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Assessment of Immunochemical Methods for Determining Low Concentrations of Albumin in Urine

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Four immunochemical methods (radioimmunoassay, RIA; radial immunodiffusion, RID; immunoturbidimetry, IT; enzyme-linked immunosorbent assay, ELISA) for measuring urinary albumin at low concentrations were assessed for their assay characteristics and practicability. Precision and accuracy were comparable between the methods when studied individually. We made a method comparison, with RIA as reference, using urine samples from diabetic patients with albumin concentrations ranging from 1 to 120 mg/L. There was no significant systematic difference between RID and RIA, but IT and ELISA gave consistently lower values than RIA, the mean differences being 1.8 ($p < 0.01$) and 9.7 mg/L ($p < 0.001$), respectively. Random error, compared with that for RIA, was in increasing order: RID (residual SD = 3.8 mg/L); IT (4.3 mg/L); ELISA (7.3 mg/L). The difference between the methods increased with the albumin concentration. Operational cost was highest with IT, lowest with RIA. Capital cost was highest with RIA and lowest with RID, which required most technical skill. ELISA had intermediate overall costs.

Additional Keyphrases: *radioimmunoassay · radial immunodiffusion · immunoturbidimetry · enzyme immunoassay · diabetes · economics of laboratory operation · early detection of diabetic nephropathy*

Diabetic nephropathy is a major cause of death in insulin-dependent diabetics (*1*). Recently, three independent studies have shown that its onset may be predicted by an increased urinary excretion of albumin that's undetectable by clinical dip-stick methods (*2–4*). An albumin excretion between 15 and 200 μg/min (equivalent to an albumin concentration of 15 to 200 mg/L, assuming a urine flow rate of 1 mL/min) has been termed "microalbuminuria," slight albuminuria. It may be reversed by strict glycemic control (*5*) or decreased significantly by treatment of concomitant hypertension (*6*). Consequently, there is an increasing demand from clinicians for the screening and monitoring of albumin excretion in diabetics.

The clinical chemistry laboratory requires a technique that is sensitive, specific for albumin, and practicable. Currently, these requirements are met only by immunochemical methods. We have studied the assay characteristics and practicability of four immunochemical techniques: radioimmunoassay (RIA) (*7*), single radial immunodiffusion (RID) (*8*), immunoturbidimetry (IT) (*9*), and an enzyme-linked immunosorbent assay (ELISA) (*10*).[6] In a method-comparison study, we used radioimmunoassay as the reference method, because it is the longest-established assay and has been used in most studies of diabetic microalbuminuria (*2, 4, 5*).

Materials and Methods

Materials

Standard: In all the methods, the standard used was "Pure Human Albumin" (Behring Diagnostics, Hoechst, U.K. Ltd., Hounslow, Middlesex, U.K.).

Antibody: The same antibody, rabbit anti-human albumin (Dako Ltd., High Wycombe, Buckinghamshire, U.K.), was used in all assays.

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[6] Nonstandard abbreviations: RID, radial immunodiffusion; IT, immunoturbidimetry; ELISA, enzyme-linked immunosorbent assay.

Test samples: For the method-comparison study, urine specimens from 92 diabetics were stored at −20 °C with sodium azide (2 g/L) as preservative. They were thawed just before analysis. The urines, all negative to dip-stick testing with Albustix (Ames Co., Stoke Poges, Buckinghamshire, U.K.), had albumin concentrations ranging from 1 to 120 mg/L.

Quality control: Pure Human Albumin standard was added to a urine sample of known albumin concentration to provide quality-control material at low, middle, and high concentrations along the analytical range of the assays.

Procedures

Radioimmunoassay. This is a "saturation assay," performed in liquid phase in the presence of excess antigen. Reaction equilibrium is reflected by the amount of bound, radioactively labeled analyte. Pure Human Albumin was labeled with ^{125}I (code 17530; Amersham International, Amersham, Buckinghamshire, U.K.) by the Chloramine T method (Dr. D. Pearson, Unit for Metabolic Medicine, United Medical & Dental Schools, Guy's Hospital, St. Thomas St., London SE1 9RT, U.K.).

