

# Toxicology News

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## OTC ANABOLIC STEROIDS POSE UNKNOWN RISKS

By Siu C. Chan and R. H. Barry Sample

**A**nabolic steroids have been misused in sports for quite some years. They are consumed in hopes of building muscle and shortening recovery time from intense training or injury. There are many documented adverse health effects from their long-term use. The International Olympic Committee has banned the use of these drugs since 1976, as have many other sports organizations.

Anabolic steroids are now classified as controlled substances, making possession without a prescription an offense, but abuse remains rampant. It was recently reported that their use is common in major league baseball (1). A recent survey indicated that as many as 550,000 adolescents, including 175,000 girls, have used anabolic steroids at least once in their lives (2). These young people use the drugs not only to enhance athletic performance, but also to improve physical appearance.

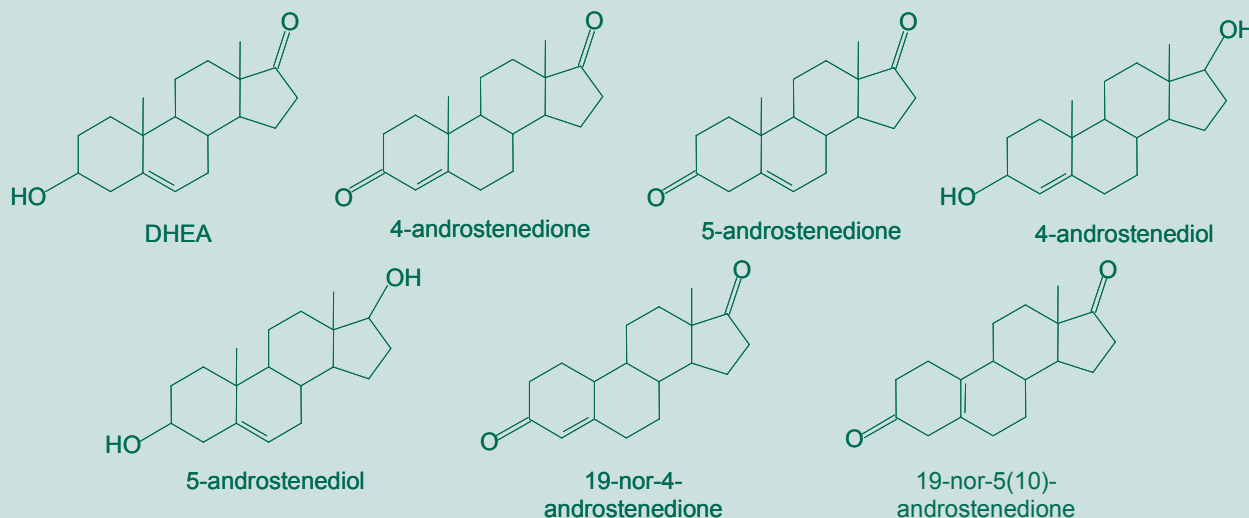
Anabolic steroid abusers procure their supplies from the black market and foreign countries. The enactment of the Dietary Supplement Health and Education Act of 1994 changed the picture somewhat by making steroid compounds marketed as dietary (nutritional) supplements available legally over-the-counter. These "supplements" are widely advertised over the Internet and in muscle magazines where unsubstantiated claims about their benefits are commonplace.

The first that came on the market was dehydroepiandrosterone (DHEA), and many others followed. The compounds for which metabolic information has been published include DHEA, 4-androstenedione, 5-androstenedione, 4-androstenediol, 5-androstenediol, 19-nor-4-androstenedione, and 19-nor-5(10)-androstenedione (Figure 1).

These steroids are metabolic precursors (prohormones) of testosterone or nandrolone, both controlled substances. Because manufacturers of nutritional supplements are not required to undertake clinical trials to show efficacy, it is not known whether oral administration of these compounds en-

*Continued on page 2*

**Figure 1. Structural formulas of some over-the-counter anabolic steroids**



## Anabolic Steroids

*Continued from page 1*

hances athletic performance or has any health benefits. However, they are supported by intense marketing efforts which are aided occasionally by high profile users. For example, a few years back, the sale of androstenedione increased significantly when a well-known player, pursuing a major league baseball record, revealed that this substance was part of his training regimen.

