Comparison of Drugs of Abuse Detection in Meconium by EMIT® II and ELISA

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Abstract

The results of meconium specimens and fortified samples screened for drugs of abuse by both enzyme multiplied immunoassay technique (EMIT® II) and enzyme-linked immunosorbent assay (ELISA) methods were compared. The sample preparation for the ELISA screen was a simple buffer extraction versus a lengthy and more laborious sample preparation procedure for the EMIT II screen. The ELISA method was automated using a TECAN Genesis. The EMIT II analysis was automated with an Olympus AU400e. The opioid screen was calibrated with hydromorphone and the benzodiazepine screen was calibrated with clonazepam to maximize detection for these analytes. Previously validated gas chromatography–mass spectrometry (GC–MS), two-dimensional GC–MS, or liquid chromatography–tandem MS methods were used for confirmation. Results from the two techniques compared well. Agreement of the ELISA assay was greater than 90% when compared to EMIT II for all drug classes except barbiturates and benzodiazepines. ELISA appears to be more sensitive than EMIT II for the detection of amphetamines, methadone, propoxyphene, and cocaine. ELISA compared well to EMIT II for cannabinoids, opioids, and PCP. Specificity of the ELISA assay was slightly better for PCP and opioids. EMIT II appears to be more sensitive for the detection of barbiturates and benzodiazepines. The ELISA method reduced turnaround time by 50% compared to the EMIT II method.

Introduction

According to the SAMSHA National Survey on Drug Use and Health Report from June 2, 2005, 4.3% of all pregnant women aged 15 to 44 surveyed (n = 1100) admitted to using illicit drugs in the past month. The number was almost double, 8%, for pregnant women aged 15–25 (1). Newborns exposed to drugs of abuse in utero may exhibit symptoms of drug dependence and withdrawal and can also suffer from short- and long-term health problems. Early detection can lead to effective management of withdrawal symptoms, proper treatment, and a more successful outcome (2). Results are also used in child-custody proceedings if removal from the home is in the best interest of the child. Specimens available for newborn drug testing include urine, blood, hair, and meconium. Collection of urine and blood from newborns can be logistically difficult, uncomfortable, and/or invasive. In addition, urine or blood taken from the baby shortly after birth only detects drugs that the mother took within a week or so prior to birth. Collection of hair can be met with opposition, and often very little hair is present. In addition, incorporation of drugs into hair can vary by hair color or type, and accurate detection can be compromised by external contamination or cosmetic treatments (3). Meconium, first introduced as a potential specimen in the 1980s, is currently the specimen of choice for determining fetal exposure to drugs of abuse. Meconium is the black, tarry stool passed by the newborn for the first 1–5 days after birth (4). It begins to form in the digestive tract between 12 and 16 weeks of gestation and accumulates until birth. Drugs and their metabolites collect in meconium beginning at approximately 5 months gestation, and at term it can effectively identify exposure during the last 4 months of pregnancy (5,6). Meconium has also been shown to be marginally more sensitive than hair for the detection of cocaine and cannabis in newborns (7). Meconium is a very complex specimen matrix. It is heterogeneous with a variable composition consisting of water, epithelial cells, lanugo, mucus, amniotic fluid, bile acids and salts, fatty material from the vernix caseosa, cholesterol and sterol precursors, blood group substances, enzymes, mucopolysaccharides, sugars, lipids, proteins, trace metals, various pancreatic and intestinal secretions, drugs, and other compounds ingested or otherwise used by the mother (8). Consequently, the incidence of matrix interferences is greatly increased in meconium specimens when compared to urine specimens (9–12). This complexity can create challenges when developing drug screening methods. Immunossay techniques developed for urine or blood have been used successfully to detect drugs of abuse in meconium (11). We have used enzyme multiplied immunoassay technique (EMIT II, Dade-Behring/
Siemens Healthcare Diagnostics, Deerfield, IL) for screening of meconium specimens, but the need for lower cutoffs and higher throughput led us to consider the use of enzyme-linked immunosorbent assay (ELISA, Immunalysis, Pomona, CA) and an automated diluter/pipettor (TECAN Genesis, Männedorf, Switzerland). The calibrators were changed from morphine to hydrocodone for opioids and from oxazepam to clonazepam for benzodiazepines in the ELISA method to maximize detection for these drugs.

