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Development and validation of a particle-enhanced turbidimetric inhibition assay for urine albumin on the Dade *aca*® analyzer

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The measurement of urine albumin now has a well-established role in the monitoring of patients with diabetes mellitus. We have developed a particle-enhanced immunoturbidimetric inhibition assay for urine albumin on the Dade *aca*® analyzer. The inhibition approach removes any of the potential antigen excess difficulties that could be expected from the wide clinical range of urine albumin, but retains the sensitivity advantages of latex-enhanced immunoturbidimetry. Human serum albumin (HSA) is covalently attached to 40-nm poly(chloromethyl)styrene-modified latex particles. This reagent, along with monoclonal antibody to HSA, is aliquoted into the *aca* reagent pack along with polyethylene glycol 8000 in a tablet form (giving a final reaction concentration of 15 g/L). A 150 mmol/L phosphate buffer, pH 7.8, is used to fill the reagent pack in the instrument and the agglutination reaction is monitored at 340 nm. The sample volume is 100 μL and the calibration curve covers the range 2–250 mg/L. Evaluation of commercial scale reagents against the Beckman Array nephelometric immunoassay system gave a Deming regression correlation of *aca* = 0.87 × Beckman + 8.5, r = 0.995, n = 145. Mean analytical recovery was 104 ± 4.5%, n = 20, and there was no evidence of a lack of parallelism. Interassay precision was 8.8% at 10.0 mg/L and <2.5% at >65 mg/L. Calibrator stability was in excess of 60 days. A small reference range study (24-h urine collections, n = 27) gave a mean of 5.6 mg/L with a range of 0.5–16.2 mg/L. Analytical sensitivity (2.5 SD from zero) was 0.40 mg/L.

INDEXING TERMS: calibration stability • computer cooptimization

Diabetic nephropathy is one of the commonest causes of end-stage renal disease (ESRD) *[1–3]*.[3] Enormous economic savings and quality-of-life benefits would be achieved if those patients at risk of developing nephropathy could be identified (30% of diabetics develop nephropathy *[4]*) and an appropriate treatment, other than improved glycemic control, given to slow the rate of progression to ESRD. An increase in albumin in urine is a predictor of renal failure in diabetics, and angiotensin-converting enzyme inhibitor therapy can slow the rate of progression towards ESRD *[5–10]*.

This has led to the need for reliable, rapid, and automated assays for urine albumin with good sensitivity, an interassay imprecision of <5% over the range 25–250 mg/L, and good calibrator stability *[9]*. We previously reported such an assay with particle-enhanced immunoturbidimetry *[11]*; however, one additional characteristic a urine albumin assay requires is a very wide working range. Urine albumin concentrations range from 5 mg/L in healthy individuals to up to 5 g/L in patients with nephrotic syndrome. Direct immunoaggregation assays could give falsely low values at these high concentrations, because of antigen excess. This led us to devise a new method with particle-enhanced turbidimetric inhibition, where antigen excess would not be a problem but good assay imprecision at the upper limit of the reference range could be achieved.

Such an assay also enables the use of a urine albumin method in clinical conditions other than diabetic nephropathy, where it can be used to replace the measurement of

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[3] Nonstandard abbreviations: ESRD, end-stage renal disease; HSA, human serum albumin; CMST, chloromethylstyrene; PEG, polyethylene glycol; and BSA, bovine serum albumin.

urine total protein. The immunochemical measurement of the predominant urinary protein offers improved analytical and potentially clinical sensitivity and specificity and would be useful in monitoring such conditions as hypertension and preeclampsia *[12, 13]*.

We report the development of a sensitive and reliable method for urine albumin measurement on the Dade *aca*® that has excellent calibration and reagent stability and no problems caused by antigen excess.

Materials and Methods

We performed all experiments with a Dade *aca* IV discrete analyzer (Dade International, Wilmington, DE) with manual pack construction for development of the method. We used packs produced by the manufacturer to validate the method.

Protocol for latex particles/human serum albumin (HSA) reagent synthesis. The 40-nm latex particles, which contained a polyvinyl naphthalene core with a chemically reactive shell of chloromethylstyrene (CMST), were provided by Dade International *[14]*. Particles were covalently coupled to HSA (code ORHA 20/21; Behring Diagnostics, Milton Keynes, UK) to give a final concentration of 1 g/L HSA to 20 g/L particles in coupling buffer containing 15 mmol/L sodium phosphate buffer, pH 7.4, with 0.5 mL/L Gafac RE610 surfactant (Gafac Corp., Wythenshaw, Manchester, UK). After an overnight incubation in a shaker/incubator, with constant shaking at 37 °C and 240 rpm, the reagent was sonicated with an MSE Soniprep 150 (Fisons, UK) and stored at 4 °C, ready to use. We performed no centrifugation or washing procedures.

