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ORIGINAL ARTICLE

Methodological considerations with the use of urine samples for assessment of mercury excretion and markers of renal damage

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Abstract

Objective: To provide recommendations for design and analysis of studies using urine specimens to evaluate renal function or mercury excretion in children.

Methods: An analysis of mercury, albumin, γ-glutamyl transpeptidase (γ-GT) and *N*-acetyl-β-D-glucosaminidase (NAG) concentrations was carried out.

Results: Mercury concentration and creatinine-corrected renal markers were higher in daytime compared with overnight samples. Excretion rates increased with urinary flow rate. γ-GT and NAG concentrations decreased with storage time at −20°C. Differences by age, sex and race were noted.

Conclusions: We recommend use of these creatinine-corrected markers and collection of timed overnight urine samples, stored at −70°C, with control for urinary flow rate, age, sex and race in statistical models.

Keywords: *methodological study; albumins; gamma-glutamyltransferase; acetylglucosaminidase; child*

Introduction

In epidemiological studies of exposure to nephrotoxic compounds, kidney injury is commonly assessed by measuring the excretion of various proteins in the urine (Cardenas et al. 1993,WHO 2003). For example, glomerular integrity is generally assessed by the excretion of albumin, while renal tubular damage is evaluated by low-molecular-weight proteins, such as β2-microglobulin, α1-microglobulin (A1M, also called protein HC), or retinol-binding protein or enzymes, such as *N*-acetyl-β-D-glucosaminidase (NAG) or γ-glutamyl transpeptidase (γ-GT). In addition, urinary measurements are often used for assessing kinetics or body burden of potentially nephrotoxic agents. One such agent that has been of interest to numerous epidemiological studies is inorganic mercury (Clarkson 2002). Exposure to inorganic mercury may occur at high levels, often from occupational hazards, or at low levels, such as from dental amalgam fillings. Indeed, numerous epidemiological studies have used urinary mercury excretion measures to assess long-term exposure to and/or body burden of inorganic mercury (Dye et al. 2005, Bast-Pettersen et al. 2005, Echeverria et al. 2006, Kingman et al. 1998, Factor-Litvak et al. 2003,WHO 2003).

Ideally, to assess kidney damage or mercury body burden in epidemiological studies, the average excretion rates of these markers, e.g. the mass excreted per 24 h or per hour, would be measured. In practice, however, it is difficult and inconvenient to collect such 24-h or other timed samples. Instead, spot samples are used, either first morning urine or daytime samples, which are 'corrected' for dilution using creatinine (Boeniger et al. 1993) or specific gravity. An important limitation of the use of spot samples, which are collected at various times of the day, is that urinary flow rate (ml h^{-1}) often varies substantially over the course of a day (and night). Several small studies have suggested that urinary flow rate may

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affect obtained measures of creatinine, γ-GT, NAG and mercury (Araki et al. 1986, Greenberg & Levine 1989, Jung & Schulze 1986, Jung et al. 1986, Wellwood et al. 1975, Piotrowski et al. 1975). As these prior studies have not definitively determined the magnitude of the influence of urinary flow rate, the extent to which urinary flow rate may complicate the interpretation of results or decrease validity remains unclear.

Another complication with the use of urine samples to assess renal damage is that, in many studies, urine samples are stored for long periods of time before analysis. Although storage at –20°C is common, many recent 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 (MacNeil et al. 1991, Schultz et al. 2000, Matteucci et al. 1991, Klasen et al. 1999, Loeb et al. 1997, Erman et al. 1988, Manley et al. 1992, Tencer et al. 1997). Finally, marker levels may vary based on age, sex and/or race (Agirbasli et al. 1996, Davies et al. 1984, Jones et al. 2002, Jung et al. 1990, Mattix et al. 2002, Skinner et al. 1996). Therefore, to interpret test results and to make valid comparisons in epidemiological studies, it is necessary to take these factors into account.

The objective of this article is to provide much-needed methodological recommendations concerning specimen collection, storage and data analysis for studies using urine samples to assess renal damage and mercury body burden. To develop these recommendations, we use findings from the New England Children's Amalgam Trial (NECAT), a large, racially diverse sample of healthy children prospectively followed for 5 years. This article highlights results from five previous NECAT publications (Trachtenberg & Barregard 2007, 2010, Trachtenberg et al. 2008, 2009, Dunn et al. 2008) with practical suggestions to aid in design of epidemiological studies to collect urine samples to measure mercury levels or renal function.