We used small polypropylene cuvettes (no. 2174-701; LKB Clinicon Ltd., Lewes, Sussex, U.K.), adding to each 100 μL of ^{125}I-labeled albumin tracer (specific activity about 200 mCi/g); 100 μL of albumin standard, test, or control samples; and 100 μL of antibody that had been previously diluted 400-fold in phosphate-buffered (0.2 mol/L, pH 8.0) isotonic saline. Tubes were also included for total counts (100 μL of tracer only) and nonspecific binding (100 μL of tracer, 200 μL of the phosphate-buffered saline). Addition of analyte and reagents was mechanized by use of a sample processor (Model 2071; LKB Clinicon Systems Ltd.). After mixing, we incubated the reactants overnight at 4 °C. Polyethylene glycol 6000 (BDH Chemicals Ltd., Poole, Dorset, U.K.) and bovine γ-globulin (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) were added to phosphate-buffered saline at 4 °C to give concentrations of 125 and 0.5 g/L, respectively, and 1.5 mL of this solution was added to each assay tube. The reactants were mixed, then centrifuged (2000 × *g*, 20 min, 4 °C), and the supernatant fluid was rapidly decanted. The radioactivity in the tubes was counted (1270 Rackgamma II Counter; LKB Nuclear Ltd., Croydon, London, U.K.) at the rate of 30 to 50 000 counts/min. A built-in microprocessor constructed an albumin standard curve of activity vs log concentration (range 1.25 to 80 mg/L), with linear interpolation of data points, from which the test and control values were derived.

Radial immunodiffusion. This assay takes place in an antibody-containing agar gel. One monitors the distance an antigen travels to reach equilibrium, at which point antigen–antibody complexes precipitate in the presence of antibody excess. The distance is measured manually by assessing the diameter of a stainable ring of albumin–anti-albumin complex.

Agar gel media were prepared by pouring 10 mL of a 10 g/L solution of agarose (BDH Chemicals) in diethyl barbital buffer (50 mmol/L, pH 8.6) onto 8.5 × 8.5 cm slide-coverglass plates (Kodak Ltd., London, U.K.) We added 10 μL of anti-human albumin antibody to the agarose just before pouring (i.e., antibody dilution was 1000-fold). After gelling was complete, we punched sixty 2-mm-diameter wells in each agar plate, using a gel cutter attached to a rotary vacuum pump (RE2 single stage; Baird & Tatlock, Romford, Essex, U.K.). To each well we added 2.5 μL of standard, test, or control; let the plates stand in a moist environment for at least 48 h; then dried them at 37 °C with moist, overlying filter paper. After staining the plate with Coomassie Blue (Sigma Chemicals), we differentiated the rings with dilute acetic acid (BDH Chemicals), 50 mL/L in distilled water, and measured the ring diameters. Readings were entered into a microprocessor, which constructed an albumin calibration curve (line of best fit) based on diameter^2 vs arithmetic concentration (range 2.5–80 mg/L), from which the test and control results were derived.

Immunoturbidimetry. In this kinetic assay the rate of precipitation of antigen–antibody complex in solution and in the presence of antibody excess is detected by the increase of absorbance of transmitted light at 340 nm. Using a Hamilton Digital Dilutor (VA Howe Co. Ltd., London, U.K.), we diluted 50 μL of standard, control, and test samples 9-fold in phosphate-buffered saline (0.1 mol/L, pH 7.4) containing 40 g of polyethylene glycol-6000 per liter. Anti-human albumin antibody was diluted 12-fold in the same buffer and 100 μL of this was added to diluted standard, test, or control samples in a Model 2086 reaction rate analyzer (LKB Clinicon Systems Ltd.), the reaction taking place in 7-mm-diameter cuvettes (Sarstedt Co. Ltd., Leicester, U.K.), and the absorbance change at 340 nm was monitored for 2 min. Reaction profiles were recorded graphically on a flat-bed recorder. The albumin calibration curve was plotted manually for peak height vs concentration (range 2–80 mg/L), and test and control results were read from this curve.

