Zinc Reduces the Detection of Cocaine, Methamphetamine, and THC by ELISA Urine Testing

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Abstract

Federal workplace drug testing was initiated during the late 1980s. Since then, numerous methods have been employed to subvert these drug tests, adulteration of urine samples being the most common. A wide variety of adulterants has been reported to date along with suitable methods of their detection. Recently, websites have claimed that zinc sulfate can be an effective adulterant to bypass drug testing. Herein, these claims are investigated using standard drug detection kits and urine samples adulterated with zinc. Drug-free urine samples were fortified with different amounts methamphetamines and benzoylecgonine, to which zinc sulfate was added to study its effect. Urine samples from acute marijuana smokers were also obtained in order to study the effects of zinc supplements on THC drug testing. All urine drug testing was performed using ELISA detection kits manufactured by Immunalysis. Both zinc sulfate and zinc supplements are effective in interfering with the detection of all three drugs by Immunalysis drug detection kits. Also, no suitable method could be established to detect zinc in urine samples. Zinc can be an effective adulterant in urine for some illicit drugs that are commonly screened under routine drug testing.

Introduction

When federal drug testing was initiated in the late 1980s, the National Institute of Drug Abuse (NIDA) established the first set of guidelines for federal workplace drug testing and standards for accreditation, which were published in the *Federal Register* on April 11, 1988, and were later revised on June 9, 1994, and again on November 13, 1998 (1,2). Federal guidelines state that specimens initially must be screened for specific drugs by antibody-based FDA-approved immunoassays. If an immunoassay test is negative, the urine sample is reported as negative and no confirmative test is permitted. However, if an immunoassay test is positive for a particular drug, then the presence of drug or drug metabolite must be confirmed and quantified using gas chromatography–mass spectrometry (GC–MS).

From the moment routine drug testing programs began, methods have been employed to circumvent them, including adulteration, substitution, and dilution. Testing laboratories have reported observing numerous additives in urine samples, and websites such as www.passusa.com and www.passthedrugtest.com provide materials and discussion forums dedicated to the successful adulteration of urine samples in order to pass routine drug testing (3). Some of the common adulterants first associated with invalid urine specimens are simple household chemicals readily available to all. In 1988, Mikkelsen and Ash (4) studied the effects of eight additives that were thought to produce false-negative results on immunoassays. This study included NaCl, Visine®, hand soap, Drano®, bleach, vinegar, lemon juice, and goldenseal tea. The authors concluded that each of these adulterants, except lemon juice, could produce a false-negative on an immunoassay. However, they observed that specimens adulterated with vinegar, bleach, and Drano had a pH outside the physiological range; specimens containing NaCl had specific gravity outside the normal range (> 1.035); samples containing goldenseal tea appeared dark in color; and urine samples containing hand soap were found to be very cloudy. Visine was the only adulterant that could not be detected based on pH, specific gravity, and appearance. The authors argued that appearance of urine samples must be evaluated at the time of collection to ensure validity.

In 1999, Wu and colleagues (5) discovered that pyridinium chlorochromate (PCC), at a concentration of 100 g/L, was effective at interfering with a variety of immunoassay-based drug testing techniques, but could be detected as an oxidant using a spot test. In 1994, Goldberger and Caplan (6) reported that glutaraldehyde, marketed as UrinAid®, was effective in producing false negatives in some drug screens. In 2001, Cody and Valtier (7) scrutinized the effects of Stealth™, another advertised urine adulterant, which contains a combination of per-

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oxidase and peroxide. The authors conclude that the presence of Stealth in urine samples cannot be detected by measuring pH, specific gravity, creatinine, color, chloride, blood, sugars, or nitrites and was effective in masking the presence of several drugs commonly sought in urine testing.

Most of the adulterants described cause a characteristic change in the pH, specific gravity, and/or creatinine levels, which can be easily measured when urine validity is questioned. However, more specific procedures are needed to detect nitrites or other oxidants. The most common ways of detecting such adulterants is by a spot test or using urine test strips. In 2002, Dasgupta and colleagues (8) developed rapid spot tests using simple lab reagents for the detection of PCC and nitrites in urine samples. In 2004, the same group analyzed the effectiveness of the urine test strips Intect®7 and AdultaCheck®6 and found that both were very effective in detecting PCC, nitrites, and glutaraldehyde in urine samples, for which no separate spot test exits (9).

