Immunonephelometric Determination of Retinol-Binding Protein in Serum and Urine

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An immunonephelometric method developed for measurement of retinol-binding protein (RBP) in serum and urine can detect it in concentrations of about 30 µg/L, which is in the lower limit of its normal concentration in urine (range 0–0.56 mg/L; mean ± SD 0.19 ± 0.15; n = 44). Urinary RBP was increased (range 0.93–29.5 mg/L) in all 25 urine specimens from 13 subjects being treated with aminoglycosides (tobramycin). Urinary excretion of RBP was correlated (r = 0.83) with the excretion of β2-microglobulin. The within-assay and day-to-day precision (CV) was determined over the detection range of 0.03–8 mg/L. Within these limits the corresponding CVs varied from 4 to 27% and from 8 to 30%, respectively. The method had fairly good precision within the optimal measuring range of approximately 0.4 to 4.5 mg/L for both urine and 20-fold diluted serum samples. For various RBP concentrations our analytical recovery was 89–114% of added RBP. Results by this method correlated well (r = 0.96, n = 24) with those by a radical immunodiffusion method.

Additional Keyphrases: nephrotoxicity · tobramycin · reference interval · vitamin A · renal tubular dysfunction

Retinol, the principal vitamin A metabolite in the circulation, is excreted and transported from hepatic stores bound to a specific transport protein, retinol-binding protein (RBP), which was first isolated by Kanai et al. (1) in 1968. RBP, synthesized in liver hepatocytes, is released to the circulation in amounts depending on the hepatic concentrations of vitamin A (2). A low molecular mass RB (21 430 daltons) (3) circulates in the plasma together with thyroxin-binding prealbumin (TBPA) as a protein–protein complex, which prevents excretion of RBP in the urine. Uptake of retinol to target cells diminishes the affinity of RBP for the TBPA complex; the dissociated RBP is freely filtered through the glomerular capillary membrane and is reabsorbed and catabolized in the proximal tubules (2, 3).

Urinary excretion of RBP is increased similarly to β2-microglobulin in dysfunction of the proximal tubules (3, 4) and in malignancies (5). Peterson and Berggård (6) found that large amounts of RBP can be isolated from the urine of patients with tubular dysfunction. Furthermore, Bernard et al. (4) suggested measuring urinary RBP instead of β2-microglobulin to screen renal tubular function, especially when the pH of collected urine cannot be kept above 5.5.

The normal mean concentrations of RBP in plasma are reportedly on the order of 40–50 mg/L, and correlate significantly with that of plasma retinol (3, 7). Under normal circumstances about 85% of RBP is believed to circulate in the form of a protein–ligand complex (holoprotein) (7).

Here we describe a relatively simple and fast immunonephelometric method for analyzing RBP in serum or plasma and in low concentrations in urine samples. We used this method to determine urinary RBP concentrations in normal subjects and, because aminoglycosides are nephrotoxic, in patients who were receiving tobramycin (6).

Materials and Methods

Materials

Serum and urine samples. Serum and urine specimens were obtained from apparently healthy laboratory workers, ages 20 to 40 years. Serum and 24-h urine collections were obtained from 13 hospitalized patients who were receiving therapy with aminoglycoside (tobramycin). The patients had normal values for creatinine clearance and no increased systemic production of β2-microglobulin or lysozyme. The samples were stored at −70 or −20 °C before assay of urinary and serum creatinine (9), RBP in serum and urine, and serum retinol. β2-Microglobulin in serum and urine was determined by Phadebas β2-micro Test RIA (Pharmacia, Uppsala, Sweden) according to the standards and recommendations of the manufacturer. Our laboratory reference values for β2-microglobulin in apparently healthy individuals are <400 µg/L in urine, and 1.0–2.5 mg/L in serum.

RBP antiserum. Rabbit antihuman RBP-antiserum (titre as antigen binding capacity 45 µg/mL) was obtained from DAKO, Copenhagen, Denmark. The specificity of the antiserum was tested by immunoelectrophoresis on agarose gel in
pH 8.6 barbital buffer. We studied a pooled serum sample (RBP 45 mg/L), a serum sample with increased RBP (RBP 75 mg/L), and a urine sample with increased RBP (RBP 27 mg/L, total protein 0.10 g/L). A single weak precipitin line in the α-region was found after staining with Amido Black 10 B. This agrees with the findings of Kanai et al. (1) that RBP can be electrophoretically separated from TBPA and has α-globulin mobility.

Procedure

This immunonephelometric determination of RBP is based on a similar method for serum vitamin D-binding globulin (10). The main modifications are as follows:

Polyethylene glycol-6000 concentration in the assay buffer was 70 g/L for routine use in urine analysis and 40 g/L in serum analysis. The urine samples were centrifuged and assayed undiluted; serum samples were diluted 20-fold in saline (NaCl, 0.15 mol/L). The standard curve was constructed from serial dilutions of protein standard serum (32 mg/L RBP; Behringwerke AG, Marburg, F.R.G.) in saline, to produce RBP concentrations ranging from 31.3 μg/L to 16 mg/L. Rabbit antihuman RBP-antisemur was diluted 10-fold in the polyethylene glycol-6000 assay buffer, and filtered through a 0.45-μm (av. pore size) filter. The buffer alone, for blank determinations, was also filtered before use.

