Measurement of Urinary Retinol-Binding Protein by Enzyme-Linked Immunosorbent Assay, and Its Application to Detection of Tubular Proteinuria

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An enzyme-linked immunosorbent assay (ELISA) for urinary retinol-binding protein (RBP) has been developed and compared with urinary $\beta_2$-microglobulin for the detection of tubular proteinuria. The assay has a working range of 10 to 250 $\mu$g of RBP per liter of urine. The within-assay CV was 3.2–7.1%, the between-assay CV 12.5%. A control population of 118 male subjects gave a geometric mean urinary RBP concentration of 7.7 $\mu$g per millimole of creatinine and a 95th centile of 22 $\mu$g per millimole of creatinine. Comparison of urinary RBP and $\beta_2$-microglobulin concentrations in 80 control subjects and 117 subjects exposed to cadmium fumes gave correlations of $r$ = 0.59 and 0.91, respectively. Of the 117 subjects exposed to cadmium fumes, 103 gave both RBP and $\beta_2$-microglobulin concentrations on the same side of the upper 95th centile values of 22 and 38 $\mu$g per millimole of creatinine for RBP and $\beta_2$-microglobulin respectively (Chi-square analysis $p < 0.001$), demonstrating that RBP and $\beta_2$-microglobulin detect tubular proteinuria with equal sensitivity and specificity. ELISA and an established latex immunoassay gave well-correlated results.

Additional Keyphrases: $\beta_2$-microglobulin, latex immunoassay compared  exposure to cadmium  occupational hazards

Nephrotoxic agents such as gentamicin, analgesic drugs, and cadmium damage the kidney proximal tubular parenchyma, resulting in increased urinary excretion of proteins of low molecular mass. Tubular function is usually assessed by measuring specific marker proteins in the urine. One such protein, present in low concentrations in normal urine but one of the major urinary proteins in subjects with renal tubular dysfunction, is the low-molecular mass protein, $\beta_2$-microglobulin ($Mr$, 11 600) (1). This protein is a sensitive indicator of renal tubular function, and radio-, latex-, and enzyme-linked immunoassays have been developed for its measurement (2–4). However, routine measurements of $\beta_2$-microglobulin, particularly in the monitoring of cadmium workers, revealed a major disadvantage with its use as a marker for renal tubular function; it is unstable if the urinary pH is less than 5.5 (5). Further, the protein is rapidly degraded at 37 °C, and therefore loss of $\beta_2$-microglobulin can occur in the bladder (6, 7). This finding prompted some workers to look for a more stable protein to use as a monitor of renal tubular function. One of the proteins studied was retinol-binding protein (RBP, $Mr$, 21 000). This protein occurs in serum complexed to prealbumin and retinol, but after retinol is delivered to target cells RBP rapidly dissociates from prealbumin, is filtered through the glomerulus, and is reabsorbed by the tubule (8). A latex immunoassay has been developed to measure urinary RBP and was used to demonstrate that RBP is stable at all urinary pH values. In urines with pH > 5.5 the RBP concentration correlates well with that of $\beta_2$-microglobulin, and it is equally as sensitive as $\beta_2$-microglobulin for detection of tubular dysfunction (6, 9, 10).

We have developed an enzyme-linked immunosorbent assay (ELISA) for urinary RBP, involving common clinical chemical instrumentation and commercially available reagents, and have compared its performance in the detection of tubular dysfunction, over a wide range of urinary RBP concentrations, with that of $\beta_2$-microglobulin measured with a $\beta_2$ microtest kit (Pharmacia, Uppsala, Sweden). Further, we have compared the ELISA and the latex immunoassay for measurement of urinary RBP.

Materials and Methods

Materials

Urine specimens. Urine specimens were obtained from 118 men not occupationally exposed to cadmium and from 152 men exposed to cadmium fumes, either from smelting or soldering with cadmium/silver rods. Urinary pH was measured, and an aliquot was diluted fivefold in $\beta_2$ microtest buffer (Pharmacia) within 24 h of collection. The diluted aliquot was stored at 4 °C for $\beta_2$-microglobulin measurement; the rest of the specimen was stored at ~70 °C for RBP measurement.

Reagents. Rabbit antibody to human RBP (cat. no. A040) and to RBP conjugated to horseradish peroxidase (P304, EC 1.11.1.7) were purchased from Dako Immunoglobulins, Copenhagen, Denmark. Purified urinary RBP (142 mg/L), prepared as described previously (11), was used as a standard for the immunoassay. Plasma (OCU) containing 94 mg of RBP per liter was purchased from Behringwerke, Marburg, F.R.G.

