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Evaluation of a Newly Formulated Enzyme Immunoassay for the Detection of Hydrocodone and Hydromorphone in Pain Management Compliance Testing

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Abstract

A new Hydrocodone Enzyme Immunoassay (HEIA; Lin-Zhi International, Inc.) was evaluated for the detection of hydrocodone and its main metabolite, hydromorphone. All specimens were tested with two different cutoff calibrators, 100 and 300 ng/mL, on an ARCHITECT Plus c4000 Clinical Chemistry Analyzer. Controls containing −25% (negative control) and +25% (positive control) of the cutoff calibrators and a drug-free control were analyzed with each batch. All 1,025 urine specimens were previously analyzed by ultra-performance liquid chromatography–mass spectrometry/mass spectrometry (UPLC–MS-MS) for opiates. Approximately, 33% (337/1,019) of the specimens yielded positive results by the HEIA assay at a cutoff concentration of 100 ng/mL and 19% (190/1,025) yielded positive results at the 300 ng/mL cutoff concentration. Of these presumptive positive specimens, UPLC–MS-MS confirmed the presence of hydrocodone and/or hydromorphone >100 ng/mL in 241 specimens and >300 ng/mL in 162 specimens, for each respective cutoff. With the 100 ng/mL cutoff, the HEIA demonstrated a sensitivity of 0.959, a specificity of 0.846 and an overall agreement with the UPLC–MS-MS of 87%. At 300 ng/mL cutoff, the HEIA demonstrated a sensitivity of 0.880, a specificity of 0.966 and an overall agreement of UPLC–MS-MS results of 95%. The Lin-Zhi HEIA 100 ng/mL cutoff assay demonstrated sensitivity for the detection of hydrocodone and hydromorphone in urine. The 300 ng/mL cutoff was less sensitive, but more selective, and should be part of an initial immunoassay screen, particularly in pain management compliance testing.

Introduction

Hydrocodone and hydromorphone are widely used analgesics for the treatment of acute and chronic pain and cough suppression (1). They also have a moderate to high abuse potential and are often subjected to widespread drug diversion by opiate abusers (1). Thus, hydrocodone and its main metabolite, hydromorphone, are an integral part of pain management compliance testing (PMCT) and urine drugs of abuse (DOA) testing. Hydrocodone and hydromorphone are reported to be 1–2 times and 7–10 times of morphine's potency, respectively (1). Like other μ-receptor agonists, overdose symptoms are characterized by pin-point pupils, respiratory depression and coma; tolerance to therapeutic and toxic effects is observed with chronic administration. Hydrocodone and hydromorphone have been important analytes in urine drug testing as part of pre-employment urine drug screening, *postmortem* drug screening, testing emergency department drug screening and recently PMCT. The plasma half-life of hydrocodone and the immediate release formulation of hydromorphone is approximately 3–9 hours, and the extended release formulation of hydromorphone is approximately 10–22 hours (1). Other hydrocodone metabolites include

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norhydrocodone, dihydrocodeine, isodihydrocodone, dihydromorphone and isodihydromorphone (2). Hydrocodone is metabolized by cytochrome P4502D6 (CYP2D6) into hydromorphone through demethylation (2, 3). Urinary excretion of hydrocodone plus hydromorphone may account for up to 26% of an administered dose (2, 3). Factors like CYP2D6 polymorphism, age, sex, urinary pH and concurrent use of a CYP2D6 inhibitor may influence the concentration of hydrocodone and its metabolites in urine (3). Continuous administration may also cause an induction of hydrocodone metabolism resulting in increasing hydromorphone concentrations in urine.

