IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 16, Issue 4 Ser. I (April 2023), PP 06-14
www.iosrjournals.org

Influence of Sodium Fluoride Concentration on Quantitative Analysis of Ethanol by headspace Gas Chromatography in Urine Samples.

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Abstract
Evidentiary blood and urine samples is preserved by adding Sodium fluoride (NaF) because NaF inhibits microbial growth and prevent production of, and/or metabolism of ethanol in evidentiary samples between the time of their collection and testing in the laboratory. Addition of NaF may also have adverse effects on the accuracy of measurement and interpretation of ethanol concentrations because NaF is known to cause salting-out of ethanol from blood and aqueous solutions. Presence of high concentrations of NaF in evidentiary blood and urine samples is common because the containers used for sample collection contain a fixed amount of NaF and small sample volumes are often collected in containers designed to hold relatively large volumes. Urine evidence collection kits include leak-proof 100 ml plastic bottles containing 1000 mg NaF. Evidentiary urine samples collected in these bottles vary from 1-100 ml and thus can result in NaF concentrations of 10-1000 mg/ml. The effect of NaF (10 to 200 mg/ml) was tested on a range of ethanol concentrations (50-400 mg/100 ml) in urine samples incubated at room temperature for up to 336 hr. The urine samples, post incubation with NaF were analyzed for ethanol concentrations by headspace gas chromatography. Ethanol concentrations measured in the urine samples incubated with relatively high concentrations of NaF (>40 mg/ml) were significantly lower (4-15%) (p< 0.004) than those in urine samples treated with 0-20 mg/ml NaF. The reduction in ethanol concentrations observed in the urine samples treated with >40 mg/ml NaF was due to NaF-induced salting-out of ethanol occurring between the time of NaF addition and testing of samples for ethanol in the laboratory.

Key words: Ethanol, Salting out, Sodium fluoride (NaF), Headspace Gas Chromatography and Urine

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Date of Submission: 26-03-2023 Date of Acceptance: 08-04-2023

I. INTRODUCTION

Globally, traffic accidents cause ~1.35 million deaths per year. A major cause for such traffic accidents is driving under the influence or driving while intoxicated (DUI/DWI). Ethanol is the most prevalent psychoactive substance used by drivers involved in such traffic accidents. **[1,2]**. Hence, the harmful effects of ethanol on a person's performance and behavior is a matter of serious concern for public safety. Accordingly, DUI/DWI is illegal in almost all countries around the world. In fact, most countries have *per se* laws specifying ethanol concentrations in blood, breath and urine samples. Therefore, the analytical methods used for the measurement of ethanol concentrations must be validated and reliable **[3-5]**. Based on the above facts, the accuracy of measurement of the ethanol concentrations in evidentiary samples (blood, urine and breath) at the time of collection are extremely important.

Urine is a preferred biological sample collected when the police officers suspect a driver is under the influence of drugs, in addition to consuming alcohol and the drugs cannot be detected by the analysis of breath. The other circumstance when urine sample is collected for alcohol analysis is if the driver is injured/hospitalized and breath test cannot be administered **[6-9]**. Although precautions are taken, occasionally, the accuracy and interpretation of the ethanol concentrations in evidentiary samples may be compromised for several reasons **[10-20]**. One such example is presence of relatively high concentrations of sugar and contamination of evidentiary samples by ethanol producing/metabolizing bacteria and/or yeast. Further examples include (i) storage of contaminated evidentiary samples at room temperature for prolonged periods of time in the absence of any preservative, and (ii) presence of relatively high concentrations of NaF that often result from collection of small sample volumes in containers having fixed amounts of NaF. Bacterial species, *e.g.*, *Klebsiella pnuemoniae, Escherichia coli* and *Proteus mirabilis*, and/or yeast, *e.g.*, *Candida albicans* and *Candida parapsilosis,* produce

ethanol from sugar present in evidentiary samples via fermentation, thus resulting in the measurement of erroneously high ethanol concentrations [**12,15,16,18**]. On the other hand, bacterial species, e.g., *Serratia marcescens* and *Psuedomonas* species, use ethanol in evidentiary samples as carbon source, thus, resulting in the measurement of erroneously lower concentrations of ethanol [**5**]. To prevent such *in vitro* ethanol production by fermentation of glucose or consumption of ethanol as a carbon source in evidentiary samples, NAF (10 mg/ml) is added as an antimicrobial agent [**12,13,15,18**]. Further, the *in vitro* production/consumption of ethanol is temperature dependent; hence the evidentiary samples are stored at 0-4°C [**12-16, 18**].