In 2004, new federal guidelines were established by the Substance Abuse and Mental Health Services Administration (SAMSHA) to address the concern of urine validity and to detect the presence of adulteration of samples (for thorough review, see references 2 and 10). For example, a urine specimen is said to be adulterated if the pH is less than 3 or greater than or equal to 11, if the concentration of nitrites greater than or equal to 500 µg/mL, or if the concentration of chromium ion (VI) is greater than or equal to 50 µg/mL. At the same time, a urine sample is defined as substituted if the creatinine concentration is less than 2.0 mg/dL and the specific gravity is less than 1.0010, or the specific gravity is above 1.0200. A urine specimen is defined as dilute if either the concentration of creatinine is below 20 mg/dL (but greater than or equal to 2.0 mg/dL) and the specific gravity is greater than 1.0010 but less than 1.0030. Urine samples containing oxidizers, often bleach, may be classified as invalid specimens for drug testing. In addition, the temperature of the sample must be checked within 4 min of collection and must range between 90°F and 100°F. In addition, the chain of custody must be maintained from the point the sample was collected to the point when the sample reaches the lab.

Nevertheless, the commercial market flourishes for dietary supplements and adulterants which claim to produce false negatives in routine drug testing. Two websites, www.erowid.org and www.angelfire.com, have both recently referenced the use of zinc sulfate as a possible means to evade drug testing, although the mechanism of such an effect is unclear. Thus, the current study seeks to examine the ability of zinc, either as a dietary supplement or a urine adulterant, to mask the presence of illicit drugs in routine drug testing.

Materials and Methods

Materials

Drug standards (1 mg/mL in methanol) of methamphetamine and benzoylecgonine drugs were obtained from Sigma Aldrich (St. Louis, MO). Zinc sulfate was obtained from Fisher Chemicals (Pittsburgh, PA). ELISA kits for testing methamphetamines, benzoylecgonine, and THC metabolite in urine samples were obtained from Immunalysis (Pomona, CA); their catalog numbers are 211-0192, 212-0192, and 205-0192 respectively. Adultacheck©10 strips were obtained from Sciteck (Arden, NC).

Drug standards

The drug standards were transferred to small glass vials with screw caps. The glass vials were uncapped and the methanol was allowed to evaporate completely. The drugs were then resuspended in Milli-Q water to obtain a final concentration of 1 mg/mL.

ELISA assay

Initial studies involve diluting positive calibrators (obtained from Immunalysis) to obtain different drug concentrations and treating them as samples for ELISA assay. Later, drug-free urine was obtained from a healthy volunteer. The urine was fortified with different volumes of drug standard (in water) in order to obtain different concentrations of the drug in urine. Different concentrations of zinc sulfate solutions were prepared in water. Zinc sulfate was then added to urine samples fortified with drugs in the ratio 1:10 (v/v). An ELISA assay was then performed on these fortified samples. The procedure provided by Immunalysis was followed for the assay. Tecan Columbus Plus and Tecan Sunsrise remote were the plate washer and plate reader, respectively, that were used for this assay.

Effect of pH

Drug-free urine was obtained from three healthy volunteers and split into two separate tubes. To one tube, zinc sulfate solution (15 mg/100 µL) was added at the ratio 1:10 (v/v). The pH of the urine samples were then measured using a Accumet pH meter (Fisher Scientific).

Drug testing for acute THC use

Four separate volunteers were recruited and urine was collected according to the time course indicated in the figure legends. The volunteers consumed approximately 0.75–1.0 g of marijuana and, if indicated, four 50-mg tablets of dietary zinc supplement (Chelated Zinc, Nature's Bounty™). Urine collection continued, as indicated in figure legends.

Spectrometry

Drug-free urine was obtained from four healthy volunteers and split into two separate tubes. To one tube, a zinc sulfate solution (15 mg/100 µL) was added in the ratio 1:10 (v/v). An absorption spectrum in the UV and visible range was then taken from the samples using a UV Pharmaspec-1700 spectrometer (Shimadzu, Tokyo, Japan).

Use of human subjects

This project and its protocols were approved by the Institutional Review Board of John Jay College (protocol 10-08-113-0137), and urine donors signed informed consent forms before participating in this study.

Results

Within the discussion forums of a website aimed at circumventing routine drug testing (www.erowid.org), it was observed that multiple website visitors claimed that zinc supplements or zinc sulfate added to urine can be used to escape detection of certain drugs in the urine. In a simple effort to test this claim, a solution of zinc sulfate was added to the positive control standard of an ELISA kit used to detect the presence of cocaine in urine. Figure 1 shows a dose-related effect when zinc sulfate is added to the calibrators before the assay is performed. Unexpectedly, the zinc solution appears to have an effect with both the positive and negative calibrators, which argues against the possibility that zinc interferes with the assay by chelating the drug and making it unavailable for antibody binding. In any event, in two separate experiments (Figure 1 and additional data not shown), the addition of zinc increased the ELISA signal of the calibrators, with maximal effect at 10 mg/mL. This increase in signal causes the positive calibrators to yield signal intensities similar to that of negative calibrators, indicating that zinc may indeed be an effective adulterant at preventing the detection of cocaine metabolites in an ELISA urine screen.

