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Simple sequential injection analysis system for rapid determination of microalbuminuria

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ABSTRACT

A simple, specific and sensitive sequential injection analysis (SIA) system based on non-immunoassay fluorescent detection has been developed for the determination of urinary albumin. The specific binding of the dye Albumin Blue 580 (AB 580) to albumin in urine generated high emission fluorescent signals. The excitation and emission wavelengths were set at 590 and 610 nm, respectively. The analytical range was obtained from 1 to 100 mg L^{-1}, with a detection limit of 0.3 mg L^{-1} (S/N = 3). The SIA system gave high precision with relative standard deviations (R.S.D.s) of 0.9% and 1.4% when evaluated with 15 and 100 mg L^{-1} albumin (n = 15), respectively. The method exhibited good reproducibility, as assessed by performing four analytical curves on different days, and intra-run CVs (2.3–3.3%) and inter-run CVs (3.8%) were obtained. Rapid operation was achieved with a sample throughput of 37 h^{-1}. This method was successfully applied to the determination of urinary albumin, and the method was highly correlated with the immunoturbidimetric method (r^2 = 0.965; n = 72).

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1. Introduction

Microalbuminuria is defined as the detection of an albumin concentration of 30–300 mg from a 24-h urine collection [1]. Early detection of microalbuminuria is a sensitive forecast of the outcome of nephropathy complications in diabetic patients [2]. The importance of microalbumin detection has been realized by the American Diabetes Association, who issues guidance for the annual detection of microalbuminuria in every diabetes patient [3]. Additionally, it is a powerful predictor of non-diabetic coronary artery disease patients [4–6].

Unlike in blood plasma, albumin concentrations in urine are usually low, and therefore require a sensitive and selective method for an accurate assay. Numerous methods have been described in the literature for the determination of urinary albumin. Among those methods, the most routinely used methods are based on immunoassays, including radioimmunoassay [7], immunoturbidimetry [8], immunonephelometry [9,10], enzyme-linked immunosorbant assay (ELISA) [11], fluorescence immunoassay [12], and immunoresonance scattering spectral assay [13]. However, most of the existing methods have significant drawbacks. The radioimmunoassay is notorious for its health hazards, and thus is not widely used, although it is claimed to be a gold standard method [14]. ELISA is known as the most sophisticated immunoassay because of its numerous time-consuming and tedious washing steps. However, immunological methods are accepted for use in most laboratories on the basis of high sensitivity and selectivity. On the other hand, it is commonly known that most of the immunoassay-based methods consume large amounts of expensive reagents, especially the antibody specific for albumin. Therefore, non-immunoassay methods that offer satisfactory sensitivity and selectivity are of great interest for low cost determination of microalbuminuria.

Recently, Kessler et al. [15,16] proposed a simpler dye-binding assay that utilizes the fluorescent dye Albumin Blue 580 (AB 580). The dye can specifically bind to human albumin, and the protein–dye complex can generate dramatic emission fluorescent signals at 610 nm [15]. Upon binding to albumin, the fluorescence quantum efficiency of the dye AB 580 increases by two orders of magnitude [17]. Several papers have demonstrated that the AB 580 fluorescent dye is able to quantify low levels of urinary albumin with appreciable characteristics [14–17]. Although the AB 580 was first evaluated by Kessler et al. [15] for the determination of urinary albumin in a conventional spectrofluorometer, several dif-

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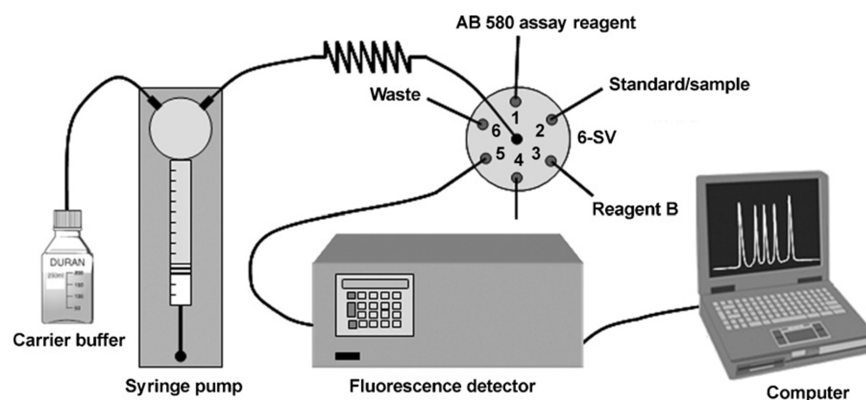

