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Reanalysis of Forensic Urine Specimens Containing Benzoylecgonine and THC-COOH

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ABSTRACT: Drug testing laboratories are often requested to retest specimens that have tested positive. The reproducibility of analytical retest data for delta-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and benzoylecgonine in specimens that had previously been analyzed and then frozen by the Navy Drug Screening Laboratory, Great Lakes is examined in this study.

All specimens were tested by gas chromatography/mass spectrometry (GC/MS). Retest values generally showed a decrease in concentration but exhibited considerable variability. Eighty-five THC-COOH positive urine specimens stored frozen for 1 to 10 months (average 2.3 months) declined an average of 25% (range, +30% to −80%) and 61 benzoylecgonine positive urine specimens stored for 1 to 8 months (average 2.3 months) declined an average of 19% (ranged +20% to −100%) from initial GC/MS test results.

Drugs were found to partition into strata when frozen in urine because of the thermodynamics of the freezing process. To assure a homogenous solution for repeat testing, specimens that have been frozen and thawed were gently mixed before analysis.

KEYWORDS: toxicology, urine, chromatographic analysis, drug testing

When a forensic drug testing laboratory reports a urine specimen as positive under the guidelines provided by the Department of Defense or Department of Health and Human Services, the laboratory should be able to substantiate the original test result by reconfirming the initial result [*1,2*]. To prevent specimen deterioration, positive specimens are frozen and stored in a secure freezer until a request for a retest is received. While almost all retests performed by the Navy Drug Screening Laboratories confirm the presence of the drug, many retest results show a decrease in drug concentration compared to the original GC/MS results. To determine the variability of analytical values of retest specimens relative to the initial GC/MS concentration, this study examines the retest results of 146 specimens that were requested for retesting by military commands and legal counsel.

While there has been research on the stability of delta-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and benzoylecgonine in blood, there is very little information about the stability of these drugs in urine. Several studies have shown that under certain conditions benzoylecgonine and THC-COOH are stable for months. When benzoylec-

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gonine was stored at room temperature in hemolyzed blood, the concentration decreased by 25% after one month, but when benzoylecgonine was stored at refrigerator temperature there was little change in concentration [3]. When whole blood containing tetrahydrocannabinol (THC) was stored frozen in plastic containers for 4 weeks there was a 60% to 100% decrease in THC concentration. In contrast, if whole blood containing THC was stored frozen in glass containers for the same amount of time there was no decrease in THC concentration [4]. THC-COOH was found to be stable for six months when stored at −10°C in blood or plasma [5]. One study has shown that frozen urine samples containing THC-COOH are stable for more than one year [6].

Methods

Drug Standards

THC-COOH (0.1 mg/mL), d_3-THC-COOH (0.1 mg/mL), d_3-benzoylecgonine, d_6-morphine, and d_5-PCP were purchased from Research Triangle Institute. Benzoylecgonine, d_3-benzoylecgonine, PCP, d_5-PCP, morphine, d-amphetamine HCl, and methamphetamine were purchased from AllTech Applied Science. d_6-amphetamine, d_9-methamphetamine and d_6-codeine were purchased from Merck Sharp and Dohme. 1.0 mg/mL stock solutions of benzoylecgonine and d_3-benzoylecgonine were prepared in methanol.

Radioimmunoassay

Specimens were screened with the Abuscreen radioimmunoassay (RIA) manufactured by Roche Diagnostic Systems, Nutley, New Jersey. The cutoffs employed were 100 ng/mL for THC metabolites and 300 ng/mL for cocaine metabolites in both the RIA and repeat RIA.

GC/MS

Specimens were analyzed using a Hewlett Packard 5890 GC and 5970 MSD. The methyl derivative of THC-COOH was analyzed by GC/MS with using a modification of the method of Paul et al. [7]. The monitored ions were 313, 357 and 372 for THC-COOH derivative and 360 and 375 for d_3-THC-COOH derivative. The propyl derivative of benzoylecgonine was analyzed by GC/MS according to the Navy Standard Operating Procedure Manual [2]. The monitored ions were 210, 272 and 331 for the benzoylecgonine derivative and the 213 and 334 ions of d_3-benzoylecgonine derivative. The GC/MS procedures for amphetamines, PCP, morphine and codeine were according to the Navy's Standard Operating Procedure Manual [2].

