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Enzyme-immunoassay for the determination of metallothionein in human urine: application to environmental monitoring

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The objectives of this study were to develop an enzyme immunoassay for metallothioneins in human urine using a polyclonal antiserum and to demonstrate a possible relationship between the level of this biomarker and heavy metal exposure. The antiserum was raised in sheep against horse metallothionein conjugated to carboxylated bovine serum albumin. The antibody was used to construct a two-step competitive ELISA procedure. Human urine was treated with activated charcoal powder to remove traces of metallothioneins and known amounts of pure metallothioneins were added to provide standards for a standard curve. Metallothionein levels were measured in two groups of children living in areas of mild and high environmental pollution due mainly to heavy metals. A comparison was made between the biomarker levels and the levels of cadmium and lead in urine samples in the two groups. A group of children from a non-polluted area acted as controls. The results show that the detected levels of metallothioneins appear to correspond to levels of the two heavy metals studied and that there was an apparent relationship to the environmental exposure. Thus according to results of this study the increase in the metallothionein excretion seems to provide an indication of previous of exposure to metals. The ELISA procedure is sensitive and robust and can be used to screen large numbers of samples and is more rapid than the physical procedures currently used for analysis of these proteins. The assay can therefore be used as an additional tool for screening at-risk populations where either environmental or occupational exposure to divalent heavy metals is suspected.

Keywords: metallothioneins, biomarkers, enzyme immunoassay, children, heavy metals.

Introduction

The ability of living organisms to cope with reactive heavy metal ions in the environment and to utilize them in metabolism is essential to life (Kagi and Schaffer 1988). Metallothioneins are low-molecular weight, highly conserved, cysteine-rich proteins with high affinity for nonessential and essential metal ions. This family of proteins is involved in many physiological systems in addition to metal homeostasis including cell proliferation, apoptosis and protection against oxidative damage (Janssen *et al.* 2002). Synthesis of metallothioneins can be induced by various stimuli such as cadmium, mercury, zinc, glucocorticoids

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and anticancer agents (Sato and Kondoh 2002). When renal function is normal the proximal tubular epithelial cells reabsorb the majority of the filtered metallothioneins and only a small fraction is excreted in urine. Exposure to low levels of lead can result in renal damage (Fels *et al.* 1998) while cadmium exposure affects the proximal tubular cells (Roels *et al.* 1993). A range of different biomarkers are now available for monitoring the effect on kidneys following exposure to metals (Mueller *et al.* 1998). However, the measurement of metallothioneins in urine has potential as a useful biomarker tool since they are ubiquitous proteins and therefore can be studied in most living organisms (Geffard *et al.* 2002).

Metallothionein determination has many advantages over the direct measurement of metals in urine and serum for monitoring human exposure to environmental contamination and in occupational health. Although metallothioneins can be determined by high pressure liquid chromatography (Baranowska *et al.* 2002), immunological methods are more sensitive (Summer and Klein 1991, Tang *et al.* 1999) and would provide a more convenient analytical tool for high throughput screening and for use by a wider range of investigators. Radioimmunoassay (Shaikh 1991) is a protracted procedure and it also has the disadvantage of the incorporation of radioisotopes. Enzyme-immunoassay methods have the advantage of sensitivity and of ease of performance (Cousins 1991) particularly when large numbers of samples need to be processed.

A fundamental problem with analysis of components present in biological fluids is the effect of the complex and variable mixture of proteins, carbohydrates, lipids, urea, and small molecules and salts also present in the sample. The effect of these components on analytical techniques is termed the "matrix effect" and may seriously affect the measurement (Selby 1999). The most appropriate biological matrix in which to determine MT concentration is still under discussion (Mourgaud *et al.* 2002). Urine sampling is convenient and reflects levels of target metallothioneins as well as conditions of the kidney. However, it presents some disadvantages including dilution factor issues and possible deterioration of target analytes if storage conditions are not adequate (Tang *et al.* 1999).

In the present study an immunoassay procedure for human metallothioneins was developed using animal metallothioneins which are widely available in pure form from commercial sources. The assay was initially based on the determination of standards in buffer systems and then modified to use analyte reference standards in metallothionein-free human urine. In addition to the construction of the ELISA, the potential use of urine metallothioneins as a biomarker of exposure to metals was demonstrated by showing a possible relationship between urine levels of the biomarker proteins and living in areas where metal pollution was high. The immunoassay for human metallothioneins can be used in conjunction with other analytical procedures currently in use in monitoring and rapid screening programmes.

