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BETA-2 MICROGLOBULIN AND OTHER URINARY PROTEINS AS AN INDEX OF CADMIUM-NEPHROTOXICITY

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One of the most detrimental effects of cadmium exposure pertains to the renal function. The tubular and/or the glomerular function will be affected resulting in an elevated excretion of low molecular mass and/or high molecular mass proteins respectively. Of these proteins, a few have been selected from each group. The relationships between protein concentration and cadmium concentration in kidney tissue, liver tissue and urine are discussed. Critical concentration values for each parameter are given. A preferred analytical method for the determination of the low molecular mass proteins is described and a recommendation is made as to which protein(s) should be determined.

INTRODUCTION

β_2-Microglobulin (β_2M) is commonly regarded as the most sensitive index of early detection of effects on the body due to excessive cadmium exposure (refs. 1-3). However, it has been questioned whether determination of this small molecular protein is the most reliable method of assessing cadmium exposure (ref. 4). At pH values below 5.5, β_2M degrades very rapidly at 37°C and it is likely that this degradation can be initiated in the bladder. This means that when decision is made to determine β_2M, then the pH must be controlled. This can be done immediately after urine collection, or preferably by ingestion of sodium bicarbonate and collection of the urine after several hours. As an alternative index retinol binding protein (RBP, molecular mass 21.4×10^3 daltons) can be determined, as this protein is stable in urine down to a pH of about 4.5.

SPECIFIC METHODS AND SEPARATION METHODS

Proteins such as β_2M can be determined by a specific method or by using a separation technique such as electrophoresis. The electrophoretic methods which have been used include iso-electrofocussing (ref. 5) and sodium dodecylsulfate polyacrylamide gel (SDS-PAGE)(ref. 6). The advantage of these separation techniques is that a "fingerprint" pattern can be derived so as to indicate the kind of kidney damage, i.e. tubular and/or glomerular. The disadvantage of separation methods is that they are labour-intensive and mainly qualitative (ref. 6).

Bernard *et al.* (ref. 6) found poor correlation between the area of the densitogram of SDS-PAGE and the amount of protein relative to creatinine: n=32, r=0.59, $(1-r^2)$=0.65. Contrary to this findings, Kammüller (ref. 7) found a good correlation between the area of the densitogram and protein concentration for a small number of samples: n=13, r=0.97, $(1-r^2)$=0.07, the concentration here however was expressed in the same units, while Bernard *et al.* (ref. 6) expressed their densitogram concentration in the uncorrected value of mg/l, but the protein concentration in a corrected mg/g creatinine. Kammüller (ref. 7) also found a good correlation between β_2M and RBP: n=7, r=0.97, $(1-r^2)$=0.06 when determined by SDS-PAGE. This was consistent with the findings of Bernard and Lauwerys (ref. 4) concerning the correlation between β_2M (radioimmuno assay, RIA) and RBP (latex immuno assay, LIA). The conclusion is that determination of β_2M with RIA can be compared with SDS-PAGE, provided both methods use the same units, i.e. either mg/l, *or* mg/g creatinine.

β_2-MICROGLOBULIN (β_2M)

The problem of the urinary pH has been mentioned already in the Introduction; for pH < 5.5 (sometimes the case with morning urine) it is not useful to determine β_2M. Such a sample should be rejected for β_2M determination. Before the next urine sample is taken from the same person, sodium bicarbonate may be given orally at least a few hours before. However, in screening industrial workers it is only practicable to collect spot urines. Since dilute urine can give rise to poor precision or even erroneous results, it is useful to determine the creatinine concentration or specific gravity, not only to correct for the dilution, but also to give a threshold value below which β_2M should not be determined. Thus a creatinine concentration of 0.7 g/l can be regarded as the threshold level for deciding whether or not

the urine can be analyzed for β_2M.

Since 1981, three methods have been available (Table 1) to determine β_2M - the widely used radioimmuno assay (RIA), latex immuno assay (LIA) according to Bernard *et al.* (ref. 8) and enzyme immuno assay (EIA).

