Papain Adulteration in 11-nor-Δ⁹-Tetrahydrocannabinol-9-carboxylic Acid-Positive Urine Samples*†

Scott J. Larson¹, Justin M. Holler¹, Joseph Magluilo, Jr.¹, Christopher S. Dunkley¹, and Aaron Jacobs²

¹Division of Forensic Toxicology, The Armed Forces Medical Examiner System, Armed Forces Institute of Pathology, Rockville, Maryland 20850 and ²Air Force Medical Operations Agency, Brooks City-Base, Texas 78235

Abstract

The adulteration of urine samples is an ongoing problem in forensic drug-testing laboratories, even in the military where the practice of observed collections is performed. These adulterants are used to produce a false-negative result when samples are analyzed for drugs of abuse. It has been reported that papain, a cysteine protease, could be successfully used as a urine adulterant, altering the concentration of 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) in urine samples. The current study analyzes the effects of latex papain (Sigma, 10 mg/mL) and Lawry's® Adolph's Meat Tenderizer (papain is an active ingredient, 10 mg/mL) on immunoassays (FPIA, EMIT, KIMS) and gas chromatography–mass spectrometry (GC–MS) analysis for biological samples. The samples were analyzed initially between 2 and 4 h and then at 1-, 3-, 7-, and 10-day time intervals after the addition of papain. A decrease in response averaged over the course of the study was observed with FPIA (Abbott, 22%) and EMIT (Syva® Dade Behring, 26%, Microgenics, 10%) screening assays by the addition of latex papain to the samples. An increase in response was found using the KIMS (Roche) assay (156% increase). In addition, the GC–MS results (27% decrease) demonstrate that papain affects both the screening and confirmation assays. The addition of meat tenderizer caused a decrease in the FPIA (Abbott, 11%) screening assay and GC–MS results (22%) similar to the latex papain while having varied results on the other screening assays. This study confirms papain could be a potential problem for urine drug-testing programs.

Introduction

Adulteration is the tampering of specimens with the purpose of altering the test results. Successful adulteration produces a false-negative result and is an ongoing issue for forensic drug testing laboratories. It can occur with drugs-of-abuse screening immunoassays, as well as with gas chromatography–mass spectrometry (GC–MS) confirmation assays. The adulterant disrupts the screening assay or alters the drugs present in the sample to make them undetectable.

The Department of Defense's military drug-testing program serves as a deterrent for drug abuse among service personnel. A component of this program is detection of 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (THCCOOH), the primary metabolite of the active component in marijuana. It has been reported that papain, a cysteine protease purified from papaya fruit, could be successfully used as a urine adulterant for detection of THCCOOH in spiked synthetic urine samples (1). Papain consists of 212 amino acids stabilized by three disulfide bridges and two distinct structural domains with the active site between them (2). It can hydrolyze esters and amides while being used in the enzymatic synthesis of amino acids and peptides (3–7). Papain possesses a broad range of activity between pH 3 and 12 (8) and can be activated by urea or cysteine (8–10).

The current study focuses on the effect of papain on biological samples. Because of the presence of numerous cannabinoids and metabolites other than THCCOOH following marijuana use, it was determined that biological samples would provide results that are more accurate. In addition, the previous study (1) stored specimens at 4°C, whereas Department of Defense samples are sent from bases throughout the world to six laboratories in the United States. The specimens are not temperature controlled during shipment, so the present study analyzed the effects at room temperature (22°C). The samples were analyzed using Fluorescence Polarization Immunnoassay (FPIA), Enzyme Multiplied Immunoassay Technique (EMIT), and Kinetic Interaction of Microparticles in Solution (KIMS) screening immunoassays followed by confirmation using GC–MS. A counterculture magazine referred to meat tenderizer as a potential source of papain (11). Therefore, Lawry's® Adolph's Meat Tenderizer was used in the study as an alternative source of papain.

The investigation of these factors seemed pertinent to further understand the role of papain as a potential adulterant effecting the detection of THCCOOH. This is particularly im-
important since the adultered samples tested normal and would not be rendered invalid in specimen validity testing (pH, specific gravity, creatinine, chromate, nitrite, and oxidants) in this laboratory and others (1).