Enzyme-linked immunosorbent assay. This ELISA is a "two-site" immunoassay in which antigen is fixed by binding to excess first antibody that has been previously immobilized on a solid phase. Complexes are detected and quantified by their reaction with a second antibody conjugated to an enzyme label. The conjugate, prepared by conjugating horseradish peroxidase (EC 1.11.1.7; Sigma Chemicals) to rabbit anti-human albumin antibody as described elsewhere (*11*), is stored at 4 °C in an equal volume of buffered glycol (per liter: 600 g of glycerol, 0.1 mol of borate, pH 7.4).

We incubated 200 μL of rabbit anti-human albumin antibody diluted 1000-fold in carbonate–bicarbonate buffer (50 mmol/L, pH 9.6) at 4 °C in all but two wells of a covered polystyrene microtitre plate (Nunc Immunoplate II; Gibco Europe, Uxbridge, U.K.). The plates were washed three times in an automatic plate washer with phosphate-buffered (0.1 mol/L, pH 7.2) isotonic saline containing Tween-20 (0.5 mL/L) and gelatin (500 mg/L), i.e., PBS-Tween buffer. We then filled 94 of the 96 wells with 200 μL of albumin standards (concentrations 2 to 512 μg/L), controls, and test samples (pre-diluted 200-fold in PBS-Tween buffer), and incubated the plate for 1 h at room temperature. After washing the plate three times as before, we added 200 μL of conjugate to each well. We again washed the plate three times and then, in subdued light, added 200 μL of a fresh solution of *o*-phenylenediamine (Sigma Chemicals), 0.3 g/L in citrate–phosphate buffer (150 mmol/L), and 10 μL of 300 g/L hydrogen peroxide solution. After incubating the plate for 20 min in the dark, we stopped the color reaction by adding 20 μL of 340 g/L sulfuric acid reagent to each well. We read absorbance at 420 nm with a microplate reader (MR 580; Dynatech Laboratories, Billingshurst, Sussex, U.K.) with 200 μL of substrate in the two unused wells as blanks. The calibration curve of absorbance vs log concentration was constructed by the reader's microprocessor by linear interpolation of data points, from which tests and controls were calculated.

Analytical Variables

Assay characteristics. The analytical range of each method was considered to be the concentration range of the respective calibration curve for which the inter-assay CV was <12% (n = 20) and included the detection limit of the assay. The detection limit was the lowest concentration of urinary albumin that was consistently distinguishable from blank (n = 20). Intra-assay variation was calculated from duplicates at concentrations covering the analytical range (n = 50). Inter-assay variation was measured over 20 separate runs: for RIA, RID, and IT, albumin concentrations were 5, 15, and 30 mg/L; for ELISA, 23, 77, and 92 μg/L. Analytical recovery was assessed by measuring known albumin concentrations (5, 15, and 30 mg/L for RIA, RID, IT; 25, 50, and 100 μg/L for ELISA) added to five separate urine samples and comparing observed and expected values within the same run. We have reported the ranges of inter-assay CV and of percentage recoveries.

Practicability. To assess practicability, we considered several criteria. We subjectively graded technical skill from 1 to 4 in increasing order of skill required. Costing was divided into capital cost and operating cost per 100 tests (1985 prices; £1 sterling = $1.42 US). The cost of a microprocessor was not included for RID and IT assessment because it was not an integral part of their instrumentation. Operating cost included labor cost, which we calculated from the hourly wage for a State-registered Medical Laboratory Scientific Officer employed in the National Health Service in the U.K. We also evaluated the practicability of sample size, amount of radioactivity per 100 tests, number of reagent additions, number of centrifugation steps, and turnaround time.

Statistical Methods

Urinary concentration of albumin obtained with RID, IT, and ELISA were independently compared with RIA as reference by plotting the differences between paired samples against the mean of the two values (*12*). Systematic error was evaluated by performing a paired *t*-test on the mean difference between methods. Random error between methods was assessed as the residual standard deviation by a two-way analysis of variance (*13*). Because the absolute variation between methods increased with the albumin concentration, we assessed systematic and random errors for low and high concentrations. We arbitrarily divided results above and below 30 mg/L, an albumin concentration greater than this having been shown to predict diabetic nephropathy (*14*).

