Evaluation of an Enzyme Immunoassay for the Detection of Methadone Metabolite EDDP [2-Ethylidene-1,5-Dimethyl-3,3-Diphenylpyrrolidine] in Urine

Carl E. Wolf,^{1*} Ashley Goldstein,² Justin L. Poklis,³ and Alphonse Poklis^{1,2,3}

¹Department of Pathology, Virginia Commonwealth University, Richmond, Virginia

²Department of Forensic Science, Virginia Commonwealth University, Richmond, Virginia

³Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia

Background: We evaluated a new EDDP [2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine] enzyme immunoassay (EDDPI; Lin-Zhi International, Inc., Sunnyvale, CA) for the detection of this primary methadone urinary metabolite. **Methods:** All specimens were tested with two different cutoff calibrators at 150 and 300 ng/ml EDDP on an ADVIA 1200 Chemistry System auto-analyzer. Controls containing 0, –25% (negative control), and +25% (positive control) of the cutoff calibrators (Lin-Zhi) were analyzed with each batch. All urine specimens were then analyzed by high-pressure liquid chromatography/mass spectrometry/mass spectrometry (HPLC-MS/MS) for EDDP. **Results:** Approximately, 42% (151) of the 362 specimens yielded positive results by the EDDP assay at

150 and/or 300 ng/ml cutoff values. Of these specimens, HPLC-MS/MS confirmed the presence of EDDP > 25 ng/ml in all 151 specimens. No specimen yielding negative EDDPI results contained EDDP by HPLC/MS/MS. At 150 ng/ml cutoff, the EDDPI demonstrated a sensitivity of 1.00, a specificity of 0.986, and an overall agreement of HPLC/MS/MS of >99%. At 300 ng/ml cutoff, the EDDPI demonstrated a sensitivity of 1.00, a specificity of 0.959, and an overall agreement of HPLC/MS/MS results of 97.5%. **Conclusion:** The Lin-Zhi EDDPI provides a precise, reliable method for the routine detection of methadone metabolite in urine specimens, particularly in pain management compliance testing. J. Clin. Lab. Anal. 28:136–140, 2014. © 2014 Wiley Periodicals, Inc.

Key words: enzyme immunoassay; EDDP; urine drug testing; pain management compliance testing

INTRODUCTION

Methadone was originally developed in Germany during the Second World War as an alternative analgesic to morphine. The drug is an equipotent to morphine and demonstrates wide range of morphine-like effects including orthostatic hypotension, cough suppression, and slowing of gastrointestinal propulsion (1). Methadone overdose, like other mu-receptor agonist, is characterized by pin point pupils, respiratory depression, and coma. Tolerance develops to these therapeutic and toxic effects with chronic administration, and naloxone is an antidote for methadone poisoning. Methadone exists as a racemate of R and S isomers, with the levo form (R) isomer being 8–50 times more potent than the S isomer depending upon the particular effect (2). Pure racemic isomer is expensive to produce and as a result only the racemate has

been available in the US pharmaceutical market. The primary indications for methadone are analgesia for chronic pain, prevention of opiate withdrawal, and maintenance therapy for opiate-dependent individuals (3). As a result, methadone has been an important analyte in urine drug testing as part of methadone maintenance drug treatment programs, postmortem drug screening, testing emergency

Grant sponsor: National Institute of Drug Abuse (NIDA) Center for Drug Abuse; Grant number: P50DA005274.

*Correspondence to: Carl E. Wolf, Department of Pathology, Virginia Commonwealth University, P.O. Box 98-0165, Richmond, VA 23298-0165. E-mail: cewolf@vcu.edu

Received 11 March 2013; Accepted 6 June 2013

DOI 10.1002/jcla.21657

Published online in Wiley Online Library (wileyonlinelibrary.com).

department specimens, and recently, pain management compliance testing (PMCT).