We present an evaluation of a new Hydrocodone Enzyme Immunoassay (HEIA; Lin-Zhi International, Inc., Sunnyvale, CA) designed for the detection of hydrocodone and hydromorphone in urine. The assay may be performed with a 100 or 300 ng/mL hydrocodone cutoff calibrator. As with other enzyme immunoassays, HEIA is based on the competition between drug labeled with the enzyme glucose-6-phosphate dehydrogenase and free drug for a fixed amount of antibody-binding sites. In the presence of free drug in the urine, the specific antibody binds the enzyme-labeled drug causing an increase in enzymatic dehydrogenation of glucose-6-phosphate with reduction of nicotinamide adenine dinucleotide (NAD) cofactor to reduced nicotinamide adenine dinucleotide (NADH). This reaction creates a direct relationship between the drug concentration in urine and activity. The enzyme activity is determined spectrophotometrically by measuring the change in absorbance at 340 nm. Overall analytical efficiency of the HEIA was evaluated. All urine specimens were previously analyzed by a validated ultra-performance liquid chromatography–mass spectrometry/mass spectrometry (UPLC–MS-MS) assay for opiates.

Experimental

Reagents

All primary reference materials of hydrocodone, hydromorphone and other drugs included in this study were obtained as 1.0 mg/mL methanolic solutions from Cerilliant Corp. (Round Rock, TX). The 100 and 300 ng/mL cutoff HEIA assay reagents, including antibody/substrate and enzyme conjugate reagent, were obtained from Lin-Zhi International, Inc. (Catalog No. 0381 and 0391, respectively). The 100 and 300 ng/mL cutoff HEIA calibrators were also obtained from Lin-Zhi International, Inc. Drug-free urine was obtained from laboratory personnel who did not smoke tobacco or take prescription or over-the-counter medication. The collected drug-free urine was pooled and analyzed by UPLC–MS-MS for opiates, including hydrocodone and hydromorphone, and by enzyme immunoassays for DOA; all yielded negative results. All materials and reagents were stored per manufacturers' recommendations.

Controls

The 100 ng/mL cutoff HEIA urine controls (containing 0, 75 and 125 ng/mL hydrocodone) and the 300 ng/mL cutoff HEIA urine controls (containing 0, 225 and 375 ng/mL hydrocodone) were obtained from Lin-Zhi International, Inc. The controls were stored according to manufacturer's recommendation.

Study protocol

Concurrent urine specimens submitted for PMCT were used in this study. Each specimen was initially analyzed for opiates by the MULTIGENT Opiates Enzyme Immunoassay (Abbott Diagnostics, Abbott Park, IL) with a 300 ng/mL morphine cutoff calibrator. Specimens were refrigerated for up to 2 weeks, and stored frozen if beyond 2 weeks before analysis. Urine specimens and controls were removed from storage, allowed to come to room temperature and were then analyzed by the newly formulated Lin-Zhi International HEIA at cutoff concentrations of 100 and 300 ng/mL. All specimens, testing negative or positive for hydrocodone and/or hydromorphone, were previously quantified by UPLC–MS-MS.

HEIA instrumentation and analysis

All urine HEIAs were performed on an ARCHITECT Plus c4000 Clinical Chemistry Analyzer (Abbott Diagnostics, Abbott Park, IL). Analyzer parameters were as follows: for the 100 ng/mL cutoff, 18 µL aliquots of urine were sampled and mixed with 120 µL of antibody reagent and 45 µL of enzyme conjugate; for the 300 ng/mL cutoff, 12 µL aliquots of urine were sampled and mixed with 120 µL of antibody reagent and 45 µL of enzyme conjugate. The mixtures were incubated at a temperature of 37°C. The assays were calibrated with negative controls and cutoff calibrators containing 0 and 100 or 300 ng/mL of hydrocodone, respectively. Specimen aliquots yielding absorbance values equal to or greater than that of the 100 ng/mL cutoff calibrators were considered positive for the 100 ng/mL cutoff HEIA, while those yielding a reaction less than the 100 ng/mL cutoff calibrator were considered negative. Specimen aliquots yielding an absorbance value equal to or greater than that of the 300 ng/mL cutoff calibrators were considered positive for the 300 ng/mL cutoff HEIA, while those yielding a reaction less than the 300 ng/mL cutoff calibrator were considered negative. All enzyme immunoassay analyses were performed with urine controls added to each analytical batch containing target concentrations of 0, 75 (negative controls) and 125 ng/mL (positive control) of hydrocodone for the 100 ng/mL cutoff HEIA and 0, 225 ng/mL (negative controls) and 375 ng/mL (positive control) of hydrocodone for the 300 ng/mL cutoff HEIA.

