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Distinguishing Ingested Ethanol from Microbial Formation by Analysis of Urinary 5-Hydroxytryptophol and 5-Hydroxyindoleacetic Acid

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ABSTRACT: During the metabolism of ethanol, the metabolic conversion of serotonin (5-hydroxytryptamine) is altered, and, as a consequence, the ratio of 5-hydroxytryptophol (5HTOL) to 5-hydroxyindole-3-acetic acid (5HIAA) excreted in urine increases appreciably. The ratio of metabolites remains elevated for several hours after ethanol is no longer detectable. In the present study, urine specimens were supplemented with glucose and *Candida albicans,* a common human pathogenic yeast, and the formation of ethanol and the changes in the 5HTOL/5HIAA ratio were examined during one week of storage. Despite the production of high concentrations of ethanol (peak level 171 mmol/L, or 788 mg/dL), the 5HTOL/5HIAA ratio remained constant. The urinary 5HTOL/5HIAA ratio was also compared with urinary and blood ethanol levels in specimens selected at random during forensic autopsies. Elevated 5HTOL/5HIAA ratios were found in all specimens with detectable urinary ethanol. Some specimens showed elevated ratios of serotonin metabolites even though no ethanol was detected, indicating that these subjects had consumed ethanol prior to death but that the concentration had already returned to zero or was below the detection limit. In one case, postmortem ethanol formation was suspected, because blood ethanol concentration was 16.8 mmol/L (77 mg/dL) whereas urinary ethanol was zero. The urinary 5HTOL/5HIAA ratio fell within normal limits, which confirmed the suspicion of postmortem ethanol synthesis in the blood specimen. The present results indicate that the 5HTOL/5HIAA ratio in urine provides a useful method to distinguish between ethanol that might have been synthesized postmortem, or generated in vitro, from ethanol excreted in urine as a result of drinking.

KEYWORDS: toxicology, forensic science, postmortem, alcohol, urine, 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, serotonin, *Candida albicans*

The presence of ethanol in body fluids forms an important part of criminal and civil litigation when accidents on the road, at sea, or in the air are investigated. With gas chromatographic or enzymatic methods, or both, the forensic toxicologist can unequivocally identify and quantitate ethanol in the specimens received. However, correct interpretation of ethanol levels, and in particular the possibility of artifactual formation, has to be considered. Several microorganisms occasionally found in man are capable of producing ethanol by metabolic conversion of glucose or other endogenous substrates [*1–4*]. Various preservatives and enzyme inhibitors should be added to specimens submitted for analysis, but even this does not rule out artifactual ethanol production [*1,5*]. In postmortem toxicology, ethanol synthesis is a constant dilemma and regardless of the conditions of storage of the specimens after collection, ethanol could still have been produced between time of death and the autopsy.

During hepatic oxidation of ethanol, many of the normal metabolic processes in the liver and other organs are altered. The biogenic amine serotonin (5-hydroxytryptamine) is oxidized into an intermediate aldehyde (5-hydroxyindole-3-acetaldehyde) which is either oxidized further into an acid by the action of aldehyde dehydrogenase or reduced into an alcohol by aldehyde reductase or alcohol dehydrogenase. Studies have shown that during the metabolism of ethanol, the alcohol metabolite 5-hydroxytryptophol (5HTOL) is formed in preference to the acid metabolite 5-hydroxyindole-3-acetic acid (5HIAA) which is normally the predominant end product [*6–8*]. Consequently, the ratio of 5HTOL/5HIAA excreted in urine rises appreciably and does not return to normal values for several hours after ethanol is no longer detectable [*8*]. On the basis of these observations, the 5HTOL/5HIAA ratio in urine has been used as a biochemical marker to monitor abstinence in alcohol rehabilitation programs [*9*].

The concentrations of 5HTOL and 5HIAA in urine are relatively stable upon storage [*10,11*] and, therefore, by analyzing these metabolites, one might be able to decide whether any ethanol present originated from ingestion or represented artifactual formation caused by specimen adulteration by ethanol-producing microorganisms. In this study, urine samples were supplemented with glucose and *Candida albicans,* a common human pathogenic yeast, and the formation of ethanol and effect on the 5HTOL/5HIAA ratio were examined. In addition, the urinary 5HTOL/5HIAA ratio was compared with the urinary and blood ethanol levels in specimens selected at random during forensic autopsies.

Materials and Methods

Analytical Methods

The concentration of ethanol in blood and urine was measured by head-space gas chromatography [*12*]. Urinary 5HTOL and 5HIAA

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were determined by gas chromatography-mass spectrometry and high-performance liquid chromatography, respectively, as described in detail elsewhere [7,11]. The chemicals used were of analytical grade from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO). All solutions were prepared in deionized water from an Elgastat UHP system (Elga, Lane End, England).