Next, these results were repeated using actual urine samples fortified with drugs. Urine samples were obtained from healthy drug non-user volunteers and fortified with increasing

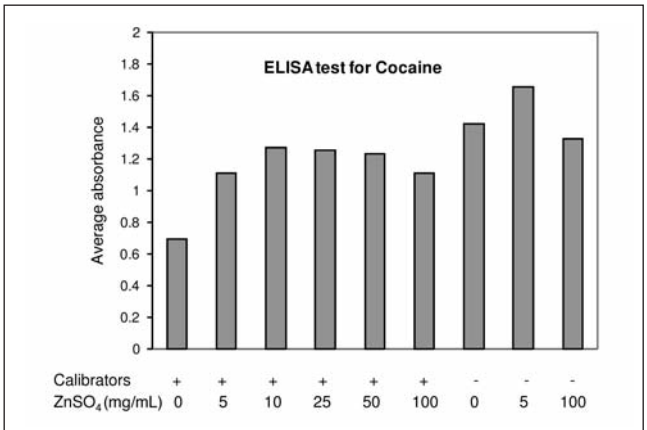

Figure 1. Effect of zinc on calibrators for cocaine drug testing by ELISA. ELISA tests for cocaine (Immunalysis) were performed on the positive (50 ng/mL) and negative calibrators provided with the detection kit, previously fortified with different concentrations of zinc sulfate, as indicated.