Evaluation of enzyme immunoassay performance

The analytical performance of the immunoassay at the 100 and 300 ng/mL hydrocodone cutoffs were determined by calculation of assay specificity, sensitivity and percent concordance with the UPLC–MS-MS results. The specificity of the assay was investigated by adding known amounts of commonly prescribed therapeutic agents, as well as popular DOA and/or their metabolites to drug-free urine at concentrations ranging from 1,000 to 33,000 ng/mL and analyzing these specimens by HEIA at both cutoff values. Specificity was calculated by TN/(TN + FP), where TN is defined as a true negative result (negative for both hydrocodone and hydromorphone by the immunoassays and UPLC–MS-MS) and FP is defined as a false positive result (positive for hydrocodone and/or hydromorphone by either immunoassay and negative for hydrocodone and/or hydromorphone at a combined concentration equal to or less than 100 or 300 ng/mL by UPLC–MS-MS).

Sensitivity was calculated by TP/(TP + FN), where TP is defined as a true positive result (positive for hydrocodone and/or hydromorphone by the immunoassays and UPLC–MS-MS) and FN is defined as a false negative result (negative for both hydrocodone and hydromorphone by immunoassays and positive for hydrocodone and/or hydromorphone at a concentration equal to or greater than 100 or 300 ng/mL by UPLC–MS-MS). The percent concordance of the immunoassay equaled the sum of TP and TN results divided by the total number of specimens tested.

UPLC–MS-MS

All the samples in the study were analyzed on a Waters AcQuity Xevo-TQD LCMSMS (Waters, Milford, MA) System, equipped with an UltraBiphenyl 3 µm, 2.1 × 50 mm (Restek, Bellephonte, PA) column and utilizing a method adapted from Danaceau *et al.* (4). The mobile phase was composed of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B). The linear gradient was 5–40% B from 0 to 1.5 minutes, 40–100% B from 1.5 to 3.0 minutes, 100% B from 3 to 3.5 minutes and 100–5% B from 3.5 to 3.6 minutes with an equilibration time of 1.0 minute. The flow rate was 0.6 mL/min and the injection volume was 5 µL. Patient urine samples were hydrolyzed using β-glucuronidase and diluted if the concentration of opiates obtained by immunoassay screening was higher than the highest control value.

Results

Control crossover study

The HEIA controls and calibrators were analyzed in triplicate by UPLC–MS-MS and yielded the results summarized in Table I. All hydrocodone controls and calibrators were within ±20% of their target values. All UPLC–MS-MS controls containing less than the cutoff values of the hydrocodone assays yielded negative results. The UPLC–MS-MS low opiate control (target 100 ng/mL) and medium control (target 500 ng/mL) tested positive with the 100 ng/mL HEIA, while only the medium control tested positive at the 300 ng/mL cutoff assay.

