Comparison and development of two different solid phase chemiluminescence ELISA for the determination of albumin in urine

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Abstract

Two chemiluminescence enzyme-linked immunosorbent assay (ELISA) methods based on avidin–biotin system and Fluorescein-isothiocyanate (FITC)-anti-FITC system as two different solid phase for the determination of albumin in urine were optimized, characterized and compared. Avidin or anti-FITC antibody was coated in the microplates to provide a universal solid phase which improved the variability and sensitivity. Alkaline phosphatase (ALP) labeled albumin and albumin from standard or patient samples compete for biotinylated-antibody or FITC labeled antibody binding sites. Enzyme activity in the bound fraction was detected with the chemiluminescence substrate 4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) (AMPPD). The influence of several physico-chemical parameters, such as incubation time, detergent concentration and solid phase conditions were also studied. For the two solid phase, both the linear range and the limit of detection of albumin were 0.15–15 and 0.089 $\mu\text{g/mL}$, compared with the commercially ELISA kit, a good correlation were obtained. Moreover, three different solid phases include avidin–biotin system, FITC–anti-FITC system and the anti-albumin Ab directly coated the microtitre plates were compared mainly from the cost and precision, the results showed: (1) when the avidin–biotin system as solid phase, the amounts of the antibody used was only the 1/10 than those of the anti-FITC antibody as solid phase and antibody directly coated the microtiter plates; (2) the C.V. of these two solid-phase CL ELISA methods were lower than that of antibody directly coated solid phase.

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1. Introduction

Quantification of the urinary albumin excretion rate is useful for identifying diabetic patients at risk for developing diabetic nephropathy [1]. Albuminuria, defined as an albumin excretion rate between 30 and 300 mg/day, is one of the earliest indicators of diabetic patients [2–6]. Early interventions including calcium channel blockers and antihypertensive agents and improvements in glycaemic control may slow or even prevent progression to persistent albuminuria and end-stage renal disease [7–11]. Therefore, routine screening to detect patients at risk of developing microalbuminuria is

crucial to patient care. The concentration of albumin in urine has traditionally been detected by various immunochemical methods such as radioimmunoassay (RIA), immunoturbidimetry (IT), radial immunodiffusion, immunonephelometry (IN), and enzyme-linked immunosorbent assay (ELISA).

Most of the presently used immunoassay utilized binding of a specific antibody or antigen on the solid-phase followed by the removal of nonbound analyte or sample matrix through the washing procedure [12]. Commonly used solid-phase was 96-well microtitration plate prepared with polystyrene. For the immunoassay purposes, the microplates were precoated with capturing protein like antibody to allow analyte immobilization. However, direct adsorption of antibodies on the plastic surface may be adversely influencing the test performance by the list parameters: (1) solid

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phases could be heterogeneous in their binding behavior, causing major problems with reproducibility of binding capacity and the long incubation time; (2) the binding sites of coating proteins (antibodies or antigens) could be less accessible than those in solution. Therefore, proteins could be partially inactivated by denaturation due to the coating process (even down to 10% of the coated protein amount); (3) proteins bound to solid phases in varying amounts dependent on their hydrophobicity, isoelectric point, and reagent preparation. Recently, the studies showed protein activity could be preserved when the capturing antibody was immobilized as a secondary layer over a primary coated layer such as via the second antibody system reaction (fluorescein-isothiocyanate (FITC)-anti-FITC system or avidin-biotin linkage). Therefore, anti-FITC antibody or avidin-coated wells were preferred to antibody or antigen coated wells. In addition, avidin is a tetrameric protein consisting of four identical subunits each about 14 kDa in size, when the protein was adsorbed on the solid phase through avidin-biotin technology, one or two of these sites might be lost but still two to three remain active. That is to say, the binding capacity of antibody or antigen onto the micro-well through avidin-biotin technology was at least two times than that of antibody or antigen directly adsorbed on the solid phase. Therefore, using the avidin-biotin system as solid phase, the antibody concentration should be reduced and the costs would be lower. About these, there was few report to test.