### Unknown effects

The effects of long-term use of these substances are not known, but because they belong to the same class of compounds and are metabolically related to the controlled anabolic steroids, they are likely to have the same adverse health effects. Such effects include skin, heart, and liver problems, disruption of the hormonal system, stunted growth in adolescents, and psychological effects such as rage and depression.

DHEA and its sulfate analog are adrenal hormones. They are interconvertible and are the most abundant circulating steroids. DHEA has received a lot of attention in the medical community and was touted as the fountain of youth. The serum concentration of DHEA peaks between ages 25 and 30, and decreases progressively with age to 10% to 20% of the peak between ages 70 and 80. This drop occurs in both men and women. Because of this temporal decline, it has been postulated that the hormone plays a vital role in the aging process, and it may act as an anti-aging agent. However, the scientific evidence on the subject is equivocal (3).

### Testosterone precursor

4-Androstenedione is the immediate precursor of testosterone; therefore, one would assume that intake of 4-androstenedione would increase circulating testosterone levels. The supplement industry has been capitalizing on this assumption in promoting the substance as an anabolic. However, in a randomized controlled trial on young men, King did not observe any increase in serum testosterone concentration after 8 weeks of daily 300-mg doses of 4-androstenedione (4). This is in contradiction to the patent filing for this compound, which indicated a significant increase in serum testosterone concentration, although there is no specific information on the subject population (5).

In a study involving two healthy women, serum testosterone concentrations increased several-fold an hour after administration of 100 mg of androstenedione (6). Other studies showed some increase

in serum testosterone in young men after supplementation with 4-androstenedione, although the effect was much less (7, 8).

A study on the effect of 4-androsten-3 $\beta$ ,17 $\beta$ -diol contained in an herbal supplement on the hormonal profiles of men did not observe any increase in serum total testosterone (9). There was a small increase in serum free testosterone (37%). Interestingly, there was a more prominent increase in serum estradiol (86%), the female sex hormone. In another study, an increase in serum testosterone was observed after oral ingestion of this compound, but the effect was less pronounced than that of 4-androstenedione (8).

### Quality control problems

The Food and Drug Administration does not regulate nutritional supplements, and the quality control during their production is not as stringent as that for regulated drugs. Consequently, there are three possible adverse scenarios: The supplement contains unstated ingredients; it does not contain the correct amounts of the stated ingredients; or it does not contain the stated ingredients.

A study by Green found numerous problems in the formulation of nutritional supplements (10). One supplement contained 10 mg of testosterone that was not listed on the label, and it contained from 163 mg to 170 mg of 4-androstenedione, instead of the stated amount of 250 mg. Another supplement was supposed to contain 50 mg of 19-nor-4-androstenediol but none was found.

It is also alarming that many of the non-steroidal supplements actually do contain steroids. In a recent study for the Medical Commission of the International Olympic Committee, Schänzer analyzed 634 non-hormonal nutritional supplements from 13 countries, including 240 from the United States (11). He found 94 of them were contaminated with pro-hormones of testosterone and/or nandrolone. Forty-five of these supplements were from the United States.

### Testing for supplements

The use of steroid supplements is banned by many sports organizations, such as the International Olympic Committee, the National Football League, and the National Collegiate Athletic Association. They are either named on the banned list, such as DHEA, or banned as related compounds.

For urine drug testing in sports, the only applicable rule for the detection of the administration of the pro-hormones of testosterone (DHEA, the androstenediones, and the androstenediols) is the ratio

of the concentration of testosterone to that of an inactive stereoisomer, epitestosterone (T/E ratio). A ratio higher than 6.0 is deemed positive.

It has not been established whether the administration of DHEA will result in a positive test. Bosty observed no positive test in seven subjects given 50-mg or 250-mg oral doses of DHEA (12). Similar observations were made in a study using a 50-mg oral dose of DHEA (13). On the other hand, Bowers observed an increase in T/E ratio from 2.4 to 8.1 in one subject who had taken 50 mg of DHEA (14). Three other subjects did not show a similar increase. Uralets had the same observation in a study with three subjects taking 200 mg of DHEA (15). The T/E ratio of one subject increased from 4.5 to 16, while the T/E ratios of the other subjects remained below 6.0. With the limited data in the literature, one could surmise that the T/E ratio may increase beyond the administration limit of 6.0, depending on the baseline ratio of the subject and on the amount of DHEA taken.

