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Blood and Urine Bioindicators for Methanol Exposure: Effect of Chilled and Frozen Sample Storage

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Measurements of methanol concentrations in blood and urine may be used as bioindicators of methanol exposure that account for all exposure pathways. These measurements will be practical if the integrity of samples from the time of collection to the time of analysis can be maintained. This study was designed to test the stability of methanol in blood and urine samples stored at 4° and −20°C for various periods of time up to 7 months. Methanol recoveries of the stored blood samples were found to fit a first-order decay model, with the best estimates for the half-life of methanol in chilled and frozen blood of 114 ± 14 and 240 ± 58 days, respectively. A half-life of 562 ± 145 days is estimated for chilled and frozen urine. These long half-lives enhance the utility of methanol bioindicators. While freezing increased the recovery in blood, it also decreased the reproducibility of results. Thus, refrigeration of samples is recommended if the analysis will be completed within about a month of sample collection, and freezing of samples is suggested otherwise. For blood, sample preservation was not enhanced using an all-glass storage system, and a conventional sampling container yielded equivalent results. BATTERMAN, S.A.; XIAO, H.; FRANZBLAU, A.: BLOOD AND URINE BIOINDICATORS FOR METHANOL EXPOSURE: EFFECT OF CHILLED AND FROZEN SAMPLE STORAGE. APPL. OCCUP. ENVIRON. HYG. 11(1):25–29; 1996.

Biologic exposure indices (BEIs), or bioindicators, of contaminant exposures potentially offer accurate assessment of chemical uptake in a wide range of occupational and environmental settings. A major advantage of bioindicators, in addition to accuracy, is that all exposure pathways are accounted for (i.e., percutaneous absorption, inhalation, and ingestion). While the collection of samples of blood, breath, tissue, urine, hair, etc. may be inconvenient or difficult, the measurement of chemicals and their metabolites in such samples can eliminate much of the uncertainty regarding dose estimation that typically accompanies indirect exposure measures (e.g., airborne concentrations). Such uncertainties arise from unknown exposure patterns, individual variation in breathing rates, body weight, exposed skin area, etc., and unknown or omitted exposure pathways. Bioindicators avoid such questions and reveal the actual absorbed dose.

Key questions for the use of bioindicators include protocols for sample collection and analysis. In particular, what constitutes appropriate sample storage conditions, and how soon after sample collection must analyses be completed? If samples are stable for extended periods, then bioindicators may be quite practical, and it may even be possible to "bank" samples for possible later analysis if warranted by future information. Conversely, if sample degradation is rapid, then a bioindicator may have little practical value. Disregard for sample storage conditions may introduce significant artifacts that can lead to subsequent misinterpretation of data.[1]

We evaluated the stability of bioindicators for methanol as part of a study examining their relationship to inhalation and dermal exposures. Methanol is of interest because of its toxicity and its wide use in industrial applications. While present opportunities for human exposure to methanol are limited because the processes are usually closed and the number of workers is small,[2] methanol has been proposed as an alternate automobile fuel or gasoline additive, which would greatly increase the potential for human exposure.[3] Due to methanol's solubility, volatility, and partitioning characteristics, dermal absorption,[4,5] ingestion,[5,6] and inhalation exposure[7] pathways can be important. Once absorbed, methanol is metabolized rather slowly and exposures are readily measurable in samples of breath, blood, and urine.[8,9]

This article presents an analysis of methanol in human blood and urine samples that have been stored for relatively long periods. Little information in the literature addresses effects of storage conditions. In what is available,[10] stringent recommendations for urinary methanol determinations are specified: collection of urine samples in glass bottles with ground glass stoppers, or polyethylene bottles, and immediate analysis, or refrigeration at 4°C with analysis in 3 days. No recommendations have been identified for the collection and analysis of methanol in blood samples. The potential importance of appropriate storage is suggested by the bacterial alteration of urinary formic acid, another methanol bioindicator, demonstrated in samples stored at room temperature.[1]

Methods

The study was designed to test the stability of methanol in human blood and urine samples that might be collected and stored in occupational or other studies. Four sets of experiments were conducted for blood and urine using both chilled (refrigerated) and frozen storage in widely used sample collection/storage containers. In a fifth experiment, blood samples were stored in all-glass containers. These containers were designed to minimize leakage, wall loss, sorption, etc. to help

separate losses attributable to biochemical degradation from losses associated with the container.

