Evaluation of Two Enzyme Immunoassays for the Detection of the Cocaine Metabolite Benzoylecgonine in 1,398 Urine Specimens

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Background: Benzoylecgonine (BE) is the primary urinary metabolite of cocaine. Two enzyme immunoassays were evaluated for the detection of BE in urine with a 300 ng/ml cutoff: the DRI[®] Cocaine Metabolite Assay and Lin-Zhi International's (LZ) Cocaine Metabolite Enzyme Immunoassay. **Methods:** This study involved 1,398 urine specimens from criminal justice and pain management programs. Gas chromatography/mass spectrometry (GC/MS) data were obtained for presumptive positives, and for negative urine specimens yielding responses significantly above the negative control. **Results:** Approximately 46% (644) of the specimens yielded positive results by DRI, and 47% (664) were positive by LZ. One specimen screened positive with both assays but was found to have a non-

detectable BE concentration by GC/MS, indicating one false positive for each assay. Twenty-one specimens yielding negative DRI results contained BE above 300 ng/ml, and 29 specimens yielded false negatives with the LZ assay. Therefore, the overall agreement between both immunoassays and GC/MS results was 98%. Assay sensitivity was 0.968 (DRI) and 0.958 (LZ); the selectivity for both assays was 0.999. Urine specimens containing cocaine, additional cocaine metabolites, and other drugs were also tested. No cross-reactivity was observed. **Conclusion:** Both the DRI and LZ assays provide a precise, reliable method for the routine detection of BE in urine. *J. Clin. Lab. Anal.* 26:130–135, 2012. © 2012 Wiley Periodicals, Inc.

Key words: enzyme immunoassay; benzoylecgonine; cocaine; urine drug testing

INTRODUCTION

Perhaps no other drug of abuse has had the magnitude of adverse consequences on the individual and on American society as cocaine has, particularly in the form of crack (1). The illicit drug trade that cocaine fueled in the United States beginning in the mid-1970's continues to have a socially destructive impact throughout North and South America. Concern over cocaine abuse resulted in the drug being included among the five drugs or drug classes in the Substance Abuse and Mental Health Services Administration (SAMHSA) federally regulated workplace urine

drug testing program in 1988 (2). More than 20 years later, SAMHSA data show that cocaine use is still involved in more than 20% of emergency room visits related to drug misuse or abuse (3).

Homogeneous enzyme immunoassays remain the method of choice for cocaine screening when rapid, high-volume testing is required for workplace, criminal justice, substance abuse treatment, pain management, or emergency department urine testing (4). As benzoylecgonine (BE) is the primary urinary metabolite of cocaine, accounting for 65–90% of an administered dose, it is commonly the target analyte (1). In this study, we evaluated two enzyme immunoassays for the detection of BE in urine: the DRI[®] Cocaine Metabolite Assay (DRI) from Siemens Healthcare Diagnostics (Tarrytown, NY), and the Cocaine Metabolite Enzyme Immunoassay from Lin-Zhi International, Inc. (LZ) (Sunnyvale, CA). Both assays use a 300 ng/ml BE cutoff calibrator. As with

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other enzyme immunoassays, these assays are based on competition between drug labeled with glucose-6-phosphate dehydrogenase (G6PDH) and free drug in the urine for a fixed number of antibody binding sites. In the absence of free drug in the urine, the specific antibody binds the enzyme-labeled drug causing steric hindrance and a decrease in enzymatic dehydrogenation of glucose-6-phosphate with the reduction of NAD cofactor to NADH. This reaction creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is determined spectrophotometrically by measuring the change in absorbance at 340 nm. To evaluate the analytical efficiency of these assays, client urine specimens were tested by both the DRI and LZ assays, and subjected to quantitative gas chromatography/mass spectrometry (GC/MS) for BE. The immunoassays' analytical performance was assessed by calculating their sensitivity, specificity, and percent concordance with GC/MS results, and their cross-reactivity to licit and illicit drugs.