Materials and methods

Horse kidney and rabbit liver metallothioneins were obtained from Sigma-Aldrich Co Ltd., which according to the supplier had been purified by ion-exchange chromatography and contained about 5%

Table 1. Comparison of amino-acid sequences of human, horse and rabbit metallothioneins prepared using the Wisconsin Package program 'pileup' (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA).

	1						69
mt3_human	MDPETCPCPS	GG . SCTCADS	CKCEGCKCTS	CKKSCCSCCP	AECEKCAKDC	VCKGGEAAEA	EAEKCSCCQ
mt3_horse	MDPETCPCPT	GG . SCTCSGE	CKCEGCKCTS	CKKSCCSCCP	AECEKCAKDC	VCKGGEGAEA	EAEKCSCCQ
q8tdc4	MDP . NCSCAT	. GVSCACTGS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAHGC	VCEGA	. LEKCNCYA
q8tdn3	MDP . NCSCTT	. GVSCACTGS	CTCKECKCTS	CKKSCCSCCP	VGCAKCAHGC	VCKGT	. LENCSCCA
mt2a_rabit	MDP . NCSCAA	AGDSCTCANS	CTCKACKCTS	CKKSCCSCCP	PGCAKCAQGC	ICKGA	. SDKCSCCA
mt2c_rabit	MDP . NCSCAT	AGDSCTCANS	CTCKACKCTS	CKKSCCSCCP	PGCAKCAQGC	ICKGA	. SDKCSCCA
mt1h_human	MDP . NCSCEA	GG . SCACAGS	CKCKKCKCTS	CKKSCCSCCP	LGCAKCAQGC	ICKGA	. SEKCSCCA
q9bxg3	MDP . NCSCAA	GG . SYACAGS	CKCKKCKCTS	CKKSCCSCCP	LGCAKCAQGC	IRKGA	. SEKCSCCA
mt1l_human	MDP . NCSCSP	VG . SCACAGS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGT	. SDKCSCCA
q8wvb5	MDP . NCSCSP	VG . SCACAGS	CKCNECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGT	. SDKCSCCA
mt1a_rabit	MDP . NCSCAT	. GNSCTCASS	CKCKECKCTS	CKKSCCSCCP	AGCTKCAQGC	ICKGA	. SDKCSCCA
mt2b_rabit	MDP . NCSCAT	. GDSCTCASS	CKCKECKCTS	CKKSCCSCCP	AGCTKCAQGC	ICKGA	. SDKCSCCA
mt2d_rabit	MDP . NCSCAT	. RDSCACASS	CKCKECKCTS	CKKSCCSCCP	AGCTKCAQGC	ICKGA	. SDKCSCCA
mt2e_rabit	MDP . NCSCAT	. RDSCACASS	CKCKECKCTS	CKKSCCSCCP	AGCTKCAQGC	ICKGA	. LDKCSCCA
mt2_human	MDP . NCSC . A	AGDSCTCAGS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGA	. SDKCSCCA
q8te66	MDP . NCSC . A	AGDSCTCAGS	CKCKECKCTS	CKKSCCSCCP	MSCAKCAQGC	ICKGA	. SEKCSCCA
mt1g_human	MDP . NCSC . A	AGVSCTCASS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGA	. SEKCSCCA
mt1i_human	MDP . NCSC . A	AGVSCTCAGS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGA	. SEKCSCCA
mt1f_human	MDP . NCSC . A	AGVSCTCAGS	CKCKECKCTS	CKKSCCSCCP	VGCSKCAQGC	VCKGA	. SEKCSCCD
mt1k_human	MDP . NCSCAA	AGVSCTCASS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	ICKGA	. SEKCSCCA
mt1b_horse	MDP . NCSC . V	AGESCTCAGS	CKCKQCRCAS	CKKSCCSCCP	VGCAKCAQGC	VCKGA	. SDKCSCCA
mt1e_human	MDP . NCSCAT	GG . SCTCAGS	CKCKECKCTS	CKKSCCSCCP	VGCAKCAQGC	VCKGA	. SEKCSCCA
mt1r_human	MDP . NCSCAT	GG . SCSCASS	CKCKECKCTS	CKKSCCSCCP	MGCAKCAQGC	VCKGA	. SEKCSCCA
mt1a_human	MDP . NCSCAT	GG . SCTCTGS	CKCKECKCNS	CKKSCCSCCP	MSCAKCAQGC	ICKGA	. SEKCSCCA
ctc7_human	MDP . NCSCTT	GG . SCTCAGS	CKCKECKCTS	CKKSCCSCCP	MGCAKCAQGC	VCKGA	. . . . CSCCV
mt1a_horse	MDP . NCSCPT	GG . SCTCAGS	CKCKECRCTS	CKKSCCSCCP	GGCARCAQGC	VCKGA	. SDKCSCCA
mt1b_human	MDP . NCSCTT	GG . SCACAGS	CKCKECKCTS	CKKCCCSCCP	VGCAKCAQGC	VCKGS	. SEKCRCCA
mt4_human	MDPRECVCMS	GGI . CMCGDN	CKCTTCNCKT	CRKSCCPCCP	PGCAKCARGC	ICKGG	. SDKCSCCP