Fig. 1. Configuration of the AB 580 SIA system. The syringe pump was used for delivering the carrier buffer; 6-SV (six-port selection valve); position 1 on the 6-SV was connected to the AB 580 working solution, position 2 was connected to samples/standards, position 3 to reagent B, 5 to the fluorescent detector, and 6 to the waste.

ferent assay formats based on AB 580 were later established, such as automated fluorescence assays [16], capillary electrophoresis [18] and lab-on-a-chip (LOC) [17]. However, none of these methods could perform rapid analysis. In the capillary electrophoresis platform, the assay time requires at least 4 min for one sample [18]. In the LOC format, the microchannel must be flushed with water for 10 min for de-adsorption of the albumin [17].

The sequential injection analysis (SIA) technique has the potential to be a fully automatic operation, with computer control for the aspiration or dispensation of samples and reagents [19]. The well-known advantageous characteristics of the SIA system have been widely reviewed, e.g., the simplicity of fundamental principles, inexpensive instrumentation, automated sampling and analytical procedures, limited sample consumption, short analysis time, and on-line performance of difficult operations [20].

Recently, there has been growing interest in the development of a fast and automatic analytical method. The purpose of this work is to establish a SIA system by exploiting the AB 580 dye for the sensitive and selective determination of microalbuminuria. Combining the SIA system and utilization of AB 580 for the chemical reaction, we obtained a low cost, simple and rapid operation method for the microalbumin assay. In this work, we successfully configured the AB 580 SIA system for the determination of urinary albumin, covering both the normal and clinical levels of microalbuminuria. The effects of various parameters affecting the fluorescence signals were studied and optimized. The applicability of the system for real sample analysis was also evaluated.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical reagent grade. Albumin Blue 580 potassium salt solution (AB 580) was purchased from Fluka (Buchs, Switzerland). N-morpholinopropanesulfonic acid (MOPS free acid), EDTA disodium salt, human IgG, human serum albumin, and 2-propanol were obtained from Sigma (St. Louis, USA). The microalbumin assay kit used for method validation was supplied by Randox Laboratories (United Kingdom).

All solutions were prepared in Milli-Q water, except the albumin standards, which were prepared daily in the calibrator diluents as described in the literature [15]. The carrier buffer (pH 7.4 ± 0.20) used in the SIA system was prepared following the method described previously [15] with a slight modification; MOPS sodium salt was omitted and the solution was adjusted to pH 7.4 ± 0.20 with $1\ \mathrm{mol\ L^{-1}}$ NaOH. The buffer solution was filtered with a 0.45-μm cellulose acetate membrane (Sartorius, Germany) and kept at 4 °C with tight sealing to prevent 2-propanol evaporation. The carrier buffer was degassed for 5 min to remove air bubbles before use.

Unless otherwise stated, the AB 580 working solution for the SIA system was prepared by mixing the purchased AB 580 solution with the carrier buffer in a 1:25 ratio. The AB 580 working solution was stored in an amber bottle for light protection throughout the experiments.

2.2. Apparatus

The SIA system consisted of a syringe pump (Cavro XL 3000, Cavro Scientific Instruments Inc., USA) equipped with a 2.5-mL syringe, a six-port selection valve (Cavro Smart Valve, Cavro Scientific Instruments Inc., USA), and a fluorescent detector (RF-10AX Shimadzu, Japan) incorporated with a 12-μL quartz flow cell. The system components were arranged as shown schematically in Fig. 1. Fluorescent peak heights were recorded and quantified using Class-LC 10 software from Shimadzu. All tubing used to connect the different components of the flow system was PTFE with 0.8 mm i.d. (Cole-Parmer Instrument Company, USA) and all connectors used were made of PEEK (Upchurch Scientific, USA).

A UV-VIS spectrophotometer (Evolution 600, Thermo Scientific, USA) was used for the immunoturbidimetric method.