Results

Assay characteristics. The analytical ranges for RIA, RID, and IT were approximately similar, reflecting the same order of assay sensitivity (Table 1). The ELISA had the lowest detection limit (6.25 μg/L) and analytical range. Intra- and inter-assay imprecision and analytical recovery were comparable for all methods.

Table 1. Comparison of the Assay Characteristics of RIA, RID, IT, and ELISA

	RIA	RID	IT	ELISA
Analytical range[a]	1–40 mg/L	2.5–40 mg/L	2.5–60 mg/L	6.25–200 μg/L
Imprecision (CV), %				
Intra-assay (n = 50)	3	3	3	3
Interassay (n = 20)	8–9	5–7	3–10	8–10
Analytical recovery, %	93–101	94–97	98–102	92–102

[a] For albumin concentrations.

Methods comparison. As Figure 1 illustrates, the absolute variance between methods increased with the size of the albumin concentration. In the comparisons of RIA results with those by IT and ELISA, most plotted values are above the zero-difference line, suggesting that IT and ELISA gave consistently lower readings than RIA, particularly for samples with albumin concentrations >30 mg/L. Table 2, illustrating the mean differences between methods and the residual SD, clearly shows that readings by ELISA deviated most from the RIA results. The ELISA readings were consistently lower for both low and high albumin concentrations, and the residual SD was greatest between ELISA and RIA. IT also gave lower readings than RIA, but only in the upper range of albumin concentrations (i.e., >30 mg/L). RID readings were closest to those by RIA, but were consistently higher than RIA in the low range (i.e., <30 mg/L).

Practicability. As summarized in Table 3, turnaround time per 100 tests was longest with RID and shortest with ELISA or IT. Technical skill required was greatest with RID and least with IT. Operating cost per 100 tests was largest with IT and smallest with RIA. Capital cost was disproportionately low with RID and greatest with RIA.

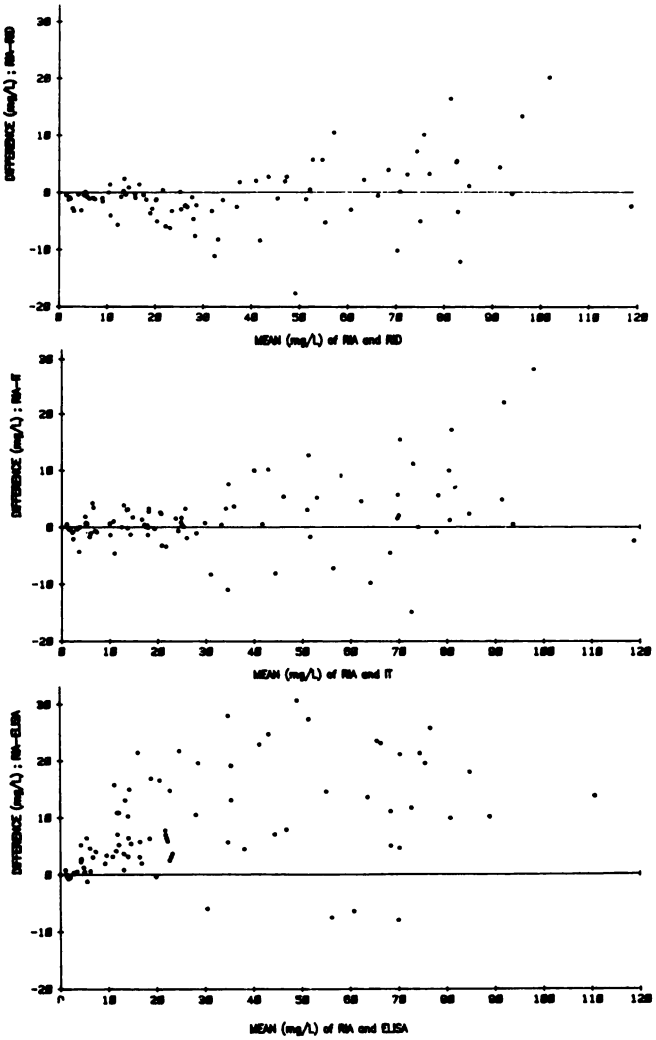

Fig. 1. Differences in the concentration of urinary albumin as measured by RIA and by (*top*) RID, (*middle*) IT, and (*bottom*) ELISA, compared with the mean of the concentrations measured by each method

