Radioimmunoassay for Urinary Albumin

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We describe a rapid, sensitive, and precise radioimmunoassay for urinary albumin (Uab). Aliquots of diluted urine were incubated at room temperature for 1 h with 125I-labeled albumin and a rabbit antiserum monospecific for human albumin. Phase separation was effected by the double-antibody technique. The dose–response curve was linear in the range of 15.6–10 000 ng, equivalent to 4 to 3000 mg/liter of urine. The limit of sensitivity was 16 ng of albumin. The coefficient of assay variation was 4.8%, both at 44 mg/liter and at 1304 mg/liter. A displacement curve obtained with a serially diluted urine sample of high albumin concentration was completely superimposable with the curve for which human albumin was used as a standard. In 26 normal individuals the range for Uab was 2.2–12.6 mg/24 h, and for albumin clearance (Cabl), 1.8 \( \times 10^{-5} \)–19.6 \( \times 10^{-5} \) ml/min. After renal homografts in 25 patients, Uab ranged from 16.9 to 9926 mg/24 h, and Cabl from 2.7 \( \times 10^{-4} \) to 1.7 \( \times 10^{-1} \) ml/min. Both increased Uab and Cabl correlated well with the severity of renal homograft rejection.

Additional Keyphrases: renal allograft homografts proteins renal glomerular disease nephrotic syndrome

Although urinary protein excretion is routinely determined in the diagnosis and assessment of renal disease, clearances of macromolecules provide a superior estimate of the degree to which glomerular permeability is altered. Because albumin is one of the smallest molecules that is almost totally excluded by the intact glomerular membrane (1, 2), renal excretion of albumin, measured as albumin clearance (Cabl), has clear merit in the detection and monitoring of renal disease, particularly minimal glomerular disease such as occurs in the early stages of renal allograft rejection. Precise evaluation of glomerular permeability has also been shown to be of clinical value in the management of the nephrotic syndrome (3). Therefore, determination of Cabl has had limited clinical application because of the lack of a sufficiently specific, sensitive, and precise method for measuring urinary albumin that is also rapid and efficient. Radial immunodiffusion (4) and radioimmunoassay (5) are sensitive and precise but also time consuming and cumbersome. Radioimmunoassay procedures reported thus far either lack the requisite sensitivity (6) or require long incubation times (7, 8). A rapid analytical method is particularly needed for the urgent planning of therapy involved in renal transplantation.

We describe a radioimmunoassay for urine albumin (Uab) involving the double-antibody technique. The procedure is simple, sensitive, and precise, requiring only 3–4 h for assay of up to 20 samples.

Materials and Methods

Reagents and Solutions

Phosphate-buffered saline, 10 mmol/liter. Ten millimoles of Na2HPO4, 150 mmol of NaCl, and 1.5 mmol of NaN3 (1.41 g, 8.8 g, and 0.1 g, respectively) were dissolved in 900 ml of reagent-grade water. The pH was adjusted to 7.0 and the solution was diluted to 1 liter.

Assay buffer. Normal rabbit serum, 7.5 ml, was mixed with 500 ml of phosphate-buffered saline.

Human albumin (Miles Laboratories, Elkhart, Ind. 46514). This material was used both as standard and for iodination, without further purification. A stock albumin standard was prepared by dissolving 12.5 mg of albumin in 50 ml of assay buffer. Working standard solutions, freshly prepared before each assay, were made by diluting the stock standard to a concentration of 5000 \( \mu \)g/liter with assay buffer and then making serial two-fold dilutions with assay buffer to yield solutions containing 1000, 500, 250, 125, 62.5, 31.3, and 15.6 ng of albumin per 200 ml.

Human albumin antiserum, rabbit. Normal human serum was added to an equal volume of saturated solution of ammonium sulfate. An alcohol–ether extract of the supernate was purified on Ultragel AcA 34 (LKB Instruments, Inc., Rockville, Md. 20852) and subsequently by anion-exchange chromatography on DEAE Biogel A (Bio-Rad, Richmond, Calif. 94804). The eluted albumin fraction, when concentrated to 5 g/liter, gave only one precipitin line characteristic of albumin on immunoelectrophoresis developed with three different antisera to normal human serum proteins. The albumin thus prepared was analyzed by electrophoresis in polyacrylamide (100 g/liter) containing 1 g of sodium dodecyl sulfate per liter. The relative molecular mass of the protein was assessed by its migration with use of pure preparations of \( \beta \)-galactosidase 135 000 daltons, transferrin 80 000 daltons, human serum albumin 65 000 daltons, ovalbumin 45 000 daltons, retinol binding globulin 21 000 daltons, and lysozyme 14 400 daltons. The albumin was diluted to 1 g/liter and mixed with equal volume of Freund's complete adjuvant and injected at monthly intervals into three New Zealand white rabbits. A final bleeding was done after five months of immunization. The antiserum was monospecific for human albumin on immunoelectrophoresis. The working titer of the antiserum was determined by performing an antibody dilution study according to the procedure to be described later. An antiserum dilution of 8000-fold yielded an optimal initial binding \((B/T)_0\)
of 50%. The antiserum diluent contained 50 mmol of ethylenediaminetetraacetate per liter of assay buffer.