Sample Preparation

Blood and urine samples were prepared at three methanol concentrations (low, medium, and high). Because methanol is found endogenously at varying but generally low levels, all samples used the same blood or urine stock. Based on earlier analyses, the background level of methanol was expected to be below 3 mg/L. Stock solutions were prepared using two units (~1 L) of compatible anticoagulated whole blood obtained from the Red Cross (containing 63 ml of citrate–phosphate–dextrose anticoagulant per 450 ml whole blood) and ~6 L of urine collected from unexposed subjects. Stock solutions were cooled to 4°C, well mixed, and split into three approximately equal portions in flasks. Two of these flasks were spiked with high performance liquid chromatography (HPLC)-grade methanol via syringe injections. The spiked concentrations (~10 and ~30 mg/L for medium and high levels) reflect methanol levels found in exposed populations. The spiked samples bracket the recommended BEI for methanol of 15 mg/L.

Following stock preparation, 60-ml aliquots of the urine/methanol solution were pipetted into 30 5-oz (115 ml) sterile urine specimen containers (Sage Products, Inc., Crystal Lake, Illinois) at each concentration level. These plastic containers were sealed with standard metal screw caps and foam seals supplied with the urine specimen container. For blood, 5-ml aliquots were pipetted into 30 7-ml gray-top Vacutainer® tubes containing potassium oxalate and sodium fluoride (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey) at each concentration level. In addition, 20-ml aliquots of blood were placed into 50-ml mini-impinger glass tubes (Ace Glass, Vineland, New Jersey) equipped with ground glass stoppers. Four of these all-glass containers were used at each concentration level. The total sample consisted of 90 urine samples, 90 gray-top blood samples, and 12 all-glass blood samples. Half of the urine containers and Vacutainer blood tubes were stored in a laboratory refrigerator at ~4°C; the other half were stored in a freezer set at −20°C (measured temperatures ranged from −20°C to −15°C). All 12 of the blood samples in all-glass containers were frozen.

Sample Analysis

Samples were analyzed using head space gas chromatography at 1, 2, 7, 30, and 210 days following preparation. Six samples each of urine and blood at each concentration and storage condition were selected randomly. The all-glass containers were sampled at 210 days. Frozen samples were thawed at room temperature. Using an autopipetter, 1 ml urine and 20 μL isopropanol standard (1000 mg/L, HPLC-grade) were placed in a clean, previously labeled 20-ml vial. The vial was immediately sealed with a butyl-rubber septum-equipped crimp cap and well mixed. Vials were then loaded onto the carousel of a model 7050/7000 (Tekmar, Cincinnati, Ohio) automated head space sampler with a 50-sample capacity. After a 30-minute incubation at 60°C and 2 minutes of mixing, a 1-ml head space sample was injected into a 2 m × 0.125 inch packed column containing 5 percent Carbowax 20M 60/80 mesh Carbopack B (Supelco Inc., Bellefonte, Pennsylvania) of a model 3700 gas chromatograph (Varian, Mountain View, California). The column was operated isothermally at 90°C. Eluting methanol was detected by flame ionization. The detector output was collected on a PC data acquisition system and processed for baseline correction, peak integration, etc. Blood samples were analyzed similarly except that 0.5 ml blood and 20 μL isopropanol standard were placed in each vial.

Five- to seven-point calibration curves for methanol were obtained for each set of analyses. Standards (0 to 60 mg/L) were prepared using HPLC-grade methanol, distilled water, and the isopropanol standard. In addition, each carousel included at least two blanks (distilled water) and five quality control (QC) standards. All standards and samples included the internal isopropanol standard. Methanol concentrations calculated by integrating the methanol peak on the chromatogram were verified by methanol/isopropanol ratios. Acceptable analyses achieved QC accuracies and precisions better than 10 and 5 percent, respectively. The limits of detection for methanol in blood and urine depend on the matrix, but are below 0.5 mg/L in all cases. The coefficient of variation for calibrations was typically 3 to 5 percent.

