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REVIEWS

POSSIBLE REASONS FOR THE OCCURRENCE OF FALSE-NEGATIVE RESULTS IN URINE DRUG SCREENING

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ABSTRACT

INTRODUCTION: Immunoassay screening of urine samples play a central role in the monitoring and fight against ever-increasing drug abuse. Thus, the aim of the present work was to clarify the reasons for deliberately or unintentionally causing of false-negative screening results.

MATERIALS AND METHODS: For the purpose of the study, an analysis of Google Scholar, PubMed, and Science Direct databases was conducted.

RESULTS: The exact number of false-negative results in the analytical practice cannot be precisely determined because of the impossible confirmation of each screening test. In this regard, the screening of drug abuse appears to be a huge challenge these days due to multiple reasons. On the one hand, it is necessary to take into account the physicochemical properties of sometimes an unknown analyte, as well as the physiological characteristics of each individual. On the other hand, the test antibodies available to date do not have the necessary specificity for absolutely all drugs, especially designer ones. At the same time, there is an unlimited access to information and products supporting the manipulation of urine samples, respectively the achievement of false-negative results. In response, analytical chemistry offers a variety of methods to address the problem of filtering out abusers.

CONCLUSION: Because of their high throughput and low cost, immunoassay techniques continue to be a cornerstone of drug screening, whether for clinical or criminological purposes. However, the risk of false-negative results requires efforts to improve their specificity in parallel with the implementation of countermeasures against the manipulation of the biological samples.

Keywords: *immunoassay, false-negative, drugs, adulteration, urine sample*

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INTRODUCTION

According to the latest report presented by the United Nations Office on Drugs and Crime, drug use continues to grow worldwide. Over the past 10 years, the consumption of narcotic substances has increased by 26%, with approximately 284 million people (aged 15–64) (1). Social isolation during the COVID-19 pandemic further exacerbated the problem presented (2). In this context, the control and mon-

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itoring of drug abuse becomes a difficult task with high and socially significant priority.

The detection of illicit substances in biological samples is an important tool for the implementation of adequate diagnostic and therapeutic measures, as well as for the enforcement of the legal framework related to their use and distribution. Their analysis could be applied by various jurisdictions to ensure the achievement of a drug-free work environment (e.g., in the military sector); in the transport industry; industries where heavy machineries are used; to detect doping in sports and others (3). In addition, drug testing is used in criminology; toxicology clinics; in order to minimize abuse among adolescents, as well as in persons involved in rehabilitation programs (4,5).

The methods for analysis of psychoactive illicit substances are divided into screening and evidentiary (confirmatory) ones (6). Although they have no evidentiary and judicial value, the former have a central role in all presented areas of drug screening (3). The reason is that in practice it is not physically and financially possible to directly test all biological samples with the more limited evidentiary analysis—most often gas chromatography with mass detection (GC-MS) or liquid chromatography with mass or tandem mass detection (LC-MS or LC-MS/MS) (7,8). Typically, screening methods are based on immunoassay in which antigen-antibody reactions are used to detect the presence of specific drugs and/or their metabolites (9). Urine is considered the most popular test matrix because its collection is non-invasive and allows the detection of a relatively wide window of narcotics (4).

Screening methods are characterized by insufficient specificity, but their easy use, together with their high productivity and low cost, make them perfect for filtering suspected positive samples (10). However, recently, the public attention has been focused on problems related to the possibility of spurious results (11). In this regard, the increasing strategies used by drug abusers to circumvent drug monitoring should not be overlooked. The motives for this are many—for example, fear of imposing legal sanctions, restriction of freedom, undertaking different therapeutic interventions, temporary or permanent suspension from work, exclusion from sports qualifications or rehabilitation programs (11–13). Thus, the present work aims to clarify the currently known causes of false-negative screening tests, options for their manipulation, as well as strategies to deal with malicious assay interference.

