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Efficacy of 1% Sodium Fluoride As a Preservative in Urine Samples Containing Glucose and *Candida albicans*

ABSTRACT: Whether urine samples used in forensic science DUI testing can be compromised by endogenous ethanol production is a recurrent and yet unresolved issue. This study first assessed unpreserved urine samples that were collected, processed, and analyzed repeatedly over 13 to 41 days using a standard gas chromatographic procedure for ethanol analysis. Despite extensive microbial growth, ethanol was not detected in any test sample. The extent of ethanol production in samples supplemented with glucose, *Candida albicans*, or both was determined to evaluate the potential for ethanol production in urine samples associated with pathological conditions such as urinary tract yeast infections and diabetes mellitus. Ethanol production under each of the above treatment conditions was assessed in the presence and absence of 1% sodium fluoride as a microbial suppressant.

Mean ethanol concentrations were determined for unpreserved samples containing urine only (0.003 ± 0.005 g%), urine plus yeast (0.006 ± 0.009 g%) and urine plus glucose (0.067 ± 0.070 g%). Unpreserved samples supplemented with both yeast and glucose attained mean ethanol concentrations of 0.164 ± 0.057 g% ($P < 0.01$). Ethanol could not be detected in any corresponding duplicate samples, which were preserved with 1% sodium fluoride. A lack of ethanol production in any of the unpreserved urine samples indicates that false DUI convictions due to endogenous ethanol production are very unlikely. And while endogenous ethanol production is possible in the presence of both glucose and contaminating *C. albicans*, 1% sodium fluoride completely eliminated microbial fermentation.

KEYWORDS: pathology and biology, sodium fluoride, urine, *Candida albicans*, DUI testing, ethanol

A major area of concern in forensic science cases of driving under the influence of alcohol involves the potential for specimen contamination by ethanol-producing microorganisms. Ethanol-producing organisms have been identified in specimens in which preservatives used apparently had little effect in deterring their growth [1–3]. Fluoride is a microbial growth inhibitor commonly used in forensic specimens, although concentration and storage conditions have been reported to alter its efficacy. It acts by inhibiting the activity of phosphoglucomutase, which prevents cell polysaccharide synthesis and has

been shown to inhibit in vitro growth of *Candida albicans*, which readily converts glucose to ethanol. *C. albicans* is a common human pathogenic microorganism and a potential contaminant of urine samples [4]. Whether variable storage conditions alter the inhibitory effect of fluoride on growth of and alcohol production by *Candida albicans* remains a critical yet unresolved issue in forensic science.

Blume and Lakatua [1] reported that a 1% concentration of sodium fluoride in blood samples was effective against several microorganisms, however, it did not prevent growth of an ethanol production by *C. albicans*. Chang and Kollman [2] confirmed these findings and also found sodium fluoride to be ineffective against ethanol production by *C. albicans* in inoculated blood samples. Ethanol was detected in preserved samples within 3 days at 37°C, 5 days at 22°C, and only trace levels after 6 months under refrigerated conditions, which suggests that the formation of ethanol in blood samples is temperature dependent. The effectiveness of various sodium fluoride concentrations in preventing microbial growth has also been assessed at various pH levels. Harper and Corry [3] were able to grow *C. albicans* in a heart infusion broth containing 0.5% glucose and 1.2% sodium fluoride, at pH 7.4 and 6.0. Ethanol production was reported from these samples when incubated at 25°C. While these studies have evaluated the action of sodium fluoride on microbial growth in various specimens under a variety of conditions, a comprehensive evaluation of sodium fluoride as a microbial inhibitor in urine samples has not been reported.

Microbial ethanol production in urine samples was evaluated in samples subjected to several combinations of potential conditions under which such samples might be stored. Unpreserved samples maintained in nonsterile containers at room temperature served as controls. Matched samples were used to test the effectiveness of sodium fluoride as an inhibitor of ethanol production in the presence of *C. albicans*, exogenous glucose or both.

