RESEARCH

A Procedure for the Detection of Stealth™ Adulterant in Urine Samples

SANDRA VALTIER, JOHN T CODY

Stealth™ is an adulterant that is advertised as not only preventing a positive drug test in urine, but also to be undetectable by currently available adulteration testing. It has previously been described as a peroxidase and peroxide that is added to urine for the sole purpose of preventing a positive drug test. The product was found to have a significant impact on the ability to detect several drugs of abuse, however, detecting the presence of the adulterant in urine had not yet been reported. A simple procedure to detect the presence of this adulterant in urine was developed. This simple color test procedure using commercially available reagents commonly used in clinical laboratories is based on the use of a chromogen to detect the peroxidase reaction in urine samples. If Stealth is present in the urine, the test sample will show an immediate color change from clear to dark brown. This qualitative test can also be adapted for use with a spectrophotometer or autoanalyzer.

ABBREVIATIONS: GC/MS = gas chromatography/mass spectrometry; LSD = lysergic acid diethylamide; PCP = phencyclidine; THC-COOH = 11-nor-delta-9-tetrahydrocannibinol-9-carboxylic acid; TMB = 3,3',5,5'-tetramethylbenzidine.

INDEX TERMS: adulteration; peroxidase detection; Stealth.

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***Sandra Valtier MS** is Research Medical Technologist, Clinical Research Squadron, Lackland AFB TX.*

***John T Cody PhD** is Director of Biomedical Science Division, Interservice Physician Assistant Program at Academy of Health Sciences, Fort Sam Houston TX.*

***Address for correspondence:** Sandra Valtier MS, 59th MDW/MSRL, 59th Clinical Research Squadron, 1255 Wilford Hall Loop, Lackland AFB TX 78236-5319. (210) 292-6172, (210) 292-6053 (fax). sandra.valtier@59mdw.whmc.af.mil*

The Reports and Reviews Section seeks to publish information on important clinical laboratory-related topics such as technological, clinical, and experimental advances and innovations. Case studies and literature reviews are also included. In addition, brief reviews of books, computer programs, audiovisual materials or other materials of interest to readers are appropriate for this section. Manuscripts and literature reviews published as a Report are peer reviewed. Direct all inquiries to Isaac Montoya PhD, Affiliated Systems Corporation, 3104 Edloe, Suite 330, Houston TX 77027-6022. (713)439-0210, (713)439-1924 (fax). imontoya@affiliatedsystems.com

Adulterants have always posed a problem in drug testing laboratories. In the past, many of these adulterants were easily detected in urine either by appearance, smell, pH or specific gravity measurements.[1-4] In more recent years, the substances ingested or added to urine to prevent a positive drug test have become more difficult to identify and detect. Some of the more commonly used products include the fixative glutaraldehyde (UrinAid and Clear Choice), strong inorganic acid (Amber-13 and THC-Free), and strong oxidants such as nitrite (Klear, Whizzies, and Randy's Klear) and chromate (Urine Luck, LL 418, Sweet Pee's Spoiler, and Randy's Klear II).[2,5-15] These adulterants were designed specifically to avoid detection of illicit drugs in urine.

One of the newer adulteration products, Stealth, was reported to consist of two vials, one containing a powder (peroxidase) and the other vial containing a liquid (peroxide).[16] Combining the contents of both vials results in a strong oxidation potential, capable of oxidizing many compounds including several drugs and drug metabolites. The peroxidase catalyzes transfer of electrons between peroxide and another compound. This coupled reaction mechanism is used as the basis of a number of different clinical laboratory tests, e.g., cholesterol, glucose, etc. There are a number of commercially available assays designed to detect peroxidase activity, however, they are not routinely used to assess samples for adulteration. Often times, laboratories that perform drug screening assays are unaware of the various adulterants, detection methods, or their effects on the assay. Adulterants often have varying effects on different drugs-of-abuse testing assays. Even when there is reason to believe a sample has been adulterated, chances are that most clinical laboratories are not equipped with the appropriate materials to test for a specific adulterant.

In many cases, laboratory tests have been developed to detect the presence of some of the more commonly used adulterants and in some cases, manufacturers have designed test kits for use on autoanalyzers to detect the active component of these adulterants. However, most of the test kits provided for these purposes are designed for high volume drug testing laboratories. An automated test for detection of peroxidase will more than likely be available in the near future; however, for the small volume or clinical laboratories doing relatively few drug tests, purchasing a test kit for a few samples may prove to be unattractive due to the cost. A manual procedure using commercially available reagents for the detection of Stealth in urine was developed as part of this study. The color test is a simple and inexpensive procedure that can quickly and easily be performed in nearly any laboratory.