Table 1

Analytical techniques for the specific determination of β_2M in urine (After Schaller (ref. 11))

Technique	Analytical procedure	Measurement instrument
radio-immuno-assay (RIA)	commercial available Phadebas-β_2 microtest (Pharmacia Diagnosis AB)	γ-counter liquid scintillation counter
enzyme-immuno-assay (EIA)	commercial available Phadezym-β_2 microtest (Pharmacia Diagnosis AB)	spectrophotometer
latex-immuno-assay (LIA)	not commercial available (ref. 8)	particle counter or spectrophotometer

Radioimmuno assay (RIA)

Principle of the method. Radioactive iodine bound to anti-β_2M competes with natural β_2M on Sephadex particles to which β_2M is bound. Measurement of the number of counts due to radioactive iodine complex bound to β_2M, expresses the competition between this compound and natural β_2M in the sample.

Critical aspects. Zainuddin *et al.* (ref. 9) tested several critical features of the RIA method. They concluded that the standard counting time of 3 min can be reduced to 1 min without loss of precision. However, the zero-effect of the blank must be taken into account. They also concluded that it is desirable to compute the S-shaped calibration curve instead of approximating by freehand drawing and they recommended that only the linear part of the curve should be used. Both procedures can lead to considerable improvement in precision and perhaps also in accuracy. Van der Sluijs Veer and Kennedy (ref. 10) developed a calibration curve program for RIA, based on the Simplex optimization method and using a simple desktop calculator. The procedure used a logit-log function, being a synthesis of an empirical and a theoretical model. Zainuddin *et al.* (ref. 9) concluded also that a three-fold washing procedure before counting was the best compromise between the amount of residual γ-activity on one hand and the instability of the RIA-complex on the other hand. Recoveries were somewhat higher than 100%, both in urine and serum. A dilution up to 1:400 could be used without affecting the recovery. A disadvantage of this method is the limited shelf life of the isotopically labelled material.

Latex immunoassay (LIA)

Principle of the method. The sample containing β_2M is incubated with latex particles to which an antibody is absorbed. The reaction of β_2M with the antibody results in agglutination of the latex particle proportional to the concentration of the protein. The density of the suspended particles can be determined either with a particle counter, or by measuring the absorbance with a spectrophotometer. To date, only one group of authors has experience with the method, the originators Bernard *et al.* (ref. 8). However, see Conclusions and Recommendations section below.

Critical aspects. The agglutination reaction depends on many variables and some critical features must be optimized. In particular the antibody loading has to be optimized for each antigen-antibody system. The stability of antibody-coated particles is another critical factor. Normally, agglutination appears to be due to protein-protein interaction but Bernard *et al.* (ref. 8) stated that this phenomenon can be prevented by saturating the latex particles with negatively changed albumin molecules. However, this can prevent the binding of proteins to the particles also. The stability of the latex particles is also strongly pH dependent: there is a rather broad optimum for pH > 9.6.
Urine must be diluted several times to achieve specificity.

Comparison of LIA with RIA

Bernard *et al.* (ref. 8) found a good correlation ($r=0.97$, $(1-r^2)=0.06$) and a fair proportional relationship (LIA=0.21 + 0.89 × RIA, (µg/l))between the LIA method and the RIA method for urine specimens. This means that according to the regression equation the difference between RIA and LIA is for higher values 11% (LIA < RIA) and for values near 1 µg/l the difference is about 0.2 µg/l (LIA > RIA). However, the experimental differences can be as high as 200 µg/l in the range 100-1000 µg/l. This discrepancy can be explained as the correlation and regression are

calculated for a very large range (from 10-10000) and the correlation coefficient is known to be a function of the range.
It should be stated that for the important range of 100-1000 µg/l the achieving of a new comparison is desirable. Bernard *et al.* (ref. 8) found for the within-assay precision of LIA for a concentration of 416 µg/l, 4.6% (RSD) and for a concentration of 442 µg/l this was 8.7% (RSD). The between-assay precision for a concentration of 171 µg/l was 10% (RSD). The main source of variability seems to be the reading of the particle counter: the precision of this apparatus was 5% (RSD) for a ten-fold replication.