Materials and Methods

Analysis of Ethanol

Urine samples were analyzed using an automated, direct injection gas chromatographic procedure. A Fisher "Dilumat" automatic dilutor was used to withdraw 50 μL of urine from each sample. Each sample was automatically diluted with 1600 μL of a 0.01 percent v/v solution of n-propanol (the internal standard) and the mixture then expelled into a test vial. The test vials, along with ethanol-negative and ethanol-positive controls, were placed in a Hewlett-Packard automatic sampler (Model 7671A) for analysis. Samples were injected directly into a Hewlett-Packard gas chromatograph (Model 5710A) equipped with a flame ionization detector, and a 6 foot long and ⅛ inch (OD) diameter, stainless steel column packed with 0.2% Carbowax on Carbopack ⁸⁰/₁₀₀ mesh. The carrier gas was chromatographic grade nitrogen. The flame support gases were hydrogen and zero grade air. The analysis was performed as an isothermal run with an oven temperature of 120° Centigrade. Injection port and detector temperatures were 200° Centigrade.

The gas chromatograph was calibrated prior to use with secondary ethanol standards. The concentration of ethanol in the standards was determined by oxidimetric titration against NBS grade potassium dichromate, which provided accuracy to within ± 0.005 g%. The detection limit of the gas chromatograph was 0.003 g%. Three secondary ethanol standards were used to define linearity between 0.100 g% and 0.300 g% in compliance with the State of California Administrative Code Title 17. One μL of the diluted urine sample was injected into the instrument for analysis. Results were calculated by an Hewlett-Packard Model 3390A internal computing integrator and reported as g%.

Sample Collection for Control Study

Seventeen subjects provided initial morning void urine samples in 1-oz glass bottles (Fisher #03-521-1A). The collection bottles were clean and preservative free. These samples were analyzed for ethanol on the day received, as described above. The samples were then stored in the original containers at room temperature and reanalyzed one to five times over a period of 13 to 41 days.

Sample Collection for Treatment Study

Seventeen additional subjects provided urine samples in plastic, 4-oz collection bottles (Fisher #11-840A). The sample from each subject was aliquoted into eight 10 mL plain vacutainer blood collection tubes (Becton Dickinson #6530) containing appropriate additives for each treatment group (described below). All treatment groups contained samples from each of the 17 subjects.

Treatment Groups

Eight mL of urine were aliquoted per tube. Additives were distributed to each group as follows: (A) no additives, (B) 1% sodium fluoride (80 mg/tube sodium fluoride; Mallinckrodt #7636), (C) yeast (*Candida albicans*, laboratory strain #3540352) in which half of the subject samples received a direct inoculation of a visible quantity of cells on the end of a flame sterilized probe while the remaining half received a 10-mL injection of a saline suspension of inoculated organisms (data from these groups were combined after analysis showed similar results), (D) 0.5% glucose (40 mg/tube glucose, Sigma Chemical Co. #G-5000), (E) 0.5% glucose plus yeast (exogenous *C. albicans*) (F) yeast (exogenous *C. albicans*) plus 1% sodium fluoride, (G) 0.5% glucose plus 1% sodium fluoride and (H) yeast (exogenous *C. albicans*) plus 0.5% glucose plus 1% sodium fluoride. All aliquots were analyzed for ethanol content on the initial day using the method previously described and then stored at room temperature. Subsequent analyses were performed daily for up to eight days until a plateau in ethanol concentration was established.

Statistical Analyses

The results were compared by Analysis of Variance and the Student-Newman-Kuels range test [5].

Results

Initial analysis of control urine samples lacking sodium fluoride preservative revealed no ethanol in any sample. Nearly all of the samples, however, exhibited microbial growth as determined by visual examination. Most samples showed evidence of fluffy colonies, red spheres, or a generally turbid appearance. Some produced gasses and all developed noxious odors. In spite of the presence of microbial growth in these unpreserved samples, ethanol failed to be detected upon subsequent analyses of all control samples throughout the duration of the testing period.