Coupling reproducibility. Five separate syntheses of albumin particle reagent were performed with one lot of albumin and one lot of particle raw material, and calibration curves run with the chosen method protocol; the imprecision of the signal at each calibrator point was <5%. We also assessed the effect of different lots of raw particle material; good between-lot agreement was shown (<5% variation in signal).

Calibrators. We prepared a series of five calibrators from 1000 mg/L HSA dissolved in 9 g/L sodium chloride containing 0.01 g/L Brij 35 and 0.1 g/L sodium azide. We then diluted the stock in the same saline/Brij solution to give calibrators of 5, 25, 50, 100, and 200 mg/L together with a zero value.

Antibody. We generated the monoclonal antibody by immunizing mice with purified HSA. The clones were grown in ascites, which was concentrated and dialyzed against PBS, pH 7.4, containing 0.1 g/L thimerosal, and protein A purified (provided by Pallaiah Thammana, Dade International, Glasgow, DE).

Experimental Procedures and Results

Method optimization. An initial linear optimization was done on the basis of other latex labeled immunoassays *[11, 15, 16]*; the major variables were the amount of antibody, the amount of albumin-conjugated latex particle, the concentration of polyethylene glycol (PEG 8000 Pharma; Hythe Chemical, Southampton, UK), and the sample volume. All work was undertaken to optimize assay performance within the constraints of the *aca* analyzer and with existing reagents available for the analyzer; the reaction buffer used in the assay was 150 mmol/L phosphate buffer, pH 7.8 (in the final reaction mixture).

A final reagent optimization was done with the BBN/Catalyst computer-assisted cooptimization experimental design (BBN Software Product; Cambridge, MA) on the basis of the work of Rautela et al. *[17]* describing a response surface approach to simultaneous optimization of multiple interdependent variables. In the Box–Behnken approach, with four variables (identified above), the model includes a range of each variable, with a total of six samples—in this case calibrators at 0, 5, 200, and 300 mg/L albumin, a normal urine, and a normal urine supplemented with the equivalent of 40 mg/L HSA. The range of each of the variables studied was 19–36 μg of antibody per reaction pack, 30–45 μL of particle reagent per pack, 0–3% of PEG 8000, and 50–250 μL sample volume.

The screen presentation of data provided plots of absorbance change against the concentration of variable for several combinations of the samples, most attention focusing on the normal urine and zero calibrator (for potential nonspecific effects), the 0–5 mg/L difference (for the sensitivity at low analyte concentration), and 0–300 mg/L difference (for the range of the assay). Each set of raw absorbance data plotted against the variable yielded two lines based on least-squares regression, one reflecting the most positive effect, the other the most negative effect. If the slopes of the two lines were the same, i.e., parallel lines, the variable in question did not interact with the other; nonparallel lines indicated interaction. Our objective was to choose conditions in which the slope was the same for both maximum and minimum effect and the influence small, i.e., a change in the variable did not greatly influence the absorbance change.

The results are illustrated in simplified form for the 0–5 mg/L and 0–300 mg/L absorbance differences in Fig. 1; PEG concentration has the major influence on both the sensitivity at low concentration and the assay range parameters. The particle volume and sample volume variables had little influence on the assay range parameter, i.e., 0–300 mg/L absorbance difference.

Final chosen protocol. We added an aliquot of 100 μL of sample with 4.9 mL of phosphate buffer and a single PEG tablet (15 g/L final concentration) to 45 μL of HSA/particle reagent (1 g/L + 20 g/L CMST particle). The

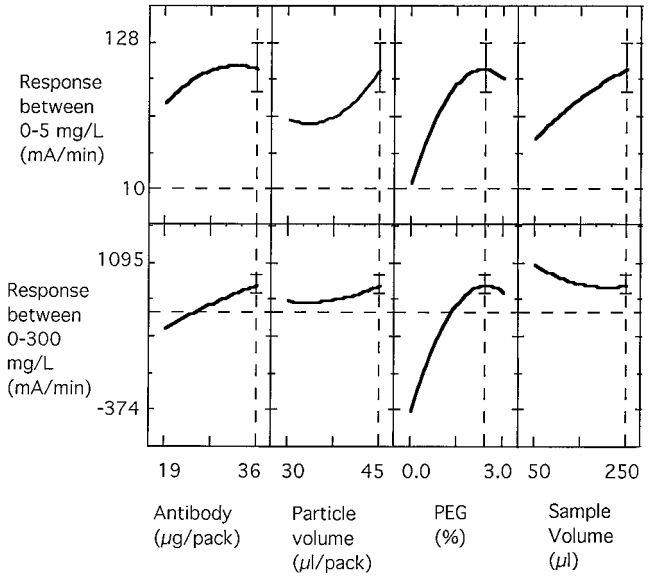

Fig. 1. Summary of data from the cooptimization study indicating the response for the 0–5 mg/L and 0–300 mg/L calibrators plotted against each of the selected variables, reflecting the influence of each while the others are fixed at the optimal concentration.