NaF, at concentrations of 5 mg/ml or higher in blood have been shown to increase vapor-phase ethanol concentrations (~5-9%) as compared to those measured in vapor-phase of heparin-treated blood [**10**] suggesting salting-out of ethanol by NaF. It is important to note that this study was not designed to determine blood concentrations of ethanol by analyzing headspace vapor. Further, there was no dilution of the blood with an internal standard. In another study, ethanol concentrations measured in the liquid-phase of NaF-free whole blood and whole blood treated with 2-10 mg/ml NaF by headspace gas chromatography (HSGC) were essentially identical [**11,17**]. On the other hand, ethanol concentrations in the liquid-phase of aqueous solutions quantified by HSGC were significantly lower upon treatment with 50-200 mg/ml NaF as compared to those treated with 0 or 10-20 mg/ml NaF [**11**].The offered explanation for the latter is that NaF added to the sample effected disproportionate salting-out of n-propanol, as compared to ethanol, during HSGC analysis due to sample incubation at 40°C for 30 min. This affects ethanol to n-propanol peak areas and their ratios, and thus results in the measurement of significantly lower concentrations of ethanol **[11]**. In yet another study, itis concluded that the measured amount of ethanol in blood samples slightly decreases as the concentration of NaF increases from zero to 30 mg/mL **[21]**. This is explained based on the salting out of n-propanol when compared to ethanol due to the presence of additional methyl group in n-propanol **[22,23]**.

Whether such an ethanol salting-out effect occurs in urine samples treated with NaF, thus accounting for decrease in ethanol concentrations as reported for blood [**10,17,21**], remains to be clearly explained with extensive experimentation. In this regard, a technical report and brief study by Jones **[24]** shows decreasing concentration of ethanol. Whether this decrease is related to disproportional salting out of n-propanol is not clear.

Blood, urine and/or breath samples are routinely used to determine ethanol concentrations in drivers under its influence in the state of Minnesota (USA) and elsewhere. Annually, ~10,000 blood and urine samples are tested in the state of Minnesota (USA) for ethanol. Among the samples tested, ~40% are urine samples. Urine samples are typically collected in 100 ml leak-proof plastic bottles containing 1000 mg NaF. The urine sample volumes in these bottles vary from 1-100 ml; majority (~70%) are >50 ml. Approximately 5% of evidentiary urine sample kits received in this laboratory contain 5-10 ml of urine. When the volume of urine in the bottle is <50 ml the resulting NaF concentration will be >20 mg/ml. Such an increased concentration of NaF is thus expected to result in salting-out of ethanol either in the transit and/or during HSGC analysis [**10,11**]. The transit of samples lasts up to 72 hr and during this time urine samples remain at ambient temperature. If the NaF-induced salting-out of ethanol occurs only during transit, the ethanol concentrations determined by HSGC in the liquid-phase will be significantly lower due to salting-out of ethanol into the headspace of the storage container. On the other hand, if NaF-induced disproportionate salting-out of n-propanol, as compared to ethanol, occurs during HSGC analysis, as a consequence of equilibration time and sample temperature (40°C) [**11**], the resulting ethanol concentrations, again, will be further significantly decreased. Not known is whether any of these scenarios occur in ~5% of the urine samples, in which the urine volumes are 5-10 ml and the resulting NaF concentrations will be 100-200 mg/ml, received in the laboratory. The study described herein was designed to address the above questions with the aid of pooled urine samples spiked with 50-400 mg ethanol/100ml, treated with 10-200 mg/ml, and incubated for up to 336 hrs at room temperature.

II. EXPERIMETAL

2.1 Materials and methods

Standard aqueous solutions of ethanol and positive control aqueous solutions of ethanol prepared in distilled water were purchased from College of American Pathologists (Northfield, IL), New England Reagent Laboratory (Providence, RI), and/or Radian International (Austin, TX). Absolute ethanol (95.6%) was purchased from National Institute of Standards and Technology (Gaithersburg, MD). All other chemicals and reagents used in this study were of the highest analytical grade available commercially. An aqueous solution of n-propanol (5 mg/ml) with or without NaF (30 mg/ml) was used as internal standard solution. Leak-proof plastic bottles (100 ml) used in this study were same as the evidentiary urine collection bottles used by the law enforcement agencies in the state of Minnesota. They were purchased from Tri-Tech, Inc. (Southport, NC). Each of these bottles contained 1000 mg NaF.