Trend Analysis

A first-order decay or loss in methanol concentration is

$$C_t = C_o \exp(-k\,t) \qquad (1)$$

where:

C_t = concentration (mg/L) measured at time t (days) after sample generation
C_o = initial concentration measured without storage (mg/L), that is, using fresh samples
k = decay rate (day^{-1})

The time for the concentration to decrease to half the initial level C_o is the half-life $t_{1/2} = 0.693/k$. Since samples were prepared at three concentrations, it is convenient to express Equation 1 in terms of sample recovery R_t (%):

$$R_t = C_t/C_o = 100\% \exp(-k\,t) \qquad (2)$$

Recoveries permit direct comparison of results from experiments using different initial concentrations. The decay rate k was estimated using a least-squares estimate of pooled recovery data. This assumes that the decay rate is independent of methanol concentration.

Results

Table 1 lists means, standard deviations, and sample sizes for the blood and urine analyses using the conventional containers. While most analyses use six replicates, in a few cases sample sizes are smaller due to a few clearly erroneous measurements which occasionally resulted from carry-over (memory) effects or leaking septa. Table 2 summarizes the reproducibility (expressed as an average coefficient of variation) for the methanol analyses. At medium and high concentrations, the average reproducibilities of methanol determinations in blood and urine are 10.1 and 7.1 percent, respectively. In most cases, urine analyses are more reproducible than blood analyses, and chilled blood and urine samples tend to be more reproducible than frozen samples. The only exception was at the highest methanol concentration in blood. Although not shown in

TABLE 1. Results of Methanol Analyses in Stored Blood and Urine

	Methanol Concentrations in Blood and Urine (mg/L)														
	1 Day			2 Days			1 Week			1 Month			7 Months		
Sample and Level	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**
Refrigerated blood															
Low	1.67	0.25	4	2.47	0.80	5	2.53	0.45	3	2.83	1.16	6	0.91	0.17	6
Medium	8.59	0.71	6	9.74	0.36	6	8.26	0.92	6	6.07	1.53	6	1.62	0.22	6
High	25.08	1.05	6	27.55	1.03	6	24.30	1.23	5	21.24	3.37	6	10.46	0.77	6
Frozen blood															
Low	NA			2.57	0.97	4	1.17	0.32	6	1.90	2.00	6	0.79	0.15	6
Medium	NA			6.31	0.74	6	8.99	0.60	6	5.73	2.32	6	4.26	0.35	6
High	NA			21.58	0.42	6	26.51	0.21	6	18.91	1.84	6	18.05	0.61	4
Refrigerated urine															
Low	2.42	0.19	6	1.89	0.15	5	1.68	0.30	5	2.14	0.32	5	2.15	0.07	6
Medium	10.27	0.32	6	9.64	0.38	6	9.19	0.27	6	9.22	0.31	6	9.39	0.30	6
High	32.83	1.33	6	32.11	0.58	5	30.66	0.74	6	29.55	1.23	6	26.93	0.98	6
Frozen urine															
Low	NA			1.85	0.24	5	1.41	0.27	4	2.27	0.33	6	1.52	0.14	5
Medium	NA			9.73	0.21	6	12.08	2.56	6	10.81	2.92	5	8.41	0.40	6
High	NA			31.88	0.96	6	32.95	2.62	6	NA			23.67	3.02	6

NA = not available.

Table 2 (the data are presented in Table 1), the magnitude of the reproducibilities has no consistent trend with respect to sample storage time. If errors are on the order of 10 percent and random (thus the uncertainty of the mean decreases as the square root of sample size), differences in mean concentrations as small as 4 percent should be observable using six replicates.

Time Trends

Figure 1 shows time trends of the mean observed and modeled recoveries for the four stability experiments. Because the ordinate axis uses a logarithmic scale, exponential decay plots as a straight line. With the exception of frozen blood, these models use results obtained for the medium and high concentration samples. Low concentration samples were less reproducible, thus small changes or trends are not reliably observed. For example, methanol concentrations in urine at low concentrations tended to increase with time; however, changes were small (typically below 0.5 mg/L). Trends are most clearly identified at the higher concentrations, which have the best experimental reproducibilities.