2.3. Sequential injection procedure

The operating sequence of the SIA system for the analysis of albumin is listed in Table 1. The analytical cycle started with the aspiration of carrier buffer into the syringe. Next, the AB 580 working solution and the standard/sample solution were aspirated into the holding coil, in which the fluorescent signals were generated as

Table 1
Operation sequence of the SIA system for the determination of urinary albumin.

Step	Operation	Valve of pump	Port of selection valve	Volume (μL)	Flow rate (μL/s)
1	Aspirate carrier buffer to syringe pump	In	–	1200	200
2	Aspirate AB 580 working solution to holding coil	Out	1	100	100
3	Aspirate standard/sample to holding coil	Out	2	100	100
4	Aspirate AB 580 working solution to holding coil	Out	1	50	50
5	Dispense mixture to fluorescent detector	Out	5	1450	25

soon as the dye reacts with albumin. The flow was then reversed, and the mixture was propelled through the fluorescent detector. Each point of the analytical plot represents the average peak height for four successive injections.

2.4. Sample preparation and analysis

Unidentified urine samples were collected from diabetes patients at the King Chulalongkorn Memorial Hospital. The samples were centrifuged at 3,000 rpm for 10 min, and the supernatant was then analyzed with the SIA system. The supernatant urine samples were aspirated to the SIA system for analysis of the albumin content. The analytical results were compared with those obtained by the immunoturbidimetric method [21]. Since urine samples can generate fluorescent signals by themselves, the sample blank peak height was also quantified. The fluorescent signals of the urine sample was measured and subtracted from those of the urine–dye mixture.

2.5. Immunoturbidimetric method

The proposed method was validated for accuracy by comparison to the immunoturbidimetric method using the commercial microalbumin kit from Randox Laboratories. A UV–vis spectrophotometer set at 340 nm was used according to the manufacturer's instructions with minor modification by minimizing the volume to 50% of original protocol. The quality control of the assay was performed using the two levels of control samples provided with the kit. Agreement between the results obtained by the SIA and immunoturbidimetric method was evaluated by Pearson analysis using the SPSS 11.5 program, in which the p values <0.05 were considered significant.

3. Results and discussion

3.1. Optimum ratio for preparation of AB 580 working solution

The optimum ratio between the purchased AB 580 solution (here, reagent A) and the carrier buffer (here, reagent B) for preparation of the AB 580 working solution was investigated at the ratios of 1:10, 1:25, and 1:50. The aspiration volumes were 25 and 75 μL for the 25 mg L^{-1} albumin and AB 580 working solution, respectively. It was observed that the ratio of 1:10 gave the highest sensitivity, whereas the signals were decreased about 10% for the ratios of 1:25 and 1:50 (data not shown). This result is different from that obtained by the previous cuvette-based method, in which the ratio of 1:50 was used for the preparation of the working solution [15]. The cause of this differing result can be explained by the dispersion of analytes and reagents that occurs during flow to the detector, due to the nature of the flow-based method, which is different from the batch-based system. In addition, the flow-based method are generally affected by the occurrence of Schlieren effect resulted from poor mixing and presence of analyte concentration gradient [22].

Although the ratio of 1:10 gave the highest signal, it was disregarded because of the relatively large amount of AB 580 dye that was consumed at this ratio. To clarify the optimum ratio between 1:25 and 1:50, the analytical curves obtained from both ratios were compared as shown in Fig. 2. These analytical curves indicated that using a more diluted dye reagent yielded a narrower analytical range. In addition, the dilution of the AB 580 working solution affected the sensitivity of the assay, especially in cases of a high albumin concentration. For example, the sensitivity at both ratios were similar for albumin concentrations less than 10 mg L^{-1}, but the sensitivity for the 1:50 ratio was lower than that of the 1:25 ratio for 200 mg L^{-1} albumin. Therefore, the ratio of 1:25 was selected as a compromise between sensitivity and the cost of AB 580 for the preparation of the working dye solution for all subsequent experiments.