2.2 Urine sample preparation:

Urine Samples were collected from two healthy male volunteers, age 34y and 36y, over a period of 7 days. Collection of urine was in clean glass bottles free of any preservative. Thus collected urine was pooled (~7.5 L) and stored at 4°C. The pooled urine was confirmed to be negative for the presence of ethanol and other volatile substances. The urine pool was first split into 5 fractions (1.5 L each). The fractions were then spiked with absolute ethanol (95.6%) at theoretical concentrations of 50, 100, 160, 200, and 400, mg/100 ml, respectively. Aliquots (5 ml) of each fraction were removed and stored in additive-free Vacutainer® glass tubes with rubber stoppers to serve as negative controls of NaF. The remaining ethanol spiked urine samples were fractionated into leak-proof 100 ml plastic bottles containing 1000 mg NaF as shown in **Table 1**. Post-fractionation, the samples were stored at room temperature for up to 336 hr. Aliquots (0.5) of samples stored at room temperature were analyzed for ethanol content by HSGC at 24, 48, 72, 168 and 336 hr., respectively.

2.3 HSGC analysis of ethanol:

Quantitative analysis of ethanol was performed as described by Anthony and co-workers [**26**]. Perkin-Elmer 8420 gas chromatograph equipped with a Perkin-Elmer HS-101 headspace analyzer or a Perkin-Elmer AutoSystem XL gas chromatograph equipped with a Perkin-Elmer HS-40 headspace analyzer (Perkin-Elmer Corporation, Norwalk, CT) was used for this purpose. Perkin-Elmer 8420 gas chromatograph was equipped with a 6ft x 2mm ID glass column with 60/80 mesh Carbopack B/5% Carbowax 20M (Supelco, Bellefonte, PA). Perkin-Elmer AutoSystem XL gas chromatograph was equipped with a 30m x 0.32mm ID Rtx®-BAC-2 capillary column (Restek Corporation, Bellefonte, PA). In each case, the column was connected to a flame ionization detector. The gas chromatograph, in each case, was connected to computer via a PE Nelson (900 series) analog digital converter. The detector signals were integrated and analyzed with the aid of Turbochrome® software (Perkin-Elmer Corporation, Norwalk, CT).

Sample dilutions were performed with the aid of a Hamilton Microlab 500 dispenser/diluter (Hamilton Co., Reno, NV). Aliquots (0.5 ml) of aqueous ethanol standards, positive controls, negative controls, ethanol spiked urine samples with different concentrations of NaF and NaF negative ethanol spiked urine controls were diluted (1:5) with aqueous n-propanol (5mg/ml) internal standard (with or without 30 mg/ml NaF) and dispensed into 20 ml headspace vials. These vials were then capped with rubber stoppers and sealed with an aluminum ring crimped over the rubber stopper and placed into a headspace analyzer.

The headspace analyzer was set to the following parameters: sample temperature -40°C, needle temperature -70°C, headspace transfer temperature -70°C, sample equilibration time - 12 min, vials pressurization time with the carrier gas - 0.5 min, injection time - 0.08 min (0.04 min in the case of HS-40) and sample withdrawal time - 0.2 min. The cycle time was 6 min in the case of Perkin-Elmer HS-101 headspace analyzer and it was 4 min in the case of Perkin-Elmer HS-40 headspace analyzer. The auto sampler injector needle was set to pierce through the vial septum and allow the internal pneumatic system to force the headspace gas onto the column at the end of equilibration time. The carrier gas used was chromatography grade helium. Flame support gases were hydrogen and air. The gas chromatograph was set to the following parameters: injector temperature – 200°C, oven temperature – 75°C (40°C in the case of AutoSystem XL), detector temperature 200°C (300°C in the case of AutoSystem XL), carrier gas flow - 10 ml/min and all other gas flows (air and hydrogen) were set for optimal signal and separation.

The ethanol standards used for the calibration of the chromatograph contained 50, 100, 150, 200, 300, 500, 1000, 2000, and 4500, mg ethanol/100 ml water. The gas chromatograph was calibrated with each analysis and ethanol positive controls were used to check the performance of the gas chromatograph during each analytical run. Under the conditions described herein, the ethanol concentrations determined with the aid of Perkin-Elmer 8420 or AutoSystem XL gas chromatographs were identical. Each sample was analyzed in duplicate and the duplicate runs were performed on the same day but at different times of day.

2.4 Data analysis:

The Macintosh-based STATView II (Brainpower, Inc., Calabas, CA) computer program was used to generate means, standard deviations, compare means (two-tailed, paired, Student's *t*-test), linear regression lines and *p*-values.