The plasma half-life, renal clearance, and ultimately urinary concentrations of methadone are greatly affected by urinary pH. The plasma half-life of methadone is approximately 20 hr at an acidic urinary pH due to ion trapping and approximately 42 hr in alkaline urine due to renal tubular reabsorption (4). Nine methadone urinary metabolites have been identified; however, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) is formed by *N*-demethylation and spontaneous cyclization of the primary urinary metabolite (5, 6). EDDP is physiologically inactive. Urinary excretion of methadone plus EDDP may account for up to 57% of an administered dose. Continuous administration causes an induction of methadone metabolism resulting in increasing urinary EDDP. In a study of six patients following initial dosing, urinary EDDP/methadone ratios ranged from 0.40 to 1.3 with a mean of 0.77; however, after 4 weeks of steady state, the mean EDDP/methadone had increased to 2.90 with a range of 0.60–9.0 (5). Following continuous administration, EDDP would more likely be present in greater concentrations of methadone. Therefore, EDDP is the analyte of choice in urine testing of patients on low doses of methadone, particularly PMCT.

We present an evaluation of a new EDDP enzyme immunoassay (EDDPI) designed for the detection of EDDP in urine (Lin-Zhi International, Inc., Sunnyvale, CA). The assay may be performed with a 300 or 150 ng/ml EDDP cutoff calibrator. As with other enzyme immunoassays (EIA), the EDDPI is based on competition between drug labeled with glucose-6-phosphate dehydrogenase and free drug in the urine for a fixed amount of antibody-binding sites. In the absence 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 enzyme activity. The enzyme activity is determined spectrophotometrically by measuring the change in absorbance at 340 nm. Overall analytical efficiency of the EDDPI was evaluated. All urine specimens, whether testing positive and negative by the EDDPI, were then analyzed by a validated direct injection high-pressure liquid chromatography/mass spectrometry/mass spectrometry (HPLC/MS/MS) assay (7).

MATERIALS AND METHODS

Reagents

All primary reference materials of methadone and EDDP and other drugs were obtained as 1.0 mg/ml

methanolic solutions from Cerilliant Corporation (Round Rock, TX). In-house drug-free urine was used for the preparation of some control specimens for both EDDPI and HPLC/MS/MS assays. Urine was obtained from laboratory personnel who did not smoke tobacco, take prescription or over-the-counter drugs. These urine specimens were analyzed by HPLC/MS/MS for methadone and EDDP, and by EIA for drugs of abuse and yielded negative results. These urine specimens were then pooled to create the in-house drug-free urine.

Controls

The 115 ng/ml EDDP control was prepared with in-house drug-free urine. All other EDDPI controls were obtained from Bio-Rad Laboratories, Irvine, CA.

Control Crossover Study

EDDPI controls were analyzed by HPLC/MS/MS over multiple batches yielding the following results: the prepared target 115 ng/ml EDDP control was determined to contain 112 ± 2.6 ng/ml (coefficient of variation [CV] = 3%, $n = 8$). Lin-Zhi-provided controls yielded the following by HPLC/MS/MS results: 205 ± 16.8 ng/ml (CV = 8.3%, $n = 8$) for the target 225 ng/ml control and 325 ± 12.5 ng/ml (CV = 3.8%, $n = 8$) for the target 375 ng/ml control. HPLC/MS/MS analysis of the EDDPI cutoff calibrators yielded the following: 309 ± 5 ng/ml (CV = 2%, $n = 3$) for the 300 ng/ml and 145 ± 8.4 ng/ml (CV = 5.6%, $n = 3$) for the 150 ng/ml calibrator. All EDDPI controls and calibrators were within $\pm 20\%$ of their target values. All HPLC/MS/MS controls containing less than the cutoff values of the EDDPI assays yielded negative results. The HPLC/MS/MS lower limit of quantification (LLOQ) control (target: 25 ng/ml) and low-quality control (target: 75 ng/ml) were negative when tested at the EDDPI cutoffs of 150 and 300 ng/ml. The HPLC/MS/MS medium quality control (target: 500 ng/ml) and high quality control (target: 2,000 ng/ml) tested positive by the EDDPI at both cutoffs.

Study Protocol

Three hundred and sixty-two urine specimens submitted for drugs of abuse testing, from criminal justice clients, substance abuse treatment, and PMCT patients were used in this study. Many of the urine specimens had previously screened positive for parent methadone and/or drugs of abuse by immunoassays. Batches of 10–50 specimens were stored and refrigerated for up to several weeks. These batches of specimens were removed from storage, maintained room temperature, and were then analyzed

for EDDP by new Lin-Zhi EDDPI. Each specimen was tested by EDDPI at two different cutoff values, 300 and 150 ng/ml EDDP. All specimens, testing both positive or negative for EDDP by the EDDPI assay, were then analyzed for EDDP by HPLC/MS/MS at an LOQ of 25 ng/ml. Testing of these specimens is not regulated by federal or state urine drug testing statutes; therefore, we applied a rapid, direct injection, validated HPLC/MS/MS method that determines the presence or absence of methadone and EDDP (7).