In another study, Uralets administered 50 mg of 4-androstenedione to three volunteers (16). Urinary testosterone concentration increased in all three subjects significantly (two-fold or more) and returned to the pre-dose level in about 20 hours. The T/E ratios in two subjects increased but only the one with a high baseline T/E ratio increased beyond the limit of 6.0. The effect of the oral administration of 100 mg of 4-androstenediol was also investigated. The increase in urinary excretion of testosterone was much more dramatic than that in the administration of 4-androstenedione. However, there was a concomitant increase in the urinary excretion of epitestosterone, and as a result, only one subject's T/E ratio increased beyond the allowable limit.

For the detection of the administration of nandrolone, the marker is the urine metabolite norandrosterone. The ingestion of the pro-hormones of nandrolone, 19-nor-4-androstenedione, and 19-nor-5 (10)-androstenedione results in the excretion of nonandrosterone, and therefore a positive drug test (15, 16, 17, 18).

In summary, pro-hormone nutritional supplements are pharmacologically related to controlled anabolic steroids. Their efficacies have not been scientifically proven and the risk of their long-term use is unknown. The administration of these substances is banned by most sports organizations, and may result in a positive drug test.

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## Creatinine Provides Validity Measure in Urine Drug Tests

By Jeri D. Roper-Miller

Muscle contraction leads to the spontaneous cyclization of creatine and creatine phosphate to yield creatinine, a metabolic degradation product that is excreted by the kidneys (Figure 1). The amount of creatine per unit of muscle is relatively consistent (about 0.5 mmol creatinine per kg muscle), and it breaks down at a constant rate, so serum and urinary creatinine concentrations change little in the absence of disease.

Creatinine is eliminated from the body at a rate of about 1.0–2.5 g/day (depending largely on muscle mass), making it a clinically useful measurement of renal function and a forensically useful measurement to detect diluted urine and potentially substituted specimens (1–5).

Because the excretion of creatinine is constant, the rate can also be used as a normalization tool when measuring other biochemical products or drugs excreted into the urine. As a result, its measurement is recommended for monitoring subjects in drug treatment programs, in employee-assistance programs, and on parole.

Normally, an individual's daily urinary creatinine output varies by less than 20% and largely depends on dietary intake of protein, exercise, hydration, and renal function; however, clinical reference ranges are gender-sensitive due to inherent differences in muscle mass. Similarly, creatinine values are lower for infants, children, and the elderly. Creatinine levels for these age groups can be 20 to 80% less than a normal adult concentration (1). Tables 1 and 2 summarize both clinically and forensically relevant creatinine values.

Since the introduction of urine drug testing, individuals have labored to hide their use of drugs by

adulterating their urine by both in vivo and in vitro techniques in attempts to lower the drug concentration below the established cutoff level. Some try to dilute their urine by ingesting large quantities of fluid, taking diuretics, or adding water or other fluids to their specimens.

### Determining adulteration in drug urinalysis

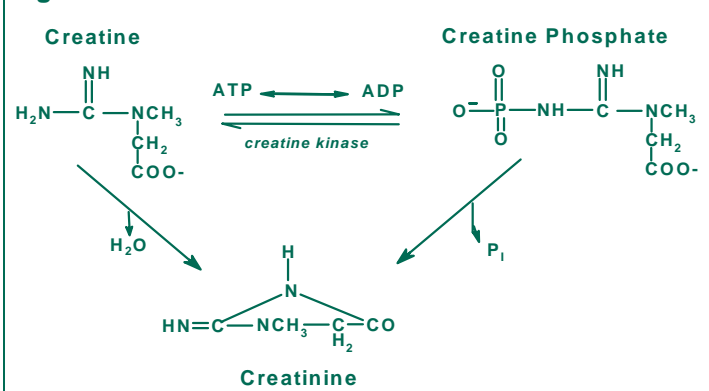
Forensic urine drug-testing programs have many protocols in place to prevent or detect specimen adulteration or substitution. Measures at the collection site include direct observation of collection, removal of excess clothing, hand washing, water bluing agents, and monitoring of specimen color and temperature. At the laboratory, integrity checks are routinely performed to detect dilution or substitution. One such specimen integrity assay is the measurement of urinary creatinine.

