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Intra-individual variability in markers of proteinuria for normal subjects and those with cadmium-induced renal dysfunction: interpretation of results from untimed, random urine samples

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The project aimed to help interpretation of urinary protein measurements, namely β-2-microglobulin, retinol-binding protein, albumin and total protein in untimed, random urine samples as indicating significant changes in renal tubular reabsorption and glomerular permeability in an individual. A standard methodology used in clinical laboratory medicine was applied to calculate the intra-individual biological variation for these analytes. This parameter in conjunction with a laboratory's analytical variation allows definition of uncertainty about a single urine protein measurement, significant changes above normal variation in serial measurements within an individual and a defined level of maximum acceptable analytical imprecision. Repeat urine samples were obtained over a period of one week from a group of cadmium-exposed workers, 90% of whom had long-term tubular proteinuria, and a group of five unexposed volunteers with normal renal function. Dilute samples defined as having creatinines less than 3 mmol l^{-1} were excluded, as were urines with pH less than 5.5 for β-2-microglobulin. Samples were analysed twice after randomisation in large batches. There was no evidence of any diurnal variation in the four protein measurements from samples collected between early morning and 16:00 hours. Creatinine or specific gravity correction of urine results for all four proteins only marginally reduced the uncertainty associated with an individual measurement as reflecting the true excretion value. For those subjects with defined tubular proteinuria, variability in retinol-binding protein excretion was less than that for β-2-microglobulin. About 30% of the samples had urine pHs of 5.5 or less where β-2-microglobulin degradation occurs. Using our laboratory analytical precision the minimum changes between serial creatinine-corrected measurements that are needed to be considered statistically significant ($p < 0.05$) is 110% for retinol-binding protein, 177% for β-2-microglobulin, 70% for total protein, and 81% for albumin. Unlike published data for cadmium and mercury, the use of creatinine or specific gravity correction of random untimed urine samples for the urinary proteins does not make a large improvement to the interpretation of data by reducing the uncertainty associated with a measurement. There are significant advantages to the use of retinol-binding protein in contrast to β-2-microglobulin as an indicator of renal tubular damage. Levels for defined acceptable analytical precision are calculated for laboratories undertaking protein estimations. The data in this report will help in the interpretation of urinary protein measurements without monitoring cadmium workers.

Keywords: retinol-binding protein, beta-2-microglobulin, urinary total protein, urinary albumin, analytical imprecision.

Introduction

There has been a considerable investigation over a number of years into the effects of excessive cadmium exposure on renal function (Friberg *et al.* 1985, Elinder and Jarup 1996, Savolainen 1995). The increased excretion of low-

molecular weight proteins in urine has been extensively studied and the measurement of a urinary low molecular weight protein, indicating proximal tubular damage, has been accepted as part of the practical biological monitoring strategy of workers exposed to cadmium. There is some evidence that urinary increases in higher molecular weight proteins normally retained in blood plasma by the glomerular barrier can occur, unrelated to renal tubular effects of excessive cadmium exposure (Bernard *et al.* 1979). In occupational and environmental medicine, monitoring of ambulatory subjects is usually performed using untimed urine collections as the collection of accurate timed urine samples is problematic. Correction of an analyte in an untimed urine sample for the influence of urine concentration or dilution is often carried out using creatinine or specific gravity, but the value of such corrections has been questioned (Boehniger *et al.* 1993).