Methods

Study design

This study was performed as part of the New England Children's Amalgam Trial (NECAT, clinicaltrials.gov identifier: NCT00065988) (Anon 2003, Bellinger et al. 2006). The study was approved by the institutional review boards of all clinics, the Forsyth Institute and the New England Research Institutes. All participants provided informed consent and child assent. The NECAT study was designed to examine, over 5 years, the effects of amalgam dental fillings in 534 children in Boston and Maine, aged 6–10 years at the beginning of the study (1997–1999). Eligibility criteria for the trial included no evidence of kidney disorders, and the sample was gender balanced and racially diverse. Outcome measures of this trial included effects on the kidney, as measured by creatinine-corrected albumin, γ-GT, NAG and A1M excretion from urine samples. Urinary mercury concentration was also measured to assess body burden from the amalgam.

The initial NECAT protocol called for yearly timed overnight urine samples over 5 years, with spot samples collected from those children who did not provide overnight samples. However, it became clear that too few children were providing timed overnight samples, and thus the study switched to collecting only spot urine samples in the middle of the trial. Considering the mixture of data from overnight and daytime specimens, a paired data collection substudy of 82 children was imbedded at the 4-year visit to examine the effects of overnight versus daytime spot samples and to determine how to analyse and interpret longitudinal findings with a mixture of overnight and daytime specimens. These samples allowed comparison of the creatinine-corrected measures between overnight and daytime spot samples, as well as examination of the influence of urinary flow rate on the timed overnight samples (Trachtenberg et al. 2008, 2009).

Laboratory methods

Annual urine specimens were sent to Rochester General Hospital and Strong Hospital clinical laboratories in Rochester, NY for analysis of creatinine, total mercury and γ-GT. At years 3 and 5, specimens were also sent to the Sahlgrenska University Hospital in Gothenburg, Sweden for analysis of creatinine, albumin, NAG and A1M; specimens were also sent there at year 4 for the substudy analysis, which included total mercury as well, analysed at Lund University, Sweden. When the specimen was not sufficient for both laboratories, priority was given to the laboratories in Rochester, NY.

Creatinine was determined at both the Rochester, NY and Goteborg, Sweden laboratories, for use in the creatinine corrections, by the photometric 'Jaffe' method. The creatinine concentrations reported here are those from the laboratory in Rochester, NY.

In the laboratory in Rochester, NY, total mercury determination was based on the rapid conversion of Hg compounds into atomic Hg suitable for aspiration through the cell of a flameless atomic absorption monitor (Laboratory Data Control Model 1235; Thermo Separation Products, Waltham, MA, USA) (Magos & Clarkson 1972, Barber & Wallis 1986). Biological samples were digested in 45% (w/v) NaOH solution in the presence of 1% cysteine. In the presence of $SnCl_2$ at high pH, $CdCl_2$ breaks the carbon bond, with a subsequent reduction of Hg^{2+} to Hg^0. The detection limit, initially 1.5 μg l^{-1}, was reduced to 0.45 μgl^{-1} after 1 February 2000 as a result of increasing the

volume of urine analysed from each child. Only samples after this date are used in analysis, with non-detectable concentrations (<0.45 μgl^{-1}) imputed as $0.45/\sqrt{2}$ (Hornung & Reed 1990).

In the laboratory in Sweden, total mercury was determined using an automized cold-vapor atomic fluorescence technique (Bergdahl et al. 1995). Samples were digested using a mixture (5:1) of concentrated perchloric and nitric acids for 1 h at room temperature. The detection limit was 0.1 μgl^{-1}. Reference samples were included in all analysis series (Centre de Toxicologie du Quebec Interlaboratory Comparison Program, batch D-04-03, certified; target value 2 μgl^{-1}, obtained value 2.18 μgl^{-1}, SD 0.16 μgl^{-1}, n =4). The precision for duplicate analyses was good with a relative standard deviation of 8% at levels of 0.5–1 μgl^{-1}, and lower at higher mercury concentrations.