To call a specimen positive in the initial GC/MS analysis, a cutoff of 15 ng/mL was used for THC-COOH and 150 ng/mL for benzoylecgonine. The presence of the drug above the limit of detection, 1.9 ng/mL for THC-COOH and 25 ng/mL for benzoylecgonine is required to confirm a positive result for retests.

Storage of Specimens

After specimens are confirmed positive they are stored in the original urine specimen bottles (DLS 120-87-C-5652, Great Age Container, Inc., Bronx, NY) in a freezer at −15°C.

Selection of Specimens

Only those specimens that were requested to be retested by legal counsel or the submitting military command are included in this study. THC-COOH retest data were gathered from April 1986 to August 1987 and from August 1989 to December 1990. Cocaine retest data were taken from August 1989 to December 1990. One additional specimen from the Navy Drug Screening Laboratory in Norfolk, Virginia was chosen to evaluate specific properties (see below) and was not included in the statistical study.

Evaluation of Unusual Specimen

One specimen that had tested positive for benzoylecgonine, did not contain any detectable amount of benzoylecgonine upon retesting by GC/MS. An aliquot of urine was removed from this bottle and spiked with 300 ng/mL of benzoylecgonine. GC/MS analysis was performed after 1 and 3 days.

To further evaluate this phenomenon a similar specimen from the Navy Drug Screening Laboratory in Norfolk, Virginia, was sent for additional tests. This specimen was initially positive for benzoylecgonine by RIA and GC/MS, had a high pH, and was negative for benzoylecgonine upon retesting. Further analysis of other components in this specimen was performed by the Navy Drug Screening Laboratory in San Diego, CA.

Partitioning of Thawed Specimens

Thirty-five to forty mL of urine fortified with either 50 ng/mL THC-COOH, 300 ng/mL benzoylecgonine, 25 ng/mL PCP, 300 ng/mL morphine, or 1000 ng/mL amphetamine and methamphetamine were frozen in 50 mL polypropylene tubes. Forty mL of water and 40 mL of 0.1 M NaCl were fortified with 300 ng/mL of benzoylecgonine and frozen in 50 mL polypropylene tubes. The tubes were allowed to thaw by standing vertically in a rack at room temperature; the tubes were not shaken. Aliquots of urine were carefully removed from the top and bottom sections of the tubes and analyzed by GC/MS. The volumes analyzed varied with the specific drug procedure: THC-COOH 10 mL, PCP 10 mL, morphine 5 mL; benzoylecgonine 3 mL, and amphetamines 2 mL.

Partially Thawed Specimens

Forty mL of urine was fortified with either 25 ng/mL of THC-COOH or 250 ng/mL of benzoylecgonine and frozen in 50 mL polypropylene tubes. The tubes were allowed to partially thaw at room temperature until approximately 50% of the urine was melted. The liquid was separated from the ice (frozen urine) and analyzed by GC/MS. The remaining frozen urine was melted at room temperature and analyzed by GC/MS.

Results and Discussion

Over 70% of the specimens that were submitted to retesting showed a decrease in concentration. The average decrease in concentration of all retest specimens was 22% with a range from +28% to −100%. Ninety percent of the specimens which were retested had a drug concentration between 50% and 120% of their original value. Only 10% of the retest specimens quantitated below 50% of their original concentration.

The average decrease of the 85 specimens retested for THC-COOH was 24% with a standard deviation of 24%. The distribution of the percentage change in concentration between the initial GC/MS analysis and the retest GC/MS analysis for THC-COOH is shown in Fig. 1. This distribution resembles a normal bell shaped distribution curve and