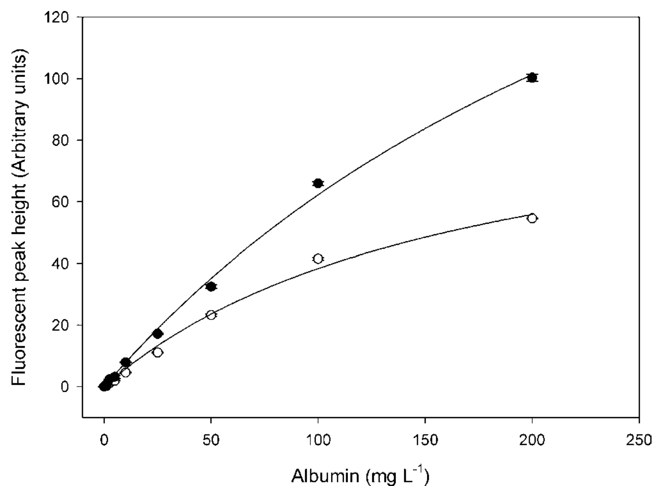

Fig. 2. The effect of the ratio of reagent A and reagent B for the preparation of the AB 580 working solution on the sensitivity and analytical range. Reagent A and reagent B ratios of 1:25 (●) and 1:50 (○).

For the AB 580 working solution at a 1:25 ratio, excellent signal stability for at least 8 h was obtained. The remaining fluorescent signal for 100 mg L^{-1} albumin was obtained at 102.5% and 92.4% after 6 and 8 h, respectively (data not shown).

3.2. Influence of sample volume

The influence of sample volume for the 25 mg L^{-1} albumin in the SIA operating sequence was studied over the range 25–150 μL. The volume for the AB 580 working solution was set at 50 μL. The relationship between the fluorescent peak height and the sample volume is shown in Fig. 3. It was observed that the fluorescent signals increased with increasing sample, the plateau appeared while injecting a volume higher than 100 μL. Therefore, the sample volume of 100 μL was chosen for further experiments.

3.3. Influence of AB 580 working solution volume

To investigate whether the volume of the AB 580 working solution at 50 μL was sufficient to react with a fixed sample volume of 100 μL, the influence of the working dye solution volume on the

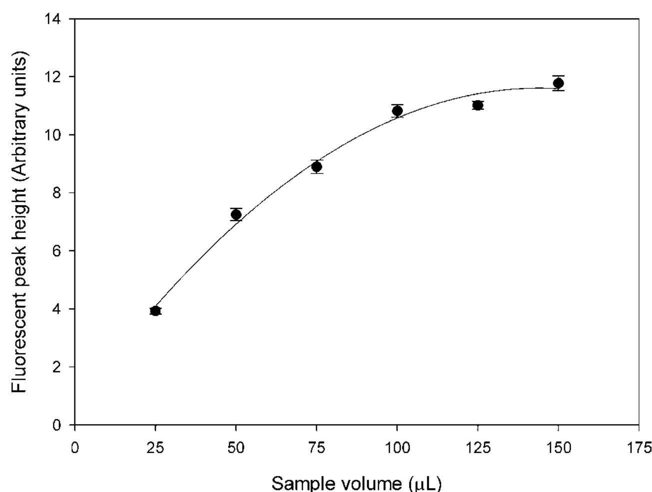

Fig. 3. The relationship between the average fluorescent peak height and the sample volume aspirated to the SIA system.

