Published in final edited form as:

Scand J Urol Nephrol. 2010 November ; 44(5): 331–336. doi:10.3109/00365599.2010.492785.

The effect of storage time at -20°C on markers used for assessment of renal damage in children: albumin, γ -glutamyl transpeptidase, N-acetyl- β -D-glucosaminidase, and alpha1-microglobulin

Felicia Trachtenberg¹ and Lars Barregård²

¹New England Research Institutes, 9 Galen St. Watertown, MA 02472, USA

²Dept. Occupational and Environmental Medicine, Sahlgrenska University Hospital and Academy, Göteborg, Sweden

Abstract

Objective—The objective of this study is to examine the influence of storage time at -20°C on the concentration of albumin, γ -GT, NAG, A1M, and creatinine in a large sample of healthy children.

Material and Methods—The New England Children's Amalgam Trial followed 534 children – aged 6–10 at baseline – for 5 years, with annual urine collections. Urine samples were analyzed for creatinine, albumin, γ -GT, NAG, and A1M concentrations. Repeated measures ANCOVA was used to model the effect of storage time on these concentrations.

Results— γ -GT concentration decreased significantly with storage time at -20°C . There was also a limited decrease in NAG. Albumin, A1M, and creatinine concentrations did not appear to be affected by storage time at -20°C .

Conclusions—If it is necessary to interpret results from samples stored for a long time at -20°C , it is advisable to account for storage time in statistical models.

Keywords

storage; renal markers; children; albumin; γ -GT; NAG; A1M

Introduction

The excretion of various proteins in urine is widely used for assessment of kidney function, in clinical practice as well as in epidemiological studies of populations exposed to nephrotoxic compounds, for example mercury (1,2). Albumin is the commonly used marker of glomerular integrity, while low molecular weight proteins, or enzymes, such as beta-2-microglobulin, alpha1-microglobulin (A1M; also called protein HC), N-acetyl- β -D-glucosaminidase (NAG), or γ -glutamyl transpeptidase (γ -GT), are used for assessment of effects on renal tubular cells.

In many cases, especially in epidemiological studies, it is necessary to store the urine samples for long periods of time before analysis. Storage at -20°C is common, although many recent

Corresponding Author: Felicia Trachtenberg, PhD, New England Research Institutes, 9 Galen Street, Watertown, MA 02472, Phone: 617-923-7747 x420, Fax: 617-926-8246, ftrachtenberg@neris.science.com.

Conflict of Interest Statement

Neither author reports any conflicts of interest.

studies have found detrimental effects at this temperature and recommend either storage at 4°C with immediate analysis or storage at -70°C long term.

We found fifteen studies of albumin yielding varying results. Nine studies (3–10) found a decrease after storage at -20°C for as little as one week (though sometimes not until after a longer time). Furthermore, two studies (11,12) found a decrease only for samples with low/normal albumin excretion, but not for those with higher (abnormal) excretion. On the other hand, four studies (13–17) found no decrease after 2 months, 6 months, 2 years, and 2 years, respectively. However, these studies were all done on diabetics or patients with renal disease (who would have abnormally high albumin excretion). Therefore, these results do not necessarily contradict the studies performed on healthy subjects, which find an effect of storage.

Two studies (18,19) have found a decrease in γ -GT concentration with storage at -20°C. The former found a 60% drop in γ -GT concentration after just one day of storage. They found γ -GT to be best preserved when urine samples were stored at -70°C after initial centrifugation.

Two studies (8,9) of NAG showed a significant decrease after 6 months, and one study (19) concluded that approximately 15% of NAG concentration is lost in the process of freezing/thawing, but there are no further decreases over time. On the other hand, another study (20, 21) of NAG found no effect of storage at -20°C for one month, but found a 50% decrease after one year.

Two studies of A1M found a significant effect of storage at -20°C. Klasen (15) found a decrease in concentration over two years of storage. Tencer (16,17) found a non-significant drop of 14% after six months, and a significant decrease of 22% after one year.

Finally, Schultz (9) found that creatinine concentration was unaffected after 6–8 months of storage at -20°C, and Manley (8) found that creatinine concentration was relatively stable after 6 months storage, but significantly decreased after 2 years.

Overall, most studies find that the concentrations of these kidney function markers are underestimated after storage at -20°C. All such studies have been performed on adults, with the exception of one study of albumin (12) and one study of albumin and NAG (9) on diabetic children. No such studies have been conducted on healthy children.

The aim of the current analysis was to examine the influence of storage time at -20°C on the concentration of albumin, γ -GT, NAG, and A1M, as well as creatinine, in a large sample of healthy children.

