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Enzyme-Linked Immunosorbent Assay for Urinary Albumin at Low Concentrations

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We describe an enzyme-linked immunosorbent assay (ELISA) for urinary albumin. It requires only commercially available reagents, can detect as little as 16 μg of albumin per liter, and analytical recovery ranges from 92 to 116%. The assay is simple, rapid, and inexpensive. Albumin excretion was 6.2 (SD 4.1) mg/24 h in healthy subjects (n = 40), 14.7 (SD 7.2) mg/24 h in albumin-test-strip-negative Type I diabetics (n = 11), and 19.7 (SD 16.2) mg/24 h in patients with essential hypertension (n = 12).

Additional Keyphrases: *diabetes · hypertension · reference interval*

Slight ("micro") albuminuria is thought to predict diabetic nephropathy in patients whose urines are albumin-negative by Albustix test strips (*1–3*), but at a stage that can be reversed by sufficient metabolic control (*4–6*). Once albuminuria is detectable by the Albustix test, the development of diabetic nephropathy usually can no longer be prevented. It is therefore important to have a sensitive, reliable, rapid, and inexpensive test available for urinary albumin. Exact estimation of microalbuminuria is also of special interest in control of hypertension and of patients with renal allografts (*7, 8*).

Various techniques have been used for measuring microalbuminuria: radial immunodiffusion (*9, 10*), immunoelectrophoresis (*11*), radioimmunoassays (*12–16*), and enzyme immunoassays (*16–21*). The main disadvantages of radioimmunoassays are isotope-related health and safety hazards and the short shelf-life of reagents.

Our aim in this study was to develop a sensitive, simple, and rapid immunoassay for measuring urinary albumin with use of only commercially available reagents.

Materials and Methods

Materials

Reagents. Human serum albumin was obtained from Behringwerke AG, Marburg, F.R.G. Bovine serum albumin was obtained from Sigma, Taufkirchen, F.R.G.; casein, gelatin, creatinine, 250 mL/L sulfuric acid, hydrogen peroxide (300 g/L), and all reagents for buffer solutions were supplied by Merck, Darmstadt, F.R.G. Tween 20 polyoxyethylene (20) sorbitan monolaurate, uric acid, urea, globulins, and the enzyme substrate *o*-phenylenediamine were from Serva, Heidelberg, F.R.G.

Antisera. Rabbit anti-human albumin antiserum (liquid; cat. no. ORCB04/05) was obtained from Berhringwerke AG, and peroxidase-labeled goat anti-human albumin antiserum (lyophilized; cat. no. 3201-034) was from Cooper Biomedical, Frankfurt, F.R.G.

Assay diluent, pH 9.6. Antigen and antibodies are diluted in 0.2 mol/L carbonate/bicarbonate buffer, prepared by dissolving 16.72 g of $NaHCO_3$ in 1 L of distilled water, and adjusting to pH 9.6 with a solution of 21.1 g of Na_2CO_3 in 1 L of distilled water.

Washing solution. Dissolve 42.71 g of Na_2HPO_4 and 53.06 g of NaCl in 4 L of distilled water, adjust to pH 7.4 with a mixture of 5.72 g of KH_2PO_4 and 6.14 g of NaCl in 0.7 L of distilled water. To 0.5 L of this phosphate-buffered saline add 2.5 g of casein and 300 μL of Tween 20.

Substrate solution. To 30 mg of *o*-phenylenediamine first add 100 mL of freshly prepared 0.1 mol/L citrate buffer, pH 5.0 (20.95 g of trisodium citrate dissolved in 0.7 L of distilled water, adjusted to pH 5.0 with 6.3 g of citric acid dissolved in 0.3 L of distilled water), then add 30 μL of the concentrated hydrogen peroxide solution. Prepare the substrate solution in, and dispense it from, a brown bottle. Fresh preparation of substrate solution before each run is recommended.

Standards. We prepared a stock 1 mg/L solution by dissolving human serum albumin in the assay buffer; we stored aliquots at −20 °C in tubes coated with the above-described washing solution. From this stock solution we prepared standards containing 0.03 to 8 mg per liter, in assay buffer.