MATERIALS AND METHODS

Materials

TMB (Tetramethylbenzidine) Substrate Reagent Set for detection of peroxidase activity was obtained from Pharmingen and horseradish peroxidase (1,100 units/mg) was obtained from Sigma. Clinical dipsticks (Multistix SG) used for testing the samples were from Bayer Corp. Stealth was obtained from the supplier and provided to these investigators by the Research Triangle Institute and the Air Force Office of Special Investigation. Drugs were obtained from the following sources: Sigma [amphetamine, phencyclidine (PCP), morphine, morphine glucuronide, lysergic acid diethylamide (LSD), secobarbital, 11-nor-delta-9-tetrahydrocannibinol-9-carboxylic acid (THC-COOH)]; Alltech (benzoylecgonine, LSD, PCP); Radian (secobarbital, LSD); and Research Triangle Institute (THC-COOH). Immunoassay reagents for THC metabolite, cocaine metabolite, opiates, barbiturates, PCP, and amphetamines were OnLine reagents from Roche Diagnostics and CEDIA from Microgenics. LSD immunoassay reagents used were EMIT II from Behring and CEDIA from Microgenics.

Methods

Sample Preparation.

Drug free urine (no preservatives) was split into two portions. One was used as the negative control and the other was spiked with the drugs listed above in the Materials section. The spiked urine was further split into two portions, where one was used as the positive control and the other portion had Stealth added. The Stealth package contains two microcentrifuge plastic vials, one containing a powder (peroxidase) and the other vial containing a liquid activator (peroxide). As per Stealth package directions, the powdered catalyst is added to the empty sample cup, approximately 60 mL of sample liquid (urine) is added followed by the addition of the liquid activator and the sample is stirred briefly. (Note: for experimental purposes, smaller volumes of urine were prepared using proportionate portions of each vial).

Reagent Preparation

Horseradish peroxidase at concentrations ranging from 0.0002 to 0.1 mg/mL prepared in 0.1 M phosphate buffer, pH 7.0 were used as control samples. As per product insert, the TMB working solution was prepared by mixing equal volumes of Substrate Reagent A (hydrogen peroxide in a buffered solution) and Substrate Reagent B (3,3',5,5'-tetramethylbenzidine in organic solvent).

Procedures

Test tube: The test was performed by adding 10 µL of urine to a test tube containing 50 µL of TMB working solution in 500 µL of 0.1 M phosphate buffer (pH 7.0). The sample was mixed and observed for an immediate color change.

Microplate: 100 µL of sample was pipetted into a microplate test well, followed by the addition of 100 µL TMB working solution and sample observed for a color change. Note: The normal procedure for these assays is to add acid to stop the reaction after a specified incubation period followed by measurement of the sample absorbance at a specific wavelength (450 nm). Addition of the acid not only stops the enzyme reaction, it changes the color of the complex to yellow. This procedure was modified by not adding the acid; thus the only samples that turned yellow were those that had strong redox potential.

Spectrophotometer: Peroxidase activity was monitored on the Beckman DU Spectrophotometer. Using the wavelength scan program, the instrument was first blanked against phosphate buffer. 10 µL sample was added to a spectrophotometer cuvette containing 50 µL TMB working reagent (TMB:peroxide solution 1:1 v/v) in 500 µL 0.1 M phosphate buffer (pH 7.0). The sample and working reagent were mixed and immediately monitored. Spectrophotometer parameters used were: 10 scans per sample, interval time of 60 seconds, scan from 260 to 800 nm. Peroxidase activity was detected by monitoring peaks at 650 and/or 450 nm.

RESULTS AND DISCUSSION

Detection of peroxidase in samples was accomplished using 500 µL of 0.1 M phosphate buffer, pH 7.0 and 50 µL of the reagent mixture. Following the addition of sample to the reagent mix, the mixture was observed for an immediate color change. Horseradish peroxidase concentrations of 0.001 and 0.0002 mg/mL (1.1 and 0.22 units respectively) were monitored and compared to the activity seen in the negative, drug positive, and adulterated urine. No color change was seen for the negative or drug positive urine controls. The presence of Stealth was easily detected in the adulterated urines. In all cases, the color change observed for the adulterated urine was rapid and dramatic from clear to dark brown (Photo 1).

