.\textsuperscript{10} At a concentration of 1500 µg ml\textsuperscript{-1}, methadone, codeine, dihydrocodeine, morphine, and morphine-3-glucuronide generate responses equivalent to approximately 80, 55, 34, 17, and 15 µg l\textsuperscript{-1}, respectively, of B.\textsuperscript{9} It was further pointed out that if the cutoff for the detection of B is set too low, false positives may result when the Microgenic product is used to test urine samples collected from patients using morphine as the substitution drug (as practised in Austria)\textsuperscript{9} and patients receiving prescribed dihydrocodeine.\textsuperscript{10}

**Characteristics of dose–response curves**

The same set of B standard solutions was used to examine the dose–response characteristics of the IAs studied. Fig. 2 shows the resulting dose–response curves exhibited by the ELISA reagents, while the corresponding curve for CEDIA is shown in Fig. 3. While converting the concentration in the ELISA plots to log-scale generates a nearly linear dose–response relationship up to approximately 7.5 µg l\textsuperscript{-1} (curves II in Fig. 2), the usable ranges for the reagents in ELISA format are significantly narrower than

![Fig. 2 Dose–response characteristics of four microplate ELISA kits: (a) Neogen, (b) IDS-B, (c) IMS, (d) Diagnostix.](image-url)
the analyzer-based CEDIA reagent (Fig. 3). With limited calibration ranges, obtaining semi-quantitative concentration data would require sample dilution for most specimens. Indeed, a study using the IMS ELISA reagent recommended the use of a 10-times dilution of urine sample for the analysis.

Correlations of IA apparent versus GC–MS B concentrations

Selection of samples for correlation studies. The nature of samples selected for the correlation studies is crucial. First, samples have to be true clinical samples, with the distribution of metabolites representative of user excretion patterns. Using controls that are spiked with the analyte alone does not take into account the effect of the IA’s cross-reacting characteristics on the parameters evaluated. Second, samples with no analyte or high concentration levels of the analyte are not good candidates either. The inclusion of true negative samples (0 ng ml\(^{-1}\) of analyte) in the study will generate much better correlations but will reduce the potential differentiation of the test methods compared. Samples with high concentration levels of the targeted analyte (and associated metabolites or parent drugs) will generate IA results that are out of the methods’ dynamic ranges. Thus, ideal samples used for correlation studies should be true clinical samples in which the concentrations of the targeted analyte are near the GC–MS cutoff level. Data generated from these samples are most suitable for the correlation of IA apparent analyte concentration with the concentration of a specific analyte (determined by a GC–MS protocol).

With these considerations in mind, a set of 33 clinical specimens, collected from patients under B-treatment, were tested using the IAs studied. These specimens were also analyzed by the GC–MS procedures established in our earlier studies.\(^{35,36}\) They have also been diluted to the dynamic range of the IAs prior to IA analysis. Since IAs are responsive to compounds structurally related to B, while GC–MS analysis is specific to B (and other metabolites when targeted), correlation of IA and GC–MS data is not expected to achieve the same level of “closeness” as dose–response curves derived from calibration standards. They are, nevertheless, extremely valuable in assessing how the responses of IAs should be interpreted.\(^{40}\)

Selection of appropriate GC–MS analytes for correlation with IA data. Cross-reacting characteristics shown in Table 2 indicate that IA responses derived from different reagents vary and perhaps can be best related to the concentrations of different analytes as determined by GC–MS. This presumption was further studied as follows. First, with IMS showing minimal and high cross-reactivities toward BG and NB, respectively, one would expect that IMS’ response can be better correlated to the concentration of “B + NB” (both analytes in their free forms). Indeed, IMS’ response cannot be reasonably correlated to the concentration of “total B” (as shown in Fig. 4(a)), i.e., specimens with significant differences in their IA responses exhibit similar concentration of “total B”. Alternatively, interpretable correlation (as shown in Fig. 4(b)) is observed when the concentration of “total B” is replaced by the concentration of “B + NB”.

On the other hand, responses derived from the other three ELISA (Diagnostix, IDS, and Neogen) and the instrument-based (CEDIA) reagents correlate well to the concentration of “total B” as determined by GC–MS (Fig. 5). It thus becomes clear that the most appropriate analytes to be targeted by the GC–MS step depend on the IA reagent adopted in the first (preliminary) test step. Specifically, B and NB should be targeted by GC–MS analysis when the IMS reagent is selected; conversely, GC–MS analysis should target “total B” when the other four IA reagents are selected. This observation clearly reflects differences in the cross-reacting characteristics of the antibodies used in these two groups of IA reagents.