cadmium and zinc. Animal metallothionein was used to prepare antibodies as they have close homology to human metallothionein (table 1) since human metallothionein is unavailable commercially.

Bovine serum albumin (BSA), rabbit anti-sheep-horse radish peroxidase conjugate, porcine gelatine, glutaric anhydride, ABTS dye, buffer reagents, activated Norit A charcoal powder, hydrogen peroxide solution (30%), carbodiimide (CMC), Tween 20, Thimerosal, and MOPS were obtained from Sigma-Aldrich Co. Ltd. Microtitre plates (high bind) were from Greiner Bio-One Ltd., Gloucestershire, UK.

Random urine samples were obtained from three groups of children located in different geographical areas in Germany and Poland. Three areas were selected-a rural environment with little possibility of exposure to industrial pollution. (Group 1, 19 children), a semi-industrial environment (Group 2, 28 children) and a heavily industrialized area (Group 3, 46 children). The children's ages ranged from 6 to 14 years (mean 9.9 ± 0.4). A detailed questionnaire concerning life-style, previous disease, intake of drugs and further relevant information was answered by parents and children in order to eliminate the possibility of earlier exposure to nephrotoxins or the presence of renal disease. Approval had been obtained for the study from the appropriate Ethical Committees at the Silesian Technical University, Gliwice and King's College London, University of London. Samples were frozen immediately at $-20^{\circ}C$ until required for assay, samples were not refrozen.

Generation of the metallothionein polyclonal antiserum

An antiserum to metallothionein was generated in sheep by immunizing with a conjugate of horse MT-BSA complex. The conjugate was prepared by coupling the metallothionein to carboxylated BSA.

Preparation of carboxylated BSA

Glutaric anhydride (0.5 g in 10 ml of dimethylformamide) was added to a stirred solution of ice-cooled BSA (500 mg in 30 ml 0.1M NaOH and 20 mL dimethylformamide containing 6M urea) over a 30-min period while maintaining the pH above 11 with 5M NaOH. After mixing for 1 h, the solution was dialyzed against water (4 l) for 72 h, changing the dialysis water every 24 h. The carboxylated-BSA protein content was estimated by UV absorbance (1 mg/ml gives absorbance value of 0.65), adjusted to 5 mg/ml and stored at $-20^{\circ}C$.

Coupling of metallothioneins to carboxylated-BSA

MOPS (120 mg) was added to carboxylated-BSA (10 mg in 2 ml of water) and after cooling on ice, CMC (100 mg) was added, and after mixing the reaction mixture was left to stand on ice for 15 min. The CMC-activated carboxylated-BSA preparation (appeared turbid at this stage) was added slowly with mixing to a solution of horse MT (1 mg in 4 ml of 3% $NaHCO_3$). After standing for about 2 h, the conjugate reaction mixture was dialyzed against 50 mM $NaHCO_3$ containing 10 mM zinc sulphate. The conjugate solution (immunogen) was divided into several aliquots, freeze-dried and stored at $4^{\circ}C$.

Immunization

The immunization, collection of blood and sera was carried out by MicroPharm Ltd., Llandysul, Wales, UK. About 1.7 mg of immunogen complex was mixed with complete Freund's adjuvant and injected into one sheep subcutaneously in four sites. Boosting injections were given at four weekly intervals (incomplete adjuvant) for 24 weeks. Collected sera samples were stored at $-20^{\circ}C$ till use.