Chemiluminescence immunoassay has become very popular in recent years, as they offer significant gains in sensitivity over most other methods at a low cost, both in instrumentation and materials. The present work systematically described the development of these two different solid-phase chemiluminescence enzyme-linked immunosorbent assay (CL-ELISA) methods for the detection of albumin in urine and compared with the directly adsorption of protein as solid phase CL-ELISA from the two aspects: variabilities and costs.

2. Experimental

2.1. Chemicals

The human albumin, goat anti-FITC polyclonal antibody and biotinamidohexanoyl-6-aminoheptanoic acid-*N*-hydroxyuccinimide ester were purchased from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase (ALP) labeled albumin was obtained from Beijing Kemei Dongya Biotechnology Co. Ltd. Rabbit anti-albumin antiscites and avidin were gift of Beijing Academy of Science technology. 4-Methoxy-4-(3-phosphatophenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) (AMPPD) was purchased from Diagnostic Products Corporation (California, USA). Bovine serum albumin (BSA), hydrogel, Tween 20, sodium phosphate, sodium chloride and sodium azide were from

Merck (Darmstadt). The 96-well white microtiter plates were from Shenzhen Jincanhua Industry Co. Ltd.

2.2. Buffers

The coating buffer consisted of phosphate buffer (50 mmol/l, pH 7.0) or citrate buffer (50 mmol, pH 4.8), 0.5% saline solution. The blocking buffer was 0.1 M Tris-HCl buffer containing 0.5% gelation and 0.1% NaN₃, pH 7.4. The washing solution was 0.1 M Tris-HCl buffer and 0.5% saline solution, pH 8.5. The assay buffer was 0.1 M Tris-HCl buffer containing 0.1% gelation and 0.1% NaN₃, pH 7.4. The biotinylated antibody dilution buffer was 0.1 M Tris-HCl buffer containing 0.1% gelation, 0.1% NaN₃ and 0.01% Tween 20, pH 7.4. The chemiluminescence substrate buffer was 0.1 M Tris-HCl (pH 9.5) with 1 mmol/l MgCl₂ and 0.02% sodium azide.

2.3. Procedures

2.3.1. The purification of rabbit anti-albumin antiscites

The rabbit-anti-albumin antiscites was purified according to a modified saturated ammonium sulfate (SAS) precipitation method [13].

2.3.2. Biotinylation of rabbit anti-albumin antibody

The conjugate of biotinylated anti-albumin antibody was synthesized and purified as described in [14]. The anti-albumin antibody purified was dialyzed overnight at 4 °C against 50 mM NaHCO₃ buffer, pH 9.3. Biotinamidohexanoyl-6-aminoheptanoic acid-*N*-hydroxyuccinimide ester (BCNHS) with an extended spacer arm was applied to react with primary amines of the protein which can reduce the steric hindrance. The 20 µl BCNHS dissolved in dimethylsulfoxide (2 mg/mL) was added to 200 µl purified anti-albumin antibody (7.5 mg/mL). After 2 h incubation at room temperature, the mixture was dialysed for 24 h at 4 °C against phosphate buffered saline (PBS), pH 7.4. The dialysed antibody was centrifuged at 3000 rpm for 30 min to discard any precipitates. Aliquots of the biotinylated antibody conjugate were stored at -20 °C until further use.

2.3.3. FITC labeled rabbit anti-albumin antibody

Anti-albumin purified antibody was dialyzed overnight at 4 °C against 50 mM NaHCO₃ buffer, pH 9.3. FITC (sigma) was applied to react with primary amines of the protein. 25 µl FITC dissolved in 50 mmol carbonate/bicarbonate buffer, pH 9.5 (2 mg/mL) was added to 200 µl purified anti-albumin antibody (5 mg/mL). After reaction with stirring 12–16 h at 4 °C, the mixture was dialysed for 4 days at 4 °C against phosphate buffered saline (PBS), pH 7.4. The dialysed antibody was centrifuged at 3000 rpm for 30 min to discard any precipitates. Aliquots of the antibody-FITC conjugate were stored at -20 °C until further use.