Results for the microplate assay were comparable to the test tube assay where all Stealth adulterated samples and peroxidase controls showed a dramatic color change to dark brown. Peroxidase activity in samples was also monitored using a spectrophotometer. Positive drug control, negative control, and Stealth adulterated urine results were based on the relationship between the sample

Photo 1. Color test for Stealth adulteration

absorbance and the absorbance of the peroxidase control. The absorbance and wavelength of peroxidase activity in samples for the spectrophotometer were established by using the parameters described earlier. Evaluation of the spectral data showed the absorbance maxima associated with TMB following reaction with hydrogen peroxide in the presence of peroxidase were 450 and/or 650 nm. Initially, following a single electron transfer, TMB forms a complex that absorbs at 650 nm. Transfer of another electron results in an increase in absorbance at 450 nm with corresponding diminution of the peak at 650 nm. The absorbance of the Stealth adulterated sample was compared and found to show rapid increase in absorbance at 450 nm. When diluted, the Stealth adulterated samples showed the characteristic initial absorbance at 650 nm followed by formation of a peak at 450 nm with corresponding decrease in the 650 nm peak. It was demonstrated that low concentration of peroxidase produced a peak at 650 nm but, unless the amount of enzyme was high, there was little or no formation of the peak at 450 nm. The peak absorbance of adulterated samples was high enough to leave no question of the presence of Stealth in the urine sample. This dramatic difference in activity was used as the basis for the qualitative assay.

Because peroxidase activity can result from blood or bacterial contamination, 167 urine samples from the clinical laboratory that tested positive for blood and/or bacteria by clinical dipstick were tested for peroxidase activity using the color test procedure. Most samples showed no color change at all; the few that did, developed a faint blue-green tint. However, the blue-green color from these samples was easily distinguished from the dark brown color seen in samples adulterated with Stealth. Whole blood and hemolyzed blood were also used to directly assess the pseudoperoxidase activity of hemoglobin. These samples, along with the two clinical samples that had shown slight color development were monitored by spectrophotometer. No absorbance was seen for any of these samples at 450 nm under the experimental conditions described above. These results demonstrate that the possibility of getting false positives from samples containing blood and/or bacteria when using the parameters described is unlikely.

Horseradish peroxidase in buffer stored in a refrigerator has an extended shelf life, however, the stability of Stealth in urine samples is, to this date, unknown. In reality, there may be no predictable stability for peroxidase in urine samples. Peroxidase activity changes over time in individual samples. For example, one sample adulterated with Stealth was monitored on the same day, 15 days, and one month after Stealth was added. At 15 days, peroxidase activity showed a slight decrease; however after approximately one month, the sample showed no peroxidase activity. Refrigeration or freezing will help to prevent degradation, but may well depend on the individual sample matrix. Enzyme activity can be affected by many different variables that cannot be changed or controlled in random urine samples.

Evaluation of peroxidase controls in urine required considering the potential effect of sodium azide, a commonly used preservative in urine control samples, on the enzyme activity. To test the effects of the azide on peroxidase activity, 100 μL of 0.01% sodium azide in urine was added to phosphate buffer containing 0.001 mg/mL peroxidase. After the addition of TMB reagent, the sample was scanned from 400 to 800 nm. Sodium azide had a significant negative effect on peroxidase activity with absorbance seen at 450 nm; therefore, urine control samples containing this preservative cannot be used to control this assay. Controls must either be prepared fresh or be in a stable and predictable matrix such as a buffer.

Parameters normally used to assess sample adulteration did not reveal any significant changes of the urine samples following addition of Stealth. The color of the urine did change to a darker amber-brownish shade after addition of Stealth; however, the color change was not significant enough to warrant suspicion and there was no change in odor of the adulterated sample. Several other parameters were measured before and after adulteration of these samples. Specific gravity, pH, creatinine, urea, and chloride in each of the samples were measured over time (0, 24, 48, 72 hr and 7, 14, 21 days). Urea is a denaturant capable of inactivating peroxidase over time by changing the structural integrity of the enzyme. Chloride can have an effect on pH by reacting to produce hydrochlorous acid. There was little or no change in either urea or chloride measurements in the adulterated samples. The pH of the adulterated samples were consistently lower than the unadulterated, but still within pH range commonly seen in the clinical laboratory. Little or no differences were seen in specific gravity and creatinine results. Clinical dipstick results showed strong positive readings for glucose, blood, and nitrite in all samples adulterated with Stealth (Table 1). It should be noted the instructions with the Stealth adulterant indicate it should not be used for physicals, which would involve clinical testing. A strong positive for glucose, blood, and nitrite in a single clinical sample is unusual and might raise the veil of concern that the sample is contaminated with this adulterant.