EDDPI Instrumentation and Analysis

The EDDP assays were obtained from Lin-Zhi International. All EDDPI tests were performed on an ADVIA 1200 Automatic Analyzer (Bayer Diagnostics, Tarrytown, NY). Analyzer parameters were as follows: 8 μ l aliquots of urine were sampled and mixed with 80 μ l of antibody reagent and 30 μ l of enzyme conjugate. The mixture was incubated at a temperature of 37°C. The assay was calibrated with negative and cutoff calibrators containing 0.0, 150, and 300 ng/ml of EDDP, respectively (Lin-Zhi). Specimen aliquots yielding a reaction rate of equal to or greater than that of the 150 ng/ml or 300 ng/ml cutoff calibrators were considered positive for EDDP, respectively. Specimen aliquots yielding a reaction less than that of the 150 ng/ml cutoff calibrator were considered negative for EDDP for 150 ng/ml test, while those yielding a reaction less than the 300 ng/ml cutoff calibrator were considered negative for EDDP for the 300 ng/ml test. All EDDPI analyses were performed with control urine specimens added to each analytical batch containing target concentrations of 0.0 and 225 ng/ml (negative controls) and 375 ng/ml (positive controls) of EDDP for the EDDPI 300 ng/ml cutoff test and 0.0 and 115 ng/ml (negative controls) and 225 ng/ml (positive controls) of EDDP for the EDDP 150 ng/ml cutoff test. All immunoassay reagents were stored and refrigerated at 2°C.

Evaluation of EDDPI Performance

The analytical performance of the immunoassay at the 300 and 150 ng/ml cutoff was determined by calculation of assay specificity, sensitivity, and percent concordance with HPLC/MS/MS results. Specificity was calculated as $TN/(TN + FP)$: where TN is defined as a true-negative result, negative for EDDP by both immunoassays and HPLC/MS/MS; and FP is defined as a false-positive result, positive for EDDP by either of immunoassay and negative for EDDP at a concentration equal to or less than 150 ng/ml or 300 ng/ml by HPLC/MS/MS. Sensitivity was calculated as $TP/(TP + FN)$: where TP is defined as a true-positive result, positive for EDDP by both immunoassays and HPLC/MS/MS; and FN is defined as

TABLE 1. Methadone, d3-Methadone, EDDP and d3-EDDP Transition Ions, and Their Cone Voltages and Collision Energies (CE)

Compound	Transition ions (<i>m/z</i>)	Cone voltage (V)	CE (eV)
Methadone	310 > 265	25	16
	310 > 104	25	30
d3-Methadone	313 > 268	25	16
	313 > 104	25	30
EDDP	278 > 249	46	26
	278 > 234	46	31
d3-EDDP	281 > 249	46	26
	278 > 234	46	31

EMDP, 2-Ethyl-5-methyl-3,3-Diphenylpyrrolidine.

a false-negative result, negative for EDDP by immunoassays and positive for EDDP at a concentration equal to or greater than 150 ng/ml or 300 ng/ml by HPLC/MS/MS. The percent concordance of the immunoassay equaled the sum of TP and TN results divided by the total number of specimens tested.

The specificity of the assay was investigated by adding known amounts commonly prescribed by therapeutic agents, as well as popular drugs of abuse and/or their metabolites to drug-free urine at concentrations of 1,000–10,000 ng/ml and analyzing these specimens by the EDDPI assay at both cutoff values.

HPLC/MS/MS Analysis

The presence or absence and/or quantification of methadone and EDDP in urine specimens was determined by a previously validated HPLC/MS/MS method (7). The HPLC/MS/MS system used a Quattro II Triple Quadrupole mass spectrometer with an electrospray ion source (ESI) attached to Water's Alliance 2695 HPLC controlled by MassLynx version 3.5 software. The chromatographic separation was performed using a Pinnacle C18 100 $\times$ 3.2 mm, 3- μ m column (Restek, Bellefonte, PA 16823). The mobile phase contained water/acetonitrile (20:80 v/v) with 1% formic acid and was delivered at a flow rate of 0.5 ml/min. The desolvation temperature was set at 250°C and ESI nebulizing gas flow rate of 15 ml/min with the drying gases flow rates of 250 ml/min. The capillary voltage was 3.5 V, cone was 40 V, and extractor was 5 V. The acquisition mode used was multiple reaction monitoring. Table 1 lists the transition ions monitored and the collision energies used for methadone, EDDP, and their corresponding internal standards. The total run-time of the method was 6 min. The calibration curves were linear from 25 to 5,000 ng/ml for both methadone and EDDP. The LLOQ for each analyte was 25 ng/ml. The intra- and interday

