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TECHNICAL NOTE

A solid phase fluorescent immunoassay for the measurement of human urinary albumin

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Human urinary albumin excretion (UAE) has been measured by radial immunodiffusion [1], nephelometry [2], and radioimmunoassay (RIA) [3]. RIA measures nanogram quantities of urinary albumin and, to our knowledge, is the most sensitive available method for quantitating UAE not detectable by Albustix. Recent studies argue that increased microalbuminuria in patients with insulin-dependent diabetes may be predictive of nephropathy risk [4, 5]. Because RIA poses potential health hazards, requires expensive equipment, and is technically complex, we evaluated a non-isotopic immunoassay to measure UAE. This fluorescent immunoassay (FIA) resembles the RIA, requiring specific antiserum and similar incubation and separation techniques [6]. The FIA is more sensitive than radial immunodiffusion methods and has been shown to accurately measure human albumin and immunoglobulin levels in saliva [7]. Herein, we report our studies on the sensitivity, accuracy, and reproducibility of the FIA for measuring UAE in normal subjects.

Methods. Thirty-two healthy adults (14 females) hospitalized for evaluation as potential kidney transplant donors served as the subjects. Thus, BP, urinalysis, urine culture, serum creatinine, 24-hr urine collection for creatinine clearance, UAE, and an intravenous pyelogram (IVP) were obtained on all subjects. These subjects, ranging in age from 17 to 61 years (mean, 32.3 years), had normal BP (mean, 116/78 mm Hg), serum creatinine levels (mean, 0.92 mg/dl), and normal creatinine clearances (mean, 105 ml/min/1.73 m^2). All other studies were within normal limits. Serum albumin levels were measured in 22 of these subjects and were normal (mean, 4.08 g/dl). Informed consent was obtained from all participants. A 5-ml aliquot from a measured 24-hr urine collection was preserved with 25 μl of 2% sodium azide (NaN_3) and stored at −20°C until evaluated.

Reagents. Immunobead reagent for antibody coupling was obtained from Bio-Rad Laboratories, Richmond, California. Both fluorescein isothiocyanate (FITC) conjugated and unconjugated goat antisera to human albumin (GAHA) (IgG fraction) were purchased from Cappel Laboratories, Cochranville, Pennsylvania. Chicken egg albumin grade V and human albumin were obtained from Sigma Chemical Company, St. Louis, Missouri. Lyophyllized immunobead matrix and antisera to human albumin were reconstituted according to package instructions and stored at 4°C and −20°C, respectively, until used.

Immunobead preparation and coupling with antibody. Covalent bonding of antibody to the immunobead matrix was carried out according to Bio-Rad Laboratory instructions [8] with the following modifications: GAHA (2 mg) was dialyzed for 4 hr against 500 ml of couling buffer (0.003 M KH_2PO_4, pH 6.3) at 4°C. The dialyzed antibody was added to 200 mg of lyophillized immunobead matrix, and the total volume of the preparation was adjusted to 20 ml with sterile water and brought to pH 6.3 with 0.5 N HCl. Following incubation at 4°C for 1 hr, 40 mg of ethyl dimethylaminopropyl carbodiimide (EDAC) were added to the mixture to facilitate covalent bonding of the GAHA to the beads. The mixture was brought to a pH of 6.3 at room temperature and maintained at that pH for 30 min with 0.5 N HCl and 1 N KOH. The mixture was then stored overnight at 4°C. Unbound material was removed by washing with buffers in the following sequence: phosphate-buffered saline (PBS) (0.01 M KH_2PO_4, 0.15 M NaCl, pH 7.2) times three; 1.4 M NaCl—PBS twice; two additional washes in PBS. The immunobead matrix was resuspended in 20 ml PBS and incubated at 4°C for 3 hr to allow renaturation of the covalently bound proteins. This step was followed by two washes in 0.005 M KH_2PO_4, pH 7.2. Each wash was followed by centrifugation at 2000 rpm for 10 min, decanting of the supernatant, and resuspension of the pellet in the appropriate buffer. A phosphate buffer (PO, 0.005 M phosphate–0.5% ovalbumin 0.1% NaN_3) was used for the last resuspension of the pellet. The immunobead reagent was then stored at 4°C until used.

Preparation of human serum albumin (HSA) standards. Lyophyllized HSA (2.5 mg) was solubilized in 25 ml of 0.005 M PO buffer to produce a 100,000 ng/ml standard. All other standards were prepared from this stock solution.

Urinary albumin FIA procedure. All urine samples were assayed at room temperature either undiluted or diluted 1:2 in 0.5% ovalbumin-PBS. All urine samples were negative for albumin by Albustix® (Ames Division, Miles Laboratories, Elkhart, Indiana), which became positive at an albumin level of 50,000 ng/ml. Urine samples that are positive by Albustix should be diluted (trace − 1:10, + − 1:50, ++ − 1:100, +++ − 1:1000) prior to measuring the albumin level. The FIA is a variation of methods previously described [6, 7]. The antibody coupled immunobead reagent (100 μl) was added to borosilicate tubes, followed by 20 μl of either a standard or urine sample and mixed by agitation on a vortex mixer. PBS (20 μl) was added to

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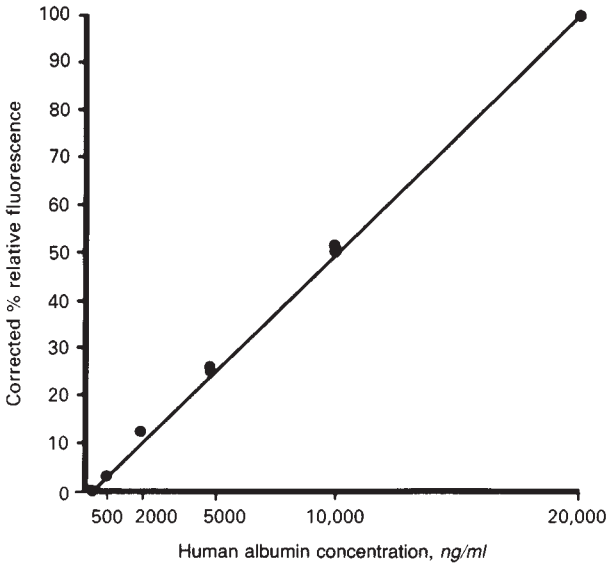

Fig. 1. *Standard curve for human albumin.* Each *closed circle* represents human albumin concentration.