Opiates 300 ng/mL cutoff assay

Approximately 49% (494 of the 999 specimens) yielded positive results by the MULTIGENT Opiate assay. Of these 494 specimens, UPLC–MS-MS confirmed the presence of hydrocodone and/or hydromorphone at ≥300 ng/mL, respectively, in 467 specimens. In testing the 999 specimens, the Opiate assay yielded 159 discordant results compared to UPLC–MS-MS, an agreement of 84% (Table II). Of the 494 specimens yielding positive results with the opiate assay, 127 specimens were found to contain hydrocodone and/or hydromorphone alone by UPLC–MS-MS analysis. Twenty-seven false positive results were obtained with this assay, in which UPLC–MS-MS analysis demonstrated the presence of opiates in the specimens, but at concentrations <300 ng/mL. The Opiate assay yielded 132 false negative results. UPLC–MS-MS demonstrated the following analyte concentrations in these specimens: 160–5,849 ng/mL of hydromorphone, 407–649 ng/mL of morphine, 20–13,065 ng/mL of oxycodone and 60–15,634 ng/mL of oxymorphone. One hundred and thirty-one of the false negatives contained oxycodone and/or oxymorphone, of which 129 contained these opiates alone.

The overall analytical sensitivity of the Opiate assay was 0.799 and its analytical specificity was 0.932.

Hydrocodone 100 ng/mL cutoff assay

Approximately 33% (337 of the 1,019 specimens) yielded positive results by the Lin-Zhi HEIA. Of these 337 specimens, UPLC–MS-MS confirmed the presence of hydrocodone and/or hydromorphone at ≥100 ng/mL in 214 specimens. There were 132 discordant results compared to UPLC–MS-MS, an agreement of 87% (Table III). Of the 337 specimens yielding positive results, 121 were found to contain hydrocodone and/or hydromorphone alone by UPLC–MS-MS analysis. One hundred and twenty-three false positive results were obtained, in which UPLC–MS-MS analysis demonstrated the presence of hydrocodone and/or hydromorphone in the specimens, but at combined concentrations <100 ng/mL. The HEIA yielded nine false negative results. UPLC–MS-MS demonstrated the following ranges of analyte concentrations in these specimens: 122–333 ng/mL of hydrocodone and 160–5,849 ng/mL of hydromorphone.

The overall analytical sensitivity of the Lin-Zhi HEIA was 0.959. In addition to hydrocodone and/or hydromorphone, 103 of the 223 UPLC–MS-MS positive specimens contained other opiates including codeine, oxycodone, oxymorphone, morphine and 6-MAM. These opiates were also present in 42% (284/675) of the hydrocodone/hydromorphone-negative specimens. In the specimens yielding negative results, other opiates were present at concentrations up to codeine ≥3,533 ng/mL, oxycodone ≥22,299 ng/mL, oxymorphone ≥10,584 ng/mL and morphine ≥5,620 ng/mL. The analytical specificity of the Lin-Zhi was 0.846.

Table I. Control crossover studies results for the 100 and 300 ng/mL HEIAs

HEIA	Target Concentration (ng/mL)	Experimental Concentration (ng/mL)	CV ($n = 3$)
100 ng/mL cutoff	75	74.33 ± 1.23	1.7%
	100	91.60 ± 3.95	4.3%
	125	130.26 ± 3.51	2.7%
300 ng/mL cutoff	225	219.18 ± 4.28	2.0%
	300	283.83 ± 9.70	3.4%
	375	372.94 ± 3.69	1.0%

Table II. Comparison of opiates enzyme immunoassay at 300 ng/mL cutoffs with UPLC–MS-MS results

	Opiates Assay		
UPLC–MS-MS		+	–
	+	467	132
	–	27	373

Table III: Comparison of Lin-Zhi HEIA utilizing the 100 ng/mL cutoffs with UPLC-MS/MS results for hydrocodone and hydromorphone.

		Hydrocodone 100 ng/mL cutoff	
		+	–
UPLC-MS/MS	+	214	9
	–	123	675

Table IV. Comparison of Lin-Zhi HEIA utilizing the 300 ng/mL cutoff with UPLC-MS/MS results for hydrocodone and hydromorphone.