TABLE 2. Comparison of Lin-Zhi EDDPI at 150 and 300 ng/ml Cutoffs With HPLC/MS/MS Results for EDDP From Testing of 362 Urine Specimens

		EDDPI, 150 ng/ml cutoff	
		+	–
LC/MS/MS	+	148	0
	–	3	211
		EDDPI, 300 ng/ml cutoff	
		+	–
LC/MS/MS	+	142	0
	–	9	211

precisions had CVs less than 15% and the accuracy was within the range from 94% to 110%.

RESULTS

The linearity, within-run, and between-run precession of the Lin-Zhi EDDPI assay were determined from the absorbance values obtained with repetitive analysis of positive and negative control urine specimens for the 150 and 300 ng/ml cutoff calibrations of the EDDP assay. The within-run CVs for 150 ng/ml cutoff negative control (115 ng/ml) and positive control (205 ng/ml) were 1.9% ($n = 12$) and 1.0% ($n = 12$), respectively. The between-run CVs determined over 3 months for 150 ng/ml cutoff negative control were 6.0% ($n = 21$) and for positive control were 7.0% ($n = 21$), respectively. The within-run CVs for 300 ng/ml cutoff negative control (205 ng/ml) and positive control (325 ng/ml) were 2.0% ($n = 12$) and <1% ($n = 12$), respectively. The between-run CVs determined over 3 months for 300 ng/ml cutoff negative control were 3.3% ($n = 21$) and positive control was 2.2% ($n = 21$).

The comparison of urine test results with the EDDPI applying 150 and 300 ng/ml cutoffs and results of HPLC/MS/MS are presented in Table 2, respectively. Approximately 42% (151/362) of the specimens yielded positive results by the EDDPI with a 150 ng/ml cutoff. EDDP was detected in these urine specimens at concentrations ranging from 26 to 234,000 ng/ml. Of these 151 specimens, HPLC/MS/MS confirmed the presence of EDDP at ≥ 150 ng/ml in 148 specimens. The apparent three false-positive results occurred with specimens that contained EDDP, but at concentrations below the cutoff values: 26, 67, and 127 ng/ml. Therefore, the overall analytical sensitivity [TP/(TP + FN)] of the EDDPI assay was 0.980. The EDDPI yielded no false-negative results with 150 ng/ml cutoff. Thus, the selectivity of the assay was 1.00 (100%). The entire set of test results for the 362 urine specimens yielded a concordance at the 150 ng/ml cutoff EDDPI and HPLC/MS/MS results of 99.2%.

Approximately, 42% (151) of the 362 specimens yielded positive results by the EDDPI with a 300 ng/ml cutoff. Of these 151 specimens, HPLC/MS/MS confirmed

TABLE 3. Drugs Tested in Urine at 100 mg/ml That Did Not Cross-React With the Lin-Zhi EDDPI at a 300 ng/ml Cutoff

Acetaminophen	Diphenhydramine	Nortriptyline
Alprazolam	Fentanyl	Oxazepam
Amitriptyline	Fluoxetine	Oxycodone
Amphetamine	Hydrocodone	Oxymorphone
Benzoylceognine	Hydromorphone	Phencyclidine
Carbamazepine	Imipramine	Phenobarbital
Chlorpheniramine	Meperidine	Propoxyphene
Chlorpromazine	Methamphetamine	Pseudoephedrine
Citalopram	Methylenedioxymphetamine	Quetiapine
Cocaine	Methylenedioxyme thamphetamine	Secobarbital
Codeine	Methylphenidate	Sertraline
Cotinine	6-Monoacetylmorphine	Thioridazine
Diazepam	Morphine	Zolpidem
Desimpramine	Nicotine	
Dextromethorphan	Nordiazepam	