There has been debate about which of the specific candidate low molecular weight proteins should be used for monitoring effects on the proximal tubular reabsorptive process. β-2-microglobulin (B2mg), α-1-microglobulin(a1mg) and retinol-binding protein (RBP), among others, have been proposed by various authors as the marker of choice (Kjellstrom *et al.* 1977), Grubb 1992). Assay systems for these three proteins are commercially available. Given the generalized nature of the physiological process that these urinary measurements are following, factors such as stability of the specific protein in urine, ease and the precision of the analytical methodology are important factors in defining the sensitivity of the test to detect small changes in this renal proximal tubular function. β-2-microglobulin rapidly degrades in urine at a pH of less than 5.5 and may be affected at pH less than 6.0. Urinary pH values around these figures are not uncommon in randomly collected spot urine samples. Thus without intervention such as giving bicarbonate to induce alkalinized urine, a proportion of urines may be unsuitable for β-2-microglobulin analysis, having undergone degradation within the bladder. Several studies have suggested that α-1-microglobulin, which is stable in acidic urine, may be as useful as β-2-microglobulin in detecting the renal tubular damage caused by cadmium (Kido *et al.* 1985) Urinary retinol-binding protein is found at much lower levels in urine than α-1-microglobulin, but can still be readily measured and is stable at the pH range found in urine.

In contrast to markers of tubular dysfunction, the measurement of urinary total protein and albumin are routinely used in a clinical environment to investigate the integrity of the renal glomerulus and thus are often used in conjunction with a low molecular weight protein to test both renal glomerular and tubular functions.

This report defines the intra-individual biological variability of urinary RBP, B2mg, albumin and total protein using creatinine and specific gravity correction in cohorts of cadmium-exposed subjects with evidence of proteinuria and normal subjects. The methodology uses techniques from clinical laboratory medicine (Fraser and Harris 1989). The influence of analytical precision on the definition of significant differences between measurements in an individual is also defined. These factors aid in the interpretation of an isolated result in an individual and the detection of a trend in these biological effect markers over time. Fraser (Fraser and Harris 1989) noted that valid estimates of the components of total measured variation, namely analytical and biological, can be obtained from relatively small numbers of samples in appropriate subjects.

As the renal dysfunction caused by cadmium is stable over relatively long periods, the results from this study may have wider application in areas of

environmental, occupational or clinical medicine where it is necessary to decide if a change in measured urinary proteins shows a significant difference from a previous result in an individual.

Methods and population

The basis of the study design is that if appropriate subjects give a number of urine samples over a short-time frame, the distribution of measurements in a subject's urine samples will describe the 'true' excretion in each individual with an associated degree of uncertainty or variability. The spread of results in an individual is a combination of both intra-individual biological variability and a component of error due to analytical imprecision in the laboratory. In those with cadmium-induced low molecular weight proteinuria any change in the degree of dysfunction is not discernible over a short-time frame of a week and multiple urine samples will reflect the variability of excretion of specific proteins in this renal physiological abnormality.

Ten male subjects with a history of chronic, high occupational cadmium exposure through their use of cadmium containing silver solder as jigmakers were studied. They were under continuing investigation by HSE and gave informed, written consent to their participation in the study. These workers' exposure to cadmium over the last years had been minimal, but nine of the workers were considered to have evidence of persistent low molecular weight proteinuria. The criteria used was of a urinary RBP consistently greater than 42 mg mol^{-1} creatinine. These subjects had also been chronically exposed to silver. Five male laboratory volunteers who had neither history of occupational cadmium exposure nor evidence of renal abnormality also gave their informed consent to take part in the study. No restriction on dietary protein intake or exercise, which may influence protein excretion, was made on the volunteers during the study.

All subjects were asked to provide five random, untimed urine samples throughout a single day. Time bands for collection of these spot urine samples were; 06:000–08:00, 08:00–10:00, 10:00–12:00, 12:00–14:00, 14:00–16:00. These time-points include several suggested times for sample collection in occupational biological monitoring strategies i.e. first urine of the day, pre-shift, during shift.

On each of the other four days of the working week, subjects provided a single urine sample at 10.00 am. Therefore subjects provided a maximum number of nine samples during one week. All subjects, except one retiree, were at work during the study and several missing samples were recorded throughout the study. Where appropriate, incomplete data-sets were included in statistical analysis after weighting for the number of duplicate measurements for each individual.