MATERIALS AND METHODS

Study Protocol

One thousand three hundred and ninety-eight (1,398) urine specimens from criminal justice and pain management programs were used in this study. This study was conducted with approval from the Virginia Commonwealth University's Investigational Review Board. Many of the urine specimens had previously screened positive for drugs of abuse, including cocaine metabolite, by immunoassays other than the DRI and LZ assays. Batches of 10–50 specimens were stored refrigerated for up to several weeks. These batches of specimens were removed from storage, allowed to come to room temperature, and then analyzed for BE at a cutoff concentration of 300 ng/ml by both DRI and LZ immunoassays. Specimens yielding a positive response by either immunoassay were analyzed by GC/MS for BE at a cutoff concentration of 150 ng/ml. Additionally, specimens tested by both immunoassays that yielded negative responses less than the immunoassay cutoff concentration, but clearly greater than the negative control were also subjected to GC/MS testing to assess possible false negative results.

The immunoassays' analytical performance was assessed by calculating their sensitivity, specificity, and percent concordance with GC/MS results. Sensitivity was calculated as $TP / (TP + FN)$; where TP is defined as a true positive result, positive by the immunoassay and a quantified BE result ≥ 300 ng/ml by GC/MS; and FN is defined as a false negative result, negative by the immunoassay and positive by GC/MS at a quantified result ≥ 300 ng/ml BE. Selectivity (specificity) was calculated as $TN / (TN + FP)$; where TN is defined as a true negative

result, negative by the immunoassay and a quantified BE result < 300 ng/ml by GC/MS; and FP is defined as a false positive result, positive by the immunoassay and a quantified BE result < 300 ng/ml by GC/MS. Percent concordance of the assay equaled the sum of the TP and TN results divided by the total specimens tested.

Immunoassays

The DRI cocaine metabolite assays were obtained from Microgenics (Siemens Healthcare Diagnostics, Tarrytown, NY). The LZ cocaine metabolite assays were donated by Lin-Zhi International. All immunoassay testing in this study was performed on an ADVIA 1200 Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY). Analyzer parameters were as follows: DRI – 3.2 μ L sample, 40 μ L antibody reagent, 40 μ L enzyme conjugate; LZ – 8 μ L sample, 80 μ L antibody reagent, 30 μ L enzyme conjugate; incubation temperature 37°C. Each assay was calibrated with its manufacturer's negative and cutoff calibrators containing 0.0 and 300 ng/ml of BE, respectively. Control urine specimens containing 225 and 375 ng/ml BE were obtained from BioRad Laboratories (Irvine, CA). Specimen aliquots yielding a reaction rate equal to or greater than that of the 300 ng/ml-cutoff calibrator were considered a presumptive positive result. All immunoassay reagents were stored refrigerated at 2–8°C.

Reagents

All primary reference materials including BE, BE-d₃, cocaine, and other drugs were obtained as 1.0 mg/ml in methanol solutions from Cerilliant Corporation (Round Rock, TX). Analytical reagent grade chemicals including ammonium hydroxide, dibasic, and monobasic sodium phosphate, as well as high-purity, glass-distilled hexane, dichloromethane, and isopropanol were purchased from Fisher Scientific (Fair Lawn, NJ). The derivatizing agents for GC/MS analysis, *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MtBSTFA) and *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 10% TMCS, were purchased from Regis Chemical Company (Rockford, IL). Cleanscreen® Extraction Cartridges (ZSDAU020) 200 mg columns were obtained from United Chemical Technologies, Inc. (Horsham, PA). GC/MS urine controls were obtained from Quality Assurance Service Corp. (Augusta, GA).

Benzoyllecgonine Extraction and Derivative Preparation Procedure

Extraction of BE from urine was by solid phase extraction using Cleanscreen® Extraction Cartridges (5).