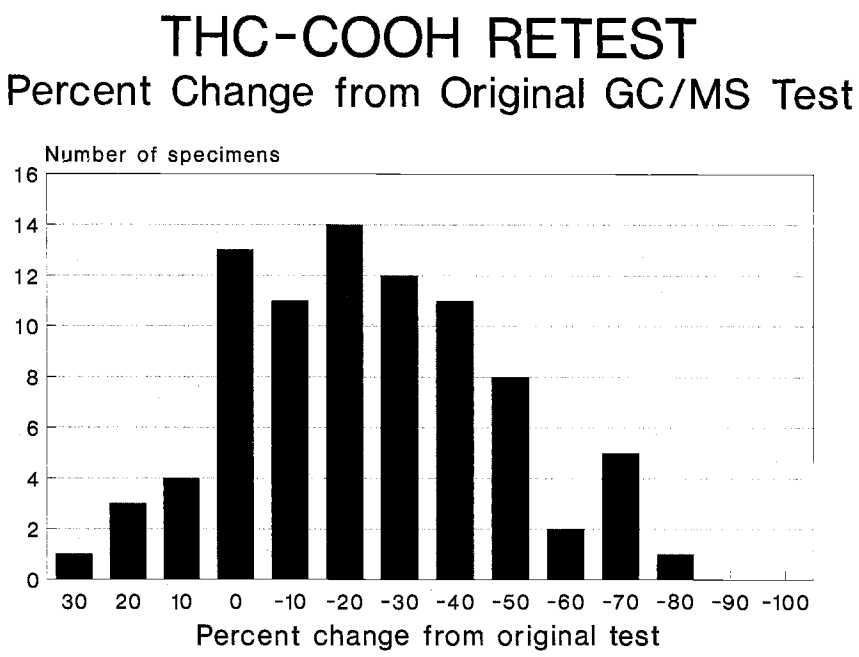

FIG. 1—*The percentage change from the original GC/MS analysis to the retest GC/MS test for 85 specimens tested positive for THC-COOH. The average storage frozen time was 2.3 months.*

ranges from +30% to −80%. Retest values ranging from +24% to −72% would fall within two standard deviations of this distribution.

The average decrease of the 61 specimens retested for benzoylecgonine was 19% with a standard deviation of 28%. The distribution of the percentage change in concentration between the initial GC/MS analysis and the retest GC/MS analysis for benzoylecgonine is shown in Fig. 2. This distribution appears to be bimodal with one distribution centered around −10% with a standard deviation of 15%, and a second distribution centered around −80% with a standard deviation of 8%. It is not known if the unusually large decreases in benzoylecgonine concentrations in the second group were due to chemical, biological or physical factors.

One of the specimens in the second group of benzoylecgonine retests failed to confirm; no benzoylecgonine was found upon retesting by GC/MS. The pH of this specimen was 8.9. When a portion of this urine was spiked with 300 ng/mL of benzoylecgonine, the benzoylecgonine concentration rapidly decreased to 0 after 3 days. Thus, the disappearance of benzoylecgonine from that particular specimen appears to be related to the condition of the urine, rather than an inaccurate initial test.

Further information on the stability of benzoylecgonine in urine was provided by the Navy Drug Screening Laboratory in Norfolk, VA. One specimen that was initially positive for benzoylecgonine by GC/MS, but upon retesting did not have any benzoylecgonine, was sent to the Navy Drug Screening Laboratory in San Diego for analysis. This specimen also had a high pH. The San Diego Laboratory showed that this specimen contained ecgonine.[3] Thus, ecgonine appears to be more stable in urine than benzoylec-

[3]Robert Czarny, Cecil Hornbeck and John Christopher, Navy Drug Screening Laboratory San Diego, personal communcation, 1993.