**Experimental**

**Reagents and standards**

All standards were of 99% purity and purchased from Cerilliant (Austin, TX). All solvents were reagent grade or better and purchased from Thermo-Fisher Scientific (Waltham, MA) or VWR (West Chester, PA). Type I water was generated using a Barnstead Nanopure Infinity ultra-pure water system (Thermo Fisher Scientific). Extraction buffer and analysis kits for the ELISA assay were supplied by Immunalysis. Analysis kits for the EMIT II assay were purchased from Dade-Behringer. Drug-free meconium was pooled from excess patient specimens that tested negative by EMIT II and used to prepare calibrators, controls, and fortified samples. Positive patient specimens were obtained from residual patient specimens that confirmed positive and were de-identified to protect personal health information according to the University of Utah’s Institutional Review Board protocol. The drugs used for calibration, the published EMIT II and ELISA cutoff concentrations, the validated confirmation cutoffs, and the technique used for confirmation are summarized in Table I by drug class. Nine assays were performed for the EMIT II drug screen; 11 assays were performed for the ELISA drug screen, because amphetamine and methamphetamine are one assay using EMIT II but separate assays using ELISA, and opioids and oxycodone are a single assay using EMIT II but separate assays using ELISA. Calibrators prepared in duplicate with drug-free meconium were fortified at the cutoff concentration for each drug class and the average value was used to determine positivity. Negative and positive controls were prepared using drug-free meconium fortified at zero and 50% (negative controls) and 150% (positive control) of the cutoff concentration. These were prepared, extracted, and analyzed with each batch of samples to assure adequate assay performance.

**Apparatus**

Homogenation of the EMIT II meconium specimens was performed with an Omni Tissuemiser Homogenizer (Thermo-Fisher Scientific), and solvent evaporation was achieved with a Jouan centrifugal vacuum evaporator (CVE) system, model RC10.10 (Thermo-Fisher Scientific). Dilution and pipetting of the ELISA samples was done using a TECAN Genesis 150 equipped with 5-mL polypropylene tubes and 96-well microtiter plates. ELISA plates were washed using a Columbus 16-well plate washer (TECAN), and read using a TECAN Sun- rise plate reader. EMIT II analysis was automated using an Olympus AU400e (Olympus, Center Valley, PA).

**Methods**

**Sample preparation and analysis: EMIT II.** Each meconium specimen (0.5 g) was homogenized in a 16 × 50-mm polypropylene tube in 3 mL of methanol, then centrifuged at 14,000 rpm and 0°C for 15 min. Two milliliters of the supernatant were transferred to 16 × 114-mm glass conical tubes and 100 µL of methanolic HCl were added. The solvent was evaporated to dryness under vacuum using a CVE at 60°C for 2–3 h. Samples were checked frequently after 2 h and dried samples were removed as they were discovered. After all of the samples were dried, they were reconstituted in 250 µL of methanol, vortex mixed, sonicated for 15 min, then centrifuged at 3500 rpm and 0°C for five min. Two-hundred microliters of each meconium extract was added to individual pre-labeled 1.5-mL microcentrifuge tubes containing 100 µL of EMIT II buffer. The extracts were centrifuged at 14,000 rpm and 0°C for