Several studies have indicated that consumption of large amounts of fluid can decrease an individual's urine creatinine levels. Needleman et al. determined that with an ingestion rate of 125–250 mL of fluid per hour, it takes 4–7 hours for urine creatinine to fall below 100 mg/dL and at least 6 hours to decrease to 50 mg/dL. With substantial fluid intakes of 2 to 6 liters, urine creatinine could decrease to 50 mg/dL in as little as 2 hours (5).

Likewise, Lafolie et al. demonstrated that after the consumption of 500 mL of water within a 15 minute period, excretion of urinary creatinine decreased to a mean of 45 mg/dL within 60 minutes and 34 mg/dL within 120 minutes. When the fluid intake was doubled, urine creatinine decreased to 25 mg/dL within 60 minutes and 15 mg/dL within 120 minutes. More significantly, 95% of the specimens remained below 44 mg/dL and 10 mg/dL for up to 5 hours after water consumption in these amounts (3).

Given these water-loading studies and others like them, the Substance Abuse and Mental Health Services Administration's (SAMHSA) Mandatory Guidelines for the Federal Workplace Drug Testing Programs and the U.S. Department of Transportation currently recommend that a specimen with a urinary creatinine below 20 mg/dL and a specific gravity of less than 1.003 be considered a diluted specimen. A diluted specimen by these criteria is not considered reasonable cause to require the subject to submit another specimen; however, the employer may require direct observation of the subject during the next specimen collection (6). Furthermore, SAMHSA developed criteria for defining a "substituted" urine sample (that is, a sample inconsistent with normal or dilute human urine) as one with a creatinine of  $\leq 5$  mg/dL and a specific gravity of  $\leq 1.001$  or  $\geq 1.020$ . Table 2 details these regulatory limits.

**Figure 1. Chemical formation of creatinine in muscle**



**Table 1. Reference ranges for creatinine and specific gravity**

Measurement	Reference Range
Serum creatinine	M: 6.4–10.4 mg/L
	M: 57–92 $\mu$ mol/L
	F: 5.7–9.2 mg/L
	F: 50–81 $\mu$ mol/L
24-hr pooled urinary creatinine	M: 1.00–2.00 g/day
	M: 8.8–17.7 mmol/day
	F: 0.80–1.80 g/day
	F: 7.1–15.9 mmol/day
Creatinine clearance	M: 97–137 mL/min
	F: 88–128 mL/min
Random urinary creatinine	M: 44–250 mg/dL F: 37–300 mg/dL
Random urinary specific gravity	1.002–1.030

Source: Reference 1; M=male; F=female

**Table 2. Regulatory limits for creatinine and specific gravity**

Test results	Interpretation/action
Urinary creatinine <20 mg/dL; specific gravity <1.003	Lab reports with "Dilute specimen" remark. No action.
Urinary creatinine $\leq$ 5 mg/dL; specific gravity $\leq$ 1.001 or $\geq$ 1.020	Lab reports "Test not performed" with comment: "Specimen substituted." Constitutes refusal to test; removal from safety-sensitive function.

Source: Substance Abuse and Mental Health Services Administration, National Laboratory Certification Program, Program Document # 35.