Enzyme immuno assay (EIA)

Principle of the method. The principle is similar to that of RIA: an anti-β_2M complex solid phase bound to Sephadex competes with natural β_2M. This competition is measured by spectrophotometry by means of an enzymatic reaction using a dye complex.

Critical aspects. Schaller (ref. 11) indicated that this method tested on about 3000 samples, did not give rise to any problems. Here - as for RIA - a computer calibration should afford improvement in precision and accuracy.

Comparison of EIA with RIA. Carlier *et al.* (ref. 12) compared their EIA with RIA and found a correlation coefficient of r=0.97, $(1-r^2)$=0.06, n=29 and EIA = 0.81 × RIA + 49.5 (µg/l) for a range < 200-7000 µg/l. Thus they found lower values for EIA then for RIA. Ferrus *et al.* (ref. 13) found for their EIA-method compared to RIA: r=0.97, $(1-r^2) \sim 0.06$, n=95 and EIA = 0.82 × RIA + 2.47 (mg/l), range 200-217000 µg/l — clearly also too low.
Schaller (ref. 11) made a comparison between the two methods and found a somewhat lower detection limit for EIA. Between-assay precision was better for EIA, but the recovery was 10% too low; this gave rise to 50 µg/l higher values for EIA than for RIA. Therefore, it seems that there is a bias in the EIA method so that there is room for improvement.

Performance characteristics of RIA, LIA and EIA

Table 2 summarizes the characteristics of the three methods. The higher the detection limit of EIA is not a serious drawback, considering the upper limit of the reference range (200 mg/l).

Table 2

Performance and comparison of different methods for the determination of β_2M

	Test	Detection limit (µg/l)	Sensitivity (relative)	Precision (RSD, %)	Day-to-day precision (RSD, %)	Relative accuracy	Sampling handling
RIA	RIA > EIA	2	1	6.8	9.0	EIA = RIA+50 (ref. 11)	EIA > RIA > LIA
EIA		15		5	8.2	EIA = 0.81+4.9 (ref. 12)	
LIA	not commercial available	1 spectrophotometer 0.5 particle counter	2	4.6-8.7	∿10	EIA = 0.81RIA+2.4 (ref. 13) LIA ∿ 0.9 RIA	

However, there is an intrinsic bias in EIA, and its accuracy in the range 10-1000 µg/l should be studied. The two advantages of EIA are its relatively low cost and the fact that no radiochemical facilities are needed. The same holds true for LIA, and so far LIA seems to have the best characteristics as no bias is observed in contrast to EIA.

RETINOL BINDING PROTEIN (RBP)

Bernard *et al.* (ref. 14) determined RBP also by latex immunoassay, which in fact is the only routine method. They found a correlation coefficient of 0.99 between LIA and rocket immunoelectrophoresis (RIE) for the determination of RBP in 26 urine specimens and diluted 20 fold. The regression equation was LIA = 0.996 x RIE-0.061. These results can be regarded as excellent. However, the range covered was 0.1-100 mg/l and the important range from 0.1-10 mg/l needs to be studied in detail. The bias could be about 10 mg/l.

ALBUMIN, OROSOMUCOID AND OTHER PROTEINS

For the most proteins, e.g. albumin, there exists many clinical methods. Bernard *et al.* (ref.

15) developed a LIA method also for this protein and compared the LIA-method with a nephelometric method (ref. 16). They found a correlation coefficient of r=0.96 for 52 urine specimens. Mostly the proteins also can be determined by electrophoretic or electrofocussing methods.

SIGNIFICANCE OF PROTEIN DETERMINATION

Before investigating proteins, a choice must be made *which* protein(s) is (are) to be determined and *how* to interpret the concentration(s) observed. As already indicated, from the analytical point of view, it is possible either to determine a "fingerprint" pattern with a separation technique like electrophoresis or to use a specific technique to determine a simple protein. Which protein is to be determined depends on the clinical circumstances or the kind of study being undertaken.