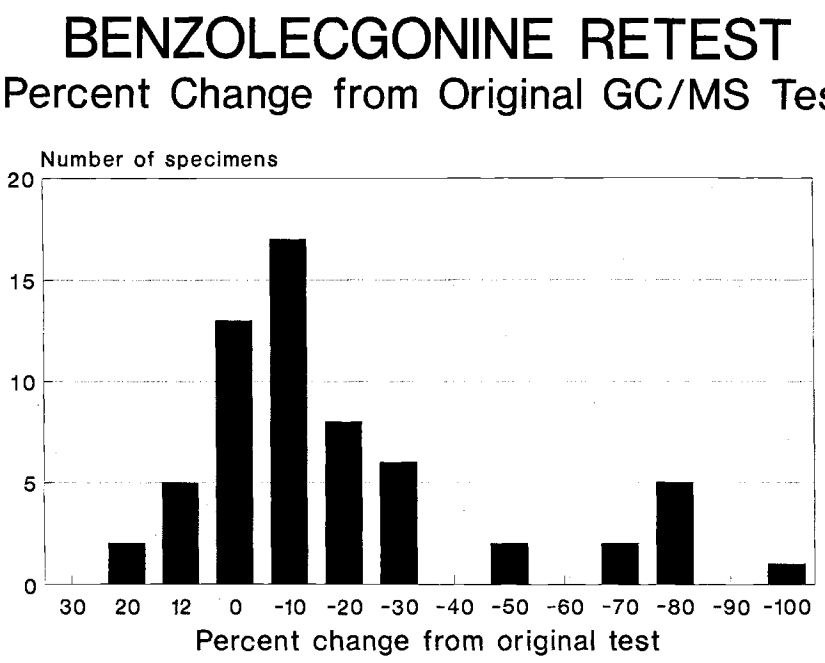

FIG. 2—*The percentage change from the original GC/MS analysis to the retest GC/MS analysis for 61 specimens tested positive for benzoylecgonine. The average frozen storage time was 2.3 months.*

gonine, and may be present in specimens that have previously tested positive for benzoylecgonine.

There was no apparent relationship between the stability of THC-COOH and benzoylecgonine in urine with the length of time spent in frozen storage. The correlation coefficient for the decrease in THC-COOH concentration versus time was 0.02 and the correlation coefficient for the decrease in benzoylecgonine concentration versus time was 0.22. While time spent in frozen storage undoubtedly is a factor affecting concentration, apparently other factors influenced the decrease in urine drug concentrations to a greater

TABLE 1—*Concentration gradient of drug urine solutions after thawing.*

Drug	Target concentration	Upper layer	Bottom layer
THC-COOH	50 ng/mL	44 ng/mL	65 ng/mL
Benzoylecgonine	300 ng/mL	67 ng/mL	585 ng/mL
PCP	25 ng/mL	49 ng/mL	8 ng/mL
Morphine	300 ng/mL	350 ng/mL	590 ng/mL
Amphetamine	1000 ng/mL	394 ng/mL	2212 ng/mL
Methamphetamine	1000 ng/mL	385 ng/mL	2228 ng/mL
Benzoylecgonine in 0.1 N NaCl	300 ng/mL	150 ng/mL	435 ng/mL
Benzoylecgonine in water	300 ng/mL	291 ng/mL	289 ng/mL

NOTE: Drug fortified solutions stored frozen in 50 mL polypropylene tubes. All solutions are in urine unless otherwise stated.

TABLE 2—*Dependence of drug concentration upon aliquot preparation.*[a]

	THC-COOH	Benzoylecgonine
Partially thawed[b]	27 ng/mL	335 ng/mL
Remaining ice[c]	19 ng/mL	166 ng/mL
Thawed, not mixed[d]	17 ng/mL	176 ng/mL
Thawed and mixed[e]	24 ng/mL	268 ng/mL
Theoretical[f]	25 ng/mL	250 ng/mL

[a]Drug fortified urine stored in 50 mL polypropylene tubes.
[b]Specimen not completely thawed; some frozen urine remaining.
[c]Remaining frozen urine after melted liquid removed.
[d]Specimen completely thawed, but not mixed; aliquot poured before mixing.
[e]Specimen completely thawed and gently mixed.
[f]Expected target value.

extent in these specimens. Factors that may affect urine drug concentration include: absorption of the drug onto the walls of the container; storage temperature; time spent at room temperature before freezing; assay variability; and bacterial, enzymatic or chemical degradation. Due to the variability of the results observed in retest specimens, these factors are probably present to different degrees in different specimens.

To assess the effect of proper mixing of specimens that had previously been frozen, urine fortified with drug was frozen in polypropylene tubes and allowed to thaw. As shown in Table 1 a concentration gradient was formed when the tubes were allowed to thaw without shaking or mixing. The concentration gradient was apparent for all of the drugs in the study. The benzoylecgonine solution that was frozen in water did not form a concentration gradient, while the benzoylecgonine frozen in 0.1 N NaCl did form the gradient. Thus, the relative differences between the observed gradients listed in Table 1 are dependent upon the ionic strength of the solution.