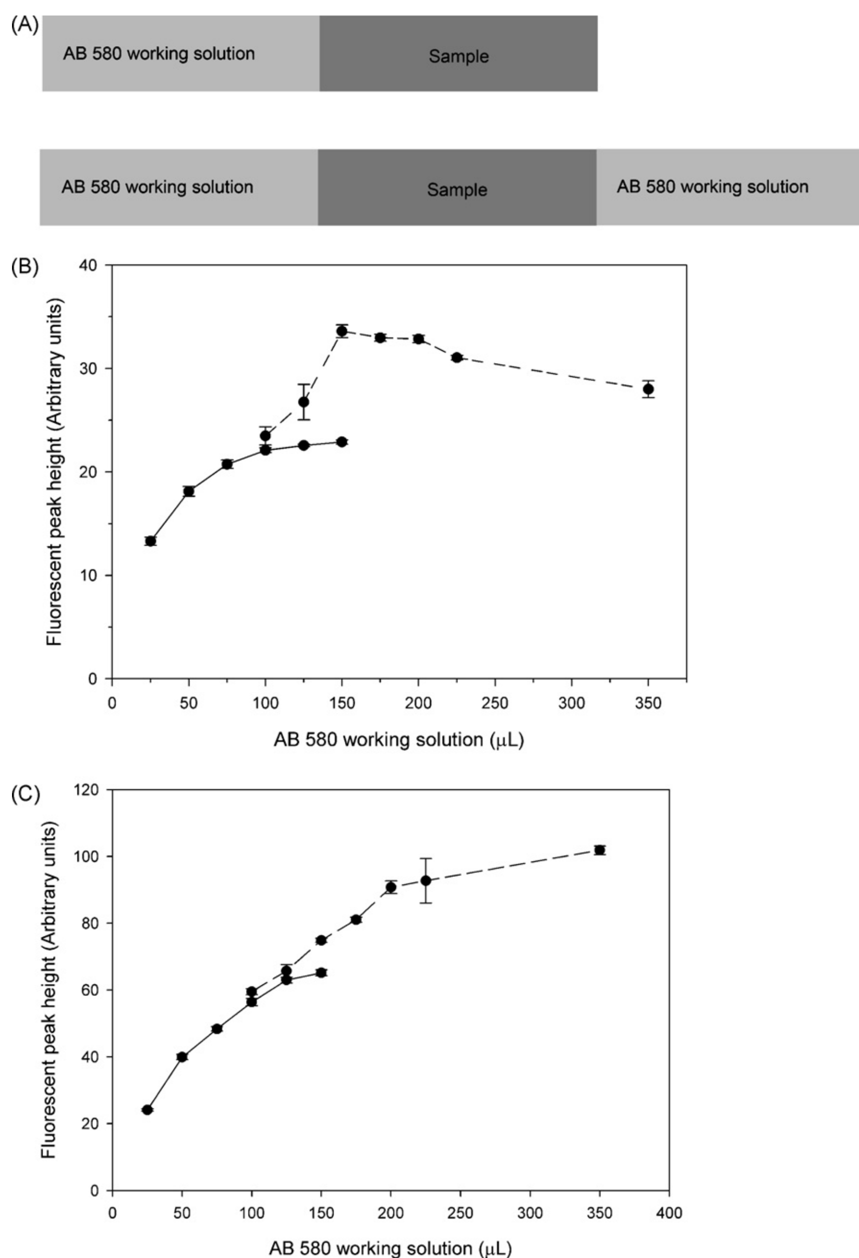

Fig. 4. (A) Aspiration sequences of the AB 580 working solution and sample. The influence of the AB 580 working solution volume aspirated to the system and reacted with 25 mg L^{-1} albumin (B) or 100 mg L^{-1} albumin (C). The aspiration sequence for (A) top (solid line) and (A) bottom (dashed line).

fluorescent signals was studied over the range 25–350 μL. In the preliminary experiment, the simple operation sequence (Fig. 4A, top) was performed by aspirating the working dye solution and the sample sequential, before dispensing the mixture through the fluorescent detector. Two levels of albumin standards were investigated, 25 and 100 mg L^{-1}. The results shown in Fig. 4B (solid line) demonstrated that increasing the volume of the dye reagent could improve the sensitivity of the assay. Clearly, a plateau signal was observed when the dye and 25 mg L^{-1} albumin were injected in a proportion volume of 100:100 μL. This ratio enhanced the fluorescent signal approximately 22% when compared to those obtained from a ratio of 50:100 μL. A similar curve pattern was observed for the injection of 100 mg L^{-1} albumin (Fig. 4C, solid line).

Furthermore, we also investigated whether the additional volume of the AB 580 working solution could improve the sensitivity of the current procedure. Using the operation sequence shown in Fig. 4A (bottom), we found that an additional step of aspirating an extra 50 μL volume of the AB 580 working solution, in which the total volume of the AB 580 working solution was 150 μL, could raise the sensitivity considerably (Fig. 4B, dashed line). The sensitivity was increased approximately 43% when compared to the original sequence. An additional volume of the AB 580 working solution higher than 50 μL was unable to improve the sensitivity of the assay. However, the results for 100 mg L^{-1} albumin were different from that of 25 mg L^{-1} albumin. As shown in Fig. 4C (dash line), the results implied that the injection of a higher dye reagent volume lead to better sensitivity of the assay, and the plateau curve was found at 200 μL of the working dye reagent. However, it was observed that the sensitivity for the total dye volume of 200 μL was increased only 20% compared to that of 150 μL. Therefore, the operation sequence of aspirating the AB 580 working solution (100 μL), sample (100 μL), and AB 580 working solution (50 μL) was selected as a compromise between sensitivity and consumption of the AB