<table>
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<th>Drug Class</th>
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<th>Confirm. Method</th>
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<tr>
<td>THC</td>
<td>(–)-9-Carboxy-11-nor-Δ9-THC</td>
<td>EMIT: 40, ELISA: 20, Confirm.: 10</td>
<td>GC–GC–MS</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>(+)-Amphetamine</td>
<td>EMIT: 200, ELISA: 20, Confirm.: 20</td>
<td>GC–MS</td>
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<tr>
<td>Methamphetamine</td>
<td>(–)-Methamphetamine</td>
<td>EMIT: 200, ELISA: 20, Confirm.: 20</td>
<td>GC–MS</td>
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<tr>
<td>MTD</td>
<td>Methadone</td>
<td>EMIT: 100, ELISA: 25, Confirm.: 10</td>
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<td>PXN</td>
<td>Propoxyphene</td>
<td>EMIT: 200, ELISA: 50, Confirm.: 10</td>
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<tr>
<td>Be</td>
<td>Benzoylcegonine</td>
<td>EMIT: 150, ELISA: 20, Confirm.: 20</td>
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<td>PNP</td>
<td>PCP</td>
<td>EMIT: 20, ELISA: 10, Confirm.: 10</td>
<td>GC–MS</td>
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<td>Barbiturates</td>
<td>Secobarbital</td>
<td>EMIT: 100, ELISA: 50, Confirm.: 50</td>
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<td>Benzodiazepines</td>
<td>Lormetazepam</td>
<td>EMIT: 100, ELISA: 50, Confirm.: 20</td>
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<td>Opioids</td>
<td>Morphine</td>
<td>EMIT: 200, ELISA: 20, Confirm.: 2</td>
<td>LC–MS–MS</td>
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<tr>
<td>Oxycodone</td>
<td>Oxycodone</td>
<td>EMIT: 200, ELISA: 20, Confirm.: 2</td>
<td>LC–MS–MS</td>
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* Changed from oxazepam to clonazepam.
† Changed from morphine to hydrocodone.
15 min. Finally the extracts were transferred to 12 × 85-mm tubes with 2-mL inserts for automated analysis on the Olympus AU400e.

The AU400e was calibrated according to the manufacturer’s instructions (13) using EMIT II reagents and kits from Dade-Behring. Two negative controls (blanks) and two calibrators (fortified at the cutoff for all drug classes) were used to calibrate the instrument. A negative control (fortified at 50% of the cutoff for all drug classes) and a positive control (fortified at 150% of the cutoff) were analyzed to verify correct instrument calibration. These were prepared by fortifying a 0.5 g aliquot of drug-free meconium with stock solutions prepared in methanol. The calibrators and controls were prepared and analyzed in the same manner as the patient specimens.

Sample preparation and analysis: ELISA. Each meconium specimen (0.25 g) was weighed into a 16 × 50-mm polypropylene tube, followed by addition of 2 mL of Immunalysis N-EXT extraction buffer. The tubes were vortex mixed for 30 s and then sonicated for 30 min. The samples were then centrifuged at 14,000 rpm and 0°C for 15 min. The supernatant was collected and poured into 5-mL polypropylene tubes. Calibrators and controls were prepared by fortifying 0.25-g aliquots of drug-free meconium at the appropriate concentration using stock solutions prepared in methanol. The ELISA extracts, calibrators, and controls were loaded onto the TECAN Genesis. Two-hundred microliters of each sample was pipetted into individual 5-mL polypropylene tubes. Each 200-µL sample was diluted with 800 µL of phosphate-buffered saline. After dilution, an aliquot of each diluted sample was pipetted into a well of each 96-well microtiter plate, one 96-well plate for each drug class (11 drug classes and microtiter plates total). The amount of sample added per well for each drug class was optimized with assistance from Immunalysis. The TECAN Genesis added 100 µL of enzyme conjugate (specific to each drug class) to each well, and the plates were incubated at room temperature for 1 h. The plates were removed, the conjugate was aspirated, and each plate was washed six times with deionized water using a Columbus 16-well plate washer. The plates were placed back on the TECAN Genesis, and 100 µL of 3,3’,5,5’-tetramethylbenzidine (TMB) substrate was added to each well. The samples were incubated for 30 min at room temperature. Next, the TECAN Genesis added 100 µL of stop solution (1 N HCl) to each well. The plates were removed from the TECAN Genesis and the plates were read using a TECAN Sunrise plate reader at 450 nm.

Method comparison. The two methods were compared by analyzing 68 patient specimens and 9 fortified samples. Samples needed to be fortified for phencyclidine (PCP) and propoxyphene (PXY) because no positive PCP specimens and a limited number (n = 3) of residual positive specimens for PXY were available. Three samples were also fortified with clonazepam. The spike concentrations were between 125% and 200% of the cutoff concentration. Samples that screened positive by EMIT II were confirmed by validated drug specific methods using gas chromatography–mass spectrometry (GC–MS), liquid chromatography (LC)–tandem MS, or two-dimensional GC (GC–GC)–MS. Samples that screened positive by ELISA but were negative by EMIT II were not confirmed because there was an insufficient quantity of residual patient specimen (quantity not sufficient or QNS). Most of the specimens screened positive for more than one drug class, and also confirmed positive for multiple analytes within a drug class (amphetamine and methamphetamine, or hydromorphone and hydrocodone for example), so the total number of confirmed positive results were 233 by EMIT and 221 by ELISA.