Precipitating antibody. Goat anti-rabbit gamma-globulin serum, lyophilized (Calbiochem, La Jolla, Calif. 92036; lot no. 539844), was reconstituted with 5.0 ml of the phosphate-buffered saline.

Procedures

Radioiodination of human albumin. Human albumin was iodinated by the Chloramine T method, by a modified procedure of Hunter and Greenwood (9). Human albumin (Miles Laboratories, Elkhart, Ind. 46514), 20 μg in 50 μl of phosphate buffer (0.4 mol/liter pH 7.4) and 5 μl of carrier-free Na125I (500 μCi, from Amersham Searle, Arlington Heights, Ill. 60005) were mixed gently in a 500-μl plastic AutoAnalyzer cup. Twenty microliters (300 μg) of a freshly prepared Chloramine T solution was added and the mixtures were allowed to react for 1.5 min. The reaction was terminated with the addition of 100 μl (500 μg) of sodium metabisulphite solution. After the specific activity was measured in a trichloroacetic acid precipitate, the iodinated preparation was purified by gel filtration chromatography on a 0.9 cm × 15 cm column packed with Ultragel AcA 44 (LKB Instruments, Inc., Rockville, Md. 20852). The column was eluted with the phosphate-buffered saline, and fractions containing the iodinated albumin were combined. A calculated amount of unlabeled human albumin was added to the purified iodinated preparation to achieve a final specific activity of 1 Ci/g. To increase the shelf-life of this reagent, 2000 units of aprotinin (Trasylol; Calbiochem, La Jolla, Calif. 92037) were mixed with each milliliter of this solution. Just before assay, an aliquot of this iodinated preparation was diluted with assay buffer to a concentration of 50 ng/100 μl (about 500 000 cpm/100 μl).

Radioimmunoassay. Assay-standard incubation mixtures in 12 mm × 75 mm disposable glass tubes were diluted to a total volume of 1000 μl with (a) 200 μl of albumin antiserum (1:8000 dilution), (b) 200 μl working albumin standard solution (15.6–1000 ng), (c) 100 μl of 125I-labeled albumin containing 50 000 cpm, and (d) appropriate amounts of assay buffer. Urine samples were each diluted 50- and 1000-fold with assay buffer by use of an Autodiluter (LKB, Model 2075). Sample incubation mixtures were prepared similarly, except that in b we substituted 200 μl of urine sample at 50- or 1000-fold dilution. To correct for nonspecific binding of the labeled albumin, we also prepared incubation tubes with antiserum diluent replacing a and with assay buffer replacing b. All standards and samples of both dilutions were assayed in duplicate. The contents of these tubes were well mixed and allowed to incubate at room temperature for 1 h. One hundred microliters of precipitation antibody, goat antiserum to rabbit gamma-globulin, was added to each tube, and incubation was continued for 1 h at room temperature. Antibody-bound and free albumin were separated by centrifugation for 15 min at 2000 × g in a refrigerated centrifuge. The supernatant fluid was drained from each tube, and the precipitate, which contained the bound fraction, was counted for 1 min in a gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill. 60515; Model 52300).

Radioimmunoassay data obtained from the bound fraction was processed with a Hewlett Packard 9830A programmable calculator and a Hewlett Packard 98622 plotter. The dose-response variables were subjected to logit-log transformation and weighted regression analysis to yield a linear slope, an 80% intercept, and a 50% intercept, all of which serve as quality-control data for assay monitoring. In sample calculation, an averaged albumin value obtained from both dilutions was converted to the unit reported, mg/24 h. Where the binding for one of the dilutions fell beyond the acceptable limits of precision, i.e. 85% < B/B0 < 10%, the final calculation was performed on one dilution only.

Specimens. Timed 24-h or 48-h urines were collected from healthy laboratory individuals known to be taking no drugs and from patients undergoing kidney transplantation. Serum was sampled from the same individuals during the course of their 24-h urine collection. Albumin was determined in the serum samples by the bromcresol green procedure, used with the Centrifichem System 400.

Albumin clearance was calculated in both normal subjects and in patients as follows:

\[ C_{alb} = \left( \frac{U_{alb} \times V}{S_{alb} \times t} \right) \]

where \( U_{alb} \) = urinary albumin excretion, mg/liter, \( S_{alb} \) = serum albumin concn., mg/liter, \( V \) = volume of 24-h urine, ml, \( C_{alb} \) = albumin clearance, ml/min, and \( t \) = time for urine collection, min.