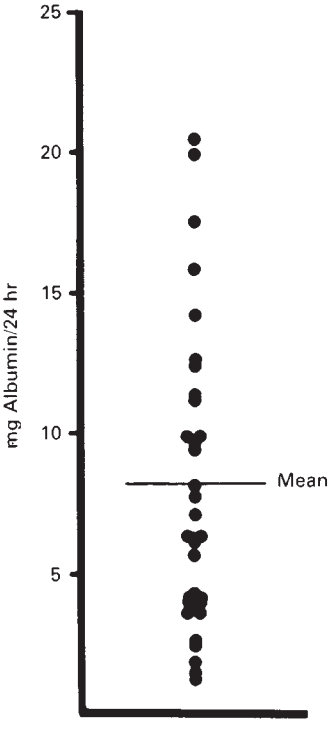

Fig. 2. *Twenty-four hour urinary albumin excretion in control subjects.* Each *closed circle* represents one individual.

the blank tubes. After incubation at 37°C for 1.5 hr, 20 μl of 1% ovalbumin-PBS, and 20 μl of FITC GAHA (1:20 dilution with 1% ovalbumin in PBS buffer) were added to each tube followed by mixing and incubation at 37°C for 2 hr. Samples were then washed in PBS followed by centrifugation at 3000 rpm for 10 min. This step was repeated twice. The remaining pellet was resuspended in 2.0 ml PBS and the percent relative fluorescence (%RF) determined using a spectrofluorometer (Aminco-Bowman American Instrument Company, Silver Springs, Maryland) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The albumin concentration of duplicate samples was calculated from a standard curve. Standard statistical formulae were used.

Table 1. Coefficient of variation within-run analysis of ten samples of three unknown urine specimens

Specimen number	Mean albumin concentration *ng/ml*	SD	Coefficient of variation (%)[a]
1	7016	538	7.67
2	9898	674	6.81
3	6763	381	5.63

[a] Coefficient of variation $= \frac{(\text{SD}) \times 100\%}{(\text{mean})}$.

Results. The standard curve was linear in the range of 500 to 20,000 ng/ml of albumin with a correlation coefficient of 0.99 (Fig. 1). The standard curve was not linear at values above 20,000 ng/ml of albumin. The mean %RF of 32 samples of a 500 ng/ml standard assayed on 16 different days was 3.7% ± 0.67 ($\bar{X}$ ± SD). The mean recovery of 2500 or 5000 ng/ml of albumin standard added to 16 urine samples was 105% ± 7%. The interassay coefficient of variation (CV) of ten samples of three different unknown urine specimens measured on the same day was 5.6 to 7.7% (Table 1). The intra-assay CV for 23 urine samples (measuring between 700 and 19,900 ng/ml) each measured on 2 to 4 different days was 7.8%. Using range statistics, we calculated the average difference between duplicates on 200 random samples. The allowable difference between duplicates was 500 ng/ml [9]. The mean 24-hr UAE rate for the subjects was 8.16 mg ± 5.24 ($\bar{X}$ ± SD; range, 1.33 to 20.50 mg) (Fig. 2). There was no significant correlation between UAE and age ($r = -0.21$), body surface area ($r = -0.19$) or creatinine clearance ($r = 0.23$).

Discussion. This study demonstrates that the FIA is a sensitive method for detecting human UAE in the normal concentration range. The inter- and intra-assay CV experiments demonstrate the good precision of the assay at nanograms per milliliter quantities. Recovery studies compare favorably to a recovery of 103 ± 9% reported in the Immuno-Fluor® instruction manual [10].

Urinary albumin has been measured previously by FIA employing fluorescein labelling of the albumin antigen and magnetizable particles to which antibodies to HSA are covalently linked [11] or albumin labelled with fluorescein and antibodies against both albumin and fluorescein [12]. However, the calibration curves for these methods are nonlinear as compared to the linear calibration curve in the method we describe; these methods are relatively insensitive measuring 1.5 million to 100 million ng/ml and 4 million to 100 million ng/ml, respectively. The method we have described in this report is a minor modification of the method previously described by Izutsu et al [7]. The major new observation is the application of the method to urine. The lower limit of sensitivity of the FIA method described by Izutsu et al [7] to measure salivary albumin levels was 1000 ng/ml, similar to the lower limit of sensitivity of 500 ng/ml with the method we have described.

The values of UAE in normal subjects obtained in this study are very similar to these obtained by RIA. Viberti et al [13] and Mogensen [14] report UAE in normal subjects to be 3.6 to 23 and 7.2 to 28.8 mg/24 hr, respectively.

The FIA procedure for UAE is reproducible over a wide range. The method is sensitive and precise and can also be applied to urine samples containing high concentrations of albumin. The assay can be performed in 4 to 6 hr. The low cost and avoidance of isotopic reagents offer significant advantages over the RIA.

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