We pipetted 100 μL of standard or sample, followed by 200 μL of diluted antiserum (or buffer for blank determination), into cuvettes of the laser nephelometer (Behring, Hoechst AG, Frankfurt, F.R.G.) and incubated them for exactly 1 h at room temperature before the light-scatter signals (in volts) were measured. Blank readings were subtracted from the total readings, and the specific scattering was plotted vs the respective standard concentration on log graph paper. The sample concentrations were read from the log curve, and the results, in milligrams per liter, were multiplied by 20 (the dilution factor) for serum analyses.

RBP was also measured by immunodiffusion (LC-Partigen-RBP; Behringwerke AG), according to the manufacturer's recommendations; the resulting precipitin rings were measured after an incubation of 72 h. The mean within-assay and day-to-day CVs, determined over the measuring range of 2 to 36 mg/L, were 6 and 8%, respectively. When the urine RBP exceeded the lower detection limit of the immunodiffusion plates, the samples were applied unconcentrated. Otherwise, they were concentrated with Minicon B Clinical Sample Concentrators (Amicon Corp., Danvers, MA 01923). The results for concentrated urine samples were corrected for the percentage of recovery.

Retinol measurements. Serum retinol was determined by the methods of De Leenheer et al. (11, 12). These methods were modified and tested in our laboratory to give reproducibility and accuracy equal to that reported in those studies.

Results

RBP assay method. Figure 1 shows results of experiments on optimization of the immunonephelometric RBP assay. A PEG-6000 concentration of 70 g/L of assay buffer was optimal for analysis of RBP in urine samples. The standard curve covers a wide RBP concentration range, 0.03-16 mg/L, that encompasses most RBP concentrations in urine. In only five urine samples of the 44 normal subjects studied was the RBP concentration less than the detection limit of the method (about 30 μg/L). For serum RBP analysis, a lower concentration of polyethylene glycol-6000 (40 g/L) in the assay buffer was optimal.

To assess the precision of the method, we assayed RBP in urine and serum within the range 0.09-4.6 mg/L. Table 1 summarizes the within-assay and day-to-day CVs with various RBP concentrations. The precision was poorer at lower RBP concentrations. Thus the measuring range of approximately 0.4 to 4.5 mg/L is optimal, both for urine and 20-fold diluted serum samples. Analytical recovery of added RBP in urine is also shown in Table 1.

Correlation of the immunonephelometric method with the immunodiffusion method (Figure 2) was excellent (r = 0.96, n = 24), and a linear regression equation y = 0.972x - 0.029 was obtained. The means and SDs for the two methods were: 36.1 ± 16.5 mg/L (y ± SDy), and 35.0 ± 15.4 mg/L (x ± SDx), respectively. Table 2 shows in more detail the validation of urine RBP analysis by comparing the results from both of the methods.

RBP analyses from serum samples. Figure 3 shows the good correlation (r = 0.91, n = 49) between RBP and retinol concentrations determined in healthy subjects, the linear regression equation being y = 0.932x + 0.275 for concentra-
concentrations in moles per liter. The mean serum RBP and retinol concentrations in these subjects were 46.1 (SD 12.9) mg/L and 587 (SD 182) μg/L, respectively. The mean molar saturation of RBP with retinol was calculated to be 94.3% (SD 11.8%, n = 49). The degree of saturation was somewhat greater in men than in women.

**RBP analyses from urine samples.** RBP in urine from 44 normal subjects ranged from not detected (in five cases) to 0.56 mg/L (mean 0.19, SD 0.15 mg/L). The RBP values in urine were corrected for urine volume by dividing the RBP concentrations (mg/L) with the respective creatinine concentrations (g/L), yielding RBP in milligrams per gram of creatinine. The resulting values ranged from not detected to 0.89 mg/g (mean 0.21, SD 0.22 mg/g).

RBP in 25 urine specimens from 13 patients receiving tobramycin ranged from 1.1 to 60.5 mg/24 h (0.93–29.5 mg/L) with a mean of 14.0 (SD 15.0) mg/24 h (26.2, SD 44.6 mg/g of creatinine). β2-Microglobulin excretion by the same patients was 14.6 (SD 20.1) mg/24 h, which correlated reasonably (r = 0.83) with the excretion of RBP.

**Discussion**

Results with this immunonephelometric assay for RBP in human serum and urine correlate well with those by the radial immunodiffusion method. The advantage of this over other methods for RBP measurement such as radioimmunoassay (7) or radial immunodiffusion (5) is its simplicity and speed. Quantitative results are obtained after an incubation interval of 1 h.