Results

Iodination of human albumin. Figure 1 shows a typical gel filtration pattern of the iodinated albumin preparation on Ultragel AcA 44. The iodination efficiency was 86%. The specific activity, determined by trichloroacetic acid precipitation, was usually 20–25 Ci/g. Stored frozen in aliquots, the working iodinated-albumin solution, prepared by adjusting its specific activity to 1 Ci/g and stabilized with aprotinin, was stable for at least 30 days.

Standard curve. Figure 2 shows a representative dose-response curve, after logit-log transformation and weighted regression analysis. The relation was linear over an absolute range of 15.6–1000 ng of human albumin per assay tube, equivalent to 4–250 mg/liter of urine at a sample dilution of 50-fold and 50–5000 mg/liter of urine at a sample dilution of 1000-fold. The assay sensitivity, as defined by \( B/B_0 \) of 85%, was 16 ng of albumin, or 4 mg/liter of urine at a sample dilution of 50-fold. The upper limit of precision, as defined by \( B/B_0 \) of 10%, was 600 ng of albumin, or 3000 mg/liter of urine at a sample dilution of 1000-fold.

Precision. Thirty-seven urine specimens, whose albumin
values ranged from 6.2–1305 mg/liter, were each assayed on two separate days for the assessment of between-run method precision. The coefficient of variation was 5.9%. Another precision study, in which two urine specimens were used as controls in 21 individual assays, resulted in a coefficient of variation of 4.8% both at 44 and at 1304 mg/liter of urine.

Accuracy. Known amounts of human albumin (25–400 mg/liter) were added to aliquots of a normal urine specimen for which the albumin concentration had previously been determined. These aliquots were then assayed for albumin. The analytical recovery of albumin from urine by this method is 90–98%.

Specificity. The cross-reactivity of the antisera to other plasma proteins such as IgG, transferrin, and bovine serum albumin was evaluated by reacting each protein separately with the assay system for measuring albumin. The extent of cross-reactivity of the proteins tested, as shown in Figure 3, is insignificant.

Assay validation. Figure 3 shows the standard curves obtained with human albumin as the standard and a serial two-fold diluted urine specimen from a renal transplant patient. Both these curves were superimposable in the range of 15.6–1000 ng/assay tube, which is equivalent to 4–250 mg/liter for a 50-fold diluted urine sample, and 80–5000 mg/liter for a 100-fold diluted sample. This suggests that the determination of albumin in urine is specific and not influenced by the other substances present in the concentration range of 4–5000 mg/liter of urine.

Quality-control parameters. Radioimmunoassay functions that are useful in quality-control monitoring are: %B/T0, per cent nonspecific, slope of the logit-log transformed standard curve, the 80% intercept, and the 50% intercept (10). Between-run variations in these are graphically presented in Figure 4. The mean and standard deviations for each function (21 runs) are: %B/T0 = 52.7 ± 4.5, per cent nonspecific = 1.5 ± 0.2, slope = -1.00 ± 0.06, 80% intercept = 26.8 ± 2.4, and 50% intercept = 107 ± 7.

Normal range and patient data. The normal range for $U_{alb}$, based on 25 healthy laboratory subjects, was 2.2–12.6 mg/24 h; the corresponding $C_{alb}$ was $1.8 \times 10^{-5}$ to $19.6 \times 10^{-5}$ ml/min. $U_{alb}$ for 26 patients after renal homografts ranged from 16.9 to 9928 mg/24 h, and the corresponding $C_{alb}$ was $2.7 \times 10^{-4}$ to $1.7 \times 10^{-1}$ ml/min. Albumin values for both groups are shown in Figure 5.

Discussion

The range of albumin concentration covered in this assay,
4 to 6000 mg/liter, is sufficient to include individuals with normal renal function, patients with minimal glomerular disease, and patients with advanced renal disease. The CV of 4.8% is reflected in the small between-run variations in the quality-control variables, i.e., the slope of the logit-log transformed standard curve, the 80% intercept as an index of sensitivity, and the 50% intercept as an index of precision.

We believe that the excellent stability of the assay system is in large measure attributable to the low specific activity (1 Ci/g) that we incorporated into the labeled albumin. The stable shelf-life is evident from the virtually constant percentage of nonspecific binding of 1.5 ± 0.2 shown in all 21 runs.

The radioimmunoassay procedure is sufficiently rapid to allow the physician to make daily, or even more frequent, decisions on patient management. Many studies thus far in our laboratory indicate that this assay is indeed sufficiently precise, sensitive, and specific to provide close monitoring of patients with renal allografts.

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References