Another factor that will affect the outcome of a retest is the proper pouring of the retest aliquot from the frozen urine specimen. A frozen urine specimen must be allowed to completely thaw and the resulting liquid must be thoroughly mixed before an aliquot is poured for testing. As shown in Table 2, aliquots of urine taken from THC-COOH and benzoylecgonine specimens that were only partially thawed (still containing frozen urine) contain a higher concentration of drug than the original specimen because the drug and salts have preferentially concentrated in the liquid phase. Aliquots taken from specimens that have completely thawed, but were not properly mixed prior to pouring the aliquot are likely to give incorrect results because urine near the top of the bottle will have a low concentration of drug due to the thermodynamics of the thawing process. Requiring that frozen specimens be completely thawed and gently mixed prior to analysis should be included in a forensic laboratory's standard operating procedure to assure reproducible and accurate results.

Decreases in drug concentration in specimens can be accounted for by assay variation and time dependent degradation of the drug by normal processes. Laboratories can use retests to confirm low concentrations of drug provided that the drug concentration is above the limit of detection [*8*].

In some instances the retest concentration may be higher than the original result. This may occur when a urine specimen is collected after recent cocaine ingestion and contains both benzoylecgonine and cocaine [*9*]. Since cocaine hydrolyses to benzoylecgonine in a pH dependent process [*3*], retests of some benzoylecgonine positive specimens may show a significant increase in benzoylecgonine concentration. Differences may also occur

due to statistical variation especially if the drug concentration is outside the limits of linearity for the assay [*8*]. Another cause of a high retest is improper thawing or aliquoting of the frozen specimen. As shown in Tables 1 and 2, large discrepancies between the original and retest concentrations may result if the specimen is not properly prepared before the aliquot is poured.

There are many factors that may affect the stability of drugs or drug metabolites in urine. These include pH, temperature, composition of the specimen, type of storage container, and the time between when the specimen was collected to when it was tested. Further studies are needed to determine which factors or combination of factors are the major contributors to observed deterioration.

References

[*1*] *Federal Register*, Vol. 53, No. 69, 2.7e, April 1988.
[*2*] Navy Drug Screening Laboratory Standard Operating Procedures, 1986–1992.
[*3*] Isenschmid, D. S., Levine, B. S., and Caplan, Y. H., "A Comprehensive Study of the Stability of Cocaine and Its Metabolites," *Journal of Analytical Toxicology*, Vol. 13, 1989, pp. 250–256.
[*4*] Christophersen, A. S., "Tetrahydrocannabinol Stability in Whole Blood: Plastic Versus Glass Containers," *Journal of Analytical Toxicology*, Vol. 10, 1986, pp. 129–131.
[*5*] Johnson, J. R., Jennison, T. A., Peat, M. A., and Foltz, R. L., "Stability of Delta 9-Tetrahydrocannabinol (THC), 11-Hydroxy-THC and 11-Nor-9-carboxy-THC in Blood and Plasma," *Journal of Analytical Toxicology*, Vol. 8, 1984, pp. 202–204.
[*6*] Fuhrmann, L. and Szasx, V. S., "Long Shelf Life of 11-Nor-Delta-9-Tetrahydrocannabinol-9-Carboxylic Acid Standard," presented at the 42 Annual Meeting of the American Academy of Forensic Sciences, Cincinnati, Ohio, February 1990.
[*7*] Paul, B. D., Mell, L. D., Mitchell, J. M., McKinley, R. M., and Irving, J., "Detection and Quantitation of Urinary 11-Nor-Delta-9-Tetrahydrocannabinol-9-Carboxylic Acid, A Metabolite of Tetrahydrocannabinol, by Capillary Gas Chromatography and Electron Impact Mass Fragmentography," *Journal of Analytical Toxicology*, Vol. 11, 1987, pp. 1–5.
[*8*] Needleman, S. B. and Romberg, R. W., "Limits of Linearity and Detection from Some Drugs of Abuse," *Journal of Analytical Toxicology*, Vol. 24, 1990, pp. 34–38.
[*9*] Ambre, J., "The Urinary Excretion of Cocaine and Metabolites in Humans: A Kinetic Analysis of Published Data," *Journal of Analytical Toxicology*, Vol. 9, 1986, pp. 241–245.

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