2.3.4. Titration levels assessment of the biotinylated antibody and FITC labeled antibody

A microtiter plate was coated with 100 μL of avidin (3 $\mu\text{g}/\text{mL}$) in phosphate buffer (pH 7.0) or 100 μL of anti-FITC antibody (10 $\mu\text{g}/\text{mL}$) in citrate buffer (pH 4.8) for overnight at 4 $^{\circ}\text{C}$. Then the wells were blocked using 300 μL blocking buffer for 1 h at room temperature. For the titration of biotinylated antibody, 50 μL of serially diluted biotinylated antibody, standard analyte and diluted enzyme tracers (1:1000) were added and incubated 1 h at 37 $^{\circ}\text{C}$. For the titration of FITC labeled antibody, 100 μL of serially diluted specific FITC-anti-albumin antibody were added and incubated 1 h at 37 $^{\circ}\text{C}$. Then, the standard analyte prepared in 0.1 M Tris–HCl buffer containing 0.1% gelatin and 0.1% NaN_3 and the 1:2000 diluted enzyme tracers were added to the wells and incubated 1 h at 37 $^{\circ}\text{C}$. After the competitive reaction, five times washing were performed with 400 μL of washing solution. Then, 50 μL chemiluminescence substrate solution was added and incubated 20 min at 37 $^{\circ}\text{C}$, the emitted photons were measured.

2.3.5. Avidin–biotin chemiluminescence immunoassay procedure

Immunoassay procedures are presented in Fig. 1. The wells of the microtiter plates were coated with 150 μL of avidin (3 $\mu\text{g}/\text{mL}$) in a phosphate buffered saline (pH 7.0). The plates were allowed to stand sealed at 4 $^{\circ}\text{C}$ for 15 h. After washing with 400 μL washing solution, a second coating was done with 300 μL of gelatin in Tris–HCl buffer for 1 h at room temperature. Another three times washing was performed. Then, 50 μL albumin standard solutions or urine samples and 50 μL diluted enzyme labeled albumin (1:1000) and 50 μL diluted biotinylated anti-albumin antibody (1:8000) in 0.1 M Tris–HCl buffer containing 0.1% (v/v) gelatin, 0.1% NaN_3 and 0.01% Tween-20 were added and incubated for 1 h at 37 $^{\circ}\text{C}$. After the competitive reaction, five times washing were performed with 400 μL of washing solution. 50 μL chemiluminescence substrate solution was added and incubated 20 min at 37 $^{\circ}\text{C}$, the emitted photons were measured. The role of Tween 20 was crucial in the assays due to its prop-

erty to unfold proteins, enabling antigens and antibodies to interact, thus, increasing enzyme effectiveness and decreasing background signal.

2.3.6. FITC–anti-FITC system chemiluminescence immunoassay procedure

The immunoassay procedure was individed into two steps: the wells of the microtiter plates were coated with 100 μL of anti-FITC antibody (10 $\mu\text{g}/\text{mL}$) in citrate buffer (pH 4.8). The plates were allowed to stand sealed at 4 $^{\circ}\text{C}$ for 15 h. Then, three times washing was performed and the plates were post-coated with 300 μL of 0.5% gelatin in Tris–HCl buffer for 1 h at room temperature. After washing three times with washing solution, 100 μL FITC labeled anti-albumin antibody (1:2000) were added into the microwell to react with the second antibody at 37 $^{\circ}\text{C}$ for 1 h. Another five times washing was performed, 50 μL albumin standard solutions or urine samples and 50 μL diluted enzyme labeled albumin (1:2000) in 0.1 M Tris–HCl buffer containing 0.1% (v/v) gelatin, 0.1% NaN_3 were added and incubated for 1 h at 37 $^{\circ}\text{C}$. After the competitive reaction, five times washing were performed with 400 μL of washing solution. Then, 50 μL chemiluminescence substrate solution was added and incubated 20 min at 37 $^{\circ}\text{C}$, the emitted photons were measured.