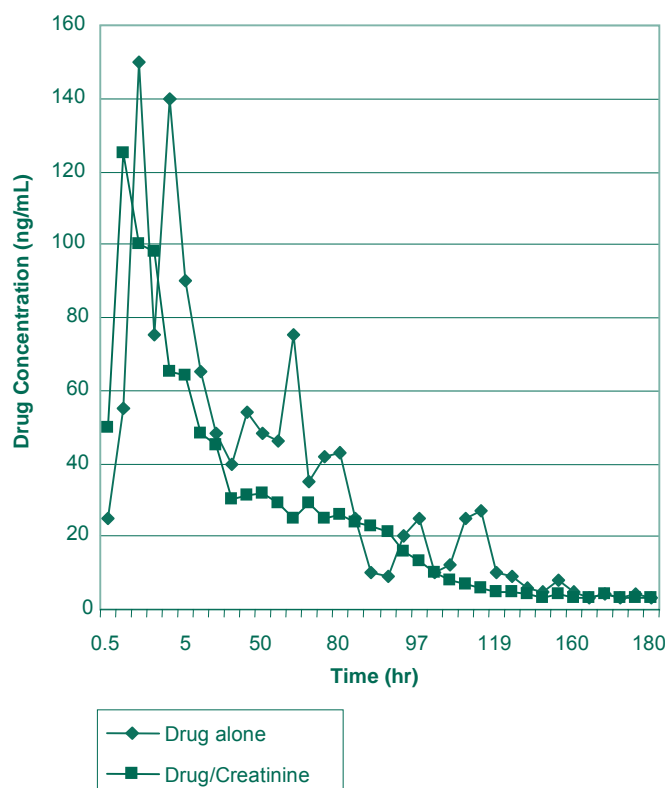
An evaluation of more than 47 studies, including general population clinical studies, reports of subjects with severe overhydration or polyuria, and water loading studies, found no exception to the criteria, thus providing analytical and physiological justification for the regulatory limits (6). SAMHSA and other regulatory agencies couple specimen integrity tests such as creatinine and specific gravity to reduce occurrence of false results. Even with this precautionary measure, given the serious consequences of the tests, the limits for "substituted" urine are continually challenged through litigation.

### Normalization of drug concentrations

Normalization of drug concentrations to urinary creatinine is used mostly in drug rehabilitation and correctional programs. If a subject has continued to use drugs and ingested excess fluids, false results may occur without normalization. On the other hand, urinary drug concentrations can appear to increase due to reduction in urinary output or dehydration, which may lead to misinterpretation of new drug consumption rather than continued excretion from previous drug use.

Drug is metabolized at a relatively constant rate, although the concentration in the urine does not decrease in a uniform pattern; rather, it fluctuates depending on urine volume. Because creatinine excretion is relatively stable, the ratio of the concentration of the drug of interest to that of urinary creatinine provides an accurate estimate of the total urinary excretion; that is, creatinine can be used to "smooth" the drug concentration profile and provide a more accurate picture of drug use history (Figure 2).

Several studies have demonstrated how creatinine normalization gives a more accurate account of drug use during a given time period. Drugs that have been investigated include marijuana, amphetamines, cocaine, nicotine, and buprenorphine (7). In one study in which creatinine was used as a marker of diluted specimens, 7.6% of the specimens identified as diluted (creatinine <20 mg/dL) had measurable carboxy-THC (11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid) concentrations and 1.5% had measurable benzoylecgonine concentrations. If these subjects had discontinued their drug use, normalizing urinary drug concentrations to urinary creatinine would have shown a steady downward trend even with more concentrated urine (4). Another report demonstrated that without normalization, a subject being monitored for marijuana use had an increased carboxy-THC concentration on day

**Figure 2. Typical urine profile with and without creatinine normalization**

15 of rehabilitation. With data normalization to urinary creatinine, a linear decline in drug concentration was observed with a terminal half-life of 10.8 days, which supported that the subject had abstained from marijuana use (8).

Ideally, creatinine normalization between multiple drug tests over time can detect episodes of new drug use by comparing the metabolite to creatinine ratios and observing abrupt increases. Huestis and Cone evaluated the specimen ratio criteria of urinary carboxy-THC to creatinine for two consecutively positive specimens to determine if the individual used marijuana between collections. Prior to this study, a change in specimen ratio criteria of 1.5 between two positive marijuana specimens suggested new drug use. After evaluating more than 530 carboxy-THC positive urines, these authors determined that a ratio of 0.5 most accurately differentiated between new use and residual excretion. They concluded that specimen ratio criteria for a specific drug program should be established based on the program's needs, taking into account sensitivity, specificity, accuracy data, and the consequences of the laboratory results (7). Thus, creatinine normalization can decrease false-positive and false-negative results in drug-testing programs by minimizing the effects of urinary output variability.