Results and Discussion

Patient specimen comparison

Results for each drug class are summarized in Table II. Table III details the number of confirmed positives, the concentration range, and the cross-reactivity for each analyte. Overall, there were 12 false-positive results for both assays. Ten confirmed-positive patient specimens were detected by EMIT II but were negative by ELISA. Twenty-one patient specimens screened positive by ELISA, but were negative by EMIT II. Detailed results summarized by drug class follow. All cross-reactivity data were provided by the

<table>
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<tr>
<td><strong>EMIT II Results Summary</strong></td>
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<tr>
<td>Confirmed positives</td>
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<td>False positives (failed to confirm)</td>
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<td>EMIT positive, ELISA negative†</td>
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<tr>
<td><strong>ELISA Results Summary</strong></td>
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<td>Confirmed positives</td>
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<td>False positives (failed to confirm)</td>
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<td>ELISA positive, EMIT negative†</td>
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</table>

† Quantity of specimen not sufficient to confirm.

* Abbreviations: THC, cannabinoids; Amp, amphetamine; Mamp, methamphetamine; MTD, methadone; PXY, propoxyphene; Be, cocaine (benzylecgonine); PCP, phencyclidine; Barb, barbiturates; Benz, benzodiazepines; Opi, opioids; and Oxy, oxycodone.
manufacturers (14,15). All methods used for confirmation of results were validated for clinical use and met CAP, CLIA, and New York Department of Health assay performance requirements.

**Cannabinoids**

The most prevalent metabolite of marijuana (THC) found in urine is 9-carboxy-11-nor-∆⁹-THC (9-THCA). Although 9-THCA is detected in meconium, 11-hydroxy-∆⁹-THC (11-OH-THC) is also readily detected in meconium; sometimes it is the only THC metabolite present (16). The ELISA assay for THC does not detect the 11-OH-THC metabolite; however, the cross-reactivity for 11-OH-THC using EMIT II is 46.5% (15). Thirty-two specimens screened positive by both methods and were confirmed for the presence of 9-THCA, with a range of 23 to > 1000 ng/g. Two of those specimens also confirmed positive for 11-OH-THC. There were no false positives using either assay technique. There was one confirmed positive specimen (9-THCA = 154 ng/g) that screened positive by EMIT II but negative by ELISA. Results were confirmed by GC–GC–MS (17). There was one specimen that screened positive by ELISA and was negative by EMIT II. Five additional specimens screened positive by both methods but had insufficient quantity to confirm.

**Amphetamine/methamphetamine**

Amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and methylenedioxyethylamphetamine (MDEA) are all detected in meconium. The ELISA assay offers better sensitivity for MDA and MDMA. MDA cross-reacts at 250% with the ELISA amphetamine antibody, but at only approximately 27% with the EMIT II antibody. MDMA cross-reacts at 135% with the ELISA methamphetamine kit, but only at 3% using EMIT II. Ten positive specimens were detected for amphetamine using both assays with a range of 62 to > 5000 ng/g. These specimens also screened positive for methamphetamine (439 to > 5000 ng/g) by ELISA. One specimen screened positive for amphetamine by both methods and methamphetamine by ELISA and was confirmed for MDA at 293 ng/g and MDA at 39 ng/g. GC–MS was used for confirmation of results. One additional specimen screened positive for amphetamine by both methods but was QNS. There were two false-positive specimens for amphetamine by both EMIT II and ELISA, and one of these also incorrectly screened positive for methamphetamine by ELISA. In addition, there were four specimens that screened positive for amphetamine, five that screened positive for methamphetamine, and one that screened positive for both by ELISA but were negative by EMIT, but were QNS to confirm.