No ethanol was detected initially in any of the treatment samples. Following incubation, samples containing urine only produced ethanol ranging from 0.000 to 0.014 (mean 0.003 ± 0.005) g%. Urine samples inoculated with yeast produced a range of ethanol concentrations extending from 0.000 to 0.032 (mean 0.006 ± 0.009) g%. Urine samples supplemented with glucose produced from 0.000 to 0.220 (mean 0.067 ± 0.07) g% ethanol.

Samples containing glucose and yeast produced ethanol concentrations ranging from 0.067 to 0.237 (mean 0.164 ± 0.057) g%. Ethanol was not detected in any of the samples containing sodium fluoride. A summary of the results of ethanol production within the treatment groups is shown in Table 1.

Ethanol concentrations in urine samples containing urine only, urine plus yeast, urine with sodium fluoride, urine with yeast and sodium fluoride, urine with glucose and sodium fluoride, and urine with yeast, glucose and sodium fluoride are not significantly different ($P \geq 0.05$) and cannot be distinguished from each other. Urine samples containing glucose produced more ($P \leq 0.05$) ethanol than any fluoride-containing samples, urine only or urine plus yeast. Samples containing urine, glucose, and yeast produced more ethanol than all other treatment groups ($P \leq 0.05$).

Discussion

This study has evaluated the possibility of endogenous microbial ethanol production and its prevention by sodium fluoride in urine samples collected under typical investigative conditions. Urine samples for this experiment were obtained from both males and females after consumption of meals and were not collected under sterile or unusually clean conditions. They, therefore, represent a cross-section of typical samples and collection conditions. The unpreserved samples were opened and reopened as multiple analyses were performed, hence they were intermittently exposed to the ubiquitous microorganisms of the environment. In this study, ethanol was not produced in control samples lacking preservatives. It has been argued that urine samples collected without preservatives but containing high concentrations of yeast may be compromised by in vitro ethanol production and this was shown to be the case with yeast-inoculated samples, which attained ethanol concentrations of approximately 0.03 g%. Because ethanol production occurs under restricted conditions, which include both adequate glucose availability and the presence of ethanol-producing organisms, is is unlikely that both conditions would be present under normal circumstances. Preservatives should, however, always be used to eliminate the remote possibility of in vitro microbial ethanol production.

The issue of microbial ethanol production in urine samples has been confounded by reports dealing with atypical samples and incubation conditions [1–3]. In this study, ethanol production from urine samples containing exogenous glucose varied from no ethanol to a maximum of 0.220 g%, which is near the theoretical maximum of 0.25 g%

TABLE 1—*Ethanol production in various treatment groups containing combinations of urine, glucose, yeast, and sodium fluoride.*

Treatment	Ethanol Production (Mean ± SD)	Range High (g%)	Low	*
U	0.003 ± 0.005 g%	0.014	0.000	a
U,SF	0.000 ± 0.000 g%	0.000	0.000	a
U,Y	0.006 ± 0.009 g%	0.032	0.000	a
U,G	0.067 ± 0.070 g%	0.220	0.000	b
U,G,Y	0.164 ± 0.057 g%	0.237	0.067	c
U,Y,SF	0.000 ± 0.000 g%	0.000	0.000	a
U,G,SF	0.000 ± 0.000 g%	0.000	0.000	a
U,G,Y,SF	0.000 ± 0.000 g%	0.000	0.000	a

*Samples having different lower case letters are significantly different ($P \leq 0.01$).

Abbreviations: U—urine only; SF—1% sodium fluoride; Y—yeast *Candida albicans*; G—0.5% glucose.

for these cultures (Fig. 1). Microscopic examination of these samples revealed the presence of yeast resembling the *C. albicans* seed cultures as might be expected since the study was conducted under routine, nonsterile conditions that are subject to cross-contamination. Few other microbes were present in some of the samples. No identification was attempted. More significant were the nearly three-fold higher mean ethanol concentrations found in samples containing urine, exogenous glucose and exogenous yeast (mean 0.164 ± 0.057 g% vs. mean 0.067 ± 0.070 g%, $P \leq 0.01$). It is clear that urine samples containing glucose provide favorable conditions for ethanol production when exposed to *C. albicans*.