Briefly, 200 μL of internal standard (4.5 $\mu\text{g}/\text{ml}$, 900 ng BE- d_3) was added to 2.0 ml aliquots of urine. The pH of the mixture was adjusted to 6.0 and then applied to previously prepared SPE sorbent beds. Each column was washed and the BE and BE- d_3 were then eluted with methylene chloride: isopropanol: ammonium hydroxide (76:19:5). The eluent was evaporated to dryness. A *t*-butyldimethylsilyl-benzoylcgonine (BE-*t*BDMS) derivative was prepared with MtBSTFA and ethyl acetate. The mixture was transferred to auto-injector vials for injection into the GC/MS. It should be noted that some of the urine specimens that yielded discrepant immunoassay responses were analyzed by modified TMS-derivative preparation method using BSTFA and ethyl acetate. The MtBSTFA derivative was used for routine confirmation as the BE-*t*BDMS derivative is extremely stable and a sealed vial may be stored at room temperature for up to two weeks (6).

Gas Chromatography/Mass Spectrometry

GC/MS analyses, in this study, were performed at either an SAMHSA-accredited laboratory or a CAP-accredited laboratory. Samples were analyzed on an Agilent 6890 GC with 5973 MSD. The column was a 10–15 m DB-35MS (or equivalent). GC temperatures were: initial 160°C, hold 0.2 min; ramp 35°C/min to 290°C, no hold; the injector and MS interface temperatures were 250°C and 280°C, respectively. The flow was run in ramp flow mode with the initial flow rate of 3.5 ml/min at an initial time of 0.2 min then ramped 1 ml/min for 4.91 min. The instrument was run in the pulsed splitless mode with hydrogen gas and pulse pressure of 25 psi; pulse flow, 40 ml/min; and pulse time, 0.20 min. MtBSTFA was the derivatizing reagent; therefore, for the BE-*t*BDMS derivative, 346 *m/z* was used as the quantifying ion with 282 and 403 *m/z* as qualifying ions. The quantifying ion for the BE- d_3 internal standard was 349 *m/z* with the qualifying ion of 406 *m/z*. The acquisition data were Single Ion Monitoring mode with a solvent delay of 3.00 min and a dwell time of 30 msec.

Analysis of samples yielding discrepant results was performed on an Agilent 6890 GC with 5973 MSD. The GC was equipped with a 5 m Restek guard column and HP-1 capillary column of 12 m $\times$ 0.25 mm id with 0.33- μm film thickness. GC oven temperatures were: initial 170°C, hold 1 min; ramp 20°C/min to 280°C, no hold; the injector and MS interface temperatures were 250°C and 280°C, respectively. BSTFA was the derivatizing reagent; therefore, for the BE-TMS derivative, 240 *m/z* was used as the quantifying ion with 256 and 361 *m/z* as qualifying ions. The quantifying ion for BE- d_3 internal standard was 243 *m/z* with the qualifying ions of 259 and 364 *m/z*. The acquisition data were Single Ion Monitoring mode with a solvent delay of 3.00 min and a dwell time of 30 msec.

Both GC/MS procedures were linear for their respective silyl derivatives from 30 ng/ml to 8,000 ng/ml. A 4-point calibration curve of 0, 60, 150, and 500 ng/ml of BE was applied to each GC/MS batch. The Lowest Limit of Detection (LLOD) and Lowest Limit of Quantification (LLOQ) of BE were both set administratively at 50 ng/ml.

RESULTS

The within-run and between-run precisions of the DRI and LZ assays were determined by the absorbance rates of the negative and positive Bio-Rad controls, Table 1. In general, for both assays the within-run precision for the negative (– 25% of cutoff value) and the positive (+ 25% of the cutoff value) controls yielded CVs of less than 3%. The between-run precision for the DRI assay was much tighter, less than 4% for both negative and positive Bio-Rad controls, than that of the LZ assay, which displayed CVs of 6.2% for the negative and 4.4% for the positive control. Interestingly, controls obtained from LZ yielded very precise between-run responses: mean absorbance of 0.840 $\pm$ 0.011 ($n = 9$), CV = 1.3% for the negative control and mean absorbance of 1.157 $\pm$ 0.019 ($n = 9$), CV = 1.6% for the positive control. Obviously, controls prepared and supplied by the manufacturer of any immunoassay would be expected to yield more precise responses than those from a third-party source.