Antibody-coated microtiter plates. Polystyrene micro-titer plates were coated with antibody by incubating 100-μL aliquots of anti-RBP antibody (500-fold dilution in 50 mmol/L carbonate/bicarbonate buffer, pH 9.6) in wells of a micro-titer plate (M129B; Dynatech, Sussex, U.K.) overnight at 4 °C. After this incubation, we washed the plates three times (100 mL per wash) during 30 min, using phosphate-buffered isosotic saline containing 1 mL of Tween 20 polyoxyethylene (20) sorbitan monolaurate per liter (washing buffer). The washed plates were either used immediately or sealed and stored at 4 °C. Plates could be stored for as long as four weeks without loss of activity.

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Assay Procedure

The standard solution of RBP was diluted in 5 mmol/L phosphate buffer, pH 7.5, containing 10 mL of Tween 20 per liter (assay buffer), to give solutions with RBP concentrations of 10, 25, 50, 100, and 250 μg/L. We added 5-μL aliquots of standards or urines, plus 95 μL of assay buffer, to the wells of an antibody-coated micro-titer plate and incubated the plate overnight at 4 °C. After incubation, the plate was washed four times with washing buffer (100 mL per wash) during 15 min, and a 100-μL aliquot of antibody-enzyme conjugate, diluted 500-fold in assay buffer, was added to each well. The plate was agitated for 1 h at room temperature, then washed four times (100 mL per wash) during 15 min with phosphate (79 mmol/L)/citrate (27 mmol/L) buffer, pH 6.0, containing 1 mL of Tween 20 per liter (substrate buffer). We then added to each well 100 μL of substrate [o-phenylenediamine, 7.4 mmol/L (Sigma P3888) and hydrogen peroxide, 5.9 mmol/L, in substrate buffer]. The plate was further agitated for 15 min at room temperature, then we stopped the enzyme reaction by adding 50 μL of a 2 mol/L solution of sulfuric acid. Finally, the absorbance was measured at 490 nm (we used a MR600 plate reader; Dynatech, Sussex, U.K.). Each standard and urine was tested in duplicate, and the RBP concentration in the test samples was calculated from a standard curve.

Comparison Methods

Urinary creatinine was determined by the Jaffé technique (12), in a Technicon AutoAnalyzer. Determination of urinary RBP by latex immunoassay was performed as described previously (9). Urinary β2-microglobulin concentration was measured with a "Phadebas" β2 microtest kit (Pharmacia) according to the manufacturer's instructions. Urinary albumin was measured by immunoturbidimetry, in a Cobas Bio centrifugal analyzer (13); values for healthy individuals are <3 mg per millimole of creatinine (unpublished data).

Statistical Methods

For linear regression analysis we used a Dec PDP 11/44 computer and a GLM statistical package (Royal Statistical Society, London). Geometric means were used to describe data found to be non-normally distributed (14).

Results

Assay Validation

**Precision and sensitivity.** The working range of the assay extended from 10 to 250 μg of urinary RBP per liter (Figure 1). The detection limit was 5 μg/L. Within-assay precision was determined for three different urines, each assayed 10 times. Between-assay precision was determined from values obtained for a urine assayed on 32 occasions during six months. The results (Table 1) show that the within-assay CV was between 3.2 and 7.1%, the between-assay CV 12.5%.

**Accuracy and analytical recovery.** Absorbance values obtained for dilutions of test urines paralleled those obtained with the standard RBP, demonstrating linearity of response over the working range of the assay. Analytical recovery of RBP standard added to urine samples exceeded 90%.

**Interferences.** An assay carried out with the diluted serum standard gave lower absorbance values for the same RBP concentration than did the standard solution of urinary RBP. This suggested that the RBP in serum had a lower affinity for the antibody than that in urine, possibly because in serum RBP is complexed to prealbumin, whereas in urine it is in the free form (9). We confirmed that the RBP-prealbumin complex was responsible for the lower affinity by adding human serum Cohn Fraction V (Sigma A2386), as a source of prealbumin, to the assay buffer to give a concentration of 20 mg/L and running a standard curve. The results (Figure 2) showed a decrease in absorbance of the urinary RBP standard to values similar to that of serum RBP, demonstrating that on addition of prealbumin a complex is formed that has a lower affinity for the antibody. Evidently the use of a serum standard for urinary RBP will result in the recording of falsely high RBP values.

![Fig. 1. Standard curve for urinary RBP](image1)

![Fig. 2. Absorbance in the ELISA of urinary RBP standard, ○; standard urinary RBP in assay buffer containing Cohn Fraction V of human serum (20 mg/L), ⬤; and serum RBP, ×](image2)
Urinary RBP and $\beta_2$-Microglobulin Concentration in Control Subjects

We determined RBP and $\beta_2$-microglobulin concentrations in urine of 118 men. The subjects covered the age range of the adult U.K. male population and were a group of laboratory workers and a group of unexposed controls from a study of cadmium smelters. The subjects were unselected - i.e., none was excluded on health grounds and none had occupational exposure to cadmium. The mean age was 49 y, range 21–75 y; 25th, 75th quartiles 36 and 61 y, respectively. Table 2 gives the geometric mean RBP and $\beta_2$-microglobulin concentrations, together with the 25th, 75th, and 95th centiles and the range. For $\beta_2$-microglobulin the results were calculated for all 118 urines and again after excluding 38 specimens with pH < 5.5.