Materials and Methods

These data were available as part of the New England Children's Amalgam Trial (NECAT) (22,23). The NECAT study was designed to examine effects of amalgam dental fillings in 534 children in Boston and Maine, aged 6–10 at the beginning of the study, for 5 years. Outcome measures of this trial included effects on the kidney, as measured by creatinine-corrected albumin, γ -GT, NAG, and A1M. Eligibility criteria for the trial included no evidence of kidney disorders, and the sample was gender balanced and racially diverse (see Table 1). The study was approved by the institutional review boards of all participating sites, and it conforms to the provisions of the Declaration of Helsinki. Written parental consent and child assent was obtained for all participants.

The original protocol called for collection of timed overnight samples, with spot daytime samples collected for those children who did not provide an overnight collection. However, as

the children got older, compliance with the overnight collection become more problematic and a decision was made to switch to spot samples at the clinic for all children. Therefore, the proportion of samples collected overnight vs. daytime varied over the course of the study, from 92% overnight vs. 8% daytime at baseline to 4% overnight vs. 96% daytime at year 5.

γ -GT and creatinine concentrations were measured yearly for all NECAT children (53% overnight vs. 47% daytime samples overall); albumin, NAG, and A1M concentrations were measured at years 3 and 5, with additional measurements for 57 children in a substudy at year 4 (12% overnight and 88% daytime samples overall).

Urine specimens were sent to Rochester General Hospital and Strong Hospital clinical laboratories in Rochester, NY for analysis of creatinine and γ -GT, as well as to the Sahlgrenska University Hospital in Goteborg, Sweden for analysis of creatinine, albumin, NAG, and A1M. When the specimen was not sufficient for both, priority was given to the laboratories in Rochester, NY. Creatinine concentration was measured in both places for use in the creatinine-adjustment of the concentrations of the kidney markers; however, the creatinine concentration reported here is that from the laboratories in Rochester, NY. Creatinine was determined by the photometric 'Jaffe' method.

Specimen storage

Urine samples were stored frozen at -20°C until analysis. The median storage time for the laboratories in Rochester, NY was 71 days, with only 7% of samples stored for longer than 6 months. The median storage time for the laboratory in Goteborg, Sweden was 6 months for albumin and NAG and 9 months for A1M. Nine percent of the samples were stored for longer than one year before analysis of albumin and NAG, with 32% stored longer than one year before analysis of A1M.

Assay methods

γ -glutamyl transpeptidase (γ -GT) was measured using the DADE BEHRING Dimension clinical chemistry system: Flex reagent cartridge.

N-acetyl- β -D-glucosaminidase (NAG) was determined with an automated photometric method based on the formation of '3-cresol purple' at the reaction catalysed by NAG, using reagents and calibrator from Roche Diagnostics (detection limit 0.1 U/L).

Urinary albumin and alpha-1-microglobulin (A1M) concentrations were determined by automated nephelometric immunochemical methods using reagents and calibrator from Beckman Coulter. Additional internal reference samples were used in each analytical run. The detection limits were 2.4 mg/L for albumin and 4 mg/L for A1M. However, since several previous studies on A1M have been performed using antibodies from Dako (24), and the concentrations in these two commercial antigens ('calibrators') differ, we corrected our A1M concentrations. Using the Beckman Coulter antigens results in 40 % higher levels compared to those obtained using the Dako reagents. In order to be able to compare our data with some previous studies, we divided our A1M levels in mg/L by 1.4.

Special measures were taken to prevent inhomogeneous albumin concentrations in the thawed urine samples. The frozen samples were thawed in a water bath (37°C), mixed and kept in the water bath for another 30 minutes. After mixing again, 10 μL of Tween20 was added to 1 mL of urine. Samples were then kept cool overnight, gently mixed on the following day, then placed in the 37°C water bath for 3×60 minutes, and gently mixed between every 60 minutes period. After this procedure they were analysed within 3 days.

Statistical analysis

Repeated measures analysis of covariance models, with compound symmetric variance structures, were fit to determine the effect of storage time on the concentrations (mg or U/L) of creatinine, albumin, γ -GT, NAG, and A1M. Log transformations were used when examination of the model residuals indicated that it was needed. The model for albumin excluded samples with microalbuminuria (albumin > 30 mg/g creatinine, 12% of samples), since these samples would have too large an effect on the results. All models controlled for age, sex, race, and lean body mass. Lean body mass was calculated as $\text{weight} \times (1 - \% \text{ body fat})$, with body fat measured by a body fat scale (model TBF-551). We also controlled for urinary collection time (overnight vs. daytime sample) and creatinine concentration (except for creatinine models) (25).