III. RESULTS

Calibration curves developed with the aid of aqueous solutions of ethanol diluted (1:5) with n-propanol internal standard solutions containing zero or 30 mg/ml NaF were very much to each other, **Figure 1**, irrespective of the gas chromatograph used. The calibration curves were linear up to 4500 mg ethanol/100 ml water (the highest concentration tested). Initial analysis of pooled control urine samples lacking NaF revealed no ethanol or other volatile substances (data not shown). Concentrations of ethanol in aqueous standard solutions of ethanol, urine samples containing no NaF but spiked with different concentrations of ethanol and ethanol-spiked urine

samples treated with different concentration of NaF (1 hr post treatment) quantified with the aid of the calibration curves in **Figure 1** are shown in **Tables 2, 3** and **4**. NaF was not completely soluble when its concentration exceeded 20 mg/ml within 1 hr, therefore the NaF concentration in urine samples shown in **Table 4** are not accurate. When NaF was not completely soluble, an aliquot of the sample was subjected to low speed centrifugation (500 g for 3 min) to avoid any incorrect measurements of volume during sample dilutions. Concentrations of ethanol in aqueous ethanol standard solutions (**Table 2**), urine samples spiked with ethanol (**Table 3**) and ethanol-spiked urine samples treated with different concentrations of NaF (1 hr post treatment) (**Table 4**) diluted (1:5) with n-propanol internal standard solutions containing zero and 30 mg/ml NaF, quantified as above were identical in each case. Ethanol concentrations in the urine samples were independent of chromatograph used for analysis (**Table-5**). At any given concentration of ethanol, presence of NaF in the internal standard solution did not affect the area under ethanol or n-propanol curves (data not shown). In addition, the ratios of peak areas of ethanol to n-propanol determined in the presence or absence of NaF in the internal standard solution were essentially identical, **Table 2**.

All of the NaF, at the concentrations reported herein, appeared to be completely soluble in ethanol-spiked urine samples by 24 hr. Ethanol concentrations in ethanol-spiked urine samples (56-406 mg/ml; **Table 2**) and treated with NaF (10 – 200 mg/ml; **Table 1**) quantified at 24, 48, 72, 168, and 336, hr after treatment with NaF and incubation at room temperature are shown in **Figure 2** and **Table 6**. At any given concentration of ethanol, as judged by paired, two-tailed Students *t*-test, ethanol concentrations determined in urine samples that contained 0-20 mg/ml NaF were essentially identical ($p \geq 0.05$). Under identical conditions, ethanol concentrations determined in urine samples treated with 40 mg/ml NaF, were somewhat lower as compared to the urine fractions containing 0-20 mg/ml NaF, but were not significantly different ($p \geq 0.05$). However, again under identical conditions, concentration of ethanol in urine fractions treated with 100 and 200 mg/ml NaF, as compared to those in fractions with 0-20 mg/ml NaF, were significantly lower ($p \leq 0.05$) (**Figure 2** and **Table 6**). The loss of ethanol from the liquid phase was more pronounced in urine samples spiked with lower concentrations of ethanol. For example, in urine fractions spiked with 56 mg ethanol/100 ml urine and treated with 200 mg/ml NaF, the concentration of ethanol was decreased by ~15%, as compared to ~8 % decrease in other fractions of urine treated with the same concentration of NaF. On the other hand, the loss of ethanol from the liquid phase was not as pronounced in urine fractions that contained 100 mg/ml NaF; the loss was about 4-5%. The peak areas under ethanol and n-propanol curves in the determinations shown in **Figure 2** (**Panel A**) and **Table 6** are shown in **Table 7**. At any given concentration of ethanol, presence of increasing concentrations of NaF did not appear to disproportionately affect the peak areas of ethanol and n-propanol (**Table 7**). The variation in peak areas of n-propanol shown in **Table 7** is most likely due to the small variations in the amount of headspace volume transferred on to the column by the auto-sampler.

IV. DISCUSSION

Even though number of accidents due to alcohol related motor vehicle crashes have reduced by 24% between 1995 and 2015, the number of fatalities due to alcohol related motor vehicles crashes is significant [25]. Hence, accuracy of measurement and interpretation of alcohol concentrations in DUI/DWI drivers continues to play an important role in apprehension and prosecution of such drivers. In this regard, forensic laboratories conduct quantitative analysis of ethanol in thousands of blood and urine samples every year. As per the recommended best practices in the State of Minnesota, the quantitative analysis of ethanol in biological specimens and other media were conducted on two different days and the duplicate results must be within 3% of each other to be acceptable, with the following exceptions. In the event of sample being limited (<3 ml of blood or <25 ml of urine) the duplicate analysis will be performed the same day, however the results of the duplicates must still be within 3% of each other. In cases where the concentration of ethanol is 0.05% or less, the acceptable difference between the duplicate ethanol results will be 0.003 or less. With the exception that the duplicate runs are performed on the same day but at different times of day, the results presented herein strictly adhere to this best practice.