Experimental

Reagents and samples
Papain from papaya latex (Sigma Aldrich, St. Louis) was received as a crude powder (2.5 units/mg) and stored at 4°C. Antipain and trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E64) (Sigma Aldrich) were stored at -20°C. Lawry's Adolph's Meat Tenderizer was purchased from a grocery store and stored at room temperature. Urine samples were supplied from the Fort Meade Forensic Toxicology Drug Testing Laboratory (after a minimum of one year frozen storage) and had previously been confirmed positive for THC-COOH. All urine samples were individually treated and analyzed.

Sample preparation
The individual urine specimens were screened and confirmed to establish baseline THC-COOH levels. Samples were then divided into 25 mL volumes, treated with latex papain (10 mg/mL), meat tenderizer (10 mg/mL), or left untreated. Next, they were analyzed between 2 and 4 h and at 1-, 3-, 7-, and 10-day time intervals. Both latex papain (250 mg) and meat tenderizer (250 mg) were added as a dry powder to the urine samples.

Screening
Two EMIT assays (Microgenics, DRI® Cannabinoid Assay (Fremont, CA) and Syva Dade Behring, Emit II® Plus Cannabinoid Assay (Deerfield, IL) and a KIMS assay (Roche, Cannabinoids II®) were used on the Hitachi Modular P instrument (Roche Diagnostics, Indianapolis, IN). The FPIA screen was performed on an Abbott Axsym instrument (Cannabinoids®, Abbott Diagnostics, Abbott Park, IL).

Confirmation
The urine specimens were confirmed for THC-COOH using a solid-phase extraction procedure. The samples were hydrolyzed with 10 N potassium hydroxide (Fisher Scientific, Fair Lawn, NJ) at 70°C for 10 min, then 1.0 M acetic acid (Sigma Aldrich) was added, and the pH adjusted to 3.5. Samples were added to a conditioned solid-phase column.
The samples were washed consecutively with 2 mL deionized water and a 2 mL acetonitrile/0.1 M hydrochloric acid (40:60) mixture. The columns were dried under vacuum for 5 min before 0.5 mL hexane was added to the column. The analytes were eluted with a hexane/ethyl acetate (50:50) solution, and the solvent was evaporated to dryness with nitrogen at 50°C. The samples were derivatized with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Sigma Aldrich, St. Louis, MO) for 30 min at 70°C and quantitated by selected ion monitoring GC–MS (Agilent 6890 GC with a 5973 MS, Palo Alto, CA) as the internal standard. THCCOOH concentrations and ion ratios were calculated using a single-point calibration method. All analyte and internal standard retention times were within ± 2% of the calibrator. The ions monitored for THCCOOH were m/z 371*, 473, and 488 and for THCCOOH-d9 were m/z 380* and 479 (* denotes quantifying ion).

Results

The Syva Dade Behring assay showed decreased detection for THCCOOH in latex papain treated samples (Figure 1, N = 46, range 16–43%, mean 26%) compared with untreated samples. Samples treated with meat tenderizer actually had an increase in detection of THCCOOH (Figure 2, N = 30). Latex papain samples screened with the Microgenics assay also resulted in decreased detection (Figure 3, N = 46, range 7–16%, mean 10%), whereas those with meat tenderizer demonstrated no measurable differences (Figure 4, N = 30). The FPIA (Abbott) assay produced results demonstrating a consistent decrease in detection for both latex papain treated samples (Figure 5, N = 32, N = 19, range 19–23%, mean 22%) and meat tenderizer treated samples (Figure 6, N = 19, range 8–15%, mean 11%) regardless of initial THCCOOH concentration or time. The KIMS (Roche) screening assay was significantly affected by the addition of latex papain. There was a large increase in re-

Analysis

Samples were analyzed as untreated versus adulterated with latex papain or meat tenderizer. A two-tailed t-test was used to assess the differences between untreated versus treated within each time interval. All values are reported as percent decrease ± standard error (* = p ≤ 0.01 and ** = p ≤ 0.05 are considered statistically significant).
response in treated samples (Figure 7, N = 43, range 123–197%, mean 156%). Samples treated with meat tenderizer had a minimal change in response (Figure 8, N = 21). Initially, there was a 15.5% increase in detection, but this gradually fell to a 12% decrease by day 10.

The samples were then analyzed using GC–MS as a quantitative assay. Latex papain treated samples decreased the concentration of THCCOOH between 18 and 37%, with a mean of 27% (Figure 9, N = 46). This decrease was immediate, with no correlation to the initial THCCOOH concentration. Meat tenderizer samples decreased the concentration of THCCOOH between 11 and 40%, with a mean of 22% (Figure 10, N = 28). This trend was similar to the latex papain treated samples.