Titration of metallothionein antiserum

Antibody binding to metallothioneins was assessed by antibody dilution response analysis using a microtitre plate solid phase ELISA procedure. Rabbit metallothionein solution (range of 250 ng/ml) in 50 mM $NaHCO_3$ was used for coating plates (150 µl/well) for 16 h at $4^{\circ}C$. After washing the plates three times with 50 mM $NaHCO_3$ containing 0.1M NaCl and 0.05% Tween 20, the wells were blocked for 50 min at laboratory temperature with 0.2% (w/v) of porcine gelatine (250 µl/well) in washing buffer containing 0.01% thiomersal. After washing the blocked plates (four times), 150 µL of serially diluted antibody (1/1000 to 1/6000) in assay buffer (20 mM sodium phosphate buffer, pH 7.4, containing 0.1M NaCl, 0.2% gelatine, and 0.01% thimerosal) was added to duplicate wells and allowed to react for 60 min at laboratory temperature. The wells were washed four times with buffer, rabbit-anti-sheep-HRP (150 µL of (1/1500 in assay buffer)) was then added and the plates incubated at $37^{\circ}C$ for 60 min. Horse radish peroxidase was assayed using ABTS-H_2O_2 substrate solution (1 mg/ml in 50 mM sodium acetate/citrate buffer pH 4.1, containing 40 µL of H_2O_2 per 100 ml), and the absorbance at 405 nm was measured after 30 or 45 min in a plate reader (Anthos 2001, Anthos Labtec, Austria).

Preparation of analyte-free urine matrix

Pooled urine from normal healthy individuals ($n=5$) was used to prepare metallothionein-free (analyte free) urine sample matrix. Pooled urine was mixed with 0.5% (w/v) activated charcoal powder for 2 h at 4°C to remove organic materials and low molecular weight proteins, centrifuged at 3000 rpm for 30 min and the clear supernatant was collected and stored at −70°C. The analyte-free urine was analysed with SDS-PAGE and Western blotting to confirm that all detectable metallothionein proteins had been removed. The analyte-free urine was then used for the preparation of metallothionein calibration standards.

Two-step competitive ELISA

A two-step competitive ELISA procedure was developed for the measurement of metallothioneins in human urine. Wells in the microtitre plate were coated with rabbit metallothionein (rabbit 250 ng/mL, excluding outside rows) and blocked as described. Metallothionein standards (0.001–10 μg/ml) of rabbit metallothionein in charcoal treated urine) or samples (150 μl) were added to test tubes containing 450 μl of assay buffer (diluent), followed with 300 μl of primary antibody solution (1/1000) and the mixture was incubated for 1 h at 37°C. Aliquots of assay mixtures were transferred to duplicate wells of Metallothionein-coated assay plate and left standing at room temperature for 1 h before washing and signal development steps were carried out as described. Duplicate assays were carried out on two different days.

Statistical analysis

Data was log transformed before statistical analysis by one-way analysis of variance ANOVA using Tukey's method. Minitabs Statistical software (13 for Windows) was used for this analysis.

Other methods

Creatinine content of the urine samples was determined by the Jaffe modified procedure (Bonses and Taussky, 1945), SDS PAGE and immunoblotting was carried out as described previously (Slade *et al.* 1996). Lead and cadmium in urine were measured by electrothermal graphite furnace atomic absorption spectrophotometry using a Perkin-Elmer Zeeman 3030 atomic absorption spectrometer (Fels *et al.* 1998). Standards were supplied by Nycomed (Garching, Germany). Heavy metal analysis was carried out in the Centrum Laboratorium, Toksyklolgii Etali Ciezkich, Miasteczko, Slaskie, Poland).

Results and discussion

The coupling of metallothionein protein to carboxylated BSA is expected to link metallothionein via amino groups (table 1) and possibly (but less preferably) thiol groups. The 2-step coupling method was used despite the inefficiency of carbodiimides in aqueous conditions, to ensure that homogenous conjugates of predicted coupling site are made. The strategy was to avoid linkages with metallothionein thiol groups which are essential to retaining the metal ion ligands in place. The antibody response (titre of 1/2000) was relatively weak despite the repeated boosting injections; however, the antiserum enabled a sufficiently sensitive assay to be developed. The competitive assay format was selected because it requires only one antibody; it is more specific than alternative usage and does not require the removal of antibodies to the carrier protein.