Presence of relatively high NaF concentrations (>40 mg/ml) in urine samples lead to the measurement of relatively lower than expected ethanol concentrations. The observed decrease in ethanol concentrations in urine samples, as compared to those in urine samples treated with 0-20 mg/ml NaF, were ~4-15%. The observed decrease in ethanol concentrations could be to be due to NaF-induced salting-out of ethanol into the headspace of the container during their storage at room temperature. Under the conditions used for quantitative analysis of ethanol in this study, based on observed ethanol and n-propanol peak areas and their ratios, there was no apparent evidence for disproportionate salting-out of n-propanol, as compared to ethanol, induced by NaF during HSGC analysis. If there were any such changes, they appear to be minimal and do not seem to affect accurate measurements of ethanol concentrations in urine samples containing relatively high concentrations of NaF. Although the samples were stored at room temperature for up to 2 weeks, the alcohol concentrations in the liquid-phase remained unchanged and there was no evidence of microbial contamination or growth.

Salting-out of ethanol from biological specimens, e.g., blood, in the presence of electrolytes, e.g., NaCl, Na_2SO_4 and NaF, has been previously reported [**10,11,27**]. The original report of Jones [**10**] first documented the measurement of increase of ethanol concentrations in the vapor-phase of blood samples treated with NaF. This report did not indicate whether the alcohol concentration measured in the liquid-phase of the blood decreased or remained unchanged as compared to heparin-treated blood. However, a later report suggested no changes in ethanol concentrations in the liquid-phase of whole blood upon treatment with different concentrations of NaF. Further, another report documented the decrease in ethanol concentrations in the liquid-phase of aqueous ethanol solutions [**11**]. The measurement of decreased concentration of ethanol in this report was attributed to differential salting-out of n-propanol and ethanol into the headspace of sample vial, as consequence of prolonged equilibration time and incubation temperature during HSGC analysis. Accordingly, the report documented the peak areas obtained for ethanol and n-propanol in the presence of different concentrations of NaF. The peak areas for n-propanol were similar at all concentrations of NaF tested, except for one trial at 200 mg/ml NaF. Moreover, that study was limited to one ethanol concentration, i.e., 100 mg ethanol/100 ml water. Thus, it was not possible to compare the salting-out effects of fixed concentrations of NaF at different concentrations of ethanol. Further, in this report the internal standard solution used apparently contained no NaF [**11**]. Thus, a comparison of the values reported by Jones [**11**] to those obtained using internal standard solutions containing NaF, which is the normal procedure in forensic sample testing for ethanol cannot be made. With the exception that the sample equilibration time at 40°C was 12 min (30 min in reference # **11**), in our study, all of these points have been addressed. Moreover, increasing the equilibration time to 30 min did not significantly affect the accuracy of measurement of ethanol concentrations in the urine samples tested in this study.[*]

As per the results reported herein, the observed decrease of ethanol concentrations in urine samples treated with relatively high concentrations of NaF was a consequence of NaF-induced salting-out of ethanol into the headspace of the storage container. As observed herein, the NaF-induced salting-out of ethanol in urine samples started as early as 1 hr after treatment and the maximum was reached within 24 hr. Beyond 24 hr, the salting-out of ethanol induced by NaF in the urine samples, tested herein, was negligible. Moreover, the peak areas measured for n-propanol in the absence and presence of different concentrations of NaF were essentially identical. Variations in n-propanol peak areas, if any, were due to small variations in volume of the headspace injected into the GC column. Given the above, disproportionate salting-out of n-propanol, as compared to ethanol, into the headspace of the sample vials during analysis appears to be unlikely, especially at high concentration of NaF. On the other hand, it is also important to note that the partition coefficient of ethanol between water and the vapor phase is 5600:1 at 25°C [28]. This suggests that ethanol has more affinity for aqueous phase as compared to vapor phase and thus argues against disproportionate salting out in the presence of NaF. The latter could be true when NaF concentrations are relatively lower (<20 mg/mL) and the results from the present study (no significant difference in ethanol concentrations) are consistent with it. However, there is significant loss of ethanol to vapor phase at large concentration of NaF in urine samples (>40 mg/mL). Consistent with this observation is the fact that increased concentrations ethanol in the vapor phase of blood samples treated with NaF has been previously reported [10]. Although blood and urine matrices are different in terms of water content, urine does contain high concentrations of urea in addition to proteins and other solutes. It cannot be ruled-out that these constituents in urine will not affect the salting-out of ethanol when the concentrations of NaF is relatively high.

Acknowledgements:

Parts of this study was published as an abstract in the proceedings of the society of Forensic Toxicologists in the year 2000 (Sreerama, L., Nelson, J. K., Meyer, R. F. and Hardin, G. G. Influence of sodium fluoride (NaF) concentration on quantitative analysis of ethanol by headspace gas chromatography (HSGC) in urine samples. Proc. Soc. Forensic Toxicologists, 2000).