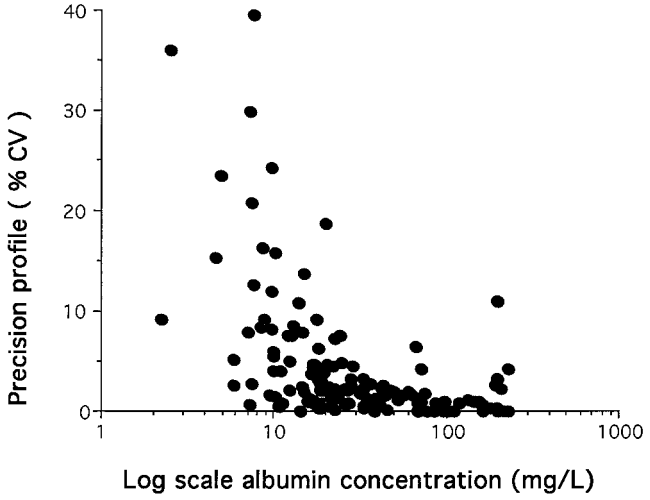

Fig. 2. Precision profile for the proposed assay derived from the duplicate analysis of 145 urine samples.

reaction was initiated with the addition of 20 μg of monoclonal antibody (dissolved in PBS, pH 7.4) and turbidity monitored at 340 nm. These were the final parameters chosen as a consequence of linear and cooptimization experimental results.

Imprecision. Three pools of urine with different concentrations of albumin were prepared, aliquoted, and stored at −20 °C to provide the quality-control materials for this study. Intraassay imprecision was assessed by running 20 replicates of each sample pool randomized in a single analytical run. Interassay imprecision was assessed by running one aliquot of each of the pools, thawed on the day of analysis, over 1 month. The data are shown in Table 1. A precision profile established from the duplicate analysis of samples used as part of the method comparison is shown in Fig. 2.

Detection limit. The detection limit, defined as 2.5 times the SD of the blank response, assessed by analyzing 20 replicates of the zero calibrator, was 0.4 mg/L.

Table 1. Imprecision data for immunoinhibition assay for urine albumin.

	Mean ± SD, mg/L	CV, %	n
Within-run	11.0 ± 6.45	6.0	20
	69.2 ± 1.73	2.6	20
	157.2 ± 2.55	1.7	20
Between-run	9.9 ± 8.89	8.8	20
	64.7 ± 1.64	2.5	20
	151.3 ± 2.25	1.4	20

Parallelism study. Urine specimens from 10 patients with albumin concentrations of up to 300 mg/L were serially diluted (five dilutions) in a solution containing 9 g/L NaCl and 0.01 g/L Brij 35 and analyzed by the proposed method. The results obtained were compared with the expected results by linear regression and showed a slope of 1.019 with regression coefficient of 0.997.

Analytical recovery. We added albumin concentrations of 50 mg/L and 100 mg/L to 10 normal urine specimens and analyzed them on the *aca* with the established protocol. The mean analytical recovery was 104% with a range of 94–112%.

Method comparison. We analyzed 145 urine samples from patients for albumin content by the Beckman Array nephelometric immunoassay system *[18]* and by our proposed method. We chose samples from patients with a range of clinical conditions to give a distribution of albumin of 0–250 mg/L, and regression statistics calculated according to the method of Deming *[19]* gave *aca* = 0.87 × Beckman + 8.5, $r = 0.995$, n = 145 (see Fig. 3).

Calibrator crossover. The *aca* calibrators were analyzed by the Beckman method and yielded a value equivalent to 80% of the stated value.

Interferences. We assessed the possibility that high concentrations of urea might interfere with the immunoreaction by supplementing five normal urine samples with urea up to a final concentration of 1 mol/L; no effect was found. Because bovine serum albumin (BSA) is present in many commercial calibrants and quality-control materials, we also checked the effect of cross-reaction from BSA in the assay; a cross-reactivity of <0.1% was found at 200 mg/L BSA.