THE CHOICE BETWEEN LOW MOLECULAR MASS PROTEINS OR HIGH MOLECULAR MASS PROTEINS

The Belgian group has stated that the effect of Cd on the kidney, formerly regarded as directed exclusively to the tubulus, also has effects on the glomerulus, even in the case of low levels of exposure (refs. 17-19). The predominance of low molecular mass protein (LMM) such as β_2M, RBP (α_2-macroglobulin) lysozyme, ribonuclease, post-protein and the light chains of immunoglobulin, is characteristic of a molecular type of effect. These proteins are incompletely reabsorbed by the proximal tubuli after filtration from plasma and subsequently occur in the urine. However, high molecular mass (HMM) proteins such as orosomucoid, immunoglobulin, albumin, and transferrin (MM > 40000 daltons) can occur in urine also, suggesting that Cd may affect the glomeruli in so far as these regulate HMM proteins.

In one study of cadmium workers, Lauwerys *et al.* (ref. 17) found that the prevalence of increase in relative clearance of β_2M was no greater than the increase in relative clearance of HMM proteins. They also found that an increase in the relative clearance of β_2M was not necessarily associated with an increase in those of the HMM proteins. The mechanism of this glomerular type of proteinuria might be an autoimmune one. An important conclusion is, that although the excretion of β_2M proportionally is more increased than the excretion of HMM proteins, the HMM remain the most important indicator of proteinuria induced by Cd.

CADMIUM METALLOTHIONEIN (Cd-Mt)

Another low molecular mass protein occurring in the urine of Cd-workers is Cd-metallothionein and this compound may be determined instead of or in addition to proteins such as β_2M and RBP.

Chang *et al.* (ref. 20) described a sensitive radioimmunoassay method for human Cd-metallothionein utilizing an antibody developed from a purified rat Cd-binding protein. They stated that Cd-binding protein may play a role in the pathogenesis of renal dysfunction associated with Cd poisoning. The method was applied by Chang *et al.* (ref. 21) who found a very good correlation (r=0.94) between the logarithms of the urinary Cd-excretion and Cd-metallothionein in urine. However, it is difficult to draw a solid conclusion from this paper, because the correlation between log CdU and log Cd-MtU has been calculated from one set of data combining three statistically different populations. These findings suggest that Cd-MtU is simply another measure of CdU which is consistent with Cousins' hypothesis (ref. 22) that after degradation of the polypeptide chains of CdMt, the liberated Cd-ions quickly are incorporated into nascent chains of thionein. This explains why the Cd content of liver and kidney increases with age and environmental exposure (see below). In the author's opinion, Cd-MtU is nothing more than CdU, derived from the kidney tissue.

RELATIONSHIP OF URINARY PROTEINS TO Cd IN THE KIDNEY

Hansen *et al.* (ref. 5) considered that the urinary excretion of protein does not only mean that the corresponding amount of protein is lost from the body, but that this may also very well affect the statistical lifespan of the protein in the body. Because of the complicated mechanisms involved in excretion, regulation and feedback, it is not easy to evaluate the consequences to health of the loss of a certain amount of protein. However, any increase in urinary protein excretion should be considered with concern. The aim should be to prevent the onset of malfunction such as renal tubular damage among Cd-exposed workers.

Brown *et al.* (ref. 23) pointed also to the problem of establishing the critical concentration of Cd in the kidney cortex. The factors related to elevated levels of Cd in human tissues in various groups of individuals should be identified, e.g. smoking, sex, age. The extrapolation of a critical cortical concentration of Cd from animal data is dubious: thus between the proposed maximal concentration of about 200 µg Cd/g in renal cortex proposed by the WHO Task Group (ref. 24) and a level, say, of 50 µg Cd/g one cannot confidently exclude all adverse health effects. Disease patterns in subjects with concentrations below 200 µg/g need to be established. Moreover Kazantzis (ref. 25) in a follow-up study of 12 workers who also had been investigated in 1962, found that they may continue symptom-free for long intervals, but in a proportion of cases serious clinical effects may develop after a number of years. He stated that proteinuria is usually only one aspect of a more generalized renal tubular