Stability of the 5HTOL/5HIAA Ratio in Urine Samples

Three urine specimens with elevated 5HTOL/5HIAA ratios were divided into two 20 mL portions and incubated in the dark in sealed polyethylene vials for 4 weeks either at 4°C or 22°C. At the start, and 4 weeks after incubation, the vials were mixed and 2.5 mL aliquots collected and stored at −80°C until 5HTOL and 5HIAA were determined.

Incubation of Urine Samples Spiked with Glucose and Candida albicans

Fresh urine samples were collected from healthy female and male control subjects (n = 6) who had not consumed ethanol or serotonin-rich foods (banana, pineapple, kiwi fruit, walnuts) for the last three days. The urine samples (20 mL) were supplemented with either glucose (20 mg/mL), *C. albicans* (10 or 1000 colony-forming units (cfu) per milliliter), or both, and incubated in sealed vials at 4°C or 22°C for a period of one week. Control samples were not fortified with exogenous glucose or *C. albicans*. Before and 1, 4 and 7 days after incubation, 2.5 mL aliquots were collected and stored at −80°C until taken for analysis of ethanol, 5HTOL and 5HIAA.

To examine the effect of various additives on the formation of ethanol, one urine sample was supplemented with glucose (20 mg/mL) and *C. albicans* (1000 cfu/mL) and stored for one week at 22°C in Vacutainer® tubes (Becton Dickinson, Meylan Cedex, France) containing potassium EDTA, sodium citrate, sodium heparin, or sodium heparin/fluoride.

Urinary 5HTOL/5HIAA Ratio in Forensic Urine Samples

Blood and urine specimens (n = 45) were selected at random from those taken during forensic autopsies in Sweden. The samples were stored at 4°C until taken for analysis of blood and urinary ethanol levels and at −80°C until taken for analysis of urinary 5HTOL/5HIAA ratio.

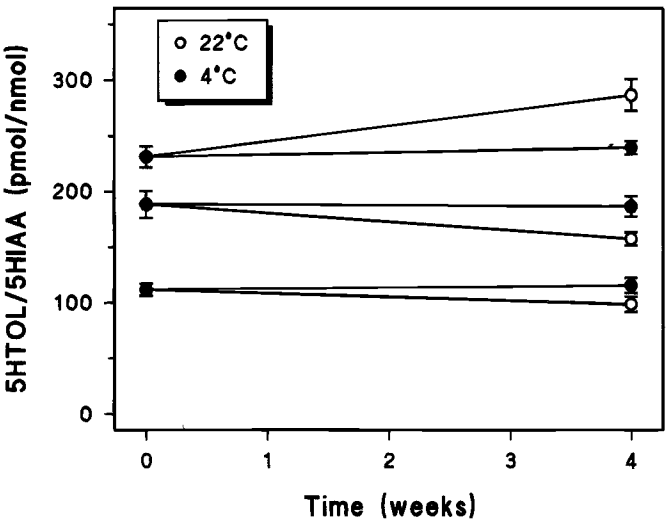

FIG. 1—*Stability of the 5HTOL/5HIAA ratio in three human urine specimens stored in the dark in sealed polyethylene vials for 4 weeks either at 4°C or 22°C. Data represent means ± SD of 5 determinations.*

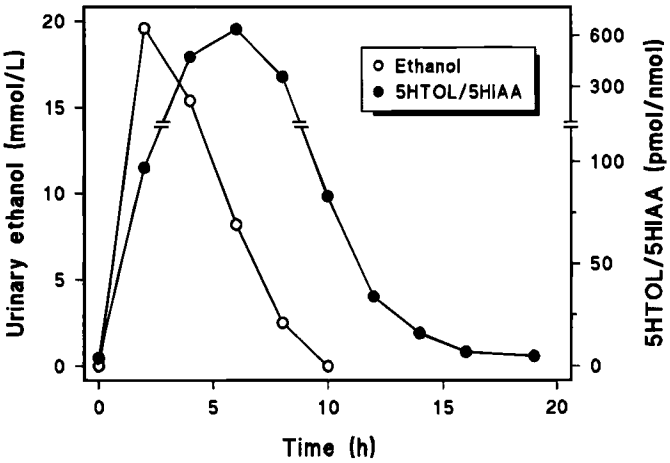

FIG. 2—*Time course effect of acute ethanol ingestion on the 5HTOL/5HIAA ratio in urine in a healthy male subject. The ethanol (0.8 g/kg body weight) was ingested as a 20% (v/v) solution over 30 min in the fasted state.*

Results

Storage of urine specimens for 4 weeks at 4°C did not significantly influence the 5HTOL/5HIAA ratio, whereas at 22°C, slightly different ratios were obtained (Fig. 1). The effect of acute ethanol ingestion on the urinary 5HTOL/5HIAA ratio is shown for one healthy male volunteer in Fig. 2. After the oral intake of 0.8 g ethanol (20%, v/v) per kg of body weight over 30 min in the fasted state, the 5HTOL/5HIAA ratio increased from a baseline level below 10 pmol/nmol to reach a peak at 640 pmol/nmol in the sample collected 6 h after ethanol intake. The ratio did not return to baseline until 16 h after drinking.