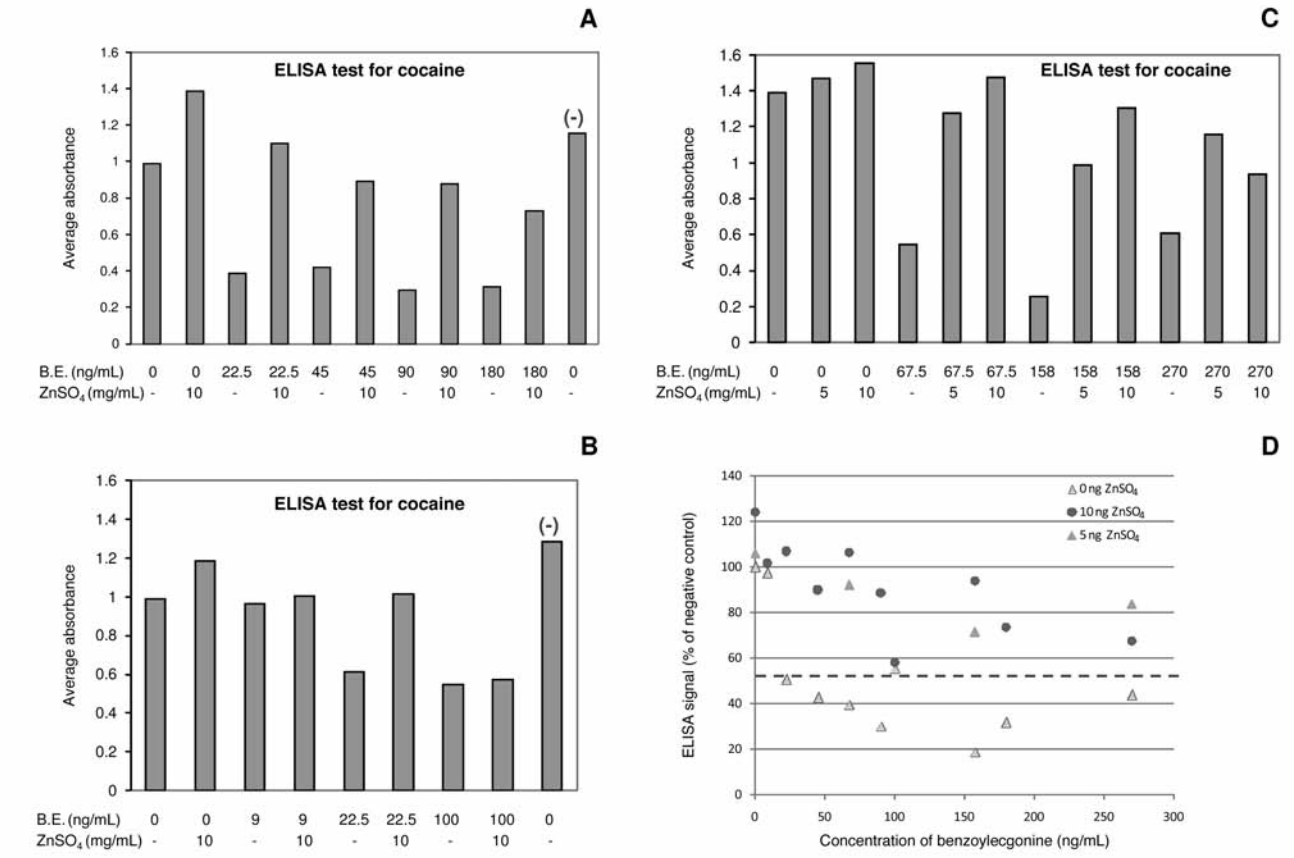

Figure 2. Effect of zinc in urine samples containing benzoylecgonine on cocaine drug testing by ELISA. ELISA tests for cocaine were performed on urine samples containing indicated concentrations of benzoylecgonine, with or without addition of zinc sulfate at indicated concentrations. A-B) Effects of zinc (10 mg/mL) on urine fortified with different concentrations of benzoylecgonine (A and B). For reference, the urine-free negative calibrator sample was also analyzed, marked with "(–)." Dose-response relationship of zinc on urine fortified with different concentrations of benzoylecgonine (C). The combined results A, B, and C expressed in one chart by expressing each absorbance value as a percent of its own negative calibrator reference sample (D). The dashed line marks the 55.7% cutoff, the average value of the kit-provided positive calibrator (50 ng/mL) in the four experiments shown in this figure.

concentrations of benzoylecgonine, the predominant metabolite of cocaine found in urine samples. Some samples were further adulterated by adding zinc sulfate at 10 mg/mL, and an ELISA screen for cocaine was performed. As shown in Figure 2A, 10 mg/mL of zinc sulfate causes an increase in the ELISA signal from samples with all examined concentrations of benzoylecgonine. This result is strong evidence that zinc sulfate could be an effective urine adulterant in masking the presence of cocaine metabolites. In order to test the robustness of this result, this experiment was repeated with urine from additional volunteers and with varying benzoylecgonine and zinc sulfate. As seen in Figures 2B and 2C, this phenomenon is both robust and reproducible across multiple individuals.

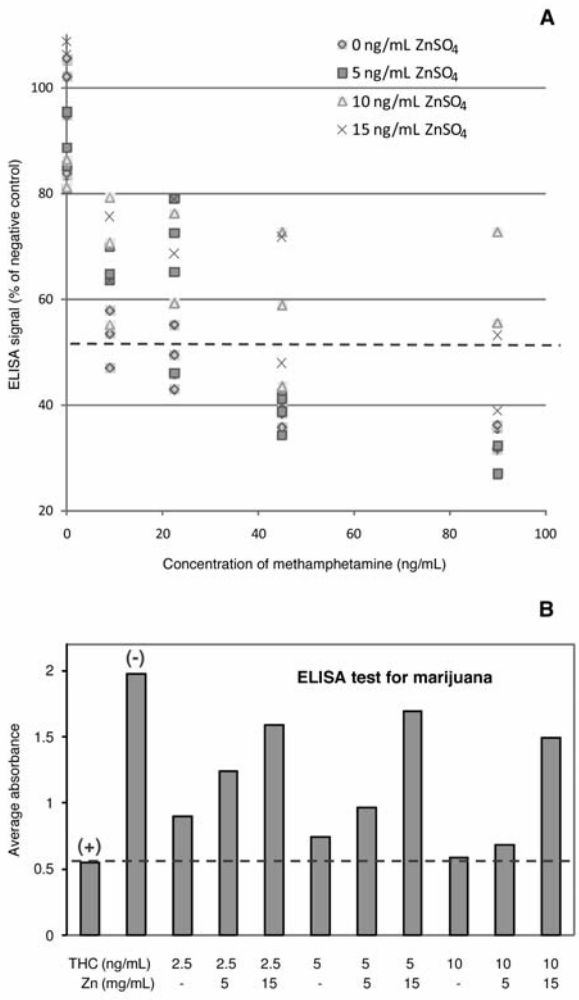

Figure 3. Effect of zinc sulfate on methamphetamine and THC drug testing by ELISA. The combined results of two separate experiments with urine from three different volunteers and involving varying concentrations of methamphetamine and zinc sulfate, expressed in one chart by expressing each absorbance value as a percent of its own negative calibrator reference sample (A). The dashed line marks the 52.3% cutoff, the average value of the kit-provided positive calibrator (50 ng/mL) in these experiments. Dose-response relationship of zinc on different dilutions of the THC positive calibrator (provided at 10 ng/mL) in urine (B). Throughout, (+) and (–) indicate undiluted and unaltered positive and negative calibrators, respectively, and the dashed line marks the (+) cutoff for this experiment.