Table 2. Statistical Comparison of Urinary Albumin Concentrations (U_A, mg/L) Determined by RIA, RID, IT, and ELISA

	Mean difference between methods	Residual SD
	mg/L	
All samples (n = 92)		
RIA − RID	−0.6	3.8
RIA − IT	1.8[a]	4.3
RIA − ELISA	9.7[b]	7.3
Mean U_A ≤30 (n = 54)		
RIA − RID	−2.1[b]	1.8
RIA − IT	−0.1	1.9
RIA − ELISA	4.9[b]	3.8
Mean U_A >30 (n = 38)		
RIA − RID	1.4	5.2
RIA − IT	4.4[a]	5.9
RIA − ELISA	16.6[b]	8.3

[a,b] Significantly different ([a] $p < 0.01$, [b] $p < 0.001$) by Student's paired *t*-test.

Table 3. Practicability of the Four Methods for Urinary Albumin Compared

Criterion	RIA	RID	IT	ELISA
Sample size, μL	100	2.5	50	20
Radioactivity, μCi[a]	12	—	—	—
No. of reagent additions	3	2	2	3
No. of centrifugation steps	1	—	—	—
Technical skill required[b]	2	4	1	3
Turnaround time, h[a]	24	48	5.5	5.5
Operating cost, £[a]	13.2	14.6	18.3	14.1
Capital cost, £1000	20	0.235	13.5	10

[a] Per 100 tests. [b] Subjective rating, with 1 requiring the least skill.

Discussion

Although RIA, RID, IT, and ELISA demonstrated satisfactory individual method characteristics, the method comparison revealed significant analytical differences. The systematic differences between RID and RIA and between IT and RIA at low (<30 mg/L) and high (>30 mg/L) albumin concentrations, respectively, are small and not clinically important. If RIA values for urinary albumin of >30 mg/L are taken to predict diabetic nephropathy (*14*), both RID and IT in this study detected these values with 100% sensitivity (no false negatives). With ELISA the larger degree of bias is considered clinically significant, particularly because ELISA detected an RIA value >30 mg/L with only 74% sensitivity. The analytical reasons for the differences between the methods are difficult to explain, given the satisfactory assay characteristics. RID reportedly gives falsely high values if citrate and phosphate are excluded from the buffer (*15*), but we have not been able to confirm this. Observer bias when reading RID ring circumferences for concentrations <30 mg/L might account for the small constant difference for this method. The large random variation with ELISA might be due to the greater pre-dilution of test urines, plate "edge-effects," between-plate and between-well variation (*16*), or the effect of environmental factors on the enzyme detector system (*17*). A previous study showing a good agreement between RIA and ELISA was confined to urine samples with albumin concentration <20 mg/L (*10*). RIA itself is prone to interference from ions and pH (*18, 19*), but for threefold diluted urine in the reaction mixture the ionic concentration is not considered to be of practical importance.

Because RIA, RID, and IT are reliable assays, giving results that are clinically comparable, a laboratory will base its choice of method for measuring albuminuria in diabetics on the method's practicability. RIA and IT are both semi-mechanized and do not require a high level of technical skill. The skill requirement in RID arises from pouring the gels, cutting wells, and reading ring diameters consistently. The radioactivity involved in RIA is small (12 μCi per 100 tests), but the limited shelf-life of the label (up to six weeks) means that RIA is most suited to large batch analyses (>100 tests). The operating cost we cite for RIA refers to an in-house method and would be considerably increased by use of a commercial kit (about £180 per 100 tests). The large capital cost of RIA should be interpreted with acknowledgment that radioactive counting equipment and a refrigerated centrifuge can be used for other laboratory analyses. The greater operating cost of IT is due to the greater amount of antibody required; however, IT has a much faster turnaround time than RIA and RID. The low capital cost of RID, almost all of which is for a suction pump for creating agar wells, is attractive; given that staff with the necessary skill are available, RID would be the method of choice, especially as it is suitable for analyses of both large and small batches. RID skill requirements could be offset by buying commercial plates, but at considerably increased operating costs (about £200 per 100 tests). ELISA is a practicable assay but, owing to its large variance in the comparison study, we cannot yet recommend it as suitable for screening for albuminuria in diabetes.