2.3.7. Data analysis

Standards and samples were run in double wells, and mean chemiluminescence intensity values were processed. Standard curves were obtained by plotting chemiluminescence intensity against the logarithm of analyte concentration and fitted to the equation of $\text{logit } Y - \log x$, in which the value of $\text{logit } Y$ was calculated according to the formula

$$\text{logit } Y = \ln[Y/(1 - Y)] = \ln [1/(1/Y - 1)]$$

2.4. Physicochemical parameter influence and optimization

Several physicochemical factors influencing immunoassay performance were studied in the two CL-ELISA methods.

Fig. 1. Schematic of avidin–biotin chemiluminescence immunoassay procedures.

Modifications of RLU_{max} and IC_{50} (the albumin concentration causing 50% inhibition of RLU_{max}) parameters of the standard curves were evaluated under different conditions.

2.4.1. Optimization of solid phase conditions

The micro-wells of the plate were coated with avidin or anti-FITC antibody using three different buffers (0.05 M sodium citrate buffer (pH 4.8), 0.05 M PBS (pH 7.0) and 0.05 M saline carbonate (pH 9.6)) and was blocked using blocking solution. Competitive assays were performed in Tris–HCl buffer (pH 7.4) containing 0.1% gelatin, 0.1% NaN_3 and 0.01% Tween-20 using the three different kinds of microplate coated avidin or anti-FITC antibody as described above. In addition, During the coating and blocking steps, the plates were examined at 4 °C, room temperature, and 37 °C through the competitive assays as described above.

2.4.2. Effect of Tween-20

Competitive assays were performed using different concentrations of Tween-20. Briefly, standard analytes were prepared in 0.1 M Tris–HCl buffer containing 0.1% gelatin and 0.1% NaN_3 , while biotinylated anti-albumin antibody were added to serial dilutions of Tween-20 (from 0.05 to 1%) in the same buffer. Therefore, assays were executed as described above.

2.4.3. Effect of incubation time

Competitive reactions were performed with different incubation time of antigen and antibody reaction. Standard analyte were prepared in 0.1 M Tris–HCl buffer containing 0.1% gelatin and 0.1% NaN_3 , while the length of competitive step were varied from 15 to 120 min and the assays were executed as described above. Moreover, the reaction time of anti-FITC antibody and FITC labeled anti-albumin antibody was also studied from 15 to 120 min.

2.5. Evaluation of the two kinds of immunoassay methods

2.5.1. Precision

The intra-assay precision of the analytical method was evaluated by analyzing five times inside a plate using three different concentration samples. Similarly, these samples were analyzed on three different days using the same protocol to obtain the inter-assay precision.

2.5.2. Parallelism

Two samples were diluted from 1 to 16 with the assay matrix and assayed, the linear correlation between expected (x) and determined (y) values were studied.

2.5.3. Recovery

Various amounts of albumin were added to urinary with albumin levels ranging from 0.15–15 $\mu\text{g/mL}$. The samples

were analysed by the CL-ELISA methods and the recovery were evaluated.

2.6. Comparison of three different solid-phase CL-ELISA methods

Three different solid phase systems include avidin–biotin system, FITC–anti-FITC system and the anti-albumin antibody directly coated the microtitre plates were studied from the two aspects: cost and precision. The different concentration of biotinylated anti-albumin antibody, FITC labeled anti-albumin antibody were added into the micro-well coated avidin or anti-FITC antibody, respectively, and different concentration anti-albumin antibody were directly adsorbed into the microwell. Then, the zero standard or samples and the enzyme-tracers were added and competitive reactions were performed as described above. The cost or precision were calculated according to the CL signal.

2.7. Analysis of urine samples

Twenty urine samples containing albumin at concentration levels ranging from 0.15–15 $\mu\text{g/mL}$, were measured using the two optimized CL-ELISA methods and compared with the commercially ELISA kit.