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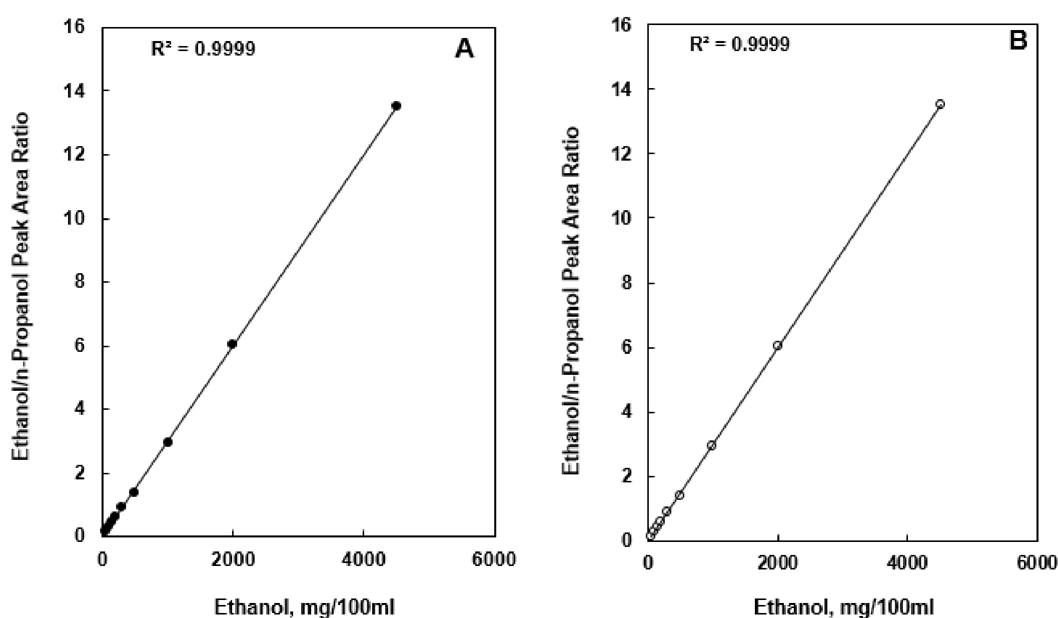

Figure 1. Ethanol calibration curves. Aliquots (0.5 ml) of commercially available standard aqueous ethanol solutions containing 50, 100, 150, 200, 300, 500, 1000, 2000, and 4500, mg ethanol/100 ml water were diluted with n-propanol internal standard solution containing zero (**Panel A**) or 30 mg/ml (**Panel B**) sodium fluoride. Quantitative analysis of ethanol was by headspace gas chromatography described in Materials and Methods. Calibration curves were constructed by plotting ratios of ethanol to n-propanol peak areas against ethanol concentrations. The data presented herein was developed with the aid of Perkin-Elmer 8420 gas chromatograph equipped with a Perkin-Elmer HS-101 headspace analyzer and a 6ft x 2mm ID glass column with 60/80 mesh Carbopack B/5% Carbowax 20M. Values are mean of duplicate determinations.

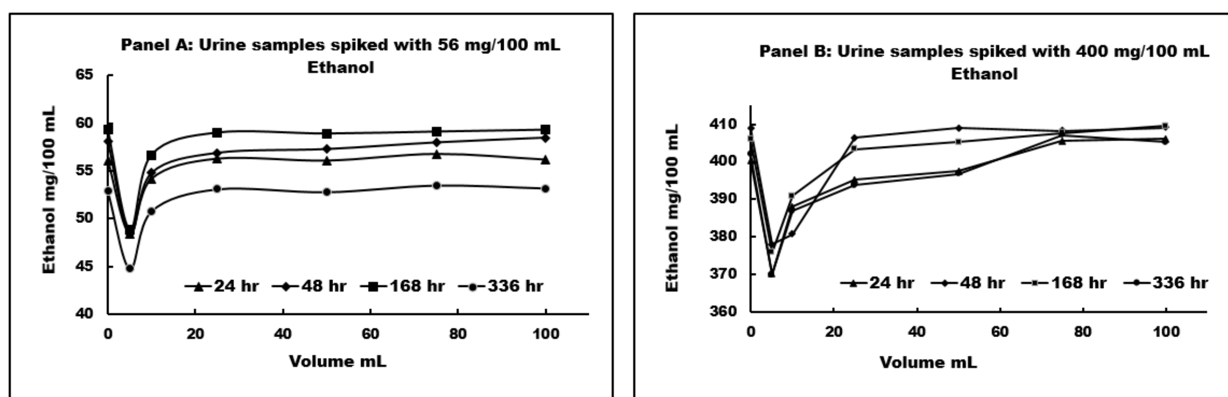

Figure 2. Concentration of ethanol in urine samples treated with two different concentrations of sodium fluoride. Pooled urine samples spiked with 0.056 (Panel A) and 400 (Panel B) mg ethanol/100 ml urine were first treated with 0-200 mg/ml sodium fluoride as described in Table 1 and Materials and Methods. Thus treated urine samples were then incubated at room temperature for 24, 48, 72, 168, and 336 hrs. At the end of each incubation period, ethanol concentrations were quantified in the above urine fractions as described in Materials and Methods. Values are mean of duplicate determinations made on each sample. Data presented in this figure has been summarized in Table 5.