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Aqueous and Serum-Based Materials Compared for Use as Simulated Calibrators for Three Ionized Calcium Analyzers

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To determine if bias between different ionized calcium analyzers could be decreased, we analyzed 10 control fluids during a study in which ionized calcium was measured in more than 150 serum and whole-blood samples. After calibrating three ionized calcium analyzers (Radiometer ICA 1, Nova 8, and AVL 980) with the manufacturers' respective calibrators, we used the between-instrument differences of the control fluids to simulate recalibration of the analyzers during each analytical run. A filtered human serum pool containing ionized calcium at 1 mmol/L concentration, with CO_2 removed and having no added buffer, was the only material that consistently decreased between-analyzer bias of both serum and whole blood. Another human serum pool containing about 1.3 mmol of ionized calcium and about 10 mmol of bicarbonate per liter was even better at minimizing analyzer biases for serum samples, but was not as effective for whole-blood samples. Some additives used to buffer pH apparently adversely affected both the accuracy and precision of some, but not other, calcium ion electrodes. We conclude that if a reference material is developed for calibration of ionized calcium analyzers, it should be tested on several analyzers for use with both serum and whole blood, and it should be at least as effective as a human serum material, such as that used here.

Additional Keyphrases: *variation, source of · serum vs whole-blood samples · reference materials*

As ionized calcium analyzers have improved, reliability has become less of an issue and more attention is being given to standardization of results and reference ranges between analyzers (*1–3*). In a previous report from this laboratory, we concluded that serum-based material was better than aqueous fluids as an index of the between-instrument differences for patients' results (*2*). Therefore, it is possible that other serum or protein-based materials would perform as well. To determine whether other fluids containing serum or protein would give comparable results, we have amplified the previous study to include analyses by three ionized calcium analyzers of 10 control fluids: two prepared from human serum, three with a bovine serum base, one aqueous with protein added, and four aqueous. Over four months, we periodically assayed these fluids, along with serum and whole-blood samples from patients. To simulate calibration we used the between-instrument differences for these control fluids to adjust the patients' results from the routine calibration for that day.

Materials and Methods

Instruments. We used three automated flow-through calcium-ion-selective electrode systems. The Nova 8 system (Nova Biomedical, Waltham, MA 02254) requires 350 μL of sample and 70 s for each analysis. Like the Nova 8, the Radiometer ICA 1 (Radiometer America, Inc., Westlake, OH 44145) controls temperature at 37 °C; the AVL 980 (AVL Scientific Corp., Pine Brook, NJ 07058) operates at ambient temperature. In the ICA 1, an ion-exchange material (calcium dioctyl phenyl phosphate) is the calcium-ion-electrode sensor, whereas both the AVL and Nova analyzers use Simon-type neutral carrier molecules (*4*), perhaps alkyl derivatives of 3,6-dioxaoctane diamide. Other details of the Radiometer and AVL analyzers have been described before (*2*). All instruments were calibrated as recommended with the appropriate calibrators supplied by the manufacturers.

Control fluids. Table 1 lists the composition of the fluids used in the study, as measured by us or obtained from the manufacturer. The first eight fluids listed are available commercially. The Nova L1, L2, and L3 controls are bovine-serum based; the buffer added was not disclosed by the manufacturer. Sera QC(quality control)1 and QC2 were prepared in our laboratory, as described before (*2*): the QC2 has 10 μL of 1 mol/L HCl added per milliliter, and the QC1

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