3. Results and discussion

3.1. Titration levels assessment of FITC labeled anti-albumin antibody and biotinylated anti-albumin antibody

Titer levels of the FITC labeled anti-albumin antibody and biotinylated anti-albumin antibody were determined using the standard *analytes* in the concentration of 0.15–15 $\mu\text{g/mL}$. As can be seen in Fig. 2, When the titration was higher than 1:2000 for the FITC labeled anti-albumin antibody and higher than 1:8000 for the bionylated anti-albumin antibody, the sensitivity were not high, this is probably due to the capture antibody excess which resulted in the superposition and denaturalization of the intra-protein. While the titration was lower than 1:2000 or 1:8000, the signal was decreased rapidly. Therefore, Titration levels of 1:2000 for the FITC labeled anti-albumin antibody and 1:8000 for the biotinylated anti-albumin antibody were set.

3.2. Physicochemical parameter optimization

Some experimental parameters (incubation time, solid phase conditions, Tween-20) were studied with two aims: (1) to improve immunoassay sensitivity, (2) to study immunoassay performance under the optimal conditions. These experiments were carried out using the proposed method described above.

Fig. 2. Titration curves of FITC labeled anti-albumin antibody (A) and biotinylated anti-albumin antibody (B).

3.2.1. Optimization of solid phase conditions

To develop a highly consistent solid phase, coating and blocking buffer and temperature were studied. The coating buffers most used are 50 mM carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; 10 mM phosphate-buffered saline (PBS), pH 7.0, citrate buffer pH 4.8 [15,16]. Different coating buffers should be investigated at the beginning of assay development. From the Table 1, when using the PBS buffer (pH 7.0) for coating avidin and the citrate buffer (pH 4.8) for coating anti-FITC antibody, the RLU_{max} and RLU_{max}/IC_{50} was high. Therefore, the PBS and the citrate buffer were respectively selected as the optimum coating buffer for avidin and anti-FITC antibody.

Table 1
Effect of different coating buffer on immunoassay

	Coating Ab	Carbonate/bicarbonate buffer (pH 9.6)	PBS buffer (pH 7.0)	Citrate buffer (pH 4.8)
RLU_{max}	Avidin	19521	27353	15012
	Anti-FITC Ab	104521	96887	124701
IC_{50}	Avidin	2.35	1.27	1.07
	Anti-FITC Ab	4.35	5.82	3.85
RLU_{max}/IC_{50}	Avidin	8306.8	21537.8	14029.9
	Anti-FITC Ab	24027	16647	32389

The choice of blocking buffer is sometimes critical for sensitive detection. Milk based blocking solutions are not recommended for using avidin-biotin system because milk contains biotin, which may directly cause competition with biotinylated antibody [17]. Bovine serum albumin (BSA), which is commonly used as blocking reagent, was not selected in this work because BSA can react with the anti-albumin antibody in the solid phase to a certain extent. Therefore, the gelation was chosen as blocking agents.

The effect of the temperature during coating and blocking steps was also examined. When the plates were coated overnight at room temperature, a large edge effect, i.e. higher standard deviation of signals in the outer wells was observed compared to the plates which were coated at 4 °C. Blocking with gelation was more efficient at room temperature than 37 °C. Therefore, plates were routinely coated at 4 °C overnight followed by a blocking step at room temperature for 1 h.

3.2.2. Effect of Tween-20

In the two immunoassay methods, we found that using avidin-biotin system as solid phase, the non-specific adsorption (NSB) was very high, while using FITC-anti-FITC system as solid phase, there are no NSB. Because surfactants (such as Tween-20) are commonly used in ELISA to reduce nonspecific interactions [18], their influence on assay performance (IC_{50} , RLU_{max} and RLU_{max}/IC_{50}) was examined in the avidin-biotin CL-ELISA. Tween-20 concentrations higher than 0.1% reduced RLU_{max} , whereas IC_{50} increased with lower Tween-20 concentrations. The reason RLU_{max} being reduced was considerably that Tween-20 itself can affect the specific and unspecific immobilization characteristics of the solid phase by interfering with the active sites of reactant molecules which had an impact on the CL signal. Fig. 3a shows the variation of the RLU_{max}/IC_{50} ratio as a function of the concentrations of the Tween-20. The 0.1% Tween-20 was selected as the optimal concentration.