The scatter plot shown in Figure 2D combines the results of Figures 2A–C, expressing each ELISA absorbance value as a percent of the negative calibrator sample for that particular experiment. Importantly, the average value given for the positive calibrator in the three separate experiments, which indicates the legal cutoff for a sample to be considered positive, is shown as a dashed line across the graph. From these results, it can easily be seen that nearly every urine sample that had been adulterated with benzoylecgonine, but not with zinc sulfate, appears positive for cocaine by this ELISA test, even at the modest concentrations of 25 and 50 ng/mL. However, every sample that had been adulterated with zinc sulfate appears negative, even at concentrations up to 270 ng/mL of benoylecgonine, nearly three times the legal cutoff for a positive result for cocaine in most jurisdictions and contexts.

In order to examine whether zinc sulfate can act as a masking agent for other drugs that are routinely included in screening, drug-free urine samples were obtained from three different healthy volunteers and fortified with varying concentrations of methamphetamine and zinc sulfate. The absorbance values were again calculated as a percent of the matched negative calibrator and combined into one scatter plot. As seen in Figure 3A, zinc sulfate also appears to be a very effective adulterant in urine samples containing methamphetamine. The masking effect is dose-dependent and quite potent at 10–15 mg/mL, which is consistent with that previously seen with benzoylecgonine.

Next, in order to test whether zinc sulfate also affects the detection of tetrahydrocannabinol (THC), varying amounts of zinc sulfate were added to samples containing the THC metabolite 11-nor-^{9}-carboxytetrahydrocannabinol (THCCOOH). Because of the poor solubility of THCCOOH in water, this experiment could only be performed by diluting the THCCOOH positive calibrator with water to obtain different concentrations of THCCOOH. For this reasons, all dilutions will obviously appear to have a lower THCCOOH concentration than the positive control reference sample. Nevertheless, as Figure 3B shows, zinc sulfate reduces the ELISA signal in THC drug testing in a dose-dependent manner, as previously observed with that for cocaine and methamphetamine.

In addition to claims about the actions of zinc as a urine adulterant, users in the aforementioned website forums have claimed that zinc supplements, taken orally, can also mask the presence of drugs or metabolites in urine. To that end, volunteers were recruited who were willing to provide periodic urine samples while also recording and reporting their use of marijuana. In addition, some volunteers were asked to consume zinc supplements when directed. Figure 4A shows the results for one such volunteer. On day 0 of the experiment, the volunteer provided urine samples and reported use of no illicit drugs in the past month. On day 1, however, s/he reported smoking marijuana the night before and estimated the amount to be between three-quarters and one gram. After taking a urine sample, the subject consumed four 50-mg tablets of zinc dietary supplement and urine samples were harvested periodically over the next three days, as shown in Figure 4A. Figure 4B contains the results of another experiment with another such volunteer. On day zero, two drug-free urine samples were

obtained at the times indicated. On day 1, the subject indicated that s/he had smoked marijuana that pervious night. A urine sample was taken and the subject then consumed four 50-mg tablets of zinc. Additional urine samples were taken, according to the indicated time course.

Given the encouraging but controvertible results in Figures 4A and 4B, a more rigorous time-course analysis was sought. A volunteer was recruited and asked to take urine samples as often as convenient, especially immediately after smoking marijuana and after taking zinc supplements. Figure 4C shows the results of the urine ELISA testing of this additional volunteer. As made visible by the additional time points, the urine of this volunteer tests positive for THC after smoking marijuana. However, two urine samples taken within 18 h of the volunteer consuming 200 mg of zinc both tested negative for THC, followed by two more samples in the following 18 h that again tested positive. Beyond this point, three days following marijuana consumption, urine THC levels fall below the positive cutoff.

Bolstered by the data from this additional subject, an additional volunteer was recruited, one who was willing to repeat the time-course urine testing twice, once taking the zinc supplements and once without doing so. Figure 4D contains the urine testing results for the time-course in which the subject