**Methadone**

Methadone (MTD) is usually found in meconium with its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). EDDP cross-reacts at less than 5% with the Immunalysis ELISA kit for MTD. Both immunoassays detected 10 positive specimens and one false positive for MTD. EDDP cross-reacts at less than 5% with the Immunalysis ELISA kit for MTD. Both immunoassays detected 10 positive specimens and one false positive for MTD. The MTD concentrations were from 157 to 1550 ng/g. All of the specimens also contained EDDP in the range of 634 to > 5000 ng/g. The results were confirmed by LC–MS–MS. ELISA screened two additional specimens positive for MTD that could not be confirmed because QNS.

**Propoxyphene**

PXY and its metabolite norpropoxyphene are both detected in meconium. Norpropoxy-
Benzodiazepines

Phencyclidine

Cocaine metabolites

Opioids/oxycodone

Phencyclidine cross-reacts at 49% with the ELISA kit and at 37.5% with the EMIT II assay. Both assays correctly identified three specimens and nine fortified samples positive for PXY. The concentration range was from 39 to 157 ng/g. Two of the three positive patient specimens also confirmed positive for norpropoxyphene. There were no false-positive specimens. Results were confirmed by LC–MS–MS. ELISA screened three additional specimens positive for PXY, but these were QNS to confirm.

The EMIT II assay has 100% cross-reactivity with lormetazepam, which is only 50% (cross-reactivity for EMIT is not available). This specimen should have screened positive by ELISA and may reflect inadequate extraction of benzodiazepines as studied here. Both assays detected two false-positive specimens. These results were confirmed by LC–MS–MS (18). ELISA screened one additional specimen as positive for benzodiazepines, but was QNS to confirm.

Both the EMIT II and ELISA assays have antibodies that are designed to cross-react 100% with secobarbital. EMIT II detected 10 confirmed positive patient specimens (five positive for butalbital, three positive for phenobarbital, and two positive for pentobarbital), ELISA only detected eight. The ELISA test failed to identify samples positive for pentobarbital (195 ng/g) and phenobarbital (565 ng/g). This is surprising, because the published ELISA cutoff is lower than EMIT II (50 vs. 100 ng/g) and the ELISA assay has cross-reactivity similar to or better than EMIT II (50 vs. 20–39% for phenobarbital and 83 vs. 79% for pentobarbital, respectively). These results may reflect the more extensive sample preparation for the EMIT II assay providing better sensitivity for barbiturates. Both assays had one false-positive specimen. Results were confirmed by GC–MS.

The EMIT II assay has 100% cross-reactivity with lorazepam; the ELISA assay cross-reacts at 100% with oxazepam. EMIT II detected eight confirmed-positive patient specimens and three fortified samples; ELISA only detected six positive patient specimens and three fortified samples. One false-negative ELISA specimen contained diazepam at 32 ng/g (below the cutoff of 50 ng/g). The cross-reactivity for diazepam by EMIT is over 200%, but only 70% by ELISA. The other false-negative specimen had nordiazepam at 48 ng/g and temazepam at 28 ng/g. The cross-reactivity of temazepam by EMIT is 140%, and 200% by ELISA. The cross-reactivity of nordiazepam by ELISA is only 50% (cross-reactivity for EMIT is not available). This specimen should have screened positive by ELISA and may reflect inadequate extraction of benzodiazepines as studied here. Both assays detected two false-positive specimens. These results were confirmed by LC–MS–MS (18). ELISA screened one additional specimen as positive for benzodiazepines, but was QNS to confirm.

EMIT II detected a total of 28 confirmed positives for opioids. ELISA detected 23 confirmed positives for opioids, and eight confirmed positives for oxycodone. Two oxycodone specimens that screened positive for opioids by EMIT II were not detected by ELISA, but one had an oxycodone concentration slightly above the ELISA cutoff of 20 ng/g. Other opioids were detected in these specimens upon confirmation. There were a total of 26 confirmed positive specimens that screened positive for opioids or oxycodone by ELISA (many specimens were positive for both), meaning that there were two confirmed positive specimens that were detected by EMIT II that were not detected by ELISA. Both of these specimens were found to have opioid concentration < 20 ng/g (the ELISA cutoff) upon confirmation. There were three false positives by EMIT II. Two of these were correctly identified as negative by ELISA. The third EMIT II false positive was also positive by ELISA. There were also two false-positive oxycodone specimens that were detected by ELISA but failed to confirm; however, both of these specimens also screened positive for opioids and were confirmed to contain opioids other than oxycodone. Results were confirmed by LC–MS–MS.