dysfunction. On the contrary, Ellis *et al.* (ref. 26) stated that a significant number of workers with normal urinary β_2M had kidney Cd values of 20-200 µg/g cortex without clinical or biochemical evidence of renal dysfunction. Table 3 summarizes the suggested critical cadmium concentrations in kidney cortex *before* in vivo neutron activation methods were developed. After the introduction of the in vivo method, the situation became somewhat more complex. The in vivo technique involves a radioactive neutron source, placed at an adequate distance from the liver or kidney, and a flux monitor for control and dosimetry. The cadmium in liver or kidney is activated by the neutrons and will emit radiation which is counted at 90 degrees to the source; this radiation is a measure of the amount of cadmium in liver and/or kidney. Ellis *et al.* (ref. 26) assumed that there was a non-linear relationship between the Cd concentration in the kidney and the Cd concentration in the liver, because of a kind of overflow; they concluded that this non-linear relationship was composed of two linear curves: one corresponding to the lower concentrations of Cd in kidney and in liver, with an inflection point at liver concentrations about 31 mg Cd, above which the Cd kidney concentration slowly decreases with increasing Cd in liver. Taking account of this value of 31 mg Cd

Table 3. Range of critical Cd concentrations in kidney cortex

Concentration (µg/g net weight)	Author
100 - 300	WHO Task Group (ref. 24)
380 - 470 (monkeys)	Nomiyama *et al.* (ref. 32)

Table 4. Proteinuria as a function of Cd in liver and renal cortex (ref. 28)

N	Liver concentration range (µg/g)	Cd in renal cortex (µg/g)	Prevalence* of proteinuria
16	0 - 25	< 50 - 100	
36	25 - 50	60 - 300	$\frac{9}{36} \approx 25\%$
25	> 50	100 - 300	$\frac{14}{25} \approx 56\%$

* Definition of prevalence:
total protein > 250 mg/g creatinine and/or β_2M > 200 µg/g creatinine, and/or albumin > 12 mg/g creatinine

in the liver, Ellis *et al.* (ref. 26) calculated a critical concentration from 319 ± 90 µg/g in the kidney cortex. In fact however, the deviation in their relation is rather large - 15-50 mg around the inflection point at 31 mg - and this means a critical kidney cortex concentration of 150-500 µg/g, which overlaps the WHO Task Group estimates. They also stated that a significant number of workers with normal urinary β_2M had kidney Cd concentrations above 200 µg/g cortex without clinical or biochemical evidence of renal dysfunction.

The other group who used the in vivo activation method was Chettle *et al.* (ref. 27) in collaboration with Roels *et al.* (ref. 28). Their findings for the critical kidney concentrations were lower. Roels *et al.* (ref. 28) evolved a three phase model for the relationship between Cd in kidney cortex and the years of past exposure to Cd. They supposed that renal cortical Cd decreases progressively after the onset of kidney damage. After the occurrence of tubular dysfunction, Cd stored in the kidney is lost, whereas Cd in liver remains unchanged. They stated that renal dysfunction will develop around a renal cortical Cd concentration of 285 µg/g. From their data, Table 4 can be derived.

Table 4 confirms in part the findings of Ellis *et al.* (ref. 26) - that it is possible on the one hand to find cortical Cd concentrations above 200 µg/g without signs of proteinuria, but on the other hand, for lower values of about 100 µg/g, proteinuria may indeed be found. This suggests that the cortical concentration of Cd in renal cortex is in the range 50-100 µg/g, in agreement with Brown *et al.* (ref. 23).
One qualifying remark, however, should be made: it is possible that the concentrations of Cd in this lower region are the result of Cd loss due to renal damage with concomitant proteinuria. In this situation it is not the cortical concentration which is a useful indicator, but rather the Cd-in-liver concentration. A "safe" limit, then, would be 20-25 µg/g Cd in the liver, which is consistent with the prevalence of proteinuria found by Roels *et al.* (ref. 28) and summarized in Table 5.