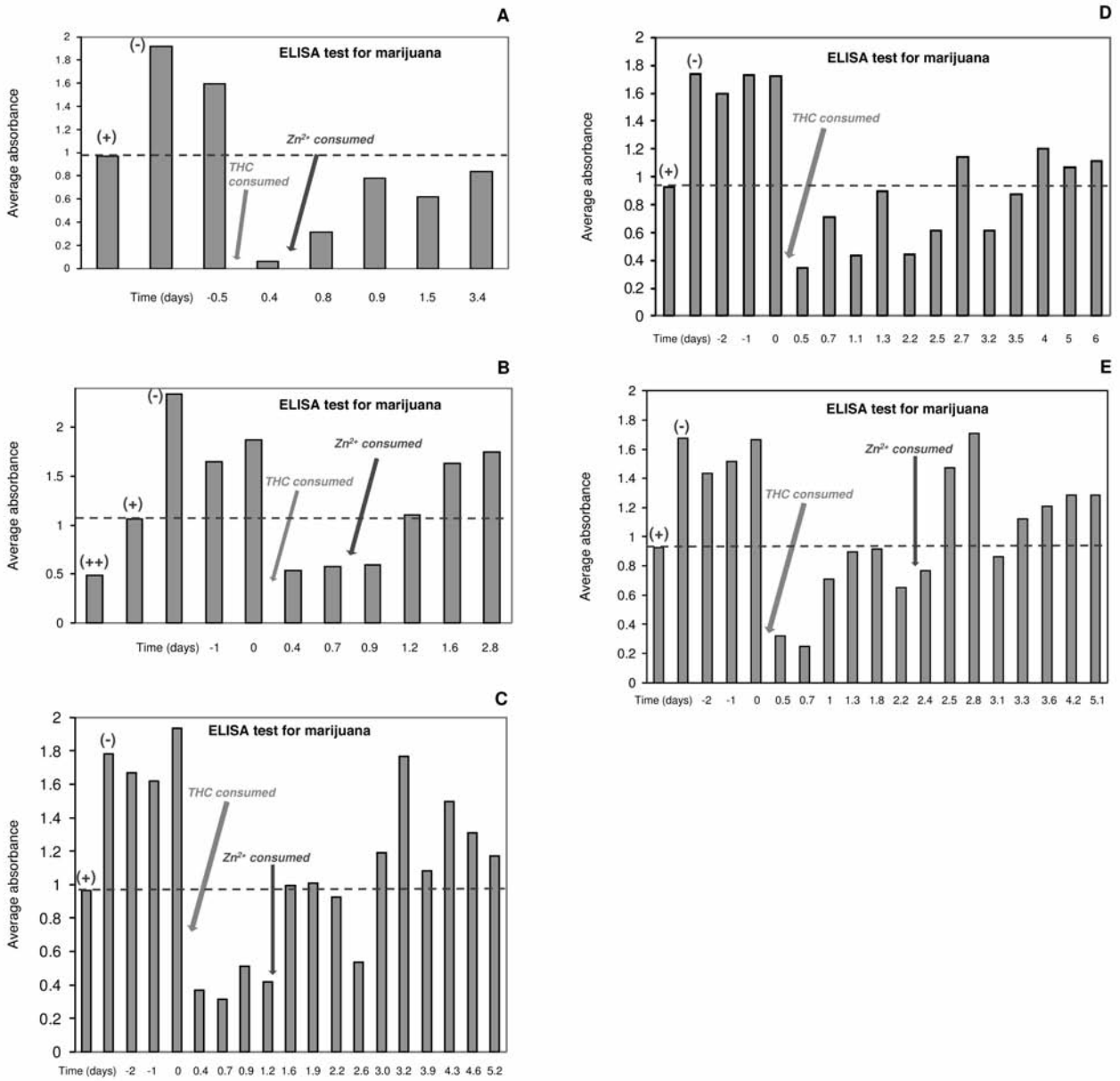

Figure 4. Effect of oral zinc supplements on THC drug testing by ELISA. Urine samples were collected chronologically from volunteers exposed to marijuana. Each graph represents separate experiments from different volunteers, except D and E, which were both from the same subject. The time scale is expressed in days elapsed from day 0.0, the point at which marijuana consumption occurred, and approximate times of marijuana (0.75–1.0 g) and zinc consumption (200-mg dose) are indicated by the respective arrows. Throughout, (+) and (–) indicate positive (10 ng/mL) and negative calibrators, respectively. In B, (++) indicates a 20 ng/mL THC calibrator.

did not consume zinc supplements, and Figure 4E contains the time-course when the same subject did consume zinc supplements. Both figures indicate an irregular but steady washout of THC from the subject's urine samples. However, as seen in Figure 4E, and previously in Figure 4C, urine samples taken in the 12 h immediately after ingestion of zinc supplement display a marked increase in ELISA signal and test definitely negative for the presence of THC. These results argue that the consumption of zinc supplements taken orally after light marijuana use can interfere with the detection of THC in urine samples for a 12–18-h period.