### Oral creatine supplementation

With the emergence of oral creatine supplements in the athletic arena in the 1990s, forensic urine drug testing faced yet another potential challenge. In an effort to improve performance and enhance strength, athletes could consume five grams of a creatine supplement mixed in a drink to receive the equivalent dietary intake of creatine and creatinine present in 2.2 pounds of uncooked meat. An average adult probably would not consume this amount of meat on a daily basis.

Earlier studies indicated that dietary contribution of creatine and creatinine could influence urinary excretion patterns of creatinine (2, 9). To date, one report has investigated the potential effects of oral creatine supplementations on urinary measurements used by laboratories to validate specimen integrity. Roper-Miller et al. evaluated 307 discrete urine specimens to determine that recommended daily doses of oral creatine supplements do not influence urine integrity tests, including pH, specific gravity, and creatinine (2). A Jaffé-based method was used in this study.

Additional studies could add information to this short-term study by investigating a larger sample size, longer period of creatine supplementation, multiple dosing regimens, and additional methodologies.

### Laboratory analysis

Creatinine is routinely measured by either a colorimetric or enzymatic method. Urine usually requires a 1:100 dilution to achieve its linear range. Most methods for creatinine measurement employ the Jaffé reaction, first described in 1886. In this reaction, creatinine reacts with alkaline picrate to form an orange-red colored complex known as the Janovski complex and its color intensity is measured by a spectrophotometer (520 nm). Advantages of the Jaffé reaction are its wide analytical acceptability, simplicity, and adaptability to automation (1).

Among the drawbacks of the original Jaffé reaction are that chromogens such as bilirubin, glucose, ascorbate, and uric acid greatly reduce the creatinine measurement while ketoacids, pyruvate, proteins, and cephalosporin antibiotic agents can increase the measurement as much as 20% (1). Various modifications have been introduced to eliminate interferences and improve specificity (Table 3).

First, acid can be added to the alkaline picrate solution to reduce interferences from noncreatinine chromogens. True creatinine is less resistant to acidification, resulting in a lighter hue, so if assays are run with and without acid (i.e., acid blanking), the difference in the two colors can be measured and the pseudocreatinine's darker hue contribution can be taken into account. Alternatively, absorption techniques such as addition of aluminum silicate (fuller's earth, Lloyd's reagent) can be used to separate creatinine from noncreatinine chromogens prior to

**Table 3. Interferent effects on creatinine assays**

Interferent	Effect on creatinine measurement
Acetoacetate	Increase or decrease
Acetone	Increase
Ascorbic acid	Increase or decrease
Bacterial products	Decrease
Bilirubin	Increase or decrease
Creatine	Increase or decrease
Drugs	
Anabolic steroids	Increase
Catopril	Decrease
Cephalosporins	Increase
Ketoprofen	Decrease
Levodopa	Decrease
Methyldopa	Decrease
Glucose	Increase or decrease
Guanidine	Increase
$\alpha$ -Ketoacids	Decrease
Proteins	Increase or decrease
Pyruvate	Increase
Urea	Decrease
Uric acid	Decrease

Sources: Young D. Effects of drugs on clinical laboratory tests, 5<sup>th</sup> ed. Washington: AACC Press, 2000.

Young D. Effects of preanalytical variables on clinical laboratory tests, 2<sup>nd</sup> ed. Washington: AACC Press, 1997.

the Jaffé reaction. However, these measures can be laborious and unnecessary for routine forensic drug testing because these types of noncreatinine chromogen interferences are not significant in this matrix.

Bacterial contamination can produce substances that retard the Jaffé reaction. Urine specimens should be kept refrigerated or frozen before analysis and a longer reaction time can reduce the effects of this type of interferent. In most instances, refrigerated

(4° C) urine specimens are stable for at least 7 days.

Creatinine can be measured alternatively using a kinetic alkaline picrate method or an enzymatic method. The kinetic, automated method employs the differential rate of color development for creatinine and noncreatinine chromogens. Several enzymatic methods coupled to different types of indicator reactions are also used to measure creatinine. Creatinine amidohydrolase (creatinase, creatinine hydrolase)

## January 2000 Workload

By Wayne R. Markus

A questionnaire was included in the AACC-CAP forensic urine drug testing survey (UDC-A) for 2000 to record workload for January 2000. One hundred two laboratories completed the questionnaire.