The calibration curve for the determination of metallothioneins in urine was linear over the range (0.05–10 μg/ml metallothionein (figure 1). The day-to-day variation was within 3–5%. The availability of a metallothionein-free human urine matrix was an essential component of the assay enabling a standard curve to be constructed under conditions closely related to those of unknown samples. Calibrations standards were included in each assay plate throughout the study. In view of the high degree of homology both among metallothioneins of individual species and between animal and human proteins resulting from conserved

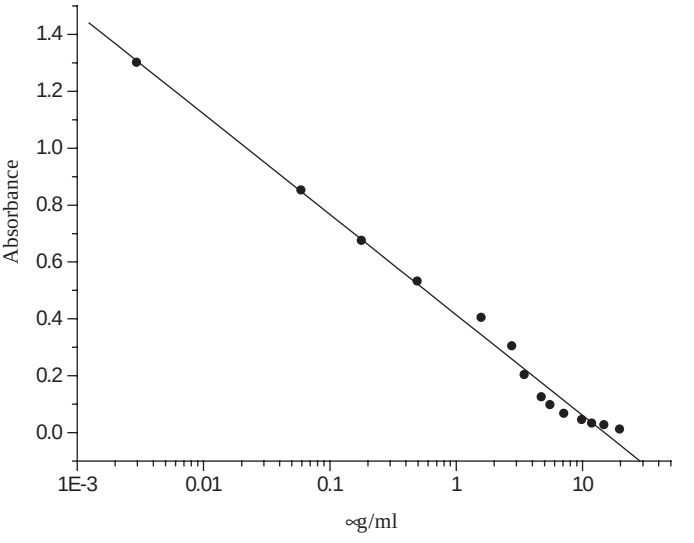

Figure 1. A standard curve generated from rabbit liver Metallothionein.

regions (Kagi and Hunzikert 1989, Norey *et al.* 1990, Koizumi *et al.* 1993, Miles *et al.* 2000, Coyle *et al.* 2002 and Boutet *et al.* 2002) it is entirely reasonable to expect that antisera raised to metallothioneins would exhibit high cross reaction levels to isoforms from the same species as well as other animals (Kojima *et al.* 1979, Winge and Garvey 1983, Kikuchi *et al.* 1990 and Leibrandt *et al.* 1991). A comparison of published amino-acid sequences confirmed the high degree of homology between human, horse and rabbit metallothioneins (table 1). In this study the antiserum generated with horse metallothionein was expected to show high levels of cross reactivity to both the rabbit and human proteins. Therefore the chosen strategy of using animal metallothionein proteins to generate the antiserum and construct an assay for human proteins is practical and overcomes the difficulties of obtaining preparative quantities of pure human metallothioneins. The competitive assay consisted of having pure rabbit metallothionein adsorbed on to assay wells (a competitor antigen) without carrier proteins. Having pure unconjugated metallothionein as the competitor antigen eliminates interference by antibodies to albumin in the competition step. It is therefore unlikely that albumin in urine samples would interfere with the assay response.

Metal levels present in the three groups

Urine samples from children living in an area with low exposure to metals (Group 2) had blood lead levels <10 μg/100 ml. The group who lived in an area exposed to high levels of metal contamination (Group 3) had blood lead levels >20 μg/100 ml. In view of the absence of comparable data in the literature it isn't possible to make comparison with other studies at present.

Table 2. Comparison of cadmium, lead and metallothionein excretion in exposed and control group expressed as mean and standard deviation. Units are μg/creatinine.

Group	N	Cd/gCreatinine	Lead/gCreatinine	Metallothionein/gCreatinine
1	19	0.13±0.40	3.45±0.42	2.94±0.36
2	28	0.12±0.40	3.35±0.37	12.85±0.51
3	46	1.10±0.53	29.69±0.5692	30.18±0.67

The cadmium, lead and metallothionein content of the samples obtained from the three groups of children were compared. The level of cadmium and lead excretion was measured by HPLC and metallothionein by the procedure described above. As random samples were used all the values were factored with creatinine to allow for variations in urine flow. The mean and standard deviation of cadmium and lead contents in each of the three groups are given in table 2 and compared in the interval plots shown in figures 2 and 3, respectively. There was only a small difference in the values found in the Control Group (Group 1) and the mildly exposed group (Group 2). However, they were significantly increased in the highly exposed group 3. The metallothionein content was found to increase when Group 1 and 2 were compared (table 2 and figure 4). Again a major increase in its concentration was observed in the heavily exposed group (Group 3). It is interesting to note that the metallothionein values increased stepwise from Group 1 to Group 3 whereas no significant differences were observed between Groups 2 and 3 when the metal content was analysed (figures 2 and 3).