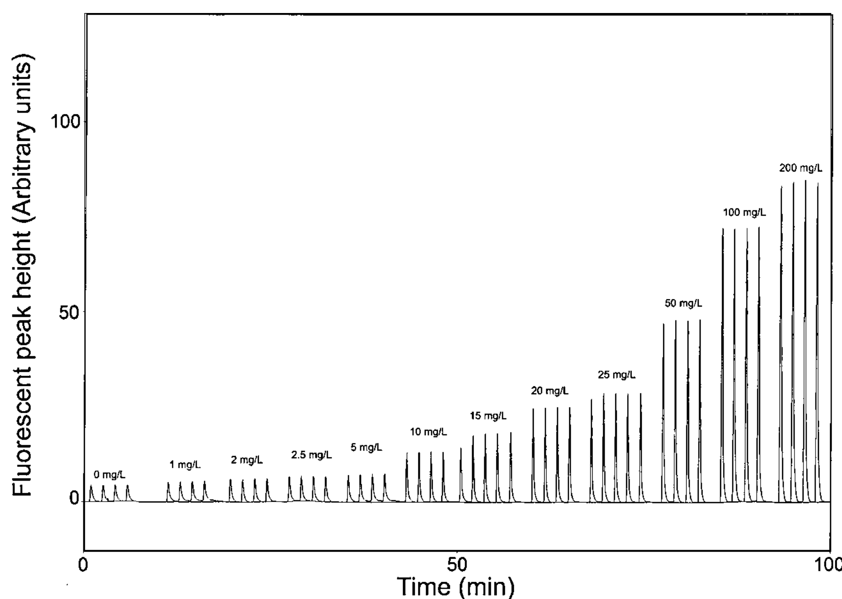

Fig. 5. Typical fluorescent signals for the microalbumin assay using the AB 580 SIA system. The fluorescent peak height is shown for four injections for each concentration.

580 working solution. This formula was used for all subsequent experiments.

Since the albumin solution was sandwiched with the AB 580 solution, the occurrence of double peaking is possible because of the exceeding content of the albumin solution or the inadequate content of the AB 580 solution. These should be considered when the volume and concentration used are different from Table 1.

With the condition above, the Schlieren effect with irregular peaks at low albumin concentrations was not found. But, the double and noisy recorded peaks were consistently observed with sample blank injection. This might have been due to the fact that sample injection into the reagent stream could lead to the appearance of a pronounced component B of the Schlieren effect as described by Dias et al. [23].

3.4. Carrier buffer and flow rate optimization

Several volumes of carrier buffer were studied over the range 600–1400 μL at intervals of 200 μL. The carrier buffer volumes less than 1200 μL were not sufficient to propel all of the chemical reaction mixture to the detector, and the carry-over was clearly observed. Another factor affecting the peak shape was the dispensing flow rate to the detector. When a slower flow rate was used, broader peaks were obtained. In this study, the optimum carrier buffer volume was found to be 1200 μL with a dispensing flow rate at 25 μL/s. Under these conditions, the sampling frequency was 37 h^{-1} (data not shown).

3.5. Analytical curve and limit of detection

The fluorescent peak height of the albumin standards and a typical analytical curve are shown in Fig. 5. The analytical curve obeyed the mass action law, in which the linear range was obtained only at the low concentration from 1 to 25 mg L^{-1} ($r^2 = 0.998$). It should be noted that this linear range was sufficiently below the range for the cut-off limit of microalbuminuria at 15–40 mg L^{-1} albumin [24].

Non-linear analytical curve obtained from this study, may be explained by the stoichiometry of the reaction of the AB 580 dye with albumin that is concentration dependent. The Scatchard analysis yielded a linear plot ($r^2 = 0.989$), which demonstrated that binding of the dye to albumin was generated at 1:1 (data not shown). The formation of 1:1 stoichiometry complexes were according to the previous report [18].