Some participants indicated that external blind proficiency testing specimens might be included, which would affect the numbers and percentages of confirmed positives in a small way.

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### Workload Questionnaire Results from 2000 UDC-A Proficiency Testing Survey

	Number of Specimens Screened	Number to Confirmation	Percent to Confirmation	Confirmed Positive Total	Percent of Confirmations Positive	Percent Confirmed Positive
Cannabinoids	705,467	38,932	5.519%	32,337	83.1%	4.584%
Ethanol	168,891	1,910	1.131%	1,668	87.3%	0.988%
Benzoylcegonine	730,385	9,567	1.310%	9,032	94.4%	1.237%
Opiate Group	700,743	9,761	1.393%	7,012	71.8%	1.001%
Codeine		15,819		7,731	48.9%	1.103%
Hydrocodone		15,715		7,244	46.1%	1.034%
Hydromorphone		4,494		1,199	26.7%	0.171%
6-MAM		1,931		379	19.6%	0.054%
Morphine		1,257		251	20.0%	0.036%
Oxycocone		671		56	8.3%	0.008%
Barbiturate Group	375,703	1,864	0.496%	510	27.4%	0.136%
Amobarbital		1,932		17	0.9%	0.005%
Butalbital		2,159		1,649	76.4%	0.439%
Pentobarbital		1,983		16	0.8%	0.004%
Phenobarbital		2,150		1,635	76.0%	0.435%
Secobarbital		2,151		48	2.2%	0.013%
Benzodiazepine Group	356,191	4,519	1.269%	1,332	29.5%	0.374%
Alprazolam metabolite		3,360		768	22.9%	0.216%
Flurazepam metabolite		1,581		26	1.6%	0.007%
Lorazepam metabolite		1,193		60	5.0%	0.017%
Nordiazepam		1,946		1,416	72.8%	0.398%
Temazepam		1,809		493	27.3%	0.138%
Oxazepam		4,524		2,450	54.2%	0.688%
Triazolam		1,252		3	0.2%	0.001%
Amphetamine Group	704,037	4,246	0.603%	1,774	41.8%	0.252%
Amphetamine		6,540		2,614	40.0%	0.371%
Methamphetamine		6,563		2,350	35.8%	0.334%
Propoxyphene	294,671	2,567	0.871%	2,267	88.3%	0.769%
Methadone	233,753	912	0.390%	705	77.3%	0.302%
Phencyclidine	671,267	756	0.113%	390	51.6%	0.058%
Methaqualone	217,246	21	0.010%	4	19.0%	0.002%
LSD	1,576	31	1.967%	6	19.4%	0.381%
All Drugs	5,159,930	75,086	1.455%			

converts creatinine to creatine in the presence of an NADH or hydrogen peroxide indicator reaction. Similarly, creatinine iminohydrolase (creatinine deaminase) reacts with creatinine to form ammonia, which is quantitated directly by an electrode or by monitoring a coupled enzymatic reaction of NADPH with  $\alpha$ -ketoglutarate in the presence of glutamate dehydrogenase (1). All methods must be thoroughly validated for linearity, precision, accuracy, and specificity prior to implementation.

### Summary

The role of creatinine measurements in drug testing is multifaceted and requires continued research to validate its use. Laboratories must perform specimen integrity assays to detect adulteration by individuals hoping to "beat" drug tests. Creatinine determinations can assist in detecting urine dilution or substitution. Creatinine is measured in conjunction with another integrity assay such as specific gravity to safeguard against false results. A specimen that fails the creatinine criteria for normal urine is subsequently subjected to a specific gravity test. Drug-use monitoring programs employ ratios of drug analyte concentration to creatinine concentration to normalize urinary output and differentiate between new drug use and residual excretion. Studies indicate that oral creatine supplements do not alter urinary integrity results, especially creatinine. Urinary creatinine can be easily measured by automated colorimetric, kinetic, and enzymatic techniques.

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