A second decay model (model 2) which uses an intercept (86 and 93% for blood and urine, respectively) to better match the data is shown for frozen blood and chilled urine. For chilled urine (Figure 1C) this model improves the fit, suggesting that the initial concentration C_o was overestimated, especially for the lower concentration samples. For frozen blood (Figure 1B), however, model 2 yields little improvement. Frozen blood samples at the low and medium concentrations are the least reproducible of samples in this study (coefficients of variation of 48 and 17%, respectively). Given the small (30%) change in concentrations relative to sample reproducibilities, the time trend for the recovery of frozen blood samples is less clear than for chilled blood. Although experimental reproducibilities were higher, urinary methanol shows only small (~10%) changes over the study period and the model provides little explanatory power. The study duration was not long enough to demonstrate meaningful decreases in urinary methanol concentrations.

While the experimental data for the refrigerated and frozen blood models (Figure 1A and B) show considerable scatter, a general downward trend is indicated and the rate constants are statistically significant ($p < 0.05$). Thus it is reasonable to conclude that methanol recoveries in blood decrease with time, and that first-order models provide a useful approximation of this decay. For urine, the decay model appears to fit most of the higher concentration data, and again rate constants are statistically significant; however, trends are not well defined. Table 3 shows the half-life $t_{1/2}$, its uncertainty, and the

TABLE 2. Reproducibility (as Coefficient of Variation) for the Methanol Analyses

	Reproducibility (%) at Concentration Level		
Sample Type	**Low**	**Medium**	**High**
Refrigerated blood	25.0	12.4	7.3
Frozen blood	47.6	16.8	4.0
Refrigerated urine	10.4	3.3	3.2
Frozen urine	14.0	13.8	7.9