3.2.3. Effect of incubation time

It has been reported from other laboratories [19–22] that, on a competitive ELISA, the time given to the immunoreagents to interact, may have a direct effect on the sensitivity of the immunoassay. For this reason, we varied the length of competitive step from 15 to 120 min for assessing the

Fig. 3. (a) Effect of tween-20 on albumin immunoassay, the tween-20 was added into antibody using serial dilutions of Tween-20 (from 0.05 to 1%). The reported results were extracted from the logit-log equation used to fit the standard curves. Standard curves were prepared using double well replicates. (b) Effect of incubation time on competitive immunoassay of albumin. The analyte, ALP-antigen and bio-antibody were incubated at different times (between 15–120 min) in the avidin coated plates. The reported results were extracted from the logit-log equation used to fit the standard curves. Standard curves were prepared using double well replicates. (c) The effect of incubation time on the formation of anti-FITC–FITC–anti-albumin antibody.

effect of incubation time on the assay performance (IC_{50} , RLU_{max} and RLU_{max}/IC_{50}). In the avidin–biotin CL ELISA, The RLU_{max} increased with the longer of incubation time in the range of 15–60 min. After 60 min, the RLU_{max} did not change evidently. This means the immunoassay between antibody and antigen reached an equilibrium. Under the conditions, no significant differences were observed for the IC_{50} values. Fig. 3b showed the variation of the RLU_{max}/IC_{50} ratio as a function of the incubation time. It was observed that the greater RLU_{max}/IC_{50} was encountered at the 60 min incubation time. Therefore, a competitive reaction of 60 min was set.

In the FITC–anti-FITC system, using the one-step reaction, the CL signal cannot be inhibited very well with the standard concentration increasing. Therefore, the two-step reaction was used which included the formation of FITC–anti-FITC complex and the formation of albumin antigen–antibody, and the reaction time of the first step was also studied. Fig. 3c showed the variation of the RLU_{max}/IC_{50} ratio as a function of the incubation time. It was observed that the greater RLU_{max}/IC_{50} was encountered at the 60 min incubation time. Therefore, in the first step, the incubation time of 60 min was set.

3.3. Calibrations and sensitivities

Under the optimal conditions, dose–response curves obtained with the chemiluminescence detection of enzyme activity were presented in Fig. 4. The linear range is 0.15–15 $\mu\text{g/mL}$. The detection limit, defined as the minimal dose that can be distinguished from zero, the minimum detected concentration (mean – 2SD of zero standard, 10 replicates) of albumin was 0.089 $\mu\text{g/mL}$.

3.4. Evaluation of the two kinds of immunoassay methods

Table 2 showed the results obtained from these experiments, and it can be observed that the intra- and inter-assay coefficients of variation were in all cases below 15%.

Two samples were diluted from 1- to 16-fold with the assay matrix. The linear correlation between expected (x) and determined (y) values were: avidin–biotin as solid phase, $y = 0.9758x + 0.0245$ ($r = 0.997$); FITC–anti-FITC system as solid phase, $y = 1.0528x + 0.079$ ($r = 0.995$).

Various amounts of unlabeled albumin were added to urinary with albumin levels ranging from 0.15–15 $\mu\text{g/mL}$. The

Fig. 4. Calibration graphs of the chemiluminescence enzyme immunoassay for albumin in urine with avidin–biotin (A) and the second antibody system (B) as solid phase.

samples were analysed by the two CLEIA methods and the results are shown in Table 3. The recoveries were in the range of 90–116%.