Samples were transported on ice and stored frozen at -70°C until analysis. Urines were randomised in large batches and duplicate measurements for each sample were performed for all analytes. Quality control material was analysed at regular intervals throughout the analytical batches. RBP and B2mg were measured by enzyme immunoassay and radio-immunoassay respectively. Total protein and albumin was measured by Coomassie-blue dye binding and immunoturbidimetry respectively on a COBAS FARA (Roche UK) automated analyser. All are, or have been, established methods in the Biomedical Sciences Group, Health & Safety Laboratory. Urine specific gravity and creatinine, by alkaline picrate methodology, were also measured and used to correct analyte concentrations for the effects of urinary concentration or dilution. Current practice within HSL disregards results in dilute urines which have a creatinine concentration less than 3 mmol l^{-1}. This criteria was applied to the study. B2mg levels in urine samples with pH less than 5.5 wee also disregarded (12% of samples).

Statistical methods used to calculate intra-individual variation and analytical variation have been well documented (Fraser and Harris 1989) and used in two published occupational monitoring studies (Mason and Calder 1994, Mason *et al*. 1998). Separate statistical analyses were carried out on those subjects with renal tubular dysfunction and normal renal function.

The influence of the time of sampling on analyte excretion was examined in the following manner. Each mean analyte result from the duplicate measurements at each of the five time points collected during the single day for a cadmium-exposed individual was normalised to the mean concentration of all five results for that individual. These normalised analyte results were used to calculate mean and distribution for each time-point for the group and to compare time-points using ANOVA, 'linear trend over time' and Bonferroni multiple range tests. The influence of the time of urine sampling on excretion of RBP, and B2mg were studied in the samples from cadmium exposed workers collected at varying times on a single day, for albumin and total protein all subjects were included in the analysis. The analyte concentrations in spot urine samples were expressed corrected for urine creatinine concentration and specific gravity (SG) as both these forms of correction are commonly used in occupational medicine. Only subjects who gave five urine collections within the time bands for a single day and whose urine samples had creatinine concentrations greater than 3 mmol l^{-1} were statistically analysed. Five cadmium-exposed subjects (25 urine samples) met these criteria for RBP and B2mg and 4/5 had evidence of low molecular weight proteinuria. For B2mg the additional criteria of discarding urine results with pH less than 5.5 led to only two subjects that could be used to study diurnal variation and this statistical analysis was not completed. For total protein and albumin nine subjects (45 urine samples) were available for investigation of diurnal variation.

Results

As reported (Mason *et al.* 1998) there was no evidence from this study of a diurnal variation for RBP corrected by creatinine or SG. The data for urine albumin and total protein showed no evidence of any discernible diurnal variation between measurements at the time-points. On this limited study there is little evidence for suggesting that the times of collection used in this study may influence the results. Therefore all urine analyses over the working week with creatinine concentration greater than 3 mmol l^{-1}, and additionally greater than pH 5.5 for B2mg, from the subjects were used to calculate intra-individual variation.

A maximum number of nine spot-urine samples per individual were collected over the five day period (some individuals provided fewer samples). A total of 118 urine samples were collected and exclusion criteria based on creatinine concentration and pH were applied. Statistical analysis used weighted averages to take account of the differences in numbers of urines collected per individual. Statistical analyses were used to calculate intra-individual biological variation (σ_I) for results expressed uncorrected, and both creatinine and specific gravity corrected. The laboratory analytical variance (σ_A^2) was calculated from the paired duplicate analyses of each urine sample. Weighted average within-subject total variance (σ_T^2) for the cadmium exposed subjects was calculated using the mean of the duplicate cadmium analyses at each time point. The mean intra-individual biological variance (σ_I^2) was calculated according to the formula:

$$(\sigma_I^2) = (\sigma_T^2) - (\sigma_A^2/2).$$

The intra-individual biological coefficient of variation (CV_I) was calculated as:

$$CV_I = (\sigma_I/(\text{grand mean of samples})) \times 100.$$

The coefficient of analytical variation (CV_A) calculated from the paired analyses was defined as:

$$CV_A = (\sigma_A/\text{grand mean of samples})) \times 100.$$

In clinical medicine it has been suggested that the acceptable precision of a method to measure an analyte should be less than half the average intra-individual biological variation (i.e. $< (CV_I)/2$) (Harris 1979).