Approximately 46% (644) of the 1,398 specimens yielded positive results by the DRI assay, and 47% (664) were positive by the LZ assay. BE was detected and quantified in 793 of the specimens with urine concentrations ranging from 58 ng/ml to approximately 902,000 ng/ml (more than 100 $\times$ dilution). One hundred fifty-six of the specimens selected for GC/MS analysis contained BE at concentrations below the immunoassay cutoff calibrator value (300 ng/ml). The DRI assay correctly indicated the presence of BE at or above the cutoff value in 643 specimens, while the LZ assay correctly yielded 663 positive results. Only one specimen that yielded a positive response by both the DRI and LZ assays was found to contain a nondetectable concentration of BE by GC/MS. A repeat analysis was performed on this specimen by both immunoassays and again they yielded false positive results. No other drug of abuse was detected in this specimen. A possible source of this false positive could not be identified. These data indicate that the DRI and LZ cocaine metabolite assays have an analytical selectivity (specificity) of 0.999.

Twenty-one specimens yielding negative responses with the DRI assay were found to contain BE above the 300 ng/ml cutoff value. Similarly, 29 specimens that contained BE above the 300 ng/ml cutoff yielded negatives responses with the LZ assay, Table 2. These data indicate that the DRI and LZ cocaine metabolite assays have an

TABLE 1. Precision of the DRI and LZ Assays as Determined by Absorbance Values of the Bio-Rad Control Urine Specimens

Control	DRI			LZ		
	Mean	±SD	%CV	Mean	±SD	%CV
Within-run						
225 ng/ml	0.677	±0.019 (<i>n</i> = 6)	2.8%	0.723	±0.017 (<i>n</i> = 8)	2.3%
375 ng/ml	1.048	±0.026 (<i>n</i> = 6)	2.5%	0.978	±0.017 (<i>n</i> = 8)	1.7%
Between-run						
225 ng/ml	0.716	±0.027 (<i>n</i> = 13)	3.7%	0.770	±0.048 (<i>n</i> = 9)	6.2%
375 ng/ml	1.098	±0.016 (<i>n</i> = 13)	1.5%	1.028	±0.045 (<i>n</i> = 9)	4.4%

TABLE 2. Comparison of DRI and LZ assays with GC/MS results for benzoylecgonine from testing of 1,398 urine specimens

DRI benzoylecgonine assay		+	–
GC/MS	+	643	21
	–	1	733
Lin-Zhi benzoylecgonine assay			
GC/MS	+	663	29
	–	1	705

analytical sensitivity of 0.968 and 0.958, respectively. However, nine of the 21 specimens yielding false negative results by DRI contained 312 to 340 ng/ml of BE, which was within + 20% of the cutoff value. Likewise, ten of the 29 specimens yielding false negative results by LZ contained 310 to 360 ng/ml of BE, which was within + 20% of the cutoff value. Plus or minus 20% of cutoff values for initial immunoassay and GC/MS confirmation testing are the accepted industry standards in urine drug testing. Had these specimens yielded positive responses, the sensitivity of the DRI and LZ assays would increase to 0.982 and 0.972, respectively. The overall agreement between both the DRI and LZ assays and GC/MS results was 98%.

Urine specimens augmented with known concentrations of cocaine, additional cocaine metabolites, and other drugs were also tested using both assays. Neither assay demonstrated cross-reactivity with cocaine, cocaethylene, ecgonine, norecgonine, ecgonine methyl ester, ecgonine ethyl ester, anhydrous ecgonine, or anhydrous methylecgonine at a concentration of 1,000 ng/ml. At 10,000 ng/ml, bupivacaine, lidocaine, mepivacaine, ropivacaine, tetracaine, and tocanine did not cross-react with either assay. Finally, neither assay yielded a positive result when analyzing samples containing 20,000 ng/ml of chlorpheniramine, brompheniramine, diphenhydramine, doxylamine, phentermine, methadone metabolites, dextromethorphan, methamphetamine, methylenedioxymethamphetamine, butalbital, or phenobarbital.