Urinary albumin and A1M concentrations were determined by automated nephelometric immunochemical methods using reagents and calibrator from Beckman Coulter (Danvers MA, USA). Additional internal reference samples were used in each analytical run. The detection limits were 2.4 mgl^{-1} for albumin and 4 mgl^{-1} for A1M. As A1M levels were below the detection limit in most of the NECAT samples (data not shown), this renal marker was excluded from analysis.

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 (Brandenburg, NJ, USA; detection limit 0.1 Ul^{-1}). γ-GT was measured using the Dimension system GGT Flex reagent cartridge from Dade Behring (Deerfield, IL, USA).

Urine samples were stored frozen at -20°C until analysis. The median storage time for the laboratories in Rochester, NY was 71 days; only 7% of samples were stored for longer than 6 months. The median storage time for the laboratories in Sweden was 6 months for albumin and NAG, and 15 months for mercury; 9% of the samples were stored for longer than 1 year before analysis of albumin and NAG.

Statistical analysis

Log-transformations were used for mercury, albumin, γ-GT and NAG in all statistical models, as these were all found to have log-normal distributions. Paired *t*-tests were used to compare mercury and renal marker concentrations and creatinine-corrected levels between (detectable) timed overnight and daytime spot samples. Urinary flow rate (ml h^{-1}) and excretions rates (mass or units h^{-1}) of creatinine, mercury, albumin, γ-GT and NAG were calculated in the timed (overnight) samples. In the timed overnight samples, analysis of covariance (ANCOVA) of excretion rates, concentrations and creatinine-corrected measures on urinary flow rate were performed, controlling for age, sex, race, lean body mass and storage time. Lean body mass was calculated as weight × (1 – % body fat), with body fat measured by a body fat scale (model TBF-551; Tanita Corporation of America, Inc., Arlington Heights, IL, USA).

Repeated measures analysis of covariance models (ANCOVA) with compound symmetric variance structures were fit to determine the effect of storage time on the concentrations of creatinine, mercury, albumin, γ-GT and NAG, controlling for age, sex, race and lean body mass, urinary collection time (overnight vs daytime sample), and creatinine concentration (except for creatinine models).

Repeated measures ANCOVA models with compound symmetric variance structures were fit to determine the effects of age, sex and race on concentrations, excretion rates and creatinine-corrected excretions. Models were controlled for lean body mass, storage time, urinary collection time (overnight vs daytime sample) and creatinine concentration (except for creatinine models). Additionally, models for race controlled for socioeconomic status. Socioeconomic status was calculated using the method developed by Green (1970) as a weighted average of education and income.

In all analyses, a p-value <0.05 was considered statistically significant.

Results

Participant characteristics

The 534 children were on average 7.9 (1.4) years old at the beginning of the 5-year study. The group was 53.8% female, 62.1% non-Hispanic white, 18.9% non-Hispanic black, 7.3% Hispanic and 11.9% of other/mixed/unknown race.

The excretion of creatinine and γ-GT increased significantly with age (p <0.001) (Trachtenberg & Barregard 2007), while mercury excretion decreased with age (p=0.01) (Dunn et al. 2008). Albumin, NAG and A1M did not significantly change with age (Trachtenberg & Barregard 2007).

Albumin and γ-GT concentrations and creatinine-corrected excretions (mg g^{-1}C or U g^{-1}C) were significantly higher for girls compared with boys (p <0.001) (Trachtenberg & Barregard 2007). Creatinine excretion was higher for boys compared with girls (p ≤0.03) (Trachtenberg & Barregard 2007). Mercury and NAG did not vary significantly by gender (Trachtenberg & Barregard 2007, Dunn et al. 2008).

Creatinine concentration was significantly higher for black children, compared with white or Hispanic (p <0.001) (Trachtenberg & Barregard 2007). The excretion of creatinine and γ-GT was significantly higher for black

and Hispanic children compared with non-Hispanic white children (p <0.001) (Trachtenberg & Barregard 2007). Being of 'other' race was associated with higher mercury excretion (p =0.025) (Dunn et al. 2008).

Normative levels of albumin, γ-GT and NAG excretions in this study population by age groups, sex and race are available elsewhere (Trachtenberg & Barregard 2007).