Direct comparison of the two techniques for opioids is difficult, because both assays contain multiple analytes with very different cross-reactivities and there is a separate assay for oxycodone in the ELISA method. Our laboratory has a single LC–MS–MS confirmation method for opioids that includes oxycodone. This confirmation method does not include enzyme hydrolysis to convert any glucuronide to the free form, nor does it directly analyze for any glucuronides (19). This approach was adopted to detect 6-monooacetylmorphine with other opioids in a single assay. In addition, the cutoff for opioids (2 ng/g) is very low. High concentrations of codeine, hydrocodone, or oxymorphone can elicit a positive screen result from the oxycodone assay, because they cross-react at approximately 30% with the oxycodone antibody. Even very high levels of morphine (> 5000 ng/g) can cause a positive screen result for oxycodone, even though the cross-reactivity is only approximately 7% (15). Because our confirmation assay includes oxycodone in addition to other opioids, this is not a concern in our laboratory. EMIT II detected two positive samples below 20 ng/g that were negative by
ELISA (as expected) even though the EMIT II cutoff is 200 ng/g. ELISA did correctly identify two opioids samples as negative that screened positive by EMIT II but were negative on confirmation. ELISA detected 26 out of 28 positive opioid/oxycodone specimens (93% agreement), and the two that screened negative had opioids concentrations below the ELISA cutoff. The ELISA method publishes lower cutoffs than the EMIT II method; however, EMIT II appears to be more sensitive than its published cutoff of 200 ng/g for opioids.

Sample preparation and analysis time
Sample preparation time was greatly reduced in the ELISA method. Total sample preparation time was 4–5 h by EMIT II, but eliminating homogenation of the samples and the dry-down step by replacing them with sonication in extraction buffer resulted in sample preparation that took only approximately 1 h by ELISA. The lengthy EMIT sample preparation was designed to minimize spectral interferences from meconium. Because detection by ELISA is not subject to the same degree of interference, the ELISA sample preparation could be reduced. Analysis time was actually longer by ELISA. The analysis time by EMIT II, which was fully automated on the Olympus AU400e, was approximately 2 h. The analysis time by ELISA took approximately 3 h: most of the procedure was automated by the TECAN Genesis, which did the dilution and pipetting of the samples, then added the conjugate, TMB, and stop solution; however, washing and reading the plates were done manually. Total analysis time was reduced from 6–7 h by EMIT II to 3–4 h by ELISA. Data analysis and review time were about the same using both techniques. The reduction in turnaround time allows for faster reporting of results, and preparation of specimens for confirmation can be started the same day.

Results from the two techniques compared well. Accuracy of the ELISA assay was greater than 90% when compared to EMIT II for all drug classes except for barbiturates and benzodiazepines. ELISA detected more positive samples than EMIT II for the detection of amphetamines, MTD, PX, and cocaine. ELISA compared well to EMIT II for cannabinoids and PCP. Specificity of the ELISA assay was slightly better for PCP, because ELISA correctly identified a sample that had screened positive by EMIT as negative ELISA also correctly identified two opioid samples as negative that screened positive by EMIT II but were negative on confirmation. EMIT II appears to be more sensitive for the detection of barbiturates and benzodiazepines, as several confirmed positive specimens were detected by EMIT II but not detected by ELISA. Since completing this work, a new ELISA kit for benzodiazepines that provides better cross-reactivity for many commonly prescribed benzodiazepines has been made available. Results from this new kit have been compared to those from the kit used in this work for detection of benzodiazepines in meconium (20).

Conclusions
The ELISA method reduced turnaround time by 50%. ELISA detected more positive samples for amphetamines, cocaine, MTD, and PX. ELISA compared well to EMIT II for detection of THC and PCP. EMIT II detected more positive samples for barbiturates and benzodiazepines. ELISA performed well for opioids, but EMIT II appears to be more sensitive than its published cutoff of 200 ng/g.

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