Enzyme-Linked Immunosorbert Assay of Retinol-Binding Protein in Serum and Urine

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This highly sensitive method for determining retinol-binding protein in human serum and urine is based on a double-antibody "sandwich"-type enzyme-linked immunosorbert assay. The assayable concentration range is 0.8–48 μg/L, the detection limit 0.2 μg/L. Within-assay coefficients of variation for 10 determinations at two different concentrations were 5.5 and 5.8%. The corresponding between-assay CVs were 7.9 and 9.2%. We saw no interference from any components of urine or serum. The mean urinary excretion by 30 healthy subjects, as determined by this method, was 101 μg/g of creatinine (SD 38.8). The concentration in serum averaged 43 mg/L (SD 12.1).

Additional Keyphrases: renal tubular proteinuria • reference interval

Retinol-binding protein (RBP) is a protein of low relative molecular mass (Mr, 21 200), isolated from plasma (1) and urine (2) of normal subjects. Its urinary excretion, like that of β2-microglobulin, may be a sensitive index for use in screening for tubular proteinuria; however, it is more stable than β2-microglobulin at normal urinary pH (3).

Among the known methods for quantifying urinary RBP, only latex immunoassay is sensitive enough to measure the low concentrations appearing in "normal" urine (4). Enzyme-linked immunosorbert assay (ELISA) is now used routinely for quantifying a wide variety of soluble antigens present in trace amounts of body fluids. Owing to its simplicity and low reagent consumption, ELISA is much more advantageous than radioimmunoassay and other methods, which require preconcentration of normal urine.

Here we propose a new ELISA for determination of RBP in human urine and serum. It is at least as precise and sensitive as radioimmunoassay and latex immunoassay.

Materials and Methods

Materials. Purified rabbit immunoglobulins against human RBP (lot no. 092A) were obtained from DAKO Immunoglo-
stand for 60 s before emptying them. Add 200 µL of sample to each well and incubate overnight at 4 °C. After washing three times with gelatin–buffered saline, add 200 µL of the conjugate diluted 200-fold with gelatin–buffered saline. Stop the reaction by washing the plates with gelatin–buffered saline and measure the amount of alkaline phosphatase by adding 200 µL of the p-nitrophenyl phosphate substrate in diethanolamine buffer. After a 15-min incubation at room temperature, measure the absorbance of the mixture at 405 nm. To do so, we used a "Titertek Multiplan" (Flow Laboratories, Irvine, U.K.).

**Results**

The standard curve is shown in Figure 1. The detection limit, defined as the least concentration of RBP that differs significantly from zero at the 95% confidence interval, is 0.2 µg/L, corresponding to the lowest standard. The working range extends from 0.75 to 48 µg/L. For higher concentrations, excess antigen leads to the "post-zone effect," which gives to the curve typical of this immunological reaction its characteristic bimodal pattern. The antigen–antibody reaction, revealed by the substrate, was measured by the absorbance at 405 nm after about 30 min, when the highest concentration on the reference curve (48 µg/L) reached an absorbance of 1.0.

**Samples.** The population examined consisted of 30 ostensibly healthy subjects, ages 28 to 40 years. The assay was performed on urine samples collected after the subjects had been standing upright for at least 2 h. To compensate for the variability due to dilution of urine, the analytical results were expressed in terms of creatinine. Under these conditions, we obtained a mean RBP value of 101 µg/g of creatinine (SD 38.8, range 37.3 to 185 µg/g). The corresponding concentrations in serum ranged between 22 and 67 (mean 43, SD 12.1) µg/L.

**Precision.** The within- and between-run precision of this ELISA were assessed by measuring two urine samples with different concentrations of RBP. The means for 10 determinations in a single run were 2.52 (SD 0.14) and 22.41 (SD 1.31) µg/L, the relative standard deviations corresponding to 0.05 and 0.06, respectively. For 10 successive replicate assays on the same urine samples, the relative standard deviations were 0.07 and 0.09 for urines containing 2.91 (SD 0.23) and 23.70 (SD 2.11) µg/L, respectively.