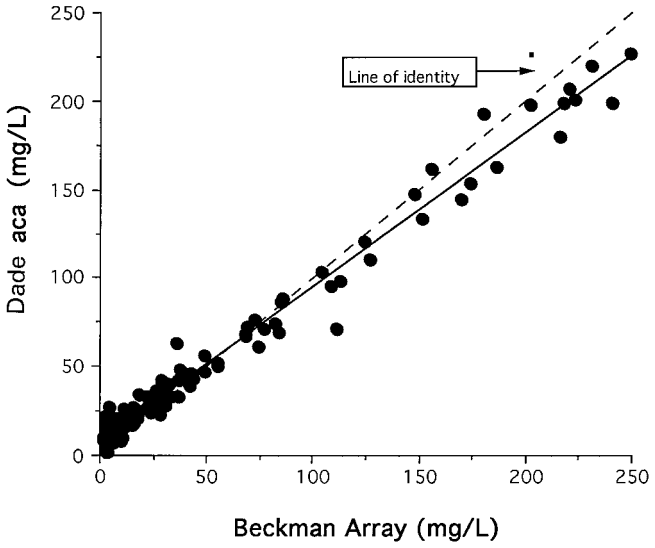

Fig. 3. Comparison of results obtained with the proposed method and the Beckman Array rate nephelometric method.

Calibration curve stability. We assessed the stability of the calibrators by constructing a calibration curve according to the manufacturers' instructions on day 1 and then measuring the calibrants as samples on subsequent days for 60 days. Over the whole study the calculated result for each of the calibrators remained within 5% of the assigned value.

Reference range. We collected 24-h urine samples from 27 healthy laboratory workers to confirm that the analytical results for our proposed method were within the published span of reference values for urine albumin. The samples gave a mean value of 5.6 mg/L with a range of 0.5–16.2 mg/L, well within the reported limits *[20]*.

Discussion

Although the measurement of albumin in urine may have originally been considered for the monitoring of gross deterioration of glomerular permeability and later as a practical alternative to the measurement of total protein, its major clinical usage lies in the routine monitoring of diabetes mellitus as a means of early detection of diabetic nephropathy *[2–5]*. Previous evidence indicates that it may be possible to reverse, or at least slow the progress of, the changes that lead to nephropathy with antihypertensive therapy *[6, 8]*. Although pathological increases in urine albumin excretion may vary from 25 to 10 000 mg/L, the clinical requirement is for an assay that can reliably detect changes of 10 mg/L in the concentration range 5–35 mg/L. The assay is not only required to be accurate and precise but also to retain this performance over many years, as it is likely that patients will only be screened once or twice a year *[21]*. Ideally, the method should also be capable of producing a result rapidly so that the patient can be given the result of the test at the time of the clinic visit.

The method described in this paper achieved the desired performance with the benefit of three key features. The choice of the inhibition format provides good sensitivity at the low analyte concentration with an avoidance of antigen excess possibilities, while the covalent coupling of albumin to the particles ensures that the particle reagent is stable; this characteristic has been demonstrated for other immunoassays where covalent coupling of protein was used *[22–24]*. Finally, the use of a unitized dose assay system such as the *aca* with highly reproducible reagent preparation also contributes to the long-term reproducibility of the assay. This is a particularly important feature when used as a regular screening test. We used both linear and multiple parameter cooptimization to achieve these aims. Although cooptimization is not generally used by researchers, we found it to be a useful adjunct to the linear process rather than a replacement. The data could also be useful as a guide to manufacturing tolerances for pack assembly.

The method described demonstrates an interassay CV of $<10\%$, well within the reference range, the precision profile *[25]* indicating a useful assay range of 5–250 mg/L (the top calibration point). This figure is well within the acceptable concentration defined by Rowe et al. *[20]*, and defined by attaining imprecision equal to, or less than, half of the intraindividual biological variation *[26]*. A doubling of albumin excretion within the reference range can easily be detected with this method. Establishment of a pathologically significant increase in albumin excretion is much harder to define because of the enormous variations in albumin excretion. Protocols have been suggested for this, usually requiring 2–3 collections over 6–12 months, giving an increase above a cutoff concentration $\sim$20–30 mg/min or 30 mg/L or 3 mg/mmol creatinine *[12]*. An assay with good imprecision within the reference range and long-term reagent and therefore calibration stability would be invaluable in the long-term monitoring of diabetics *[27, 28]*.

The method demonstrates good recovery of albumin and comparability with an established method. Furthermore, this method offers the security of avoidance of the risks of antigen excess by use of the inhibition format. It can also be used as an alternative to a total protein method with appropriate dilution of sample for accurate quantification of high concentrations of albumin excretion, as in the case of established nephrotic syndrome *[12]*.

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