		Hydrocodone 300 ng/mL cutoff	
		+	–
UPLC-MS/MS	+	161	22
	–	28	813

Hydrocodone 300 ng/mL cutoff assay

Approximately 19% (190 of the 1,025 specimens) yielded positive results by the Lin-Zhi HEIA. Of these 190 specimens, UPLC–MS-MS confirmed the presence of hydrocodone and/or hydromorphone at ≥300 and ≥375 ng/mL, respectively, in 162 specimens. The HEIA yielded 50 discordant results compared to UPLC–MS-MS, an agreement of 95% (Table IV). Of the 190 specimens yielding positive results, 97 specimens were found to contain only hydrocodone and/or hydromorphone by UPLC–MS-MS analysis. There were 28 false positive results, in which UPLC–MS-MS analysis demonstrated the presence of hydrocodone and/or hydromorphone in the specimens, but at concentrations <300 ng/mL (<375 ng/mL for hydromorphone). The Lin-Zhi HEIA assay yielded 22 false negative results. UPLC–MS-MS demonstrated the following ranges of analyte concentrations in these specimens: 315–1,193 ng/mL of hydrocodone and 80–5,849 ng/mL of hydromorphone.

The overall analytical sensitivity of the Lin-Zhi HEIA was 0.880. In addition to hydrocodone and/or hydromorphone, 73 of the 183 UPLC–MS-MS positive specimens contained other opiates including codeine, oxycodone, oxymorphone, morphine and 6-MAM. These opiates were also present in 48% (382/798) of the hydrocodone/hydromorphone-negative specimens. In the specimens yielding negative results, other opiates were present at concentrations up to codeine ≥6,800 ng/mL, oxycodone ≥55,330 ng/mL, oxymorphone ≥50,000 ng/mL and morphine ≥38,381 ng/mL. The analytical specificity of the HEIA was 0.966.

Precision

Precisions of the Lin-Zhi HEIAs were determined from the absorbance values obtained with repetitive analyses of positive and negative control urine specimens for the 100 and 300 ng/mL cutoff assays. The between-run CVs were determined with five replicated per day for 5 days. The precision studies results are summarized in Table V. The within-run CVs (n = 10) for both cutoffs were <1% and the between-run CVs (n = 25) were <2.5%.

Specificity

A total of 170 compounds were evaluated including opiates, amphetamines and analogs, barbiturates, benzodiazepines, cannabinoids, cocaine and metabolites, analgesics/non-steroidal anti-inflammatory drugs, anticonvulsants, antihistamine, antihypertension, antidepressants/anti-anxieties/antipsychotics and nicotine/cotinine. The drugs/metabolites that cross-reacted with the assay are listed in Table VI. Opiates that did not cross-react with the HEIA at both cutoffs are presented in Table VII.

Discussion

The hydrocodone and hydromorphone enzyme immunoassay evaluation data presented here represent the largest number of patient specimens containing hydrocodone and/or hydromorphone (375 confirmed positives) of any previous immunoassay evaluation involving these compounds. For example, in one evaluation of an opiate immunoassay for the detection of hydrocodone and hydromorphone, the authors detected 81 positive specimens (5). The high incidence of positive results in this study is due to the nature of the clients, pain management urine specimens. The risks of having false positive results 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. However, enzyme immunoassays are more specific by using monoclonal antibody based assays (6). This study showed that the Lin-Zhi HEIA shows cross-reactivity with high concentrations of opiates that have similar structures to hydrocodone.

Nine false negative results were obtained using the 100 ng/mL cutoff HEIA. This represents <1% of the total number of samples analyzed. Two of the 9 false negative results also had the same

Table V. Precision studies for the 100 and 300 ng/mL cutoff HEIAs

HEIA	Control	Within-run CV (n = 10)	Between-run CV (n = 25)
100 ng/mL cutoff	75 ng/mL	0.52%	2.42%
	125 ng/mL	0.61%	2.07%
300 ng/mL cutoff	225 ng/mL	0.66%	1.31%
	375 ng/mL	0.31%	0.79%

Table VI. Drugs tested in urine that cross-reacted with the Lin-Zhi HEIA

Compound	Lowest concentration to react with assay (ng/mL)	
	100 ng/mL cutoff	300 ng/mL cutoff
Hydromorphone	100	375
Oxycodone	5,000	6,000
Oxymorphone	2,000	5,000
Morphine	2,000	5,000
Dihydrocodeine	5,000	DNR[a]
Codeine	1,000	5,000

[a]Dihydrocodeine did not cross-react (DNR) with the 300 ng/mL assay in concentrations up to 20,000 ng/mL.