Discussion

This study was designed to analyze the effect of papain as a urine adulterant on multiple screening assays as well as GC–MS using biological specimens. Measurable decreases in THCCOOH detection was observed using EMIT and FPIA screening assays, and large increases were found with the KIMS assay (Table I). Addition of papain as an adulterant caused a significant number of samples to produce false-negative results (Table II), except with the KIMS assay where there were a number of false-positive results. Table II only includes samples that screened positive for THCCOOH (50 ng/mL) before the addition of papain. The FPIA screening method produced similar results to those found in previous reports (1). Samples spiked with meat tenderizer did not produce the same reduction in detection as those spiked with latex papain. This is likely caused by a lower concentration of papain in meat tenderizer (actual concentration of papain is proprietary information). The KIMS screening assay produced a different response from the other assays with a considerable increase of raw signal in treated samples. Meat tenderizer had little effect on the KIMS assay. Again, this is probably due to decreased levels of papain in the spiked sample.

GC–MS analysis was performed to determine if papain effected THCCOOH directly and not by simply altering the

### Table I. Summary of Screening Data

<table>
<thead>
<tr>
<th>Immunoassay</th>
<th>Latex Papain</th>
<th>Meat Tenderizer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Average</td>
</tr>
<tr>
<td>Syva Dade Behring (EMIT)</td>
<td>16–43% decrease</td>
<td>26% decrease (230 samples)</td>
</tr>
<tr>
<td>Microgenics (EMIT)</td>
<td>7–16% decrease</td>
<td>10% decrease (230 samples)</td>
</tr>
<tr>
<td>Abbott (FPIA)</td>
<td>19–23% decrease</td>
<td>22% decrease (121 samples)</td>
</tr>
<tr>
<td>Roche (KIMS)</td>
<td>123–197% increase</td>
<td>156% increase (215 samples)</td>
</tr>
</tbody>
</table>
screening assays. Analysis of treated versus untreated samples throughout the 10-day period showed no significant decrease in concentration for untreated samples. The effect of papain on the internal standard was also examined. A decrease in ion abundance was observed in latent papain adulterated samples. This decrease in internal standard abundance was statistically significant (p = 0.01). This decrease in response was observed in papain treated samples only and not in the untreated samples (calibrator and controls). To determine the actual influence of papain, the samples were extracted, and the internal standard was added post-extraction. The results were compared to the same samples following the regular extraction (Table III). It was determined the effect of latent papain was greater than earlier experiments had shown. The experiment was repeated using meat tenderizer, which produced varied results. There was no decrease in ion abundance in adulterated samples. Most samples spiked at 10 mg/mL meat tenderizer displayed little difference in the concentration of THCCOOH before and after normalization (Table III). The meat tenderizer concentration was increased to 100 mg/mL, and a decrease in area response was noted, similar to the latent papain (10 mg/mL). Therefore, the effect of papain is concentration dependent and immediate.

There is no established mechanism of action for the influence papain has on THCCOOH detection. Two possibilities include the nonspecific binding between papain and THCCOOH or the protease activity of papain cleaving a portion of the molecule. A previous report (1) concluded that nonspecific binding was the mechanism of action. This was based on FPIA experiments using activated recrystallized papain versus recrystallized papain with the activity inhibited by E64. There was no difference in the detection of THCCOOH between the two groups, so nonspecific binding was determined to be the mechanism of action. Experiments in the current study using papain specific protease inhibitors E64 and antipain resulted in similar results. There are potential problems with this explanation. The binding between papain and THCCOOH would likely dissipate during the extraction because of dramatic pH and temperature changes, in addition to the potential degradation of papain under those same conditions. Another potential mechanism is the protease activity of papain could alter the THCCOOH molecule through its proteolytic capabilities. Papain could theoretically degrade the THCCOOH molecule, rendering it non-detectable because urea possesses the ability to activate papain (8).

In summary, papain has a measurable effect on THCCOOH detection in urine. Both screening and confirmation assays are susceptible to the adulteration compound. Information drawn from this study will increase awareness of papain products as adulterants. If the presence of papain as an adulterant continues to increase, it would be beneficial to develop a procedure to detect it as an adulterant in urine analysis samples.

### Acknowledgments

Special thanks to Fort Meade Forensic Toxicology Drug Testing Laboratory for supplying the samples in this study. This work was funded in part by the American Registry of Pathology, Washington, D.C. 20306-6000.

### References


Manuscript received February 14, 2008; revision received March 27, 2008.