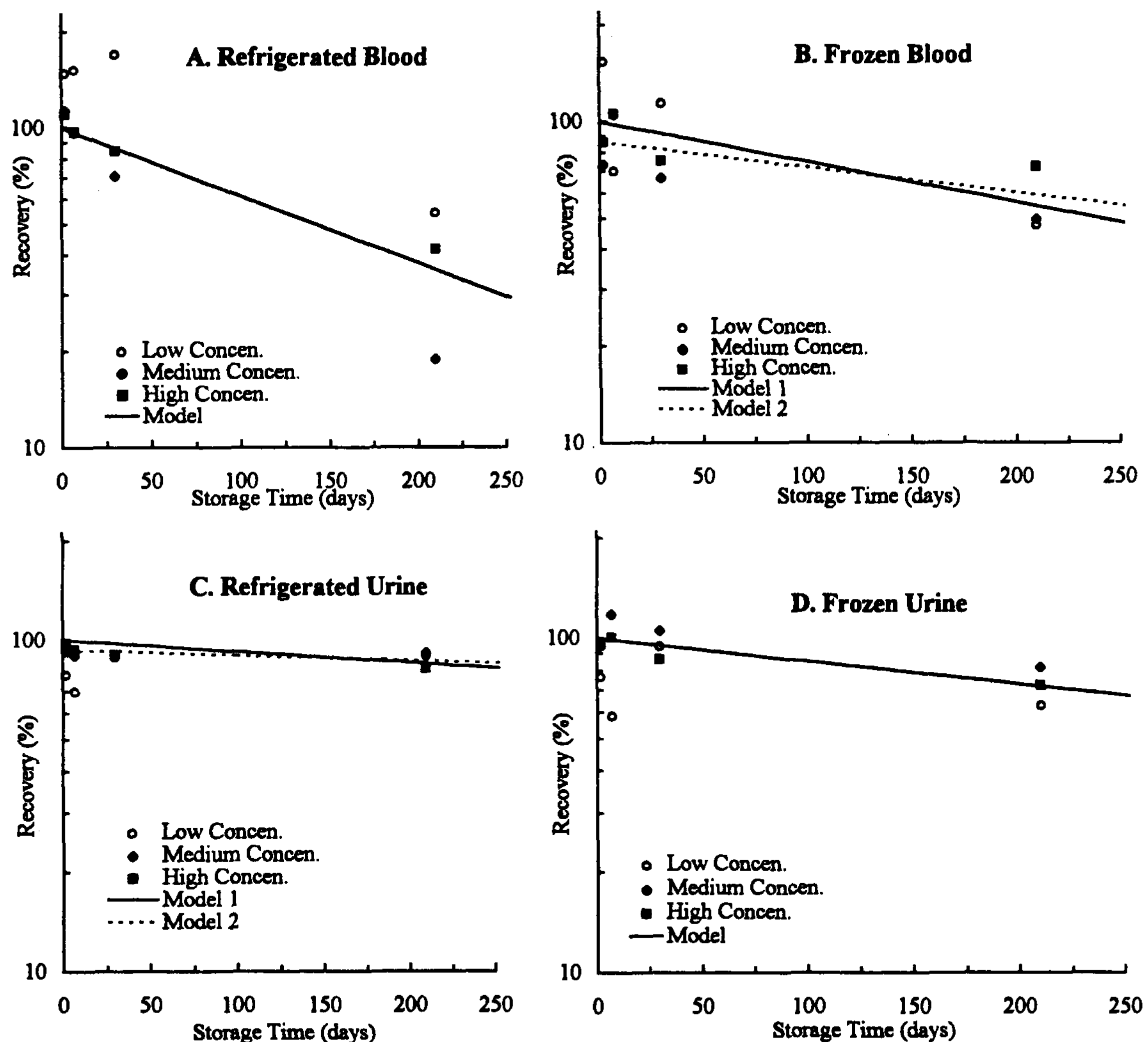

FIGURE 1. Mean recoveries and model results for the four stability experiments. Model 1 uses an intercept of 100 percent; intercepts in model 2 are least-squares estimates.

R^2 for the four stability experiments. The decay models explain 39 to 86 percent of the variance. The best conservative estimates for $t_{1/2}$ may be taken using the lower estimates in the table. For chilled and frozen blood, the suggested half-lives are 114 ± 14 and 240 ± 58 days, respectively, and 562 ± 145 days for chilled and frozen urine. Separate half-life estimates for chilled and frozen urine are not provided, as the chilled urine half-life (907 ± 294 days) overlaps the frozen urine half-life (562 ± 145 days).

TABLE 3. Half-Lives and R^2 for the Stability Experiments

	Half-Life (Days)		
Sample Type	**Mean**	**Standard Deviation**	**R2**
Refrigerated blood	114	14	0.86
Frozen blood with all data	240	58	0.44
Frozen blood with intercept	383	109	0.45
Refrigerated urine	907	294	0.39
Refrigerated urine with intercept	1960	453	0.40
Frozen urine	562	145	0.61

All-Glass Containers

Seven-month recoveries of methanol in blood for the conventional Vacutainer tubes and the all-glass containers are shown in Table 4. In both cases, losses for the unspiked (low) concentration samples were high and variable. Recoveries for the all-glass containers were slightly higher at the medium methanol concentration, but slightly lower at the high concentration. Two-sided t-tests comparing mean recoveries (results shown in Table 4) indicate that these differences are not statistically significant. Thus, the comparatively expensive and fragile glass containers did not aid sample preservation. Assuming that leakage and sorption in the all-glass containers are

TABLE 4. Average Recoveries, Standard Deviations (in Percent), and Sample Size for Frozen 7-Month Blood Samples, and p Values for 2-Tailed *t*-Tests

	All-Glass			Vacutainer			
Concentration Level	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**	**p Value**
Low	22.5	8.9	4	47.4	9.3	6	0.004
Medium	57.3	7.7	3	49.6	4.1	6	0.530
High	67.3	5.6	4	72.0	2.4	4	0.190

minimal, we infer that the decay of methanol in blood is primarily due to degradation of the sample.