Table 1: Fractionation of ethanol-spiked urine samples*

Urine, ml	Ethanol, mg/100 ml (from Table 3)				
	57	107	161	207	406
	NaF, mg/ml				
100	10	10	10	10	10
75	15	15	15	15	15

50	20	20	20	20	20
25	40	40	40	40	40
10	100	100	100	100	100
5	200	200	200	200	200

*Pooled urine (~7.5 L) was first divided into 5 fractions of exactly 1.5 L each. The fractions were then spiked with absolute ethanol (95.6%) such that the fractions 1-5 resulted in 56, 106, 161, 207 and 406, mg ethanol/100 ml, respectively, Table 3. Quantification of ethanol in these urine fractions was as described in Materials and Methods. Each of the resulting fractions was further fractionated into 5 sets of 5, 10, 25, 50, 75 and 100 ml into leak-proof plastic bottles (100 ml) containing 1000 mg sodium fluoride. Sodium fluoride concentrations in thus resulting urine fractions are as shown above. The bottles were tightly capped, the contents were mixed well on a rocker and stored at room temperature for up to two weeks. One urine sample from each subset were analyzed in duplicate after 24, 48, 72, 168 and 336 hr incubation at room temperature.

Table 2: Quantification of ethanol in commercially available standard aqueous solutions of ethanol*

Ethanol, mg/100 ml (Commercial Standard)	Ethanol, mg/100 ml (Determined by HSGC)		Ethanol/n-Propanol (Peak Ratio)	
	No NaF	NaF, 30 mg/ml	No NaF	NaF, 30 mg/ml
50	51	50	0.148	0.146
100	101	100	0.298	0.285
150	150	149	0.447	0.435
200	200	199	0.596	0.585
300	301	303	0.902	0.910
1000	989	995	2.947	2.885
2000	2009	2003	6.040	5.878

*Commercially available standard aqueous solutions of ethanol were first diluted (1:5) with internal standard solutions containing zero or 30 mg/ml sodium fluoride. Ethanol concentrations in thus diluted solutions were quantified as described in Materials and Methods. Calibration curves shown in Figure 1 were used for the determination of ethanol concentrations reported in this Table. Values are mean of duplicated determinations made on each sample.

Table 3: Concentration of ethanol in urine samples spiked with theoretically calculated concentrations of ethanol*

Ethanol, mg/100 ml (Theoretical)	Ethanol, mg/100 ml (Determined by HSGC)	
	No NaF	NaF, 30 mg/ml
50	57	56
100	106	107
160	162	161
200	206	207
400	407	406

*Pooled urine samples were processed as described in Materials and Methods and footnote to Table 1. Ethanol concentrations in these fractions were quantified as described in Materials and Methods. Calibration curves shown in Figure 1 were used for the determination of ethanol concentrations reported in this Table. Values are mean of duplicated determinations made on each sample.

Table 4: Quantification of ethanol concentrations in ethanol-spiked urine samples after 1 hr of treatment with sodium fluoride*

NaF (mg/mL)	Ethanol, mg/100 ml (Before NaF Addition; from Table 3)									
	56		107		161		207		406	
	Ethanol, g/100 ml (1 hr After NaF Addition)									
	IS (No NaF)	IS (NaF)	IS (No NaF)	IS (NaF)	IS (No NaF)	IS (NaF)	IS (No NaF)	IS (NaF)	IS (No NaF)	IS (NaF)
0	57	56	108	109	161	162	209	208	404	402
10	56	56	109	108	162	163	208	208	407	405
15	57	57	109	108	160	161	209	207	406	409
20	56	56	108	109	160	161	210	206	404	405
40	57	56	107	106	161	160	208	207	399	402
100	55	54	104	103	157	156	204	203	388	390
200	50	49	100	101	151	152	197	198	380	379

*Pooled urine samples were processed as described in Materials and Methods and footnote to Table 1. Thus processed samples were allowed to mix thoroughly on a rocker for 1 hr. Aliquots of urine samples treated with 40-200 mg/ml sodium fluoride were centrifuged at 500 g for 3 min to remove undissolved sodium fluoride before

they were diluted with n-propanol internal standard solutions. Hence, the sodium fluoride concentrations at 40, 100, and 200 mg/ml level after 1 hr of treatment are somewhat lower. Aliquots (0.5 ml) of urine, thus processed, were diluted 1:5 with internal standard (IS) solutions containing zero (No NaF) or 30 mg/ml sodium fluoride (NaF) and analyzed by HSGC, in duplicate as described in Materials and Methods. Calibration curves shown in Figure 1 were used for the determination of ethanol concentrations reported in this Table. Values are mean of duplicate determinations.