**Comparison with existing methods.** We also measured RBP concentration in 30 urine samples from healthy subjects and patients with various renal diseases by "rocket" immunoelectrophoresis (7). The correlation coefficient between results by this ELISA and Laurell's technique was 0.99, the slope of the regression line being very close to unity and the intercept negligible (Figure 2). A close relationship was also found between ELISA and radial immunodiffusion determination (8) of serum RBP (Figure 3).

**Discussion**

The range for determination of RBP by the present
method, 0.8–48 μg/L, is broad enough to measure accurately a wide range of RBP concentrations in serum and urine.

The evaluation of analytical variables and the comparison with other existing methods evidence the reliability of this procedure. ELISA also has considerable advantages over more conventional methods: no preliminary concentration of normal urine samples required, low cost per assay when one considers the working dilutions of specific antibody and conjugate, and speed, because the wells in several plates can be coated simultaneously and the plates stored at −70 °C. Then the procedure requires only 2 h to incubate the antigen at room temperature, 1 h to incubate the conjugate at 37 °C, and 30 min to incubate the substrate at room temperature. Even beyond this limit, however, we did not find any significant differences in the results. For our convenience, we therefore deposit the antigen in the evening and incubate it overnight at 4 °C, then deposit the conjugate the next morning.

The assay also has some drawbacks, but they can be easily overcome.

One is the dilution of urine samples. We diluted all urine samples 40-fold in such a way that most of them fell within our working range. Even higher dilutions will ensure accurate assay of unusually high RBP concentrations in pathological urine and serum.

Another drawback of the method is that there may be some variation in the plates (9), but this problem can be solved by performing multiple assays and by indicating the results as the mean of absorbances measured.

The mean concentration of RBP in human serum that we found in healthy subjects agrees well with that reported by other authors (2, 3). The mean urinary excretion we found for normal subjects agrees well with that measured by Peterson and Berggård (2) (110 μg/24 h), but is higher than that reported by Kanay et al. (1) and by Bernard et al. (4), even though our upper normal limit—i.e., the mean + 2 SD—agrees well with that found by the latter authors (4). ELISA may be proposed as an alternative to latex immunoassay, a well-validated and sensitive method for the determination of RBP in body fluids (4). Over this method, ELISA offers the principal advantage of a wider analytical range, the other analytical features and costs being similar or better.

**References**


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**Anti-Triiodothyronine Autoantibodies Interfere with Two Recently Commercialized Radioimmunoassays for Free Triiodothyronine**

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We examined the effect of anti-triiodothyronine autoantibodies on results of RIA of free triiodothyronine with two commercial kits (Amersham International and Diagnostic Products Corp.). Both methods gave falsely high results because the 125I-labeled analogs used in these RIAs were bound by the autoantibodies.

Interference of autoantibodies to thyroxin with measurement of free T4 in serum by RIA has recently been demonstrated (1–5), but there are few data on the effect of autoantibodies to triiodothyronine (T3) on free T3 RIA measurements (6).

Recently, two new commercial RIAs for free T3 have become available: the Amerlex free T3 assay (Amersham International Ltd., Amersham, Bucks., U.K.) and free T3 assay DPC (Diagnostic Products Corp., Los Angeles, CA). We decided to assess whether serum from patients with autoantibodies to T3 gives interference in these new RIA procedures. Both methods involve a labeled analog of T3 that is supposed not to be bound by normal serum transport proteins, and an extremely high-affinity antibody to T3, which binds the T3 analog in the same manner as T3.

Using these two procedures, we measured free T3 from 53 control individuals and from five euthyroid patients with autoantibodies to T3 in their serum. Before that, we measured total T3 by different RIA methods: double- and single-antibody RIAs and double-antibody RIA with prior extraction with ethanol (7). The presence of serum anti-T3 autoantibodies was demonstrated by incubating an aliquot of the serum with [125I]T3 and precipitation with polyethylene...