To verify overall findings, sub-analyses were performed excluding storage times greater than one year. Additionally, the effect of storage time on the detectability of albumin, NAG, and A1M concentrations was examined by logistic regression.

Results

Table 1 shows background demographic data on the 534 children, and Table 2 shows descriptive data on the kidney marker concentrations, their corresponding creatinine-corrected levels, and storage times. Creatinine and γ -GT were almost always detectable, while levels of A1M were mostly below the limit of detection (27% detectable). Albumin and NAG were 88% and 93% detectable, respectively.

Figure 1 shows plots of (detectable) creatinine, albumin, γ -GT, NAG, and A1M concentrations vs. storage times. Table 3 reports the results of repeated measures analysis of covariance models of the detectable kidney function measures on storage time. Creatinine concentration remains unchanged with storage at -20°C . γ -GT concentration decreases significantly with storage time. NAG concentration also decreases significantly with storage time; however, this decrease is no longer significant when samples with storage times greater than one year are excluded (though the slope remains similar). Samples stored for longer are significantly more likely to have non-detectable NAG ($p < 0.001$). No significant effect of storage time on albumin or A1M concentrations was found.

Based on model results, in a daytime sample from an average child (age 12), we can expect a 40% decrease in γ -GT concentration after 6 months of storage at -20°C , with a 64% decrease after 1 year. For NAG concentration, we estimate a 9% decrease after 6 months and an 18% decrease after 1 year.

Discussion

We have shown that storage time at -20°C has a large effect on γ -GT concentration, and, to a lesser extent, NAG concentration. Albumin and A1M do not appear to be affected by storage time at -20°C , apart from a possible decrease by the freezing/thawing procedure per se, irrespective of storage time. Additionally, we found no effect of storage on creatinine concentration, which suggests that both the concentrations and creatinine-adjusted levels of renal markers will be affected to a similar extent. Analyses of our creatinine-adjusted levels do in fact confirm this (data not shown). We found little information in the literature on the effect of long term storage at -70°C on these biomarkers.

Although other studies have found that albumin and A1M decrease with storage at -20°C , we do not find a significant decrease with time of storage. One possible reason for this is that the process of freezing and thawing the samples make them inhomogeneous, decreasing the

recovery of these proteins in the analysis, while there is no substantial further breakdown with time. In the present study special measures were taken to dissolve and recover all proteins after thawing. Another possibility is that because our study did not analyze the same samples over time, we may lack power to detect a significant decrease with time. Inconsistent results in the literature regarding albumin may be due to assay-dependent differences in reactivity to fragments of albumin (26).

Although we were not able to analyze the same urine samples at various storage times, our conclusions are relevant to large epidemiological studies that store urine samples for varying amounts of time before analysis. Additionally, our results extend the conclusions of previous research on adults to healthy children.

Based on the conclusions of our study and the studies of others, we recommend storing urine samples at -70°C , if storage is necessary (20). We also recommend future study of storage at -70°C to show the need for standardized freezing procedures of no less than -70°C . Furthermore, if it is necessary to interpret results from samples stored at -20°C , it is advisable to account for storage time in statistical models.

Acknowledgments

We acknowledge Elsa Cernichiari for the laboratory analyses of creatinine and γ -GT. The study was supported by the National Institute of Dental and Craniofacial research, USA (U01 DE11886), who also participated in the design and conduct of the study. Trial Registration: clinicaltrials.gov Identifier NCT00065988.

Supported by a cooperative agreement (U01 DE11886) between the New England Research Institutes and the National Institute of Dental and Craniofacial Research, National Institutes of Health.

