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Simultaneous injection effective mixing flow analysis of urinary albumin using dye-binding reaction

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ABSTRACT

A new four-channel simultaneous injection effective mixing flow analysis (SIEMA) system has been assembled for the determination of urinary albumin. The SIEMA system consisted of a syringe pump, two 5-way cross connectors, four holding coils, five 3-way solenoid valves, a 50-cm long mixing coil and a spectrophotometer. Tetrabromophenol blue anion (TBPB) in Triton X-100 micelle reacted with albumin at pH 3.2 to form a blue ion complex with a λ_{max} 625 nm. TBPB, Triton X-100, acetate buffer and albumin standard solutions were aspirated into four individual holding coils by a syringe pump and then the aspirated zones were simultaneously pushed in the reverse direction to the detector flow cell. Baseline drift, due to adsorption of TBPB–albumin complex on the wall of the hydrophobic PTFE tubing, was minimized by aspiration of Triton X-100 and acetate buffer solutions between samples. The calibration graph was linear in the range of 10–50 μg/mL and the detection limit for albumin (3σ) was 0.53 μg/mL. The RSD ($n = 11$) at 30 μg/mL was 1.35%. The sample throughput was 37/h. With a 10-fold dilution, interference from urine matrix was removed. The proposed method has advantages in terms of simple automation operation and short analysis time.

1. Introduction

The Japanese Society for Dialysis Therapy reported that in 2010 the number of people undergoing dialysis treatment was more than 297,000. Albumin is an important indicator for albuminuria and it is recognized as being the earliest clinical marker of diabetic nephropathy and other cardiovascular, renal and hypertensive diseases. To prevent progression toward persistent albuminuria and end-stage renal failure, early medical treatment should be carried out. Hence, a convenient accurate assay of urinary albumin is required.

For rapid semi-quantitative assay of albumin in the clinical laboratory, a strip test is regularly used. The test is based on the color change of an indicator in the presence of protein. Yoshimoto et al. [1,2] proposed a simple visual and sensitive method with a standard calibration chart based on a series of membrane filters using bromochlorophenol blue dye. We proposed tetrabromophenol blue (TBPB) as an ion association reagent for the determination of albumin in the range of 1–10 μg/mL [3]. A spot test using Erythrosin B and a cellulose acetate membrane filter had a detection limit of 0.5 μg/mL, the highest sensitivity of the test strip methods [4].

Highly sensitive spectrophotometry methods for urinary protein using dye–metal complexes in micellar media have been proposed [5–8]. Brandford assay is a well-known method based on color shift of Coomassie Brilliant Blue (CBB) when bound to protein. The CBB method provides concentration value of total protein as obtained from the biuret method, the reference method [9]. Bromophenol blue, bromocresol green and bromocresol purple have also been used for determination of serum albumin [10–12].

Clinical analysis usually involves large numbers of samples, and a flow based technique is suitable in terms of rapid analysis, low reagent consumption and automation. Flow injection analysis (FIA) for protein determination has been reported with CBB [13–16]. Tetrabromophenolphthalein ethyl ester (TBPEH) in Triton X-100 has also been used in a FIA system, with sample throughput of 30 samples/h [17]. However, in the FIA system, reagent consumption is relatively large because reagent solutions are being continuously pumped. Another flow based system called sequential injection analysis (SIA), which has reduced reagent consumption, has been developed [18] with detection based on protein binding

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with TBPEH [19], eosin Y [20] or albumin blue 580 (AB580) [21]. However, the system with AB580 dye had a non linear calibration curve, which is not convenient for quantitative analysis.

Recently, a new hybrid flow analysis, called simultaneous injection-effective mixing analysis (SIEMA) was designed by our group [22]. The system has the benefits of the versatility and ruggedness of SIA, since it employs a syringe pump, but maintaining the efficient radial mixing of the FIA system. A three-channel SIEMA system has been developed for the determination bilirubin in human urine [23].

In this paper, a four-channel SIEMA system was applied to the spectrophotometric determination of albumin using TBPB as the ion association reagent.

2. Experimental

2.1. Apparatus

The schematic diagram of the SIEMA system is shown in Fig. 1. The system consists of a syringe pump (P) which has a 2-way solenoid valve at its head (V_p) and five 3-way solenoid valves ($3V_1$–$3V_5$). The pump and valves are controlled by a laptop computer using an in-house software. All tubing and connections were PTFE tubing of 0.8 mm i.d. A double beam spectrophotometer (S-3250, Soma Optics, Tokyo, Japan) with a flow-through cell (8 μL volume, 10 mm path length) was used for absorbance measurements. The detector output was recorded on a chart recorder (EB 22005, Chino, Tokyo).

2.2. Reagents

All reagents were of analytical reagent grade, and deionized water from Advantec GSH-210 system was used throughout.

A TBPB stock solution (5×10^{-4} mol/L) was prepared by dissolving 0.048 g of tetrabromophenol blue sodium salt (Tokyo Chemical Industry, Tokyo) in 100 mL of water.

A Triton X-100 solution (0.5%) was prepared by dissolving 0.5 g of *t*-octylphenoxypolyethoxyethanol (Sigma–Aldrich Japan) in 100 mL of water.

A 0.5 mol/L acetate buffer solution was prepared from acetic acid (Nacalai Tesque, Kyoto, Japan) and sodium acetate trihydrate (Nacalai Tesque).

A stock solution (1000 mg/L) of bovine serum albumin (BSA, Sigma–Aldrich Japan) were prepared with deionized water and stored at 4 °C. The working solutions were obtained by diluting the stock solution with water just prior to use.

2.3. Collection of urine samples and sample preparation

Spot urine samples were collected from one diabetic patient and 6 normal subjects. All samples were stored at 4 °C. Before analysis, the urine samples were filtered through a Whatman #1 filter paper to remove particulate matter. The filtrate was then diluted 10-fold with water before aspiration into the SIEMA system.

2.4. Data analysis

Standards and sample signals were recorded, peak heights from the baseline were measured, and the peak heights were converted into the corresponding absorbance values. A calibration curve was obtained by plotting absorbance of standard/sample minus absorbance of blank against the analyte concentrations. For validation of the developed SIEMA system, the results were compared to values obtained using Brandford protein assay [24].

3. Results and discussion

3.1. System design

In this SIEMA system as shown in Fig. 1, all solutions were aspirated into the system and dispensed to the detector by a single syringe pump. The operation procedure is summarized in Table 1. At the start of the analysis, the system commenced from step A1 (without step W1) by simultaneous aspiration of TBPB, Triton X-100, acetate buffer and BSA standard/sample into holding coils HC_1, HC_2, HC_3 and HC_4, respectively. The total aspiration volume of these solutions was optimized at 800 μL (*vide infra*), and so each volume aspirated was 200 μL. Then, all aspirated zones were simultaneously pushed in the reverse direction toward the detector. Mixing of the zones starts at the 5-way connector ($5C_2$), with complete mixing occurring in the mixing coil (MC). A TBPB–BSA complex in Triton X-100 micelle is formed in the MC and subsequently detected in the flow-cell, with absorbance at 625 nm. Preliminary results are shown in Fig. 2a. However, drifting baseline and noisy blank signals were observed, which was attributed to adsorption of the TBPE–BSA complex on the inner wall of the hydrophobic PTFE tubing.