Tables 1–4 define for the four urine protein measurements uncorrected and corrected by specific gravity and creatinine, the mean intra-individual variation, the calculated maximum acceptable analytical imprecision and measured analytical precision in this study.

The estimation of a statistically significant difference ($p < 0.05$) between two successive measurements in a population is given by the formula

$$1.96 \times (\sqrt{2}) \times CV_T,$$

where CV_T is defined as

$$\sqrt{(CV_A^2 + CV_I^2)}.$$

Thus, if in this small study on cadmium workers with evidence of renal damage the calculated mean CV_T is representative of that found in a larger population, the minimum difference between two creatinine-corrected results which is statistically different at the 95% level of certainty is calculated as approximately 110% for RBP,

Table 1 Definition of intra-individual variation, acceptable analytical imprecision and measured analytical precision for retinol binding protein, both corrected and uncorrected, in renal normal and abnormal subjects.

	Retinol binding protein in abnormal group (n = 9)			Retinol binding protein in normal group (n = 6)		
	Uncorrected results $\mu g\ l^{-1}$	Creatinine corrected mg mol^{-1} creatinine	Specific gravity corrected $\mu g\ l^{-1}$ SG 1.016	Uncorrected results $\mu g\ l^{-1}$	Creatinine corrected mg mol^{-1} creatinine	Specific gravity corrected $\mu g\ l^{-1}$ SG 1.016
Grand mean	5,483	630	6,902	113	7	93
Mean intra-individual biological variation (CV_I)	48%	40%	41%	35.6%	30.6%	30.3%
Maximum acceptable analytical imprecision	24%	20%	20%	17.8%	15.3%	15.1%
Analytical precision (CV_A) calculated from duplicate measurements	13.8% mean = 5959 $\mu g\ l^{-1}$	11.0%	13.3%	13.9% mean = 154 $\mu g\ l^{-1}$	13.2%	13.9%
Analytical precision calculated for quality control measurements	9.7% at 179 $\mu g\ l^{-1}$	9.9%*	9.8%*	9.7% at 179 $\mu g\ l^{-1}$	9.9%*	9.8%*

Table 2. Definition of intra-individual variation, acceptable analytical imprecision and measured analytical precision for urinary total protein, both corrected and uncorrected, in renal normal and abnormal subjects.

	Urinary total protein in abnormal group (n = 9)			Urinary total protein in normal group (n = 6)		
	Uncorrected results mg l^{-1}	Creatinine corrected g mol^{-1} creatinine	Specific gravity corrected mg l^{-1} to SG 1.016	Uncorrected results mg l^{-1}	Creatinine corrected g mol^{-1} creatinine	Specific gravity corrected mg l^{-1} to SG 1.016
Grand mean	354.2	34.4	427.7	79.6^	4.5^	62.6^
Mean intra-individual biological variation (CV_I)	33.7%	25.1%	27.6%	43.9% (42.8%)^	38.5% (32.5%)^	34.4% (31.5%)^
Maximum acceptable analytical imprecision	16.8%	12.5%	13.8%	21.9% (21.9%)^	19.3% (16.3%)^	17.2% (8.6%)^
Analytical precision (CV_A) calculated from duplicate measurements	5.3% mean = 249 mg l^{-1}	8.0%	6.6%	9.3% mean = 80 mg l^{-1}	12.4%	11.3%
Analytical precision calculated for quality control measurements	6.0% at 105 mg l^{-1}	6.3%*	6.2%*	6.0% at 105 mg l^{-1}	6.3%*	6.2%*

^calculation after removal of single abnormal result for an individual; *calculated from analysis of variances.

Table 3. Definition of intra-individual variation, acceptable analytical imprecision and measured analytical precision for urinary albumin, both corrected and uncorrected, in renal normal and abnormal subjects.