References

1. World Health Organization (WHO). Elemental mercury and inorganic mercury compounds: human health aspects. WHO: Geneva; 2003. Concise International Chemical Assessment Document 50.
2. Cardenas A, Roels H, Bernard AM, Barbon R, Buchet JP, Lauwerys RR, et al. Markers of early renal changes induced by industrial pollutants. I. Application to workers exposed to mercury vapour. *Br J Ind Med* 1993;50(1):17–27. [PubMed: 8431387]
3. Erman A, Rabinov M, Rosenfeld J. Albumin determination in frozen urines-- underestimated results. *Clin Chim Acta* 1988;174(3):255–261. [PubMed: 3390955]
4. Elving LD, Bakkeren JA, Jansen MJ, de Kat Angelino CM, de Nobel E, van Munster PJ. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. *Clin Chem* 1989;35(2):308–310. [PubMed: 2914381]
5. Giampietro O, Penno G, Clerico A, Cruschelli L, Cecere M. How and how long to store urine samples before albumin radioimmunoassay: a practical response. *Clin Chem* 1993;39(3):533–536. [PubMed: 8448872]
6. Hara F, Nakazato K, Shiba K, Shimoda J, Kojima T, Fukumura Y, et al. Studies of diabetic nephropathy. I. Effects of storage time and temperature on microalbuminuria. *Biol Pharm Bull* 1994;17(9):1241–1245. [PubMed: 7841946]
7. MacNeil ML, Mueller PW, Caudill SP, Steinberg KK. Considerations when measuring urinary albumin: precision, substances that may interfere, and conditions for sample storage. *Clin Chem* 1991;37(12):2120–2123. [PubMed: 1764788]
8. Manley SE, Burton ME, Fisher KE, Cull CA, Turner RC. Decreases in albumin/creatinine and N-acetylglucosaminidase/creatinine ratios in urine samples stored at -20°C . *Clin Chem* 1992;38(11):2294–2299. [PubMed: 1424127]
9. Schultz CJ, Dalton RN, Turner C, Neil HA, Dunger DB. The Oxford Regional Prospective Study Group. Freezing method affects the concentration and variability of urine proteins and the interpretation of data on microalbuminuria. *Diabet Med* 2000;17(1):7–14. [PubMed: 10691153]

10. Brinkman JW, de Zeeuw D, Duker JJ, Gansevoort RT, Kema IP, Hillege HL, et al. Falsely low urinary albumin concentrations after prolonged frozen storage of urine samples. *Clin Chem* 2005;51(11): 2181–2183. [PubMed: 16244297]
11. Osberg I, Chase HP, Garg SK, DeAndrea A, Harris S, Hamilton R, et al. Effects of storage time and temperature on measurement of small concentrations of albumin in urine. *Clin Chem* 1990;36(8 Pt 1):1428–1430. [PubMed: 2387037]
12. Shield JP, Hunt LP, Morgan JE, Pennock CA. Are frozen urine samples acceptable for estimating albumin excretion in research? *Diabet Med* 1995;12(8):713–716. [PubMed: 7587012]
13. Lamb E, Dawney A. Effects of sample preparation and storage on urine albumin determination. *Ann Clin Biochem* 1989;26(Pt 6):560–562. [PubMed: 2619239]
14. Collins AC, Sethi M, MacDonald FA, Brown D, Viberti GC. Storage temperature and differing methods of sample preparation in the measurement of urinary albumin. *Diabetologia* 1993;36(10): 993–997. [PubMed: 8243884]
15. Klasen IS, Reichert LJ, de Kat Angelino CM, Wetzels JF. Quantitative determination of low and high molecular weight proteins in human urine: influence of temperature and storage time. *Clin Chem* 1999;45(3):430–432. [PubMed: 10053054]
16. Tencer J, Thysell H, Andersson K, Grubb A. Long-term stability of albumin, protein HC, immunoglobulin G, kappa- and lambda-chain-immunoreactivity, orosomucoid and alpha 1- antitrypsin in urine stored at –20 degrees C. *Scand J Urol Nephrol* 1997;31(1):67–71. [PubMed: 9060087]
17. Tencer J, Thysell H, Andersson K, Grubb A. Stability of albumin, protein HC, immunoglobulin G, kappa- and lambda-chain immunoreactivity, orosomucoid and alpha 1- antitrypsin in urine stored at various conditions. *Scand J Clin Lab Invest* 1994;54(3):199–206. [PubMed: 7518610]
18. Matteucci E, Gregori G, Pellegrini L, Navalesi R, Giampietro O. Effects of storage time and temperature on urinary enzymes. *Clin Chem* 1991;37(8):1436–1441. [PubMed: 1678320]
19. Loeb WF, Das SR, Trout JR. The effect of erythritol on the stability of gamma-glutamyl transpeptidase and N-acetyl glucosaminidase in human urine. *Toxicol Pathol* 1997;25(3):264–267. [PubMed: 9210257]
20. Matteucci E, Gregori G, Pellegrini L, Navalesi R, Giampietro O. How can storage time and temperature affect enzymic activities in urines? *Enzyme* 1991;45(3):116–120. [PubMed: 1687734]
21. Matteucci E, Pellegrini L, Uncini-Manganelli C, Cecere M, Saviozzi M, Giampietro O. More on effects of storage time and temperature on urinary enzymes: a 1-year study. *Enzyme* 1992;46(4–5): 249–251. [PubMed: 1363404]
22. Bellinger DC, Trachtenberg F, Barregard L, Tavares M, Cernichiari E, Daniel D, et al. Neuropsychological and renal effects of dental amalgam in children: a randomized clinical trial. *Jama* 2006;295(15):1775–1783. [PubMed: 16622139]
23. The Children's Amalgam Trial: design and methods. *Control Clin Trials* 2003;24(6):795–814. [PubMed: 14662283]
24. Jarup L, Hellstrom L, Alfven T, Carlsson MD, Grubb A, Persson B, et al. Low level exposure to cadmium and early kidney damage: the OSCAR study. *Occup Environ Med* 2000;57(10):668–672. [PubMed: 10984338]
25. Trachtenberg F, Barregard L, McKinlay S. The influence of urinary flow rate in children on excretion of markers used for assessment of renal damage: albumin, gamma-glutamyl transpeptidase, N-acetyl-beta-D: -glucosaminidase, and alpha1-microglobulin. *Pediatr Nephrol* 2008;23:445–456. [PubMed: 17704953]
26. Sviridov D, Drake SK, Hortin GL. Reactivity of urinary albumin (microalbumin) assays with fragmented or modified albumin. *Clin Chem* 2008;54(1):61–68. [PubMed: 17981922]