The limiting factor, however, in ethanol production is the amount of glucose available for conversion to ethanol. Since the glucose content of test samples were similar, samples contaminated with yeast would be expected to show ethanol levels similar to those inoculated with yeast, if given sufficient time for the yeast to reproduce and maximize the population size. In this study, yeast contaminated samples produced less ethanol than yeast inoculated samples (mean 0.067 ± 0.070 g% vs. mean 0.164 ± 0.057 g%, $P \leq$ 0.01) but significantly more than yeast inoculated urine lacking glucose (mean 0.067 ± 0.070 g% vs. mean 0.006 ± 0.009 g%, $P \leq 0.01$). We conclude that both sufficiently large populations of ethanol-producing microorganisms and significant quantities of glucose must be present for the in vitro production of ethanol in concentrations significant to forensic testing of unpreserved urine samples. The routine addition of 1% sodium fluoride, however, efficiently prevents ethanol production even under optimal conditions of high glucose concentrations and large yeast populations.

The conditions required for ethanol production in vitro should also apply to potential ethanol production in vivo. While urine containing glucose stored in the urinary bladder may provide favorable conditions for in vivo ethanol production, this can occur only if ethanol-producing microorganisms are present. However, the maximum ethanol produced will be limited by the amount of glucose available in the urine and the incubation time spent in the bladder [2]. Little ethanol production would therefore be expected from an individual with a yeast infection unless the individual also excreted glucose, as might

Molecular Weights: Glucose 180   Ethanol 46

Experimental Samples: 0.04 g Glucose (0.5%)

-> **Determine # of moles glucose in sample:**

(0.04 g Glucose)   (1 mole Glucose) = 0.00022 moles Glucose
                          180 g Glucose

-> **Glucose:Ethanol conversion ratio = 1:2**

0.00022 moles Glucose -> 0.00044 moles Ethanol

-> **Estimated maximal production of Ethanol in grams:**

(0.00044 moles Ethanol)   (46 g Ethanol) =   0.020 g Ethanol
                                1 mole Ethanol

-> **Conversion to g% Ethanol:**

0.020 g Ethanol   =   x gram   =   0.25 g%
8 ml sample   100 ml

FIG. 1—*Estimated maximal production of ethanol.*

be present in diabetes mellitus. Therefore, the argument that urine ethanol determinations may be in error due to a yeast infection of the urinary bladder could likely be resolved by routine clinical testing the sample for the presence of microbial metabolic products, identification of *C. albicans*, and a simple determination of unmetabolized glucose content to confirm whether conditions favorable to fermentation were present.

It should be emphasized that the ethanol levels reported from the various treatment samples in this study are cumulative and reflect the maximum levels obtained for incubation periods of up to 8 days. In a living individual, ethanol levels should not normally accumulate because the bladder will be voided periodically each day, especially when enhanced by the diuretic effect of drinking alcohol. Increased frequency and volume of bladder voiding is a classic symptom of diabetes mellitus. Both of these conditions reduce bladder retention time and minimize incubation times for ethanol production. Therefore, the results reported herein represent the maximal error possible in this testing and it is unlikely that such levels could ever be achieved in practice. We believe the inclusion of 1% sodium fluoride in aseptically collected urine samples effectively blocks the possibility of in vitro ethanol production by *C. albicans* and imparts a high degree of reliability to the urine ethanol testing procedure. It similarly will prevent ethanol production in glucose containing urine samples which may also be contaminated with yeast. The remaining possibility of in vivo fermentation would need to be established by confirming the presence of microbes, the presence of glucose, and sufficient incubation time.

Acknowledgment

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