Table 5

Relationship between liver Cd burden, prevalence of β_2-microglobulinuria and urinary excretion of β_2M in a group of 153 workers from Zn-Cd smelters with hepatic Cd $\geq$ 10 µg/g and renal cortical Cd $\geq$ 50 µg/g (ref. 28)

N	Cd liver (µg/g)	Mean β_2M (µg/g creat.)		Prevalence β_2M > 200 µg/g creat.	
		abnormal	normal	n	%
54	10 - 19	-	53	0	0
27	20 - 29	7299	55	1	4
28	30 - 39	279	68	3	11
18	40 - 49	1424	73	3	17
8	50 - 59	6995	118	2	25
5	60 - 69	4889	76	2	40
13	70 -160	6447	-	8	100

RELATIONSHIPS OF URINARY PROTEINS TO Cd IN URINE AND BLOOD AND RELATIONSHIP TO AGE

Kjellström *et al.* (ref. 29) found increased prevalence of β_2M excretion as a function of duration of employment. Smokers had about three times higher prevalence than non-smokers. Tsuchiya *et al.* (ref. 30) measured the faecal excretion of Cd in faeces (CdF) in relationship to urinary excretion of β_2M. For different groups - general population; in a Cd polluted area; Cd alloy workers - a significant correlation for CdF vs β_2M was found; however for β_2M vs age there was a higher correlation as shown in Table 6. They implicated both Cd exposure and age in the increase of β_2M, with age being the more important factor. They stated also that there might not be a "no-effect level" of Cd-exposure in respect of urinary β_2M concentration.

Table 6

Relationships respectively between β_2M, age, and cadmium in faeces (CdF) (after ref. 30)

Partial correlations	Polluted area N=107 r	Control N=70 r	Total N=177 r
β_2M vs CdF and age	0.21	0.13	0.28
β_2M vs age and CdF	0.34	0.42	0.35

Lauwerys *et al.* (ref. 17) found that when there is frank excretion of either high or low molecular mass proteins, or both, the urinary Cd excretion usually exceeds 10 µg/g creatinine and CdB exceeds usually 10 µg/l. Proteinuria is mainly observed in workers who have been exposed for more than 25 years. In agreement, Travis and Haddock (ref. 31) stated that an age-dependent biological half-life for Cd is a necessary consequence of the assumed renal damage.

CONCLUSIONS AND RECOMMENDATIONS

- Urinary β_2M and RBP can be used as sensitive indices of early tubular kidney damage. Both methods need standardization; a reference method for each is desirable. Such a method could be the latex immuno-assay method of Bernard *et al.* (ref. 8). RBP is a more suitable index for practical reasons: β_2M degrades in the bladder at pH < 5.5. However, for several years both methods should be used in parallel, to compare the results of RBP with former β_2M studies.

- Together with an index of tubular kidney proteinuria, albumin, orosomucoid or immunoglobulin in urine should be determined as an index of glomerular kidney proteinuria, as glomerular damage may happen, independently of the tubular lesion.

- For continuing studies in depth, the whole urinary protein pattern should be determined in in a qualitative and quantitative way using a separation method like electrophoresis or

electrofocussing.

- The determination of Cd-metallothionein gives no information additional to the determination of Cd in urine and can be regarded as an alternative to this assay.

- Proteins and Cd in urine should be corrected for dilution by means of the creatinine concentration.

- The Cd concentration of the liver is a more suitable indicator of renal damage than the renal Cd concentration itself. A proposed limit for the Cd in liver concentration will be 20-25 µg/g. Here the prevalence of proteinuria is low. The corresponding renal cortex Cd concentration is : 50-100 µg/g.

- Proteinuria is related to age and duration of exposure. A critical concentration for β_2M and RBP is: 200-300 µg/g creatinine. This value however, should be considered together with the Cd in urine value, which should be < 10 µg/g creatinine, and the Cd in blood value, which should be < 5 µg/l in individual workers. Cd in blood, however, is also related to smoking. For Cd in urine, an important index could be the change in concentration as a function of exposure. A fall in concentration means a diminishing exposure; a rise indicates a constant exposure or growing exposure.

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