DISCUSSION

The various types of drugs of abuse immunoassays that have been applied to the detection of BE in urine include: enzyme-immunoassay (EIA) (7–9), colloidal metal immunoassay (CMI) (10,11), fluorescent polarization immunoassay (FPIA) (12), cloned enzyme donor immunoassay (CEDIA) (13), kinetic interaction microparticle immunoassay (KIMS) (14), immunochromatography (IC) (15,16), lateral flow immunoassay (LFIA) (17), enzyme linked immunosorbant assay (ELISA) (18) and radioimmunoassay (RIA) (7). Of these, EIA is the most popular immunoassay used in urine drug screening. This is demonstrated by the College of American Pathologists (CAP) Urine Drug Testing Screening proficiency survey. In 2010, 46% (1,187/2,589) of respondent laboratories reported cocaine metabolite test results by EIA testing (19). EIA is the method of choice for high-volume analyzers. The Triage point-of-care card CMI is second most commonly reported method, at 22% of respondents.

The cocaine metabolite enzyme immunoassay evaluation data presented above represent the largest number of client/patient specimens containing BE (663 confirmed positives) of any previous immunoassay evaluation. Surprisingly, other studies, even those involving an extremely large number of urine specimens, had relatively few specimens positive for cocaine metabolite. For example, in an early evaluation of Abbott's TDx FPIA cocaine metabolite, one of the authors detected 120 BE positive specimens (12). A comparable study of 15,600 specimens tested by CEDIA yielded 138 positive BE results, a < 1% positive rate (13). Similarly, Armbruster et al. in two comparisons of EMIT II, EIA, KIMS, and RIA involving more than 53,000 urine specimens found 267 EMIT II positive results (7,8) in US Army and Air Force personnel. The overall positive rate for BE was < 0.5%. In a recent study comparing EMIT II EIA and KIMS, Lu and Taylor detected 118 positive BE specimens from 738 urine specimens collected from arrestees in Chicago, New Orleans, Seattle, and London, England, with a positive rate for BE of 16% (20). The high incidence of positive results in the presented

study is due to the nature of the clients, criminal justice urine specimens, and the selection of previously screened negative result urine specimens with absorbance rates just below the cutoff calibrator. This emphasis on positive and near-positive specimens challenged the performance of the DRI and LZ assays, and may account for the relatively low sensitivity observed in this study.

The risk of having a false positive result due to cross-reactivity of an immunoassay is largely dependent upon the nature of the antibodies, the particular drug-hapten used to create the antibodies, the labeled drug detection system, and the chemical structure of the analyte drug and cross-reactant. For example, many potential EIA cross-reactant drugs were identified with the older polyclonal antibody EIA assays. Since the late 1980's, EIA immunoassays have been more specific monoclonal antibody based assays (21). BE has a rather unique chemical structure resembling no other drugs of toxicological interest. Therefore, cross-reactivity between cocaine metabolite immunoassays and substances other than BE is nearly nonexistent (22). However, of particular interest were recent reports that urine specimens from patients receiving a 75 mg daily dose of venlafaxine or urine specimens from patients overdosing on venlafaxine yielded false positive results with some immunoassays for phencyclidine and other drugs of abuse including cocaine metabolite (23,24). These reports concerning venlafaxine have demonstrated cross-reactivity not with EIA assays like the DRI or LZ assays, but with the Abbott AXSYM FPIA and Syva Rapid-Test IC assays.

A study of the possible interferences due to nonsteroidal anti-inflammatory drugs on the EIA EMIT and FPIA TDx assays demonstrated that high urinary concentrations of feopropfen, flurbiprofen, indomethacin, ketoprofen, and tolmetin might produce false positive results with TDx benzodiazepine assay. However, only tolmetin could potentially cause false positive results with EMIT drugs of abuse assays, including the cocaine metabolite assay (25). We did not test for cross-reactivity from tolmetin as it is seldom encountered in current toxicological testing.

In conclusion, we have found that the analytical performance of the DRI and Lin-Zhi cocaine metabolite enzyme immunoassays, including precision and selectivity, was consistent with their respective manufacturers' literature. At a cutoff concentration of 300 ng/ml of BE, both assays have sufficient analytical efficiency for the detection of this cocaine metabolite in routine urine drug testing.

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