	Urinary albumin in abnormal group (n = 9)			Urinary albumin in normal group (n = 6)		
	Uncorrected results mg l^{-1}	Creatinine corrected g mol^{-1} creatinine	Specific gravity corrected mg l^{-1} to SG 1.016	Uncorrected results mg l^{-1}	Creatinine corrected g mol^{-1} creatinine	Specific gravity corrected μg l^{-1} to SG 1.016
Grand mean	36.6	3.8	44.1	12.3^	0.75^	10.3^
Mean intra-individual biological variation (CV_I)	38.4%	29.2%	30.6%	50.9% (34.9%)^	63.8% (25.8%)^	50.2% (29.0%)^
Maximum acceptable analytical imprecision	19.2%	14.6%	15.3%	25.5% (17.4)^	31.9% (12.9%)^	25.1% (12.5%)^
Analytical precision (CV_A) calculated from duplicate measurements	7.0% mean = 36.6 mg l^{-1}	9.9%	9.8%	11.6% mean = 12.3 mg l^{-1}	13.2%	13.9%
Analytical precision calculated for quality control measurements	1.7% at 42.6 mg l^{-1}	2.5%*	2.0%*	1.7% at 42.6 mg l^{-1}	2.5%*	2.0%*

^calculation after removal of single abnormal result for an individual; * calculated from analysis of variances.

Table 4. Definition of intra-individual variation, acceptable analytical imprecision and measured analytical precision for beta-2-microglobulin, both corrected and uncorrected, in renal normal and abnormal subjects.

	Urinary B2mg in abnormal group (n = 7)			Urinary B2mg in normal group (n = 4)		
	Uncorrected results $\mu g\ l^{-1}$	Creatinine corrected mg mol^{-1} creatinine	Specific gravity corrected $\mu g\ l^{-1}$ to SG 1.016	Uncorrected results $\mu g\ l^{-1}$	Creatinine corrected mg mol^{-1} creatinine	Specific gravity corrected $\mu g\ l^{-1}$ to SG 1.016
Grand mean	9955	981	11,083	153	11	144
Mean intra-individual biological variation (CV_I)	79.8	63.9	60	50.3	33.2	39.6
Maximum acceptable analytical imprecision	39.9	31.9	30	25.2	16.6	19.8
Analytical precision (CV_A) calculated from duplicate measurements	17.2	20.6	18.2	11.2	14.5	15.3
Analytical precision calculated for quality control measurements	7.8%	8.0%*	8.0%*	7.8%	8.0%*	8.0%*

70% for total protein, 81% for albumin and 177% for B2mg. The minimum difference between two results is defined as

$$(100\times(|\text{result2-result1}|)/((\text{result1+result2})/2)).$$

These calculations used the intra-individual variation from the abnormal function group and our analytical precision, derived from duplicated analyses of the samples which was in all cases worse than the estimate of analytical precision derived from replicates of a reconstituted lyophilised quality control material. Different laboratory analytical precision will influence the defined difference between two successive results in an individual that can be considered to be significant using

$$CV_T = \sqrt{(CV_A^2+CV_I^2)}$$

Discussion

A range of urinary protein measurements have been suggested to investigate integrity of the renal glomerular barrier and proximal tubular reabsorption in subjects exposed to nephrotoxins including cadmium. In the occupational or non-hospital environment this is often carried out on random untimed urine samples, as a timed urine collection is impractical or often inaccurately collected. The results are often corrected for specific gravity or creatinine to take account of the influence of urine concentration or dilution. There has been considerable debate about the value of such corrections and investigation on an analyte-by-analyte basis is probably necessary (Greenberg and Levine 1989, Boehniger *et al.* 1993). Interpretation of a single urinary protein result in an individual may be against a defined normal range. However, the reliability of a single result to reflect the true protein excretion in an individual or to detect if a result indicates a significant worsening in comparison to a previous result depends the variability or uncertainty associated with a single result. This variability depends on the inherent intra-individual biological variation of the analyte in question and the analytical precision of the laboratory undertaking the analyses. Clinical laboratory medicine has defined maximum acceptable analytical imprecision as half the defined the intra-individual biological variation (Harris 1979, Elevitch 1976). Increasing improvements in analytical precision will at some stage below this limit have little or no influence on the interpretation of a results due to the residual inherent biological variability.