Table 1. Physical effects of Stealth on urine

	Urine	**Urine + Stealth**
pH	5.264	5.135
Sp. Gr.	1.011	1.012
Creatinine	43.3 mg/dL	41.2 mg/dL
Dipstick*		
Blood	Neg	+++
Glucose	Neg	+++ (>2,000 mg/dL)
Nitrite	Neg	Positive

* Dipstick – Bayer Multistix SG

The impact of the adulterant on a sample can differ considerably depending on its methodology or on the assay used. The effect of Stealth was evaluated in several studies, including one reported by Davis that indicated this adulterant caused the screening assay for the THC acid metabolite (THC-COOH) to yield a negative result when the drug metabolite was actually present.[16] The effect of Stealth on immunoassays for several drugs-of-abuse was studied in this laboratory (Table 2). Stealth had no effect on the assays for amphetamines, PCP, benzoylecgonine (cocaine metabolite), or barbiturates. It did cause samples positive for THC-COOH and opiate (morphine) to screen negative by both the OnLine and CEDIA immunoassays. Samples positive for LSD were also negative by EMIT II and CEDIA immunoassays following adulteration with Stealth. The THC-COOH and LSD positive controls adulterated with Stealth gave values that were comparable to the negative urine control. Although the result for the sample containing 2500 ng/mL morphine yielded a negative immunoassay result, some measurable activity was seen (approximately 30% of positive control value). Samples spiked with higher concentrations of opiates (6,000 ng/mL) did test positive, indicating the effect of Stealth on the immunoassays for opiates is dependent on drug concentration. Upon confirmation testing of these samples by gas chromatography/mass spectrometry (GC/MS), THC-COOH, morphine, codeine, and LSD were undetectable. Subsequent evaluation of opiates showed the initial interference with morphine/codeine confirmation could be reversed by addition of disulfite.[17]

CONCLUSION

With new adulterants being developed at an alarming rate, it has become increasingly difficult to keep up with the development of methods to detect these products. Once a method is developed to detect presence of a specific adulterant, it will more than likely be provided commercially as a kit that may be more suitable for a high volume drug testing or toxicology laboratory. A color test using commercially available reagents for the detection of peroxidase was developed in our laboratory and found to be a reliable method in detecting Stealth in urine. The simplicity of the assay makes it ideal for laboratories that would test only a small number of samples. Using the reagents used for the manual procedure, however, the test could be adapted to most automated chemistry analyzers. The procedure is presented here to provide laboratories with a quick and inexpensive method for the detection of Stealth in urine.

Table 2. Effect of Stealth on drugs of abuse

	OnLine	CEDIA
THC	–	–
Cocaine	+	+
Opiates	–	–
Barbiturates	+	+
PCP	+	+
Amphetamine	+	+
LSD*	–	–

* LSD tested by EMIT II in place of Online and CEDIA immunoassay reagents

Most assays designed for the analysis of peroxidase activity are designed to detect relatively small amounts of activity since normal urine samples have no activity. This sensitivity makes them valuable in the laboratory for routine assays but such sensitivity is not necessary when determining the presence of Stealth. The reduction-oxidation reaction of TMB involves first a single electron transfer that yields a maximum absorbance at 650 nm. This complex has a very faint blue color at neutral pH. If there is sufficient redox potential in the sample, the TMB complex undergoes another electron transfer to yield a complex with an absorbance maximum at 450 nm. Monitoring of the reaction using a scanning UV spectrophotometer showed the development first of a peak at 650 nm. This peak continued to grow until it eventually began to diminish with the appearance of a new peak at 450 nm. Under ordinary conditions, assays are conducted by allowing the reaction to occur for a specified amount of time at which point a strong acid is added. The purpose of the acid is to stop the reaction to accommodate reading all samples at a later time. Another consequence of the acid is to drop the pH of the reaction mixture which changes the absorbance to 450 nm. In the procedure described in this study, the pH was maintained at 7.0. This allowed the reaction of samples containing components that react with the reagent to do so but the resulting reaction gave absorbances in the 650 nm range. The presence of Stealth, owing to its high redox potential, quickly changed the solution to have a significant absorbance at 450 nm. Since the pH was maintained at 7.0, the only samples that demonstrated the absorbance peak at 450 were those adulterated with Stealth.

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