Figure 1. Concentrations vs. Storage Time. (A) Creatinine, (B) Albumin (excludes cases with microalbuminuria), (C) GGT = γ -glutamyl transpeptidase, (D) NAG=N-acetyl- β -D-glucosaminidase, and (E) A1M= alpha-1-microglobulin. Regression line is log-transformed for GGT.

Table 1

Demographic Information on the 534 children

		Mean (SD) or N (%)
Age at beginning of 5-year study		7.9 (1.4)
Sex	Male	247 (46.3%)
	Female	287 (53.8%)
Race	Non-Hispanic White	323 (62.1%)
	Non-Hispanic Black	98 (18.9%)
	Hispanic	38 (7.3%)
	Other	61 (11.9%)

Table 2Concentrations and excretions for the kidney markers and their median storage time^a

	N	Median (Range)	Median Storage Time in Days (Range)
Creatinine			
Concentration (g/L)	2909	1.2 (0.1 – 4.2)	71 (4 – 1007)
Albumin ^b			
Concentration (mg/L)	813	7.2 (ND – 1320.0)	177 (42 – 468)
Excretion (mg/gC)	718	7.4 (1.5 – 773.1)	
γ -GT			
Concentration (U/L)	2912	26.0 (0.0 – 224.0)	71 (4 – 1007)
Excretion (U/gC)	2909	26.7 (0.0 – 246.7)	
NAG ^b			
Concentration (U/L)	813	1.2 (ND – 9.2)	176 (22 – 468)
Excretion (U/gC)	754	1.4 (0.1 – 7.8)	
A1M ^b			
Concentration (mg/L)	813	ND (ND – 17.0)	262 (42 – 563)
Excretion (mg/gC)	223	4.7 (1.1 – 29.5)	

 γ -GT = γ -glutamyl transpeptidase, NAG=N-acetyl- β -D-glucosaminidase, A1M= alpha-1-microglobulin, ND=not detectable.^a Creatinine-corrected excretions exclude samples with non-detectable concentrations.^b Albumin, NAG, and A1M were not measured in all years.

Table 3

Repeated measures analysis of covariance models of kidney markers on storage time, controlling for age, sex, race, lean body mass, creatinine concentration (except for creatinine), and sample time (overnight vs. daytime sample).

	Storage Time (days)	
	Coefficient (SE)	p-value
Creatinine		
Concentration (g/L)	0.00015 (0.00015)	0.30
Albumin ^a		
Concentration (mg/L)	-0.00038 (0.0037)	0.92
γ -GT		
Concentration (U/L) ^b	-0.0028 (0.00022)	<0.001 *
NAG		
Concentration (U/L)	-0.0010 (0.00044)	0.02 ^c *
A1M		
Concentration (mg/L)	-0.00043 (0.0011)	0.71

γ -GT = γ -glutamyl transpeptidase, NAG=N-acetyl- β -D-glucosaminidase, A1M= alpha-1-microglobulin, SE=standard error.

* statistically significant.

^a excludes cases with microalbuminuria (12%).

^b log-transformed.

^c in a re-analysis excluding cases stored for over one year, p=0.12 (coefficient (SE) = -0.00090 (0.00057).