We have defined the intra-individual biological variation for two low molecular weight proteins which are used in random, untimed urine to monitor proximal tubular function namely, urinary RBP and B2mg, and two measurements, albumin and total protein, which to a large extent reflect renal glomerular permeability. The data suggest that creatinine and specific gravity correction cause a small decrease in the intra-individual variability. This decrease is considerably less than has been reported for using urinary correction of cadmium and mercury (Mason and Calder 1994, Mason *et al.* 1998). This may be due to more complex physiological and biological processes involved in the renal handling of proteins without the state of urine concentration or dilution. All four proteins, but especially the low molecular weight proteins RBP and B2mg, showed considerable intra-individual variation. This implies that any single result, with or without correction, is a poor predictor of

the true analyte excretion in the individual and care has to be taken in interpreting a change from a previous result as indicating improving or deteriorating renal function. Changes between serial measurements estimated to be statistically significant can be calculated from the intra-individual variability and long-term analytical precision in the laboratory for the analyte in question. It is noted that analytical precision defined from duplicate analyses of the samples is invariably worse than that defined from replicate measurements of a reconstituted, lyophilised quality control material. The former may reflect the true level of analytical precision in real samples which are stored refrigerated or frozen compared to an idealised, treated urine sample.

B2mg has a worse intra-individual variation than RBP where tubular proteinuria is present. This is after removal of any B2mg results from samples with pH of 5.5 or less, where it is widely acknowledged B2mg degradation occurs. Twenty-five percent and thirty percent of the urine samples for the cadmium-exposed and volunteer subjects respectively had urine pH of 5.5 or less. This represents a considerable number of urine samples which were not suitable for analysis. Alkalinization of urine has been suggested, but this ignores B2mg degradation within the variable period of time urine is within the bladder. The use of bicarbonate drinks prior to urine sampling has been suggested but this is an additional complication. RBP does not suffer from pH associated degradation in urine and shows in this study smaller intra-individual variability which suggests relatively better interpretation. The poorer variability for B2mg may reflect that there is not a simple cut-off of pH 5.5 below which degradation occurs. We suggest that RBP, or other stable low molecular weight protein such as alpha-1-microglobulin, should replace B2mg measurements as the indicator of renal proximal tubular dysfunction. Commercial assays are available for B2mg, RBP and alpha-1-microglobulin.

Subjects in this study were not restricted in diet or lifestyle. One volunteer subject who undertook a five-mile run prior to collection of a urine sample had an obviously raised urinary albumin and to lesser extent total protein. Intra-individual variability was calculated for total protein and albumin both with and without this sample showing exercise-induced glomerular proteinuria. No influence on RBP and B2mg was seen for this post-exercise urine sample. This data highlights the need for interpretation of urine protein results, especially urinary albumin, in the light of any information of recent strenuous exercise or history of postural proteinuria.

The concept of a maximum acceptable imprecision for an analyte based on the extent of intra-individual variation of the analyte has been widely used in clinical medicine, but has not been widely applied in the area of occupational and environmental medicine. We consider that maximum acceptable laboratory analytical imprecision for the four proteins are taken from the normal subjects and are in the order of 13–17% for creatinine corrected values. This should be readily achievable, but we urge caution that laboratory precision derived from quality control material may not reflect the true precision for real samples.

The data suggest that for our analytical precision changes in two serial creatinine corrected results greater than 73% for total protein, 85% for albumin and 110% for RBP would be needed in a cadmium-damaged individual to indicate a significant change in renal function. These represent considerable changes in measured protein concentrations in random, untimed urines. Whilst normal ranges

for these proteins will, if properly defined, have been derived reflecting the levels of biological variability we urge caution in interpretating isolated results or improvement or deterioration in renal function in an individual from a small series of results. The data in this report, although defined from both normal and subjects with cadmium-induced proteinuria, may be applicable to any situations where renal function testing is performed using random, untimed urine samples.

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