Equipment. Polystyrene microtiter plates were obtained from Nunc, Wiesbaden, F.R.G. We measured absorbance with a micro-ELISA autoreader (MR 580; Dynatech, Denkendorf, F.R.G.).

Samples

We collected 24-h urine specimens in polyethylene containers that had been washed three successive times with the washing solution before use. We stored 2-mL aliquots of urine in tubes coated as described above at −20 °C for not longer than two months before assay. Urine samples were centrifuged at 3700 × *g* and diluted 10-fold in assay buffer before assay.

Patients

Urines from all patients and apparently healthy control subjects were negative for albumin by "Combur⁹-Test" (Boehringer Mannheim GmbH, Mannheim, F.R.G.; detection limit for albumin 60 mg/L), and for leukocytes and nitrite. Serum creatinine concentration was within the normal reference interval in all subjects, and results of routine clinical investigations were also within normal limits. Mean (±SD) age was 39.4 ±11.2 years (range 23–55, n = 40) for the control subjects, 34.4 ±10.4 years (range 22–48, n = 11) for Type I diabetics, and 55.0 ±7.6 years (45–66, n = 12) for patients with essential hypertension. For statistical evaluation of data we used Student's *t*-test.

ELISA Procedure

The ELISA was a solid-phase-binding double-antibody assay as described by Voller et al. (*22*). The procedure:

1. To sensitize the wells, incubate each well with 150 μL of rabbit anti-human albumin (diluted 5000-fold in assay buffer), keeping the plates covered for 4 h at 4 °C.
2. After emptying the wells, wash them three times with the washing solution, 150 μL per well. After the third wash

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incubate the plate for 30 min at 20 °C and then empty it, tapping it while it is inverted on paper towelling. After drying, it is ready to receive urine samples.

3. Pipette 100 μL of standard solution, sample, or assay buffer (as blank) into the wells in duplicate and incubate for 1.5 h at 20 °C.

4. Wash the wells as described in step 2, but without the incubation step.

5. To each well add 150 μL of peroxidase-labeled goat anti-human albumin (diluted 5000-fold in assay buffer) and incubate for 1 h at 20 °C.

6. Wash the wells again as described in step 4.

7. Add 150 μL of enzyme-substrate solution to each well and incubate for 20 min at 20 °C. Stop the reaction by adding 50 μL of sulfuric acid to each well.

8. Measure the absorbance of each well at 450 nm.

Results

Standard curve. The limit of detection (mean ±2 SD of readings for 21 albumin-free wells) was 16 μg/L, corresponding to a sensitivity of 1.6 ng of human serum albumin per well. The standard curve was linear over the range of 0.06 to 8.0 mg/L (Figure 1). A minimum of five standards is recommended, and standards have to be run with each plate.

Precision. Within-run precision was measured by assaying three urine samples eight times in one assay (Table 1). Between-run precision was estimated by assaying four urine samples on 10 consecutive days (Table 1).

Accuracy. To check the specificity of the ELISA system, we added urea, uric acid, creatinine, and α-, β-, and γ-globulins to the assay buffer in their greatest physiological concentrations and assayed. No cross reactivity was detected.

Analytical recovery. Recovery of low, middle, and high concentrations of human serum albumin was between 92.3 and 116.5% (Table 2). To demonstrate the absence of interfering substances we diluted, with assay buffer, urines having high, middle, and low concentrations of albumin (38.7, 5.0, 1.6 mg/L). The results of the dilution experiment are superimposable on the standard curve (Figure 1).