Table 5: Concentration of ethanol in ethanol-spiked urine samples determined by HSGC on two different gas chromatography columns*

Ethanol, mg/100ml (Theoretical)	Ethanol, mg/100 ml			
	Carbopack Column		Rtx®-BAC-2 Column	
	No NaF	NaF (30 mg/ml)	No NaF	NaF (30 mg/ml)
50	57	56	56	56
100	106	107	105	107
160	162	161	163	162
200	206	207	205	206
400	407	406	405	404

*Pooled urine samples were processed as described in Materials and Methods and footnote to Table 1. Ethanol concentrations in these fractions were quantified as described in Materials and Methods. Values are mean of duplicated determinations made on each sample.

Table 6: Ethanol concentrations in urine samples treated with various concentrations of sodium fluoride: Summary.

NaF, mg/ml	Ethanol, mg/100 ml (Before NaF Addition; from Table 3)				
	56	107	161	207	406
	Ethanol, g/100 ml (After NaF Addition)				
0	57 ± 2	108 ± 2	163 ± 3	209 ± 3	404 ± 4
10	57 ± 2	108 ± 1	164 ± 4	210 ± 2	407 ± 2
15	57 ± 2	109 ± 1	163 ± 2	210 ± 3	407 ± 1
20	56 ± 2	108 ± 1	162 ± 1	210 ± 3	402 ± 5
40	56 ± 2	106 ± 2	161 ± 2	208 ± 4	400 ± 5
100	54 ± 2†	102 ± 3†	157 ± 3†	204 ± 2†	387 ± 4†
200	48 ± 2†	099 ± 3†	148 ± 1†	196 ± 2†	374 ± 4†

*Pooled urine samples were processed and stored as described in Materials and Methods and footnote to Table 1. Aliquots (0.5 ml) of urine, thus processed and stored, were diluted 1:5 with internal standard solutions containing 30 mg/ml sodium fluoride and analyzed by HSGC, in duplicate, at 24, 48, 72, 168 and 336, hr intervals as described in Materials and Methods. Values are mean ± SD of duplicate determinations made at five different time intervals and each of ethanol and sodium fluoride concentrations.

†Significantly different (paired, two-tailed, Students *t*-test) as compared to the values at 0, 10, 15 or 20 mg/ml sodium fluoride concentrations; *p*-Values ≤ 0.004.

Table 7: Ethanol and *n*-propanol peak areas observed during HSGC analysis of ethanol spiked urine samples treated with various concentrations of sodium fluoride.

NaF, mg/ml	Ethanol, mg/100 ml (Before NaF Addition)									
	56		107		161		207		406	
	Peak Area (μV/s) x 10^3									
	E	P	E	P	E	P	E	P	E	P
0	137.5	956.7	249.4	872.3	376.3	980.5	549.9	988.0	1012.2	941.6
10	140.1	975.5	252.3	878.5	414.3	965.2	566.6	996.3	1019.9	936.5
15	134.5	925.0	292.3	1004.9	402.3	920.4	566.7	995.1	1099.7	996.3
20	129.6	911.7	274.1	956.5	405.7	955.4	563.1	989.1	1055.6	990.2
40	139.1	965.6	238.3	849.5	414.1	975.6	529.1	956.6	1060.2	998.9
100	133.3	955.0	246.3	925.9	408.3	983.7	541.1	998.2	1028.5	987.2
200	115.6	942.5	238.6	914.5	390.9	990.6	516.8	990.5	969.2	975.5

*Pooled urine samples were processed and stored as described in Materials and Methods and footnote to Table 1. Aliquots (0.5 ml) of urine, thus processed and stored, were diluted 1:5 with internal standard solutions containing 30 mg/ml sodium fluoride and analyzed by HSGC, in duplicate, at 24 hr intervals as described in Materials and Methods. Values are mean of duplicate determinations made at each of ethanol and sodium fluoride concentrations. E: Ethanol and P: n-Propanol. The variations in peak areas obtained for ethanol and n-propanol in determinations made at 48, 72, 168 and 336 hr were essentially similar to the variations reported herein, hence, they are not shown.