The two-step competitive ELISA was developed for the quantitative determination of metallothionein in urine samples. It proved to be robust easy to perform and highly sensitive. The absence of detectable metallothioneins in the matrix was demonstrated with the ELISA procedure as well as by SDS-PAGE/Western blotting prior to its use. The ELISA method can be used for determination of

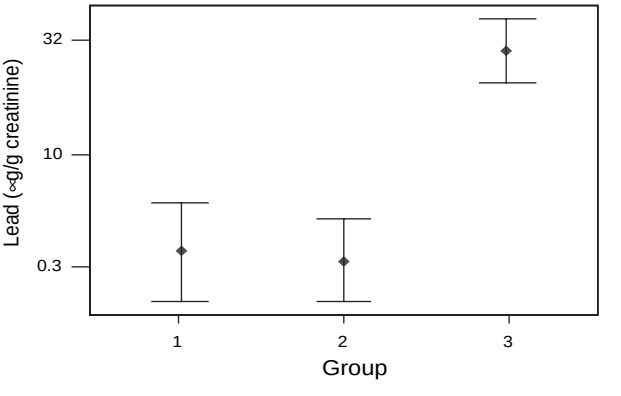

Figure 2. Interval plot showing 95% confidence limits comparing urinary lead concentration in the three groups. One-way ANOVA using Tukey showed no significant difference between Groups 1 and 2 but Group 3 was highly significantly different ($p < 0.0005$).

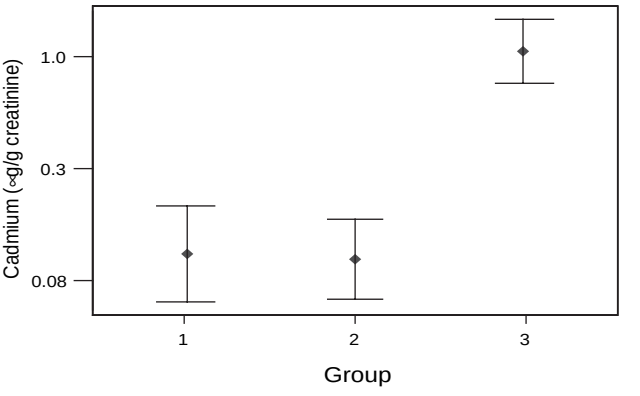

Figure 3. Interval plot showing 95% confidence limits comparing urinary cadmium concentration in the three groups. One-way ANOVA using Tukey showed no significant difference between Groups 1 and 2 but Group 3 was highly significantly different ($p < 0.0005$).

very low concentrations of metallothioneins in biological fluids. The method allows the throughput of larger numbers of samples than HPLC (Baranowski *et al.* 2002) and avoids the problems encountered by radioimmunoassay (Shaikh 1990, 1991). The availability of a sensitive, practical and cost-effective screening method for the quantification of metallothioneins in urine will add a useful tool to the monitoring of exposure to heavy metals in particular cadmium, zinc, copper and lead in polluted areas. This is particularly important in view of the need to monitor exposure of children living in or near industrial areas. The results of this study demonstrated the usefulness of the urine metallothionein ELISA and showed a correlation between heavy metal levels and history of living in known polluted environment.

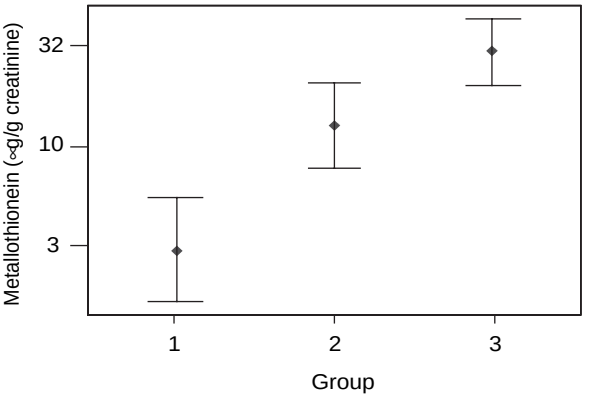

Figure 4. Interval plot showing 95% confidence limits comparing urinary metallothionein concentration in the three groups. One-way ANOVA using Tukey showed that all three Groups were significantly different ($p < 0.0005$).

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