3.5. Comparison of three different solid phase CL-ELISA methods

Three different solid phase including avidin–biotin system, FITC–anti-FITC system and the anti-albumin antibody directly coated the microtitre plates were compared from the two aspects: cost and precision. The results (as shown in Tables 4 and 5) demonstrated that: (1) when the avidin–biotin

system as solid phase, the amounts of the antibody used was only the 1/10 of those directly coated the microtiter plates. It was probably because that avidin has four identical functional tetramer binding sites for biotin [23], which results the binding capacity for the biotinylated antibody having double to four times than that of directly antibody coated. While using FITC–anti-FITC system as solid phase, Although Three or four FITC molecules can be bound to an anti-FITC antibody molecule which can be applied for the signal amplified system instead of avidin–biotin system, the amounts of the antibody used was still mostly equal to those directly coated the microtiter plate. This is probably because of two-step reaction being used. (2) The interaction of avidin–biotin is one of the tightest binding processes known. The binding of biotinylated antibody was consistent among different proteins. Moreover, the FITC–anti-FITC system has high avidity, specificity and stability, when using anti-FITC antibody as a secondary layer over a primary coated layer was immobilized, FITC labeled antibody would be have high immunoactivity. Therefore, the variability in binding to the solid phase was minimized and the C.V. of these two solid-phase CL-ELISA were lower than that of directly coated solid phase.

In addition, compared with antibody directly coated microtiter wells, anti-FITC antibody or avidin-coated wells provide a universal immobilization surface for any analyte and therefore tedious optimization of the adsorption conditions can be avoided.

3.6. Analysis of urine samples

The concentration of albumin in 20 urine samples was determined by these two developed solid phase chemiluminescence enzyme immunoassays and compare with commercially ELISA kit. The correlation obtained between the results of CL-ELISA (y) and ELISA (x) was: avidin–biotin CL-ELISA, $y = 1.0897x + 2.2436$ ($r = 0.955$); the second antibody CL-ELISA, $y = 0.9870x - 0.3354$ ($r = 0.960$).

Table 2
Precisions of the two CLEIA methods

	Sample		Mean (mIU/mL)	Range (mIU/mL)	S.D.	C.V. (%)
Intra-assay ($n = 5$)	Avidin–biotin as solid phase	1	0.74	0.65–0.86	0.082	10.7
		2	6.41	6.03–6.81	0.329	5.1
		3	14.48	13.47–15.27	0.64	4.47
	Second antibody as solid phase	1	1.78	1.56–1.98	0.187	10.5
		2	4.72	4.2–5.26	0.395	8.3
		3	13.8	12.91–16.97	1.30	9.4
Inter-assay ($n = 15$, detection of three days)	Avidin–biotin as solid phase	1	0.91	0.57–0.97	0.137	15
		2	6.33	5.49–6.81	0.435	6.8
		3	14.4	12.76–16.35	1.17	8.1
	Second antibody as solid phase	1	1.76	1.44–2.44	0.21	11.9
		2	5.04	4.2–5.72	0.487	9.66
		3	13.4	11.31–16.97	1.30	9.7

Table 3
Recoveries of the two CLEIA methods

Sample		Added ($\mu\text{g/mL}$)	Determined ($\mu\text{g/mL}$)	Recovery (%)
Avidin–biotin as solid phase	S1	0	0.24	–
		0.25	0.52	112
		7.5	7.28	93.8
	S2	0	2.15	–
		0.25	2.47	108
		7.5	9.51	98.1
The second antibody as solid phase	S1	0	1.85	–
		0.75	2.55	93
		2.5	4.14	91.6
	S2	0	3.00	–
		0.75	3.82	109
		7.5	10.96	106

Table 4
Comparison of antibody amounts used in the three different solid phases CL-ELISA

Biotin-antibody (avidin coated)	Dilutors	1:25000	1:50000	1:75000
	RLU	281881	112752	79169
FITC-antibody (anti-FITC coated)	Dilutors	1:2500	1:5000	1:10000
	RLU	275586	51236	30476
Albumin antibody (directly coated)	Dilutors	1:2500	1:5000	1:10000
	RLU	263768	49886	34140

Table 5
Comparison of variability of the three different solid phase CL ELISA

Standard concentration ($\mu\text{g/mL}$)	C.V. (%)		
	Biotin-antibody (avidin coated)	FITC- antibody (anti-FITC Ab coated)	Albumin antibody (directly coated)
0	0.4	0.6	3.4
0.15	1.3	1.5	11.5
0.5	1.1	1.2	2.8
1.5	2.0	2.5	11.7
5.0	6.3	5.2	11.9
15	1.0	2.1	8.4

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