the presence of EDDP at ≥ 300 ng/ml in 142 specimens. Nine of the specimens contained EDDP at concentrations below the 300 ng/ml cutoff. These apparent false-positive results contained EDDP at concentrations, by HPLC/MS/MS testing, of 26, 67, 127, 173, 225, 244, 266, 271, and 281 ng/ml. The overall analytical sensitivity [TP/(TP + FN)] of the EDDPI by applying a 300 mg/ml cutoff was 0.940. The EDDPI displayed a selectivity of 1.00 (100%) by yielding no false-negative results with 300 ng/ml cutoff. The entire set of test results for the 362 urine specimens yielded a concordance at the 300 ng/ml cutoff EDDPI and HPLC/MS/MS results of 97.5%.

The specificity of the assay was investigated by adding known amounts of commonly prescribed therapeutic agents, as well as popular drugs of abuse and/or their metabolites to drug-free urine at concentrations of 1–100 μ g/ml and analyzing these specimens by the EDDPI assay at both the 150 and 300 ng/ml cutoff values. The drugs/metabolites tested are listed in Table 3. The EDDPI assay with a 300 ng/ml cutoff demonstrated no cross-reactivity with these compounds at 100 mg/ml. Drugs that did cross-react with the 150 ng/ml cutoff EDDPI are presented in Table 4.

DISCUSSION

Methadone EIAs have a cutoff concentration of 300 ng/ml (8). In heroin rehabilitation programs, this cutoff concentration has sufficient sensitivity to monitor methadone maintenance patients who receive daily doses of 80–160 mg. However, a 300 ng/ml cutoff often lacks the sensitivity to detect methadone in urine from pain patients receiving 5–10 mg/day doses. The concentration of methadone in urine is dependent upon not only the daily dose, but also pH of the urine and variations in the rate of methadone metabolism (9, 10). The plasma

TABLE 4. Drugs Tested in Urine at 100 ug/ml That Yielded Positive Results With the Lin-Zhi EDDPI at the 150 ng/ml Cutoff

Drug	Concentration (μg/ml)
EMDP	100
Methadone	30
Chlorpromazine	1
Citalopram	100
Doxylamine	100
Fluoxetine	100
Quetiapine	100
Phencyclidine	10
Sertraline	100
Thioridazine	1

half-life, renal clearance, and ultimately urinary concentrations of methadone are greatly affected by urinary pH. The plasma half-life of methadone is approximately 20 hr at an acidic urinary pH due to ion trapping and as long as 42 hr in alkaline urine due to renal tubular reabsorption (10). In vitro and in vivo studies have demonstrated that CYP3A4 and to a lesser extent CYP2D6 are the major hepatic cytochrome P-450 isozymes involved in methadone metabolism (11, 12). Thus, genetic variations in the activity of these enzymes may produce wide variations of methadone metabolism and resultant urinary excretion of parent methadone and EDDP.

Of the few urine EDDP immunoassays are available, lateral flow immunoassay and enzyme-linked immunosorbent assay, presently the Cloned Enzyme Donor Immunoassay (CEDA) EDDP immunoassay is the only one independently evaluated (13). That study demonstrated the importance of EDDP as the best analyte to establish methadone use or abuse. Numerous urine specimens yielding negative results by a methadone immunoassay were positive for EDDP by the CEDA EDDP assay. All positive EDDP results were confirmed by gas chromatography/MS. Additional testing was performed on specimens yielding CEDA EDDP assay negative result. Therefore, unlike the presented study, the possibility of false-negative results was not evaluated. Homogeneous EIA, such as the Lin-Zhi EDDPI, remains the method of choice for urine drug screening when rapid, high-volume testing is required as in PMCT (14).

CONCLUSION

We have found that the Lin-Zhi EDDPI has a cutoff value of 300 ng/ml, which is highly specific for the detection of EDDP in urine and may be of particular value in emergency department drug abuse testing. Additionally, we have found the Lin-Zhi EDDPI with a cutoff value of 150 ng/ml is a highly reliable method for the detection of

EDDP in urine, particularly specimens for PMCT. From the present study, the analytical sensitivity of this assay was 0.982 and the analytical specificity was 0.987.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Drug Abuse (NIDA) Center for Drug Abuse grant P50DA005274. The EDDP immunoassays evaluated in this study were a gift from Lin-Zhi International, Sunnyvale, CA.

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