In the final phase of this analysis, whether zinc adulteration of urine can be detected using standard screening for adulterants was explored. First, urine samples from four volunteers were obtained and each were split into four vials. To the 16 vials, a solution of zinc sulfate was added at 1:10 (v/v) to a final concentration of 0, 5, 10, and 15 mg/mL. All 16 samples were tested using the Adultacheck10 strips. For three of the four volunteers, all urine samples tested completely normal/negative for all 10 adulteration checks (data not shown). Curiously, for these three volunteers, higher concentrations of zinc resulted in a slight color change on the spot for chromium adulteration, although well below the level required for the sample to be considered positive for chromium adulteration. For the remaining volunteer, even the urine sample without added zinc gave a slight color change on the chromium spot, which intensified with higher concentrations of zinc. At 15 mg/mL zinc sulfate, the reaction with the chromium spot was strong enough to be considered positive for chromium adulteration. Thus, for three of four users and for all lower concentrations of zinc, the addition of zinc sulfate was not detected using the Adultacheck10 strips. As discussed below, cross-reactivity with the sulfate ion is the likely cause of this result, but this hypothesis was not directly tested. Also, all urine samples taken during the studies with zinc supplements shown in Figures 4A and 4B were tested with Adultacheck10 strips. All samples gave indistinguishable results, testing negative for any signs of adulteration (data not shown).

Next, to what extent the addition of zinc sulfate alters the pH of urine samples was examined. Urine samples were collected from three volunteers and the pH values were recorded in triplicate both before and after adding zinc sulfate at 1:10 (v/v) to a final concentration of 15 mg/mL. As seen in Table I, the addition of zinc sulfate does cause a measurable decrease in pH, most likely caused by the heptahydrate formulation of the zinc sulfate crystals when dissolved in aqueous solution. However, all pH values fall within the normal range observed in normal unadulterated urine.

Lastly, the possibility that addition of zinc sulfate to urine might cause a detectable alteration in the absorption spectrum of the samples was examined. Urine was obtained from four volunteers, each sample was split into two tubes, and zinc sulfate (15 mg/mL) was added to half of each urine sample. Then, the urine was analyzed by UV-vis spectroscopy. As seen with one representative example in Figure 5, no observable difference was observed in the absorption spectrum of urine caused by the addition of zinc sulfate. Thus, the adulteration of urine samples with zinc, either by addition of zinc sulfate to urine or by ingestion of a zinc tablets, escapes routine screening for the presence of adulterants in urine obtained for drug testing.

Table I. Effect of Zinc Sulfate on pH of Urine Samples

	Urine pH	
Sample Source	Before $ZnSO_4$	After $ZnSO_4$
Volunteer 1	5.47 ± 0.05	4.35 ± 0.02
Volunteer 2	6.84 ± 0.02	5.48 ± 0.02
Volunteer 3	5.79 ± 0.02	4.47 ± 0.03
pH 7 buffer	7.04 ± 0.03	7.04 ± 0.03

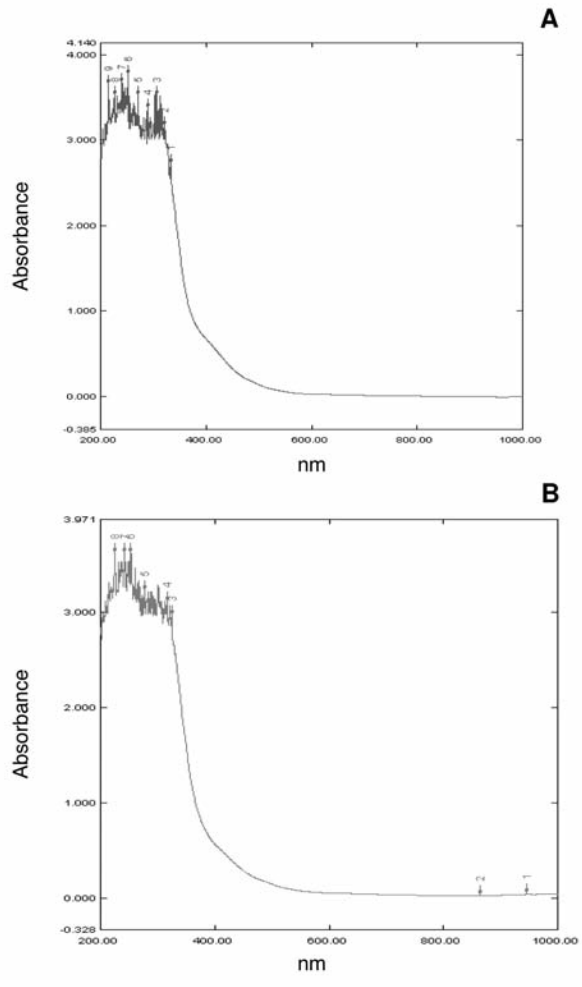

Figure 5. UV spectra of urine samples with and without zinc sulfate. Drug-free urine samples were obtained from four volunteers and each was divided into two tubes, to which zinc was added at 15 mg/mL. The absorption spectrum was then taken with the wavelength scan range indicated. Representative example of unadulterated urine (A). The same urine sample fortified with zinc sulfate at 15 mg/mL (B).