In this study, the assay range was well displayed in the range of 1–100 mg L^{-1} (Fig. 6). The equation derived from the analytical curve was $y = ax/(1 + bx)$, where y represented the relative fluorescence signals and x represented the concentration of albumin (mg L^{-1}), while a and b were obtained from fit parameters of the analytical curve. The detection limit (LOD) was calculated from ten replicate assays of the blank sample. In this work, we used the calibrator diluent as the blank sample. Based on the S/N = 3, the detection limit obtained for albumin was 0.3 mg L^{-1}. For the early detection of microalbuminuria, a detection limit of 1 mg L^{-1} is desirable. Therefore, the proposed SIA system is very promising

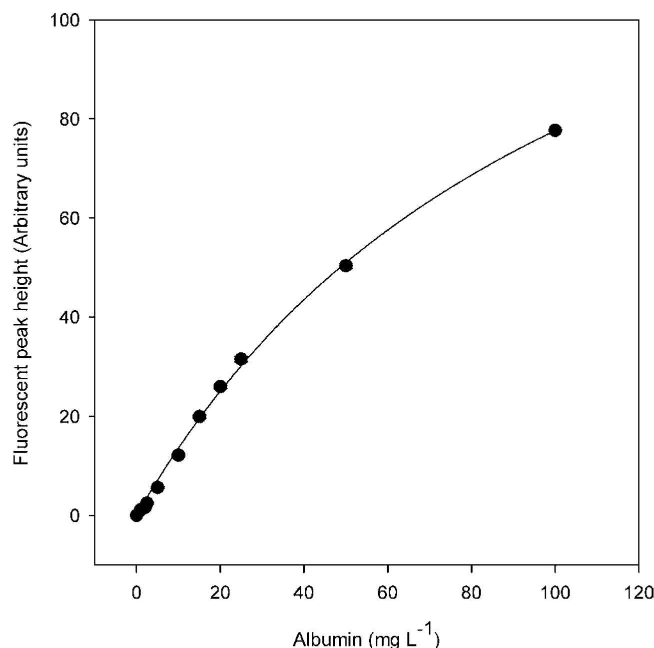

Fig. 6. Non-linear analytical curve for the determination of microalbuminuria measured by the AB 580 SIA system. The equation derived from the analytical curve was $y = 1.5802x/[1 + 0.0111x]$.

Table 2
Effects of the tested interferences on the urinary albumin assay using the AB 580 SIA system.

Added interferences	Added concentration (mg L^{-1})	% Recovery
None	–	100
Transferrin	100	97.3
2-Ketobutyric acid	1	97
IgG	1,000	102.7
Acetylsalicylic acid	100	100.0
Ibuprofen	1,000	94.7
Indomethacin	100	102.7
Acetaminophen	0.59	97.3
Pepsin	590	97.3
Glucose	20,000	100.0
Hemoglobin	200	91.8
	10	96.4
Bilirubin	80	84.9
	10	92.4
Ascorbic acid	100	95.8
NaCl	10,000	96.6

for microalbumin assays, especially in the early stages. The system was slightly more sensitive than the method described previously that was based on a centrifugal automatic analyzer using an AB 580 fluorescence assay, in which the detection limit was 0.4 mg L^{-1} [16].

3.6. Precision and reproducibility

The statistical analysis of the reproducibility study was calculated from the analytical curves performed on four different days. The method exhibited a good reproducibility, as assessed by intra-run CVs (2.3–3.3%) and inter-run CVs (3.8%). For the measurement of albumin with low (15 mg L^{-1}) and high concentrations (100 mg L^{-1}), excellent precision was observed with relative standard deviations of 0.9% and 1.1%, respectively ($n = 15$).