MATERIALS AND METHODS

The present review was done using the databases of Google Scholar, PubMed, and Science Direct. Materials were screened for the presence of the following terms: "drug screening", "immunoassay", "false-negative results", "adulteration", and "urine analysis". The sources were not chronologically limited. Irrelevance to the topics and lack of full-text articles were set as exclusion criteria. In the process of searching for information, 56 reports fell within the scope of the study. They were prepared by author teams from the United States of America, Australia, Switzerland, Korea, Italy, Sweden, Turkey, Canada, Australia, Germany, and Iran.

RESULTS AND DISCUSSION

A *false-negative* result is one in which the test incorrectly indicates the absence of the target analyte when it is actually present in the biological sample (11). Because of several circumstances, the exact number of false-negative results cannot be accurately identified. On the one hand, it is practically impossible for every screening test to be validated by an evidentiary method. Also, guided by the presumption of innocence until the establishment of guilt, usually only the positive tests are subjected to a confirmatory analysis (14). On the other hand, many designer narcotics do not show cross-reactivity with the test antibodies produced to date (15–17). On top of that, the access to unlimited information helps drug abusers educate themselves on the most up-to-date methods to compromise drug screening devices (3).

There are few independent studies on false-negative rates in the literature. According to the psychiatrist Dwight Smith, about 1 in 10 drug screening results is wrong and needs to be confirmed (18). Especially for false-negative results, it is claimed that this number can reach up to 15% (14). In another study, confirmatory LC-MS analysis of urine samples from patients admitted to a trauma unit was initiated. It has shown that 56 out of 100 of the samples tested were false-negative for the presence of psychoactive

substances. The authors emphasize that attempts to deliberately falsify the results are at a higher frequency among people using cocaine and cannabinoids (19). Usually, these are citizens who have been tested in the context of lawsuits, those who are about to be hired in a new job, those whose occupations require routine monitoring, in athletes, for pain management (with prescribed opioid analgesia), or minors (3,7). Of course, false-negative results can occur unintentionally too. These may be due to human errors, the limitations of screening test devices, properties of the drug substance, and the tested subjects. The possible causes are summarized and systematized as follows:

1. Impossibility of exceeding the cut-off concentration

Specific limit concentrations of narcotic substances and/or their metabolites in biological samples are introduced by the health authorities and implemented by the accredited laboratories, respectively (Table 1). Their excess indicates the use of psychoactive substances (7). Such values are available for each drug, as they are determined on the basis of many years of clinical experience and taking into account the analytical method used, as well as the type of biological material (urine, blood, saliva, hair) (20). They can differ depending on whether the analysis is done for clinical or criminological purposes (15). In some cases, they are also tailored to the patient population, given the fact that in children urine is more diluted and lower cut-off values should be used (21).

Limit concentration levels serve not only as a reference in issuing final decisions, but also to eliminate false results (9). Achieving lower values turns the test negative. However, this may be due to noncompliance with good laboratory practice or be intentional, as summarized below:

1.1. Exceeding the time between drug intake and urine analysis

The metabolizing and excretory organs of the human body make it a dynamic and open system. This should be taken into account when choosing a sampling time. It turns out that the choice of a biological material is also a key factor in the successful drug detection (3,22). For example, amphetamine can be detected in urine up to 72 h after intake and up to 9 days with chronic use. The same substance, however, can be found in the blood for up to 48 h, and in saliva for up to 24 h (23). The physicochemical properties of the drug itself, as well as its toxicokinetics, also determine how long after use they can be detected in the sample (3). If chronic Cannabis use can be detected by urine screening up to a month after use, a number of substances can be difficult to detect even after 24–48 h (24).

1.2. Dilution of the sample

Determining the screening result based on limit concentrations is a motive for the purposeful prevention of exceeding them. One of the methods for tampering with samples is the so-called dilution (3). Because of its simplicity, this has emerged as one of the most widely applied approaches to masking positive test results. Usually, this is done by consuming a large amount of fluids (> 2.5 L) in a short time. The aim is to increase the volume of excreted urine, respectively to reduce the concentration of the excreted drugs and/or their metabolites in the excretory system (22,25,26). Another possible method to achieve this is to take diuretic drugs leading to strong or moderate natriuresis (27). Herbal preparations (green or black tea, Hydrastis root) or substances advertised as detox products (Urine Luck®, The Cleaner®) can be used with the same intention (27). Dilution by adding water from the toilet of the laboratory facility is rare because of the common requirement to have col-

Table 1. Urine drug test cut-off concentrations for some commonly abused drugs.