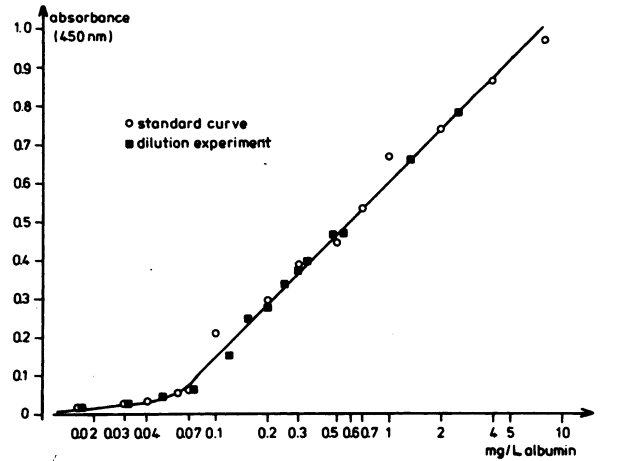

Fig. 1. Standard curve for human albumin in urine (○) and dilution curve (■)

Table 1. Precision of the Method

Albumin concn, mg/L		
Mean	**SD**	**CV, %**
Within-run		
4.19	0.386	9.2
1.05	0.053	5.1
0.10	0.008	7.6
Between-run		
1.01	0.17	16.2
2.10	0.31	14.9
3.57	0.34	9.6

Albumin in 24-h urines. Using this ELISA, we measured albumin excretion in albumin-test-strip-negative Type I diabetics, in albumin-test-strip-negative patients with essential hypertension, and in control subjects (Table 3). This excretion was significantly ($p < 0.05$) higher in the two groups of patients than in the control subjects. We could demonstrate no correlation between urinary albumin excretion and age in either the control subjects (Table 4) or the patients.

Discussion

This enzyme immunoassay (double-antibody "sandwich" technique) is simple and rapid to perform; all the reagents are both commercially available and inexpensive. Inter- and intra-assay variations and analytical recovery were satisfactory, although further improvements would probably be possible if semi-automated techniques were used. The limit of detection, 16 μg/L, corresponds to 1.6 ng of albumin per well, and is sufficiently low to detect above-normal albumin concentrations (5.0 ng per well in control subjects). ELISA-based urine-albumin assay techniques reported by several

Table 2. Analytical Recovery of Albumin

Albumin, mg/L		
Added	**Measured**	**Recovery, %**
0.5	0.58	116
1.0	1.01	101
2.0	1.94	97
3.0	2.75	92

Table 3. Albumin Excretion (mg/24 h) by Control Subjects and Patients

Mean ± SD (and range)		
Controls	**Type I diabetics**	**Essential hypertensives**
Females		
6.6 ± 5.1	14.2 ± 4.8	23.3 ± 17.2
(0.7 – 21.0)	(9.0 – 20.6)	(4.6 – 50.4)
n = 20	n = 5	n = 8
Males		
5.4 ± 3.1	15.0 ± 9.2	12.6 ± 13.3
(0.6 – 10.3)	(3.3 – 30.9)	(1.7 – 30.6)
n = 20	n = 6	n = 4
Total		
6.2 ± 4.1	14.7 ± 7.2	19.7 ± 16.2
(0.6 – 21.0)	(3.3 – 30.9)	(1.7 – 50.4)
n = 40	n = 11	n = 12

Table 4. Relation between Age and Albumin Excretion in Control Subjects

Age, y	20–30	31–40	41–50	51–60
	Albumin excretion, mg/24 h, mean (and range)			
Females (n = 5)	6.6 (2.0–14.5)	6.6 (2.0–21.0)	5.9 (2.3–9.8)	8.9 (3.1–11.7)
Males (n = 5)	6.3 (3.5–10.3)	4.5 (0.7–9.3)	7.5 (5.4–9.9)	3.5 (0.6–9.0)

investigators (*16–21*) also give satisfactory results, but most are either relatively time consuming and complicated or require reagents that are not commerically available. Using our immunoassay in clinical studies, the values we found for albumin in 24-h urine specimens from normal controls accorded well with results of other investigators (*13, 14, 20, 23*). Above-normal excretions of albumin were demonstrated in patients with essential hypertension and Type I diabetes, although routine laboratory methods (albumin-test-strip, serum-creatinine) had shown normal results for these subjects.

Others have reported conflicting results regarding age-dependence of albumin excretion in healthy children (*24, 25*); we found no such correlation in adults (Table 4).

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