3.7. Interferences

In the paper of Kessler, various substances that normally coexist in urine samples were investigated for their interferences, including common drugs, vitamins, proteins and urine metabolites [16]. The results revealed that none of the tested substances significantly interfered [16]. Therefore, the AB 580 SIA systems based on the same dye-binding principle as in the previous report was presumed to have a high selectivity to albumin. However, a few substances were tested to assure the specificity. As shown in Table 2, the results demonstrated that the interferences from proteins (transferrin, IgG, pepsin), drugs (acetylsalicylic acid, indomethacin, aminocetaphen), and glucose were acceptable with recoveries between 97.3% and 102.7%. Hemoglobin concentrations of 200 mg L^{-1} slightly decreased the fluorescent signals with a recovery of 91.8%. However, the hemoglobin concentration at 10 mg L^{-1} did not interact substantially with AB 580, which is in good agreement with the previous report [16]. Bilirubin concentrations at 80 or 10 mg L^{-1} were found to inhibit the assay signal, for which the recovery signals were 84.9% and 92.4%, respectively. This could be explained by the possible competitive binding between the bilirubin and albumin to the dye [25]. However, since bilirubin is usually absent in normal urine, this interference could be disregarded [26].

3.8. Method comparison

To evaluate the applicability of the proposed method for real sample analysis, 72 urine samples from diabetic patients were subjected to the SIA system along with albumin standards.

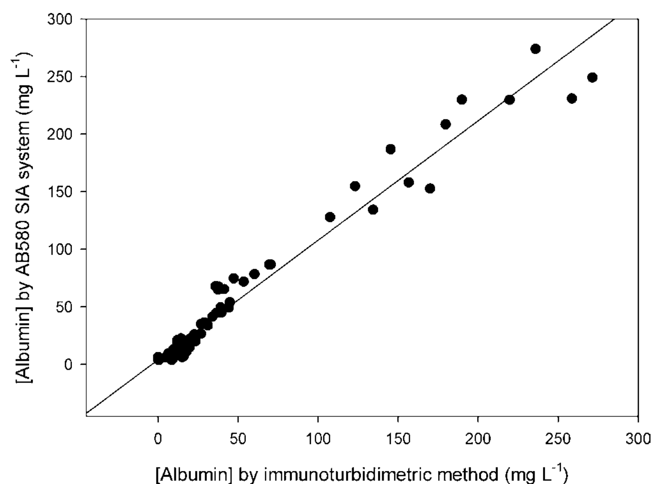

Fig. 7. The AB 580 SIA system and the immunoturbidimetric method were compared for the determination of albumin in urine. The regression analysis gives $y = 1.037 \pm 0.039x + 4.34 \pm 0.324$; $n = 72$; $r^2 = 0.965$.

The results demonstrated that the proposed AB 580 SIA system is highly correlated with the immunoturbidimetric method ($r^2 = 0.965$ and $n = 72$). The equation for the regression analysis was $y = 1.037 \pm 0.039x + 4.34 \pm 0.324$. For two methods with the ideal high correlation, the y-intercept and the slope should be close to 0 and 1.0, respectively. However, in this work, different standards/calibrators were used in the two methods, i.e., the immunoturbidimetric method used the calibrators from the kit supplied. However, the SIA system used the standards prepared in the laboratory from the commercial human serum albumin. Therefore, the y-intercept was significantly different from 0 at 4.34, and the slope obtained was 1.037, which was slightly different from 1.0. For 70° of freedom at the 95% confidence interval ($\alpha = 0.05$), the critical t-value ($t_{0.025,70}$) was ±1.994. The statistical analysis revealed that the t-value of both methods was 43.705, which was significantly higher than the 95% confidence interval. Therefore, it could be summarized that the AB 580 SIA system and the immunoturbidimetric method were well correlated (p-value < 0.05). The scatter-plot of the results obtained by both methods is shown in Fig. 7.

4. Conclusion

The proposed SIA system was very efficient for the simple and rapid analysis of microalbuminuria. Utilizing the SIA system combination with the AB 580 fluorescent dye-binding technique, the method displayed good reproducibility and high sensitivity and specificity. Since no antibodies were required and less reagent was consumed, the cost of the assay was much lower, which is in high demand for the routine analysis of microalbuminuria in diabetic patients. The analytical range obtained from this work, 1–100 mg L^{-1} albumin. This system has been applied for the determination of microalbuminuria in real samples with satisfactory results. The AB 580 SIA is very promising for use instead of an immunoassay, or it can be an alternative method for the assessment of early diabetic nephropathy. Furthermore, the SIA system has the potential to be equipped with a small detector based on the fiber optic fluorometer, which would be fully portable and very beneficial for in-field analysis.

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