	Screening Drug Test Level	Confirmatory Drug Test Level
Cannabinoids/Cannabinoid metabolites	50 ng/mL	15 ng/mL
Amphetamines*	1000 ng/mL	500 ng/mL
Cocaine/ Benzoylecgonine	300 ng/mL	150 ng/mL
Opiates	2000 ng/mL	2000 ng/mL
Phencyclidine	25 ng/mL	25 ng/mL

oring tablets in the toilet tank (28). Therefore, the increase in urine volume *in vivo* is much easier as it is done outside the sample collection site.

In order to detect the intake of urine-diluting substances in the laboratories, the physicochemical properties of the entire sample are examined. It is explained by the fact that when a diluent is added, not only the concentration of the narcotic substance is lowered, but also the excretory products normally present in the urine (28).

1.3. Patient characteristics

The physiological characteristics of the organism also have an effect on the absorption, distribution, metabolism and excretion of xenobiotics and their metabolites (9). DeFazio et al. reported an individual whose urine screened false-negative for methadone because of a genetic polymorphism. The patient was found to be heterozygous for the CYP3A5(*)1 allele responsible for high levels of CYP3A4 (one of the opioid metabolizing enzymes) (29). Likewise, intake of foods and drugs that are enzyme inducers can accelerate the biotransformation of ingested substances. For example, abuse of oxycodone (also a CYP3A4 substrate) may not be identified even by confirmatory methods in individuals taking the CYP3A4 inducer rifampin (30).

2. Low specificity of the screening test

The low specificity of immunological tests is most often commented on in the context of false-positive results. It is associated with the possibility of a non-narcotic substance participating in cross-reactivity reactions and thus deceiving the presence of a drug in the sample (31). This limitation can also cause the occurrence of false-negative results, and the following factors can be the basis of this:

2.1. Specificity of the immunoassay antibodies

Antibodies in immunoassay tests for Δ9-tetrahydrocannabinol (THC) screening are often unable to detect newer synthetic cannabinoids (32). Such problem can also be observed with some prescription psychoactive substances. For instance, most immunoassay methods for opiate screening target morphine, norcodeine, codeine, and heroin (11). On the other hand, agents that are linked to the so-called *opioid crisis*, such as oxycodone and fentanyl, do not biotransform to morphine or its derivatives and cannot be detected by these tests. For the same reason, the screening for buprenorphine and tramadol can give false-negative results for the presence of opioid drugs (3). The example with the group of benzodiazepines is analogous. The majority of enzyme immunoassays are designed to detect diazepam and/or its metabolites, nordiazepam, oxazepam, chlordiazepoxide and clorazepate. Therefore, other commonly prescribed representatives of this class such as lorazepam and clonazepam are not reliably detected by immunoanalytical screening, but only by a confirmatory method (GC-MS or LC-MS) (11). According to Gerberich (2021), in order to minimize the need for confirmatory testing, treating physicians may consider the use of detectable drugs in higher-risk patients (23).

2.2. **Adulteration of the sample**

A sample that contains a substance not inherent to the human organism, or containing an endogenous substance whose concentration does not correspond to normal physiological levels, is considered adulterated (27). This approach is often used by individuals who attempt to hide positive screening results by adding foreign substances to the test sample. Moreover, some additives are so effective that they can even compromise confirmatory analytical methods by reducing analyte extraction efficiency in sample preparation, or oxidizing and destroying target analytes (sometimes along with internal standards) (26). Significant loss or disappearance of the latter is perceived as a signal of possible sample manipulation (4).