Discussion

This study focuses on the effects of zinc sulfate as a potential adulterant in urine drug testing. The effect of zinc sulfate on the three most prevalent drugs of abuse, cocaine, methamphetamine, and marijuana, was explored. Based on the results observed in Figures 1 and 2, it is evident that urine samples containing zinc sulfate show increased absorbance signal at 450 nm in the ELISA assay, thereby leading to potential false negatives among samples screened for cocaine and its major metabolite. In addition, a decrease in absorbance can be observed in samples where zinc sulfate concentration is 25 mg/mL or higher (Figure 1). Because of this and because high doses of zinc sulfate in the heptahydrate formulation cause a substantial change in urine pH (Table I), concentrations of 15 mg/mL or lower were used throughout this study. Because an increase in absorbance can be observed in both positive and negative calibrators (Figure 1), zinc sulfate likely interacts with components in the ELISA assay, rather than interacting with the drugs themselves. As a consequence, less priority was given to preparing samples containing drug or drug metabolites that have the specific cutoff concentrations of federal workplace drug testing. Results obtained from methamphetamine and THC drug testing (Figures 3 and 4) support the notion that zinc interferes with components of the immunoassay in a robust, reproducible, and universal fashion. Lastly, results from urine samples obtained from volunteers acutely exposed to marijuana that had also consumed zinc supplements (Figure 4) support the hypothesis that zinc, rather than sulfate, is responsible for masking the presence of THC in a urine sample.

When zinc sulfate is added to actual or synthetic urine, a slightly detectable white precipitate forms, which appears as a turbid urine sample and sediments in 10–15 min at room temperature. The amount of precipitate formed varies in proportion to the amount of zinc sulfate added. According to the revised SAMSHA 2004 guidelines, abnormal color, odor, and excessive foaming are indicative of urine adulteration. However, because turbidity in a urine sample may arise due to numerous factors, it is impossible to conclude that such a physical characteristic is a sign of adulteration unless it can be associated with abnormal foaming, indicating the presence of detergents in urine samples, which is not the case with samples adulterated with zinc. Moreover, because sedimentation of white flocculate is commonly observed in unaltered urine samples, turbidity cannot be associated solely as a sign of adulteration with zinc.

Urine samples from individuals that have consumed zinc supplements show no sign of adulteration by Adultacheck10 urine test strips (Figure 4). However, abnormal levels of chromate were associated with some urine samples to which zinc sulfate was added. This is likely due to cross-reactivity of excess sulfate ions with the chromate spot test in the Adultacheck10 test strip. Lastly, neither pH testing nor UV-vis spectroscopy proved able to detect the presence of zinc sulfate in adulterated urine samples.

Although methods are available to measure the concentration zinc in a urine sample, such methods cannot easily be brought to bear in the detection of zinc adulteration. First, samples that tests negative in the initial screen for illicit drugs and their metabolites are generally not probed further, and it is not practical or economically feasible to subject all negative urine samples to detailed testing. Secondly, the concentration of zinc in the urine that is sufficient to perturb the ELISA screen may not exceed that of someone taking over-the-counter zinc supplements or certain multivitamin preparations. A possible solution to the problem of detection of zinc alteration is the development of a simple urine strip method for detection of high zinc concentration that, if positive, would prompt testing laboratories to employ more probative drug-detection methods such as gas chromatography–mass spectrometry or inductively coupled plasma mass spectrometry.

Conclusions

We conclude that zinc ion (Zn^{2+}) is a potential adulterant in urine samples tested for drugs in routine workplace drug screening under the NIDA-5 panel using ELISA. Its effect in causing potential false-negative results in drug testing is robust and reproducible. This effect appears independent of the mode by which zinc is made available in urine. Although the exact mechanism by which zinc interacts with different components of the ELISA assay is unknown, the enhanced ELISA signal increases in a dose-dependent fashion. As a consequence, it is our conjecture that zinc ion increases the binding of drug conjugate to which the active horseradish peroxidase enzyme is attached, thereby increasing the final output signal.

Although some urine samples containing added zinc sulfate may show signs of abnormal levels of chromate ions when tested using Adultacheck10 urine test strips, urine samples containing zinc by ingesting zinc supplements do not show any sign of adulteration whatsoever. In addition, such samples do not exhibit turbidity as observed with urine samples to which zinc sulfate has been directly added. Thus, we are aware of no suitable test to determine zinc adulteration in urine and conclude that zinc supplements are effective at subverting routine drug testing and undetectable by standard means.

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