In the urine specimens supplemented with both glucose and *C. albicans* and incubated at 22°C, high but varying concentrations of ethanol were formed with time (Fig. 3*a*). The highest levels were observed after 7 days of incubation with glucose (20 mg/mL) and *C. albicans* (1000 cfu/mL), and a maximum 171 mmol/L (788 mg/dL) ethanol was produced. Nevertheless, the 5HTOL/5HIAA ratios remained below the cut-off level (15 pmol/nmol) throughout the experimental period (Fig. 3*b*). Furthermore, no formation of ethanol was seen when incubations were carried out at 4°C, nor in untreated control samples or samples spiked with glucose or *C. albicans* alone and incubated at 22°C. The additives EDTA, citrate, heparin and heparin/fluoride failed to prevent microbial ethanol formation completely in urine samples incubated at 22°C for one week (data not shown).

In the urine specimens collected during forensic autopsies, a significant correlation between the ethanol level and 5HTOL/5HIAA ratio was obtained (Fig. 4). Elevated ratios of 5HTOL/5HIAA were found in all specimens with detectable urinary ethanol, although there were large interindividual variations. Elevated ratios were also found in some specimens despite the fact that ethanol was not detectable (Fig. 4).

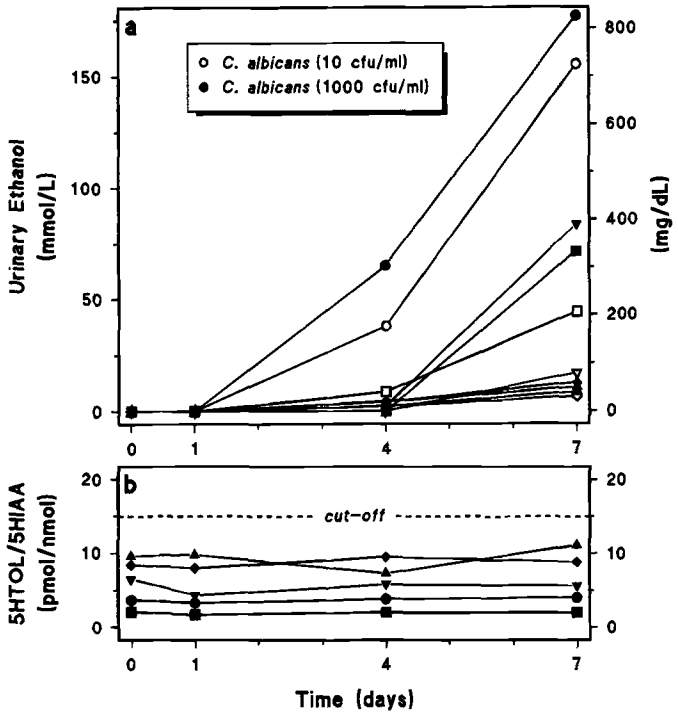

FIG. 3—a) *Ethanol production in fresh urine samples spiked with glucose (20 mg/mL) and* Candida albicans *(10 or 1000 colony-forming units (cfu) per milliliter) and incubated at 22°C, and* b) *the effect on the urinary 5HTOL/5HIAA ratio. The dashed line represents the cut-off level (15 pmol/nmol) used to discriminate between normal and elevated 5HTOL/5HIAA ratios. Data represent the results for 5 different urine samples.*

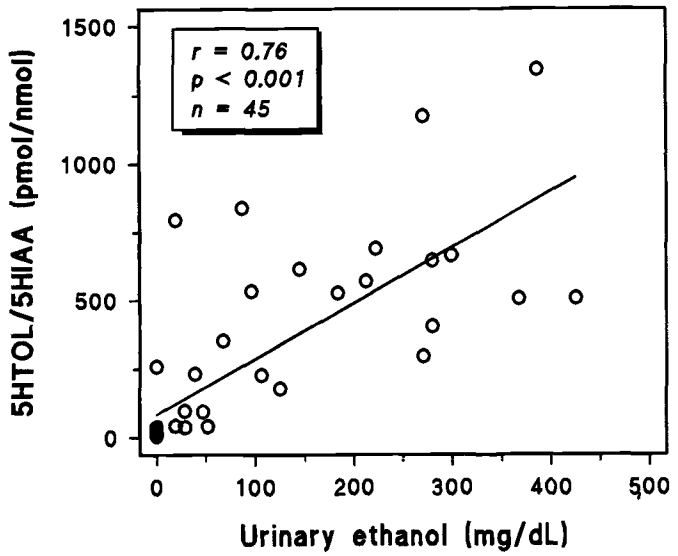

FIG. 4—*Correlation between urinary ethanol level and 5HTOL/5HIAA ratio in specimens selected at random from those taken during forensic autopsies.*