Timing of specimen collection

The NECAT data contained a mixture of timed overnight and daytime spot samples. Table 1 compares the overnight with the daytime samples in the NECAT substudy. Concentrations were higher overnight than during the day, and the difference was statistically significant for creatinine and mercury (Trachtenberg et al. 2008, 2009). In contrast, creatinine-corrected excretions were higher during the day than overnight, with statistically significant differences for albumin, γ-GT and NAG (Trachtenberg et al. 2008).

Table 2 presents results on associations between urinary flow rate and biomarkers in the timed overnight samples. Although concentrations generally decreased with urinary flow rate, the association was statistically significant only for creatinine and marginally significant for mercury (Trachtenberg et al. 2008, 2009). Meanwhile, excretion rates increased with urinary flow rate significantly for creatinine, γ-GT and NAG (Trachtenberg et al. 2008).

See Trachtenberg et al. 2008, 2009 for full details on the available sample sizes.

Specimen storage

Repeated measures ANCOVA models showed that the decreases in concentrations of γ-GT (p <0.001) and NAG (p =0.02) were statistically significant (Trachtenberg & Barregård 2010). Based on model results, for a daytime sample from a 12-year-old child, we can expect a 40% decrease in γ-GT concentration after 6 months of storage at –20°C, and a 64% decrease after 1 year. For NAG concentration, we estimate a 9% decrease after 6 months and an 18% decrease after 1 year. No significant effect of storage time on creatinine, mercury or albumin concentrations was found.

Discussion

Our prospective, longitudinal analyses showed that urinary concentration and excretion of creatinine, mercury, albumin, γ-GT and NAG often vary by time of day, urinary flow rate, specimen storage time, age, sex and race in children. Therefore, certain methodological considerations must be made in both the design and statistical analysis of epidemiological and experimental studies when urine specimens are collected and stored for these purposes.

First, studies should optimally collect urine samples from participants at the same time of day. In general, both researchers and clinicians often prefer overnight samples, although the issue is still debated (Gaspari et al. 2006, Shidham & Hebert 2006). Overnight samples are easier to obtain as they are less burdensome to patients/participants, and they include least variation in blood flow and glomerular filtration rate, both of which are affected by exercise, food intake, etc. in daytime (Gibb et al. 1989). However, the collection of urine overnight also presents several drawbacks, such asstorage of urine at the participant's home, risks of contamination and incomplete urine collection. Such samples usually require verification of the completeness of urine collection on the basis

Table 1. Comparison of overnight and daytime urine samples in a substudy of the New England Children's Amalgam Trial (2003–2004).

	n	Overnight samples, median[b] (range)	Daytime samples, median[b] (range)	*p*-value, paired *t*-test
Creatinine				
Concentration (g l^{-1})	62	1.6 (0.6–3.2)	1.0 (0.2–3.8)	<0.001
Mercury				
Concentration (ng ml^{-1})	34	0.35 (0.06–4.00)	0.23 (0.01–3.42)	0.04
Excretion[a] (ng mg^{-1}C)	34	0.26 (0.06–1.97)	0.32 (0.02–8.23)	0.29
Albumin				
Concentration (mg l^{-1})	42	6.1 (ND–36.0)	4.8 (ND–120.0)	0.62
Excretion (mg g^{-1}C)	32	4.8 (1.6–36.4)	8.6 (2.1–69.9)	0.008
γ-GT				
Concentration (U l^{-1})	62	32.0 (1.0–150.0)	30.5 (2.0–117.0)	0.44
Excretion[a] (U g^{-1}C)	62	21.3 (0.6–80.8)	39.9 (2.3–119.0)	<0.001
NAG				
Concentration (U l^{-1})	42	1.3 (ND–5.6)	1.0 (ND–9.2)	0.86
Excretion[a] (U g^{-1}C)	34	1.2 (0.2–3.3)	1.4 (0.3–4.4)	0.002

γ-GT,γ-glutamyl transpeptidase; NAG, *N*-acetyl-β-D-glucosaminidase; ND, not detectable.[a]Excretions exclude samples with non-detectable concentrations. See Trachtenberg et al. 2008,2009 for full details on the available sample sizes. [b]The geometric mean is presented for mercury rather than the median, as the medians were not reflective of overall difference.