Due to the relatively small molecular size of narcotic substances, immunoanalytical screening tests for their detection are usually of the competitive type. Thus, the narcotic substance in the sample competes with a factory-set labeled reagent for binding sites with the test kit antibodies (33). Some screening tests use the enzyme activity of an enzyme-labeled reagent, and others—fluorescence or microparticle binding. Compromise of the sample may inhibit the reaction required to report a positive result. Not infrequently, the coloration of the control line may also suffer, rendering the test invalid and the result unreported (3).

To mask the presence of the drug in their urine, addicts very often add substances found in the household to the sample:

- **Vinegar:** Vinegar intended for cooking is a dilute solution of acetic acid. For this reason, its addition to urine specimen lowers its pH. This can disrupt the antigen-antibody bond, and is often used by amphetamines and cannabis abusers (34,35). Absurdly, another falsification approach is the direct consumption of large amounts of vinegar under the delusion that this will speed up the excretion of xenobiotics. In fact, this does not affect the screening test, and if it does have any effect, it would be due to the minimal dilution of the urine in the urinary system. On top of that, it can damage the tooth enamel, as well as acidify the body (36).
- **Table salt:** Another culinary product that can be used to produce false-negative results is sodium chloride. It has the property of altering the protein structures involved in the test reaction, but tellingly increases the weight of the urine sample significantly (37).
- **Detergents:** Many products for personal use, laundry, dishwashing, and household cleaning contain surfactants as well as alkaline substances. All these affects the pH of the sample and the binding of the analytes to the factory-loaded antibodies (38).
- **Sodium hydroxide:** It is a strong base, often included in the composition of preparations for cleaning of sink drains. Like detergents, it has a highly alkaline pH that affects drug binding and solubility and gives false-negative urine results (39).
- **Bleach:** Sodium hypochlorite, commonly known as bleach, is one of the most affordable and effective methods of disguising the presence of a drug in urine. A number of reports indicate that, due to its oxidizing properties, it can lead to concentration-dependent degradation of the target substance (amphetamine, cannabis, opioids, etc.), compromising subsequent chromatographic analysis (4,40,41).
- **Visine® eye drops:** Visine® eye drops contain benzalkonium chloride. According to Pearson et al. (1989), when added to a urine sample, it can directly reduce the concentration of 9Δ-carboxy-THC (THC-COOH), causing false-negative results for cannabinoids (42).

The ever-increasing drug consumption has led to the creation of a niche market for the sale of specially developed products designed to falsify urine samples during screening. Usually these are oxidizing agents, such as:

- **Nitrites:** In clinical practice, the presence of nitrites in the urine suggests a urinary tract infection with nitrate-reducing pathogens. However, products containing potassium (Klear®) or sodium salts (Whizzies®) of nitrous acid are available on the market to alter the pH of the sample. They are often preferred by people abusing cannabis (43). Probably the reason to rely on this reagent is the fact that it does not change the color of the urine and can compromise even GC-MS, especially if a long time has passed between the sample preparation and the analysis (>4 h) (37). In relation to the latter, ElSohly et al. (1997) recommended the addition of sodium bisulfite to the sample prior to GC-MS so as to neutralize the acid additive (44).
- **Peroxide-containing additives:** It turns out that these oxidizers are very effective in masking the use of cocaine, opiates, cannabis, and LSD. An example of such a product is Stealth®. It is a kit of hydrogen peroxide and the enzyme peroxidase, divided into two vials, which are added together to the sample for analysis (45).
- **Pyridinium chlorochromate:** Pyridinium chlorochromate has been found to effectively give false-negative results in cocaine and amphetamine abuse, both in screening and chromatographic analysis. In the commercial network, it can be found in the composition of Urine Luck®, Sweet Pea's Spoiler®, and Klear II® (3). The mechanism of interference is a reduction in standard pH levels (37).
- **Gluteraldehyde:** This is one of the earliest commercial additives, but today it is being replaced by other products because does not affect GC-MS results (46). These products (Clean X®, Urine aid®, and others) cause interference with immunoassay methods by reducing the absorption rate, especially in tests with cannabis (37).