Comparison of Urinary RBP with $\beta_2$-Microglobulin

Urinary RBP concentrations were compared with $\beta_2$-microglobulin for the contr~ group and the cadmium-exposed group. Because $\beta_2$-microglobulin is unstable in urines with pH < 5.5, we excluded such urines from this analysis. The results (Figures 3, 4) show good correlation for both the control group and a cadmium-exposed group. Urinary albumin measurements showed that only five of the control subjects had concentrations exceeding the normal limit (3 mg per millimole of creatinine). In all five subjects the albuminuria was mild (<10 mg/mmol in four and 15 mg/mmol in one). Of the 117 exposed subjects, 26 had increased urinary albumin concentrations. In 18 the concentration was <12 mg per millimole of creatinine and, of these, 16 had increased $\beta_2$-microglobulin and RBP concentrations, indicating in these subjects a predominantly tubular proteinuria. The remaining eight had urinary albumin >12 mg per millimole of creatinine, six had mixed glomerular and tubular proteinuria, one had diabetic nephropathy with predominantly glomerular proteinuria, and one had a very low creatinine value, which made unreliable the albumin concentration expressed in terms of creatinine. Re-analysis of the urinary RBP and $\beta_2$-microglobulin data, omitting these eight subjects, showed there was no significant change in the correlation between RBP and $\beta_2$-microglobulin ($r = 0.89, p < 0.001$) and only a small change in the slope, not significant at the 2% level.

ELISA and Latex Immunoassay for RBP Compared

To compare the ELISA assay with the latex immunoassay, we sent 20 urine specimens, containing sodium azide (20 mg/L) as preservative, that had been analyzed in Brussels by latex immunoassay, to London for assay by ELISA. This exchange was reversed with a further 20 urine specimens. This procedure eliminated any bias in the comparison that could arise from loss of RBP activity during transit. The results (Figure 5) show an excellent correlation ($r = 0.99$) over a wide range of RBP concentrations, with a slope that is not significantly different from unity. Thus there is no proportional error, but the ELISA has a positive bias when compared with the latex immunoassay.

Discussion

This ELISA for RBP provides a simple, reliable method for assessing renal tubular function. The working range (10 to 250 $\mu$g/L) covers the range of concentrations found in all but
four of our control subjects. The geometric mean RBP concentration for the control subjects was 7.7 μg per millimole of creatinine, which falls between the previously reported arithmetic mean values of 5.0 (15), 5.3 (9), 5.6 (16), and 10.1 (17). These authors used the arithmetic mean of groups of selected healthy workers, whereas our subjects were unselected and we used the geometric mean to prevent undue weight being given to outliers. In view of these differences the values are very similar. The higher value obtained with the ELISA, 7.7, compared with the latex immunoassay, 5.3 (9), may arise as a result of the positive bias found with the ELISA as compared with the latex immunoassay.

The range of RBP concentrations for our control subjects, 1–53 μg per millimole of creatinine, is similar to that reported previously (9, 10), 1–68 μg/mmol; however, the 95th centile is higher (22 μg/mmol compared with 10.8). This difference probably arises because in this study we intentionally did not exclude subjects whose renal function may have been affected by non-occupationally related ill-health, because we wished to determine, for use in studies on the effect of occupational exposure to nephrotoxic agents, the range of RBP concentrations found in working populations.

The finding that serum RBP and urinary RBP have different characteristics in the ELISA emphasizes the need to use RBP purified from urine—and therefore not complexed to prealbumin—as a standard for the determination of urinary RBP.

Comparison of RBP concentration with β₂-microglobulin concentration showed a good correlation (unexposed subjects \( r = 0.59, p < 0.001 \), Figure 3; exposed subjects \( r = 0.91, p < 0.001 \), Figure 4), indicating that both detect tubular proteinuria with similar sensitivity. We further confirmed this by comparing the number of subjects in the cadmium-exposed group in which both RBP and β₂-microglobulin were on the same side of the upper 95th centile values, 22 and 38 μg per millimole of creatinine, respectively. Of the 117 subjects in this group, there was agreement between RBP and β₂-microglobulin in 103. In the remaining 14 subjects, both RBP and β₂-microglobulin concentrations were close to the upper 95th centile values. Chi-square analysis of the data gave \( p < 0.001 \), confirming that RBP and β₂-microglobulin detect tubular proteinuria with equal sensitivity.

In conclusion; this ELISA for RBP correlates well with previously established latex immunoassay, and for laboratories equipped for ELISA provides a cost-effective, convenient method for assessing renal tubular function.

References