Table 2. Results of analysis of covariance on urinary flow rate[a], β-coefficients and *p*-values for creatinine, mercury and kidney function markers (albumin, γ-GT, NAG) from a substudy of the New England Children's Amalgam Trial (2003–2004).

	N[†]	β-Coefficient	*p*-Value
Creatinine			
Concentration (g l^{-1})	82	-0.018	0.006
Excretion rate (mg h^{-1})	73	0.98	<0.001
Mercury			
Concentration (μg l^{-1})	36	-0.027	0.06
Excretion rate (ng h^{-1})	36	0.019	0.19
Excretion (μg g^{-1}C)	36	-0.016	0.20
Albumin			
Concentration (mg l^{-1})	49	-0.092	0.49
Excretion rate (mg h^{-1})	43	0.0040	0.09
Excretion (mg g^{-1}C)	49	-0.021	0.83
γ-GT			
Concentration (U l^{-1})	82	-0.061	0.86
Excretion rate (U h^{-1})	73	0.035	<0.001
Excretion (U g^{-1}C)	82	0.30	0.20
NAG			
Concentration (U l^{-1})	48	-0.0014	0.52
Excretion rate (U h^{-1})	42	0.0011	0.01
Excretion (U g^{-1}C)	48	0.0037	0.95

γ-GT,γ-glutamyl transpeptidase; NAG, *N*-acetyl-β-D-glucosaminidase.
[a]Analysis of covariance, controlling for age, sex, race, lean body mass and storage time; β-coefficients show the effect of an increase of urinary flow rate of 1 ml h^{-1}.

of creatinine output. In studies where urine samples have been collected at varying times of day, statistical models should control for time of day.

Another important finding was that the excretion rates of these renal markers varied by urinary flow rate; thus, studies of creatinine, albumin, γ-GT and NAG should attempt to control for urinary flow rate in all statistical models. This requires collection of timed urine samples with measurement of urinary volume. When this is not feasible, an acceptable alternative is to control for creatinine concentration in statistical models, as creatinine concentration is known to vary inversely with urinary flow rate. It should be noted, however, that if the effect of urinary flow rate on the excretion rate of a biomarker is different from the effect of flow rate on the excretion rate of creatinine, then the biomarker/creatinine ratio is not optimal, as it will also vary by urinary flow rate.

In addition, effects of storage time at –20°C were found on γ-GT and NAG. Therefore, studies of these renal markers may have improved validity if urine specimens are stored at lower temperatures; e.g. several studies have found –70°C to be adequate (Loeb et al. 1997, Matteucci et al. 1991, MacNeil et al. 1991, Schultz et al. 2000, Klasen et al. 1999). If storage at –20°C is necessary, statistical models must control for storage time.

Finally, epidemiological studies of urinary mercury and renal function should control for age, sex and race in statistical models, as these markers vary by patient characteristics. While randomized trials often have the advantage of providing approximate balance across treatment groups and therefore possibly avoiding the need for covariate adjustment, estimated variances will be increased, causing lower power.

A limitation of our study, as with any study of urinary biomarkers or analytes, is that the biomarkers may be unstable in urine. It should also be noted that the participants of the study from which these results and recommendations are based were children who met stringent eligibility criteria for a clinical trial and by no means constitute a representative community sample. Still, the observed associations between methods of urine collection and storage and measures of urinary markers are unlikely to be systematically different in broader populations of similarly healthy children.

In conclusion, methodological recommendations must consider feasibility in light of the ultimate aims of each study. As we have shown, the influence of urinary flow rate on creatinine-corrected measures of creatinine, mercury, albumin, γ-GT and NAG, is markedly less than its influence on excretion rates. This finding, together with the high correlation observed between creatinine-corrected markers and excretion rates, indicates that the use of these creatinine-corrected markers is a good choice in practice, keeping in mind that daytime and overnight samples will likely differ. For studies of mercury excretion or renal function in children using biomarkers of albumin, γ-GT and NAG, we recommend collection of timed overnight urine samples, with storage(if necessary) at –70°C, and control for urinary flow rate, age, sex and race in statistical models. If a timed overnight collection is not feasible, a spot first morning urine sample is acceptable. Certainly, if ideal conditions are not met, statistical models for these markers should also control for creatinine concentration, time of day and storage time.

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Declaration of interest

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