3. Urine substitution

In some cases, the collection of urine can take place under strict control, only after all personal be-

longings have been handed over and/or a thorough search has been conducted (27). Anyway, this is not always possible due to privacy concerns of the person being tested. Using this justification, some individuals may try to substitute their urine with that of another person who does not use illegal substances. Since this is often associated with a delay in the transmission of the freshly separated sample, often the temperature of the sample betrays attempts at adulteration (20). Other individuals resort to substitution with drinks or preparations that mimic the color of urine, which very often is identified before the actual screening analysis (47,48).

The so-called *synthetic urines* are products designed to replace real urine. They are liquids with an identical appearance and the same physicochemical properties (correct pH, specific gravity and creatinine level) as human urine (49). An example of such a product is the Incognito Belt®, which allows synthetic urine to be carried in a discreet belt hidden under clothing. The product is equipped with heating plates so that it reaches the temperature of normal urine. It has a long shelf life and can be used by both men and women (50). The Urinator® is another popular synthetic urine kit that claims to provide samples closest to human urine. The product comes with a powdered form of urine that must be dissolved prior to the drug test. Used together with an electric device, it can maintain the fake urine at the desired temperature for at least 4 hours (50). The Whizzinator® is a device suitable for concealing the administration of substitute urine in facilities with stricter controls. It is inserted into the underwear and can heat the sample to body temperature. In addition, the kit includes fake genitalia in case the sample giving needs to be observed (51).

4. Strategies for detecting intentional falsification of urine samples

Due to its high cost and significant labor-intensiveness, conducting an evidentiary analysis when screening tests are negative is not a practice. In addition, like screening methods, confirmatory test methods may be also susceptible to false-negative results (4). For this reason, significantly easier and cheaper approaches to fight against falsification of test results have been introduced in toxicological practice:

4.1. General urine examination

Urine replacement can be identified by inspecting its properties:

- **Temperature:** The temperature of the urine sample may reveal the transmission of specimens that are not separated at the time. The temperature of freshly passed urine should be 32°C to 38°C (up to 15 minutes). Normally, the detection of this parameter is done within 4 minutes after sampling (52). Temperatures outside this range may indicate a substitution of the urine sample, although it has already become clear that adulteration products with special heaters are available on the market. Therefore, several manufacturers provide collection cups with thermal strips that indicate the temperature of the sample through the container (3).
- **pH:** Normally, urine pH varies throughout the day, but is usually from 4.5 to 8.0. Values outside this range (especially <3 and >11) signal an attempt to tamper with the sample (53).
- **Urine specific gravity** is another indicator for false-negative result. Such are the cases in which its values are less than 1.002 or more than 1.020 (53). It is usually determined with test strips or refractometrically (54).
- **The concentration of creatinine** in normal human urine should be higher than 20 mg/dL. Therefore, samples with creatinine <20 mg/dL are considered diluted, while those with <5 mg/dL are defined as non-human urine (55). Creatinine is one of the most frequently investigated parameters to determine if a sample has been manipulated, which is why some authors are trying to implement new technologies to measure it. Musile et al. (2023) report on the construction of an on-site device for assessment of urine tampering, based on picric acid, 3,5-dinitrobenzoic acid, and Nessler's reagent, with color detection by a built-in smartphone camera (56).
- Some reports claim that creatine ingestion may increase urinary creatinine levels, thereby masking possible dilution of the sample. In such cases, the paler color of the urine against the background of normal creatinine concen-

trations should be viewed with suspicion by examiners (57).

- **Urinary nitrites** should not exceed 500 μg/mL. Higher levels of nitrous acid salts indicate sample adulteration, necessitating repeated urine collection under direct supervision (53).

Urine samples that do not meet all these criteria are reported as invalid.