Discussion

In agreement with previous studies [*8*], acute ethanol ingestion caused a marked increase in the ratio of 5HTOL/5HIAA excreted in urine. However, despite the production of high concentrations of ethanol in vitro by spiking urine specimens with glucose and *C. albicans,* the 5HTOL/5HIAA ratio remained constant during several weeks of storage. This observation is explained by the fact that the rise in 5HTOL/5HIAA ratio following ethanol ingestion is not a direct effect of ethanol but, instead, a result of metabolic interaction between ethanol and serotonin catabolism. This metabolic event can be attributed to competitive inhibition of aldehyde dehydrogenase by acetaldehyde, the first product of ethanol oxidation, and/or to the increased level of NADH resulting from ethanol and acetaldehyde oxidation. Both these factors promote reduction of 5-hydroxyindole-3-acetaldehyde to form 5HTOL instead of 5HIAA [*13*].

Several microorganisms are capable of producing ethanol from various endogenous substrates [*1–4*]. In agreement with previous observations [*1,5*], the amount of ethanol formed by *C. albicans* was highly temperature dependent and refrigeration (4°C) proved to be effective at preventing ethanol formation. All additives tested were unable to prevent ethanol formation completely, but one recent study reported that 1% sodium fluoride eliminated ethanol production in urine by *C. albicans* [*14*]. It should be noted that even though microbial contamination of specimens can result in false-positive or falsely elevated ethanol values, such ethanol production requires both sufficient glucose or other suitable substrate [*3,15*], and, furthermore, that the urine specimen is stored under appropriate conditions for at least a few days prior to analysis. Although the urinary level of glucose is normally very low in healthy subjects, glucosuria is not uncommon, and high levels are commonly found in diabetic patients [*4,16–18*]. Therefore, if samples are mailed to the laboratory without refrigeration, the possibility of artifactual ethanol formation has to be considered.

The ratio of 5HTOL/5HIAA in urine specimens obtained at autopsy gave results confirming that alcohol had been ingested before death. In one case, the specimen was collected from a body that had been submerged in water for more than one month, but the presence of ethanol was still confirmed by an elevated 5HTOL/5HIAA ratio. Some specimens showed elevated ratios although no ethanol was detected. Because an elevated 5HTOL/5HIAA ratio is detectable in urine for longer than ethanol is present [*8*], these subjects could have consumed ethanol prior to death but the concentration had already returned to zero, or was below the limit of detection of the analytical method. An elevated 5HTOL/5HIAA ratio was found in a urine specimen collected from a subject who had died of diabetic acidosis. The urine specimen contained no detectable ethanol but 50 mg/dL acetone. Whether the elevated 5HTOL/5HIAA ratio was also the result of prior ethanol consumption or if metabolic acidosis alters the catabolism of serotonin remains to be elucidated. In another example, postmortem ethanol formation was suspected, because blood ethanol concentration was 16.8 mmol/L (77 mg/dL) and urinary ethanol was zero. Normally, ethanol is detectable for longer in urine than in blood, mainly owing to the retention of urine in the bladder. The urinary 5HTOL/5HIAA ratio fell within normal limits which confirmed the suspicion of postmortem ethanol synthesis in the blood specimen.

In conclusion, the present results show that determination of the 5HTOL/5HIAA ratio in urine provides a useful method to distinguish between ethanol that might have been produced postmortem or generated in vitro from the ethanol excreted as a result

of drinking. The 5HTOL/5HIAA ratio is seemingly a sensitive and reliable alternative to other potential markers of postmortem ethanol synthesis such as the identification of higher alcohols (n-propanol and n-butanol) or comparing ethanol levels in urine and vitreous humor with those in blood from the heart and peripheral veins [2]. The ratio of 5HTOL/5HIAA can also be used to confirm recent ingestion of alcohol even when the concentrations of ethanol in blood and urine have decreased to reach very low or negligible levels less than 10 mg/dL. This follows from the time-lag that exists between the urine-ethanol profiles and the time-course of 5HTOL/5HIAA observed after drinking (Fig. 2). The after-effects of alcohol (hangover) might be a concern in accident investigations, and could have ramifications in connection with alcohol testing in the workplace, as several studies suggest that body-function is impaired during hangover [19]. Currently, we are investigating whether the 5HTOL/5HIAA ratio can also be used with forensic blood samples.

Acknowledgments

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