The radial immunodiffusion method is insufficiently sensitive for quantification of RBP in very low concentrations, as in normal urine samples (<0.56 mg/L), whereas with the immunonephelometric method we detected RBP in concentrations of 30 μg/L without preliminary concentration of the urine samples. The content of only five of the samples from 44 normal subjects fell below this limit of detectability. In this study we measured mean urinary concentration for 44 normal subjects was somewhat higher than that reported by Bernard et al. (4), which was 52.5 (SD 59.2) μg/g of creatinine by latex immunoassay in 150 healthy subjects, or by Peterson and Berggård (6), which was 0.11 mg/24-h urine.

The specificity of the anti-RPB in the immunonephelometric method was validated by immunoelctrophoresis, which showed only a single precipitin arc in the α2-region. Similarly, in the Ouchterlon double-immunodiffusion technique, there was a single precipitin line. Dilution of a urine sample from a tubular proteinuric patient gave a response similar to the standard serum RBP in the assay. The normal serum proteins or other constituents of urine did not interfere in the assay.

In healthy subjects we calculated the mean molar saturation of RBP with retinol to be about 94%, supporting the view that almost all serum RBP exists as a ligand–protein complex (holoprotein). The concentration of circulating uncomplexed RBP can be assumed to be very low (7). However, in comparison with the findings of Smith et al. (7), we found a better correlation between RBP and retinol, and about 10% greater molar saturation of RBP with retinol.

We found the normal urinary concentration of RBP to be as low as 0.19 mg/L. However, RBP may constitute 10% or more of the total urinary protein in patients with proximal tubular reabsorption defects (3, 6). In our preliminary study of patients with aminoglycoside (tobramycin) therapy, we found the urinary excretion of RBP to be much increased (about 150-fold normal). This increase occurred concomi-
stantly with an increase of urinary $\beta_2$-microglobulin. The exact significance of RBP as a possible marker of tubular proteinuria remains to be examined. The increase in circulating RBP and retinol might also be correlated with glomerular dysfunction, as shown by De Bevere et al. (13) for hemodialysis patients.

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References


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Liquid-Chromatographic Determination of Cephalosporins and Chloramphenicol in Serum

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A "high-performance" liquid-chromatographic technique involving a radial compression module is used for measuring chloramphenicol and five cephalosporin antibiotics: cefotaxime, cefazolin, cefoxitin, cepha- prin, and cefamandol. Serum proteins are precipitated with acetonitrile solution containing 4'-nitrocetanilide as the internal standard. The drugs are eluted with a mobile phase of methanol/acetate buffer (30/70 by vol), pH 5.5. Absorbance of the cephalosporins is monitored at 254 nm. Standard curves are linear to at least 100 mg/L. The absorbance of chloramphenicol is monitored at 254 nm and 280 nm, and its standard curve is linear to at least 50 mg/L. The elution times for various other drugs were also determined, to check for potential interferents.

Additional Keyphrases: antibiotics • drug assay • chromatography, reversed-phase • radial compression module

Several cephalosporin antibiotics are currently used in therapy. Derivatives of 7-aminopenicillopeptonic acid, these compounds are active against various organisms, both Gram-negative and Gram-positive. For cephalosporins administered intramuscularly or intravenously, the primary route of elimination is the kidney, as much as 80–90% of the dose being excreted unchanged in the urine. The rate of clearance is usually very rapid: for the cephalosporins studied in this report, the average half-life in serum ranges from 30 to 90 min in patients with normal renal function (1). Therapeutic monitoring of cephalosporin antibiotics is gaining importance in cases where high doses are being administered or where the patient may have a problem clearing the drug because of decreased renal function. The potentially hazardous side effects of chloramphenicol have already been established (2).

Although several "high-performance" liquid-chromatographic methods have been described for use in determining chloramphenicol (3) and the cephalosporins (4–10), some of them are limited (at least in the published report) to only one or two drugs, or do not measure drug concentrations that would be clinically significant. Moreover, when one must maintain a variety of columns and (or) buffer systems, each dedicated to a specific assay, changing from one to another is time consuming. Obviously, it is simpler and more economical if several different drugs can be analyzed with the same system.

Here I describe the use of a radial compression module with a reversed-phase column in analysis for five cephalosporin antibiotics (cefotaxime, cefazolin, cefoxitin, cepha- prin, and cefamandol). This same column and buffer system can also be used in analysis for procainamide and N-acetyprocainamide by decreasing the solvent flow rate from 3.0 to 2.0 mL/min (11).

Materials and Methods

Reagents and standards: Acetonitrile and methanol were distilled in glass, "HPLC" grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Triethylamine and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, MO 63178. The internal standard, 4'-nitrocetanilide was obtained from Eastman Kodak Co., Rochester, NY 14650. Cefamandole, cephalothin, and cefaclor were supplied as chemical standards by Eli Lilly & Co., Indianap-

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