4.2. Deviations in the urine color

Urine samples collected early in the morning are the most concentrated and often provide more reliable information. They have a saturated straw yellow color. Some additives may give deviations from this color. At low concentrations, Stealth® can give a slightly darker urine color, while pyridinium chlorochromate can give a deeper yellow (4). On the other hand, the presence of an abnormal urine color is not necessarily due to attempts to tamper with the sample. It is known that the use of metamizole can stain the urine in a red color due to one of its metabolites (rubazonic acid) (58). Likewise, rifampicin can stain urine in a reddish-orange color (59). Amitriptyline, indomethacin, and propofol may lead to a blue-green coloration. Deviations in the normal color can be due to food consumption (beetroot and blackberries—pink-red; green beans—brown; confectionery dyes—blue-green) or diseases (presence of blood or darkening—a sign of urinary tract infection, kidney stones or cancer of the urinary tract; dark orange urine signals a problem with the liver; green urine may be a sign of a bacterial infection in the urinary tract) (60). Therefore, it is sometimes required that the consumption of drugs, foods, or diseases be noted in the documentation that accompanies the evaluation sample (9).

4.3. Deviations in the urine odor

The smell of urine can also give away previous manipulation of the sample. Typically, samples are inspected for unusual odor of bleach, lemon, or other flavorings commonly added to detergents (20,61).

4.4. Other strategies

Some tests are equipped with reagents designed to detect the presence of oxidizing impurities (halogen from bleach, iodine or fluoride; glutaraldehyde; pyridine; surfactants, or other impurities) (20). In this regard, special devices have been developed to indicate the presence of additives. One such product is the Intect 7® adulteration test strip (Branan Medical Corp., Irvine, CA), which, in addition to basic urine parameters (pH, specific gravity, nitrites, creatinine), reports the presence of bleach, pyridinium chlorochromate, and glutaraldehyde (62). Typically, the patient's sample is divided into several fractions, one of which is analyzed with this or similar devices from other manufacturers.

This review is not exhaustive of all oxidants attempted to adulterate the sample. As can be seen, however, they can all degrade the narcotic analyte, rendering it undetectable for evidentiary analysis. In this regard, Fu et al. (2016) proposed the search for the oxidized forms of the target analytes (4). Other approaches to testing when urine samples are suspected to have been tampered with have been reported in the literature:

- Vigorous shaking of the sample should not form an excessive amount of bubbles that persist for a long time. This usually refers to the addition of detergents (liquid soap, bathroom or drain cleaner, laundry detergents) (9,47).
- Nitrites can be detected using potassium permanganate, because they decolorize the pink colored reagent. This reaction is not applicable to individuals suffering from diabetes due to the presence of glycosuria (63).
- Adulteration of the sample with peroxide-containing additives (such as Stealth®) can be detected by the addition of 10 μL of urine to 50 μL of tetramethylbenzidine, followed by the addition of 500 μL of 0.1 M phosphate buffer solution, which produces a dark brown coloration (64). Another way is to monitor the peroxidase activity using a spectrophotometer (45). According to Dasgupta (2015), a few drops of urine adulterated with Stealth® added to potassium dichromate followed by a few drops of 2N hydrochloric acid would turn the solution deep blue (64).
- Cr^{6+} in Urine Luck® (containing pyridinium chlorochromate) can be detected by adding two drops of a 1% methanolic solution of 1,5-diphenylcarbazide to 1 mL of urine, resulting in a reddish-purple color (65).
- Although less readily available and less commonly used, an alternative method for identi-

fying urine substitution is urine DNA sequencing, which is, however, more widely used in doping testing (66).

- The intake of diuretics to dilute the sample can be determined by chromatographic analysis (67).
- Despite the production of increasingly misleading synthetic urines, specific tests are available for their detection. For example, compounds that are unique to human urine, such as the steroid hormone cortisol, can be sought (64).

CONCLUSION

Screening for drug abuse is routine in clinical and forensic practice. In this regard, immunoassays are the basis of drug monitoring because of their ease of use, higher productivity, and low cost. However, the number of findings on possible sources of false-negative results in urine drug screening continues to grow. Among the reasons for this are systematic errors of the analysts, insufficient specificity of the analytical toolkit, as well as the application of various strategies to compromise the analysis by the investigated individuals. This jeopardizes the adequate interpretation of the analysis data and necessitates the introduction of reliable methods for the prevention and/or exposure of false results.

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