Table VII. Opiates that did not cross-react with the Lin-Zhi HEIA at both cutoffs

Compound	Highest concentration tested (ng/mL)	Compound	Highest concentration tested (ng/mL)
Norcodeine	20,000	O-desmethyltramadol	25,000
6-MAM	10,000	N-desmethyltramadol	25,000
Propoxyphene	33,000	EMDP	20,000
Norpropoxyphene	33,000	Methadone	20,000
Meperidine	3,000	Ketamine	20,000
Normeperidine	3,000	Norketamine	20,000
Buprenorphine	1,000	Fentanyl	5,000
Norbuprenorphine	1,000	Norfentanyl	5,000
Tramadol	25,000	Heroin	10,000
Nortramadol	25,000		

results with the opiate immunoassay, including a sample containing only hydromorphone at 5,900 ng/mL, giving anomalous results at both cutoffs. Out of the 123 false positive results at the 100 ng/mL cutoff, eight samples had at least 80 ng/mL of hydrocodone and/or hydromorphone, with one of them having a combined concentration of over 100 ng/mL. In 121 of the cases (hydrocodone 97 and 90 ng/mL), there were other opiates present in concentrations up to 240,000 ng/mL of morphine, 55,300 ng/mL of oxycodone and 50,000 ng/mL of oxymorphone, which are much higher than the ones found to cross-react with the assay (Table VI).

Twenty-two false negative results were obtained using the 300 ng/mL cutoff HEIA. This represents ~2% of the total number of samples analyzed. One of the 22 false negative results also had the same results with the opiate immunoassay. Out of the 28 false positive results at the lower cutoff, four samples had at least 275 ng/mL of hydrocodone and/or hydromorphone. In 25 of the 28 cases, there were other opiates present at concentrations up to 240,000 ng/mL of morphine, 50,000 ng/mL of oxycodone and 20,000 ng/mL of oxymorphone, which are much higher than the ones found to cross-react with the assay (Table VI).

Commercial HEIAs have a cutoff concentration of 300 ng/mL, while confirmation cutoff concentrations for non-regulated and federally regulated drug tests are 50 and 2,000 ng/mL, respectively (7). To the author's knowledge, there are no studies evaluating enzyme immunoassays specific to hydrocodone and hydromorphone. The only study involving the evaluation of a screening method for hydrocodone and hydromorphone found utilized an opiate immunoassay (5). Homogeneous enzyme immunoassays, such as the Lin-Zhi assay, remains the method of choice for urine drug screening when rapid, high-volume testing is required as in PMCT, and a specific HEIA is recommended for zero-tolerance routine testing.

Conclusion

The Lin-Zhi HEIA 100 ng/mL cutoff assay demonstrated sensitivity for the detection of hydrocodone and hydromorphone in urine, while the 300 ng/mL was more selective, but not as sensitive. The HEIA has the potential to detect hydrocodone/hydromorphone in positive specimens screening that had screened negative by the opiate enzyme immunoassay. The specificity and sensitivity for the 100 ng/mL cutoff was 0.846 and 0.959, respectively, and for the 300 ng/mL cutoff was 0.966 and 0.880, respectively. The concordance of HEIA and UPLC–MS-MS results for the 100 and 300 ng/mL cutoff was 87% and 95%, respectively. Therefore, in PMCT, a more sensitive (100 ng/mL cutoff) rather than a specific (300 ng/mL cutoff) HEIA should be part of an initial immunoassay screen.

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