Discussion and Conclusions

Methanol recoveries may decrease over time due to several mechanisms: (1) biochemical degradation of methanol in the sample; (2) leakage of sample (liquid and/or vapor) from the storage container; and (3) uptake (sorption) of methanol in the sample container itself. While our experiments did not permit all of these to be distinguished, results suggest that biochemical degradation is the major loss mechanism, at least for methanol in refrigerated blood. Freezing would be expected to reduce the rate of degradation, and this was observed in the blood experiments. However, chilled and frozen urine samples did not show meaningful differences. This may have occurred from limitations in the experimental design (e.g., the inability to see small differences that occurred over the study period), or possibly from experimental errors. The degradation rate is expected to be small at temperatures of 4°C or below. Note that large differences have been observed in other methanol biomarkers in samples stored at room temperatures.[1]

First-order models of methanol decay fitted to the data permit convenient estimation of sample half-life. The best conservative estimates for the half-life of methanol in chilled and frozen blood are 114 ± 14 and 240 ± 58 days, respectively. For chilled and frozen urine, a conservative estimate is about 562 ± 145 days. The half-life in urine may be longer because concentrations of enzymes that attack methanol are lower in urine than in blood. The validity of the decay models may be limited, however, by the small changes in methanol concentrations in urine over the study period and the relatively few storage times examined. Still, results indicate that earlier recommendations for the storage and analysis of urinary methanol[10] are unnecessarily conservative.

Recommendations

Properly stored blood and urine samples can maintain their integrity for many months. Thus, typical delays encountered between the collection and analysis of the sample, due to shipment, laboratory processing, etc., should not degrade results. While recovery will be increased by freezing samples, the reproducibility of results will be decreased. Fortunately, the latter effect appears minor (unless differences on the order of 10% must be distinguished). To avoid potential problems, we recommend refrigerating samples if analyses will be completed within a month of sample collection, and freezing of samples otherwise. As sample degradation appears to be the primary loss mechanism, there is no need for storage containers more sophisticated than those ordinarily used.

The first-order models and half-lives presented should not be used to correct or compensate for storage losses in other studies. Uncertainties and small differences in samples, sample preparation, storage, and possibly analytical techniques probably preclude such adjustments. However, results presented can be used to determine maximum storage times and the potential usefulness of previously stored samples. Allowing a 20 percent decay, storage for 0.32 half-lives would be acceptable, that is, 35 and 77 days for chilled and frozen blood, respectively, and 160 days for chilled or frozen urine.

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References

1. Sciwara, H.-W.; Siegel, H.; Gobel, A.: Increase and Decrease in Formic Acid Concentration in Urine Samples Stored at Room Temperature. Eur. J. Clin. Chem. Clin. Biochem. 30:75–79 (1992).
2. Posner, H.S.: Biohazards of Methanol in Proposed New Uses. J. Toxicol. Environ. Health 1:153–171 (1975).
3. Russell, A.G.; St. Pierre, D.; Milford, J.B.: Ozone Control and Methanol Fuel Use. Science 247:201–205 (1990).
4. Downie, A.; Khattap, T.M.; Malik, M.I.A.; Samara, I.N.: A Case of Percutaneous Industrial Methanol Toxicity. Occup. Med. 42(1):47–49 (1992).
5. Dutkiewicz, B.; Knocalik, J.; Karwacki, W.: Skin Absorption and Per Os Administration of Methanol in Men. Int. J. Occup. Environ. Health. 47:81–88 (1980).
6. Becker, C.E.: Methanol Poisoning. J. Emerg. Med. 1:51–58 (1983).
7. Ogata, M.; Iwamoto, T.: Enzymatic Assay of Formic Acid and Gas Chromatography of Methanol for Urinary Biological Monitoring of Exposure to Methanol. Int. Arch. Occup. Environ. Health 62:227–232 (1990).
8. Biological Exposure Indices Committee: New BEI Committee Policy on Establishing BEI's for Monitoring of Industrial Chemicals in Extracted Air. Appl. Indus. Occup. Hyg. J. 6(9):802–804 (1991).
9. Franzblau, A.; Batterman, S.; Sargent, N.; Gross, K.; Shreck, R.: Breath Monitoring of Inhalation and Dermal Methanol Exposure. Appl. Occup. Environ. Health 10:833–839 (1995).
10. Sedivec, V.; Mraz, M.; Flek, J.: Biological Monitoring of Persons Exposed to Methanol Vapors. Int. Arch. Occup. Environ. Health 48:257–271 (1981).