Comparison of correlation parameters derived from IAs with appropriate analytes targeted by GC–MS. The correlation plots shown in Fig. 5 indicate that responses derived from these four IA reagents can all be correlated to the concentration of “total B” as determined by GC–MS. These “IA apparent B concentration” vs. “GC–MS total B concentration (B plus BG)” plots shown in Fig. 5 reveal the following information. The Neogen product

**Fig. 4** Correlation of IMS “IA apparent B” vs. (a) “GC–MS total B” and (b) “GC–MS free B plus free NB” concentrations.
Fig. 5 Correlation of “IA apparent B” vs. “GC–MS total B” concentrations with reagent from (a) Diagnostix, (b) IDS-B, (c) Neogen and (d) Microgenic.

(Fig. 5(c)) exhibits the highest sensitivity (steeper slope of the correlation curve) among the three microplate reagents studied. Higher sensitivity implies the reagent’s ability to better differentiate specimens with slight difference in total B concentrations. On the other hand, the IDS product (Fig. 5(b)) exhibits the highest responses, indicating its ability to reach a lower detection limit; the low slope reflects this reagent’s lower sensitivity toward concentration change. The responses of the Diagnostix product (Fig. 5(a)) fall in between both parameters.

The resulting correlation equations (Fig. 5) also project varied responses from different IAs for a urine specimen containing the same amount of total B (as determined by GC–MS). For example, for a specimen containing 5 µg l⁻¹ of total B, the responses generated by the Diagnostix, IDS, and Neogen products would be equivalent to 7.4, 8.2, and 7.3 µg l⁻¹, respectively, of apparent B.

Selection of corresponding cutoffs for the IA and the GC–MS test steps. With a two-step test protocol, the cutoff concentration adopted for an IA should correspond well with the cutoff concentration of the compound targeted by GC–MS. If an inappropriately low IA cutoff value is adopted, an excess number of negative GC–MS results will be reported, causing the overall analytical procedure to be financially inefficient. On the other hand, if the preliminary test cutoff value is set too high, an excessive number of positive samples may be rejected as negatives in the preliminary screening process without being submitted to the GC–MS test step, causing the testing program to be technically inefficient.

Data derived from the instrument-based CEDIA reagent on a group of 58 clinical specimens (Table 3) is adopted to illustrate the point mentioned above. For example, if 25 µg l⁻¹ (total B) is selected as the cutoff for the GC–MS test, 35 µg l⁻¹ (apparent B) can serve well as the cutoff for the CEDIA reagent, because all specimens tested positive by CEDIA are also tested positive by GC–MS, and one specimen tested negative was found to be slightly above the GC–MS cutoff concentration. On the other hand, if 50 and 60 µg l⁻¹ are selected as the cutoffs for the GC–MS and the CEDIA test steps, the CEDIA test data would generate one false positive and one false negative.
Thus, the following observations have demonstrated potential application of this reagent to a high-volume test environment: (i) its performance characteristics derived from standard solutions; (ii) the test data obtained from the limited number of clinical specimens; and (iii) the consistent positive/negative readings resulting from specimen test data with selections of exemplary sets of cutoff values. However, before a specific analyte-pair and a specific set of cutoff values (for IA and GC–MS) are selected for a specific testing program, data derived from a larger specimen population and issues related to the analytes’ metabolism status and the underlying test policy should be fully addressed.

Conclusions

The B-System (standard solutions of B and its metabolites, IA reagents designed for the analysis of B and/or its metabolites, and clinical urine specimens collected from patients under B-treatment) serves as an effective model for fundamental study on parameters critical to the two-step test strategy. This study has demonstrated that the analyte(s) targeted in the GC–MS test step should include all analytes that generate significant responses to the IA reagent. For the exemplary system adapted in this study, analyzer-based CEDIA and three ELISA products (from Diagnostix, IDS and Neogen) generate responses that can be favourably correlated to the total B (B + BG) concentration as determined by GC–MS; while the response generated by the ELISA reagent from IMS correlates more favourably to the concentration of “B + NB” as determined in the GC–MS test step.

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