

Microalbuminuria Immunoassay Based on Antibodies Covalently Conjugated to Eupergit C-Coated Beads

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OBJECTIVE — To develop a reliable, simple, and sensitive assay for microalbuminuria, based on covalent attachment of anti-HSA to oxirane-bearing polymethylmethacrylate beads (Eupergit CB6200).

RESEARCH DESIGN AND METHODS — Anti-HSA antibodies were coupled to CB6200 beads by reaction of their amino groups with the oxirane groups of the matrix. The capability of the beads to bind HSA from standard solutions or urine was evaluated and compared with the state of the art ELISA test.

RESULTS — The new bead immunoassay is sensitive and linear in the range of 1–25 mg/L, which is considered the low microalbuminuria range. When HSA levels in urine were tested, the intra- and interassay CV values ranged between 2.7 and 3.9% and between 5.6 and 6.6%, respectively. The long-term storage stability of the antibodies covalently bound on the beads was higher than of the same antibodies adsorbed on ELISA plates. After 16 wk of storage, the CV was about 7.3% with the bead assay, compared with 14% obtained for the ELISA test under the same experimental conditions.

CONCLUSIONS — A new procedure for microalbuminuria assay was developed, with Eupergit CB6200 beads as a solid support for covalent binding of the first antibody. Accuracy, sensitivity, reproducibility, and precision of the bead immunoassay were similar to those of commonly used immunoassays, as exemplified by the analysis of HSA in 53 clinical urine samples. The bead assay retains a low degree of variability over long storage periods, and the beads may be reapplied after a simple acid-washing procedure.

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HSA, HUMAN SERUM ALBUMIN; CV, COEFFICIENT OF VARIATION; ELISA, ENZYME-LINKED IMMUNOSORBENT ASSAY; HRP, HORSERADISH PEROXIDASE; PBS, PHOSPHATE-BUFFERED SALINE; T₃, TRIIODOTHYRONINE; IgG, IMMUNOGLOBULIN G.

Diabetic patients are characterized by gradual increase in urinary albumin excretion, which at the early stages is still undetectable by routine Albustix tests. Yet, detection of urinary albumin at this phase, known as microalbuminuria, is crucial for the early prediction of diabetic nephropathy, especially when protein levels exceed 50 mg/24 h (1).

Various methods have been described for quantitative determination of low concentrations of urinary albumin. These include rocket immunoelectrophoresis (2), radial immunodiffusion (3), radioimmunoassay (4), immunoturbidimetric test, latex agglutination, immunofluorescence, and bromphenol blue binding assays (5–8). However, each of these methods suffers from some specific disadvantages, mostly inaccuracy and lack of sensitivity.

In recent years, numerous laboratories have adopted ELISA for measuring urinary albumin, using polystyrene microtiter plates (9) or beads (10). Yet, the hydrophobic nature of this carrier results in obvious deficiencies associated with interassay variations caused by non-homogeneous coating of the anti-human albumin antibodies and their leakage upon long-term storage.

In this paper, we present the application of Eupergit C-coated beads as a solid support for detection of microalbuminuria. These beads, composed of a solid polymethylmethacrylamide core coated with 1- μ m Eupergit C beads and bearing activated epoxy groups, are capable of covalent binding of antibodies (11). The validation of a bead-based enzyme immunoassay that may be adopted for albumin and other proteins in a stable and reproducible manner in the clinical laboratory is described.

RESEARCH DESIGN AND METHODS

Reagents

All reagents were analytical grade and obtained from Sigma (St. Louis, MO).

HSA and rabbit anti-HSA antibodies were obtained from Bio-Makor (Rehovot, Israel) and HRP-labeled anti-HSA was obtained from Serotek (Oxford, UK). Eupergit C-coated beads (CB6200) were obtained from the Rohm Co. (Darmstadt, Germany).

Clinical specimen

Urine samples were collected every 24 h in polyethylene containers, mixed thoroughly, and kept at 4°C for no longer than 7 days until tested. The samples were not frozen because 10–40% losses in albumin content were noted when urine was stored at –20°C because of precipitates formed during freezing and thawing (12).

Immobilization of antibodies on Eupergit C

Eupergit C-coated beads (CB6200, 6-mm in diameter) kept at –20°C were rinsed in 1 M potassium phosphate buffer (pH 7.4) and reacted with a solution of rabbit anti-HSA at a concentration of 2 µg antibody/bead. After 16 h of incubation at 4°C, the beads were rinsed in the washing buffer, and the excess of oxirane groups on the matrix was blocked by incubation with 0.2 M β-mercaptoethanol for 4 h at 4°C. After further rinse in PBS, the beads could be stored at 4°C for several months. A more detailed account of immobilization of proteins on Eupergit C-coated beads was described previously (11).

ELISA tests

ELISA tests for microalbuminuria, which used polystyrene microtiter plates, were performed as described by Feldt-Rasmussen et al. (13).

Microalbuminuria immunoassay with Eupergit C-coated beads

The beads containing covalently bound anti-HSA antibodies were incubated for 1 h at 37°C with increasing amounts of HSA (0.1–0.5 µg/bead) or 1-µL urine samples in 1.0 mL of PBS containing 5% casein, 1% Triton X-100, and 1% poly-

ethylene glycol 400, which was included to reduce nonspecific adsorption of HSA to the beads. After removal of the unbound proteins by extensive washing with distilled water, HRP-labeled anti-HSA antibodies (diluted 1:2000) in the above buffer were added and incubated. These reactions were conducted with the individual beads incubated with the various solutions and rinsed in 10-mL glass tubes. After the binding of the second HRP-conjugated antibody, the reacted Eupergit beads were transferred to the large wells of a tissue culture CLUSTER 24 plate (Nunc, Roskilde, Denmark) (1 bead per well). Enzymatic activity of HRP bound to the beads was determined with *o*-phenylenediamine (2.0 g/L in 0.05 M citrate buffer, pH 5.0, containing 2% H₂O₂). The reaction was stopped after 15 min by the addition of 100 µL of 4 M HCl. One hundred-microliter aliquots of the supernatants were transferred into the wells of a Nunc 96-well microtiter plate, and their absorbance was monitored with a Kontron reader (SLT 210, Zurich, Switzerland) at 492 nm with a 405-nm reference. All assays were conducted in duplicate. Note that laboratories equipped with an Abbott setup for various diagnostic kits may automate the above procedure using the Abbott 25- or 48-well plates adapted for the automatic bead washer Kwikwash and the Abbott automatic Quantum spectrophotometric reader.

Repeated use of the bead-antibodies conjugates in immunoassay

Ten beads containing anti-albumin antibodies previously used in the microalbuminuria immunoassay were incubated for 30 min with 0.2 M glycine buffer, pH 2.0, to remove immunologically bound antigen. The beads were washed extensively with PBS, and their performance in a calibration curve constructed with known amounts of HSA was evaluated.

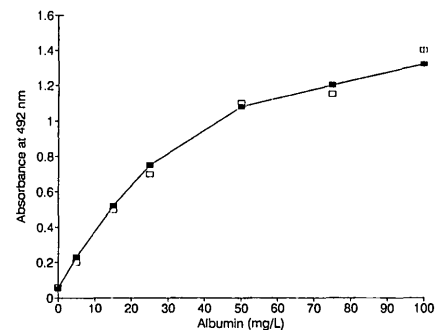


Figure 1—Calibration curve of HSA with covalently immobilized anti-HSA on fresh (■) and reused (□) antibody-coated CB6200 beads.

Long-term storage stability of antibodies coated on beads and on ELISA plates

Thirty beads and 10 ELISA plates, freshly coated with anti-albumin antibodies, were stored at 4°C. After different lengths of time (up to 16 wk), 5 beads and 1 ELISA plate were brought to room temperature and incubated with HSA (30 mg/L) as described above. The amount of bound HSA was measured by the standard assay procedure, and intraassay CV values were calculated.

RESULTS— Our aim in this study was to develop an enzyme immunoassay for microalbuminuria that would be more accurate than the commonly used ELISA tests because of the covalent binding of the respective antibodies to the Eupergit C-coated beads. A standard curve, constructed with known amounts of HSA, yielded a linear increase in bound albumin as a function of added protein to a concentration of 25 mg/L HSA, which is considered the lower microalbuminuria range (Fig. 1). The assay sensitivity, the lowest albumin concentration significantly detected, was 2.0 mg/L (95% confidence interval). Recovery of albumin from the clinical samples was tested by addition of known amounts of HSA (5 to 50 mg/L) to 5 random urine samples. The values obtained for the recovered HSA varied between 98 and 101% of the expected values.

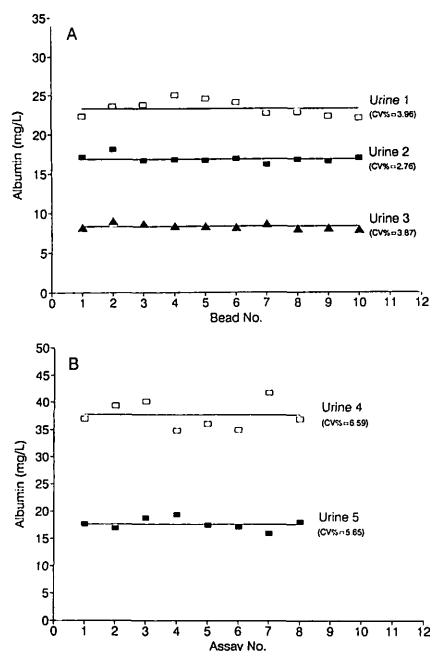


Figure 2—Precision of microalbuminuria bead immunoassay determined as intra- (A) and interassay (B) variations.

The precision of the assay in the range of 5–40 mg/L was evaluated by the intra- and interassay CVs. For the evaluation of the intraassay CV, each of 3 urine samples was tested with 10 beads in the same assay. CV values were calculated as 2.7–3.9% (Fig. 2A). Interassay CV values ranged between 5.6 and 6.6% and were calculated from measurements of albumin contents in two urine samples by 8 repetitive daily assays (Fig. 2B). Both intra- and interassay CVs are comparable with reported values obtained with ELISA and other methods (13,14). When HSA concentrations of 53 urine samples were analyzed by the bead-based assay and ELISA, a high correlation was observed between the results obtained by the two procedures in the range of 2–100 mg albumin/L (Fig. 3).

Because the anti-albumin antibodies are bound covalently to the CB6200 beads in a highly stable manner (11), their repeated use in the bead immunoassay was feasible. After removal of

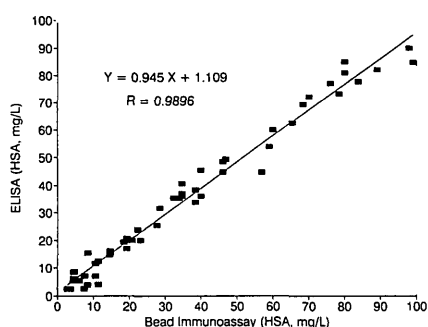


Figure 3—Correlation of albumin concentrations in clinical urine samples tested simultaneously by ELISA and bead immunoassay. Each dot represents single urine sample tested by both assays.

all the immunologically bound antigen from the beads by washing with low pH buffer, the beads were reapplied to the assay without recoating with the first antibodies. The standard curve obtained for the reused beads with known amounts of HSA was similar to that obtained for freshly coated beads (Fig. 1).

Most laboratories that perform ELISA tests are accustomed to periodically coating polystyrene microtiter plates with fresh antibodies and storing them in the cold for as long as 4 wk. At longer storage periods, antibodies tend to lose activity or desorb from the plates, resulting in a decrease in the reproducibility and sensitivity of the assay (15). Antibodies covalently linked to a solid support are expected to possess higher shelf stability than the respective antibodies immobilized by hydrophobic adsorption because 1) desorption is eliminated, and 2) the antibodies are stabilized by multipoint attachments to the solid support (16).

As shown in Fig. 4, the intraassay CV of the bead assay was less affected by long-term storage than those for the ELISA. After 16 wk of storage, the CV was ~7.3% for the bead assay compared with 14% for the ELISA under the same experimental conditions.

CONCLUSIONS—A new procedure for microalbuminuria assay is proposed

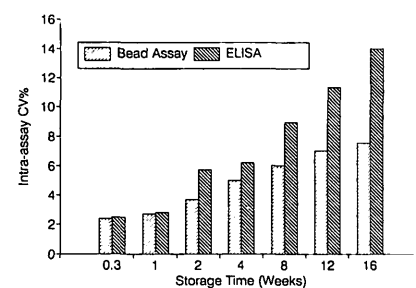


Figure 4—Long-storage stability of antibodies immobilized covalently on CB6200 beads or adsorbed on ELISA plates (see text for details).

that uses Eupergit C CB6200 beads as a solid-phase carrier for covalent binding of the first antibody. The assay performance with respect to its accuracy, sensitivity, reproducibility, and precision (low intra- and interassay CVs), is similar to that of commonly used ELISAs. In contrast to assays based on noncovalent binding of the antibodies to polystyrene surfaces, the bead assay retains a low degree of variability over long storage periods. In addition, the beads may be reapplied after a simple acid-washing procedure.

While this work was in progress, Kakabakos et al. (17) reported measurement of T₃ antigen with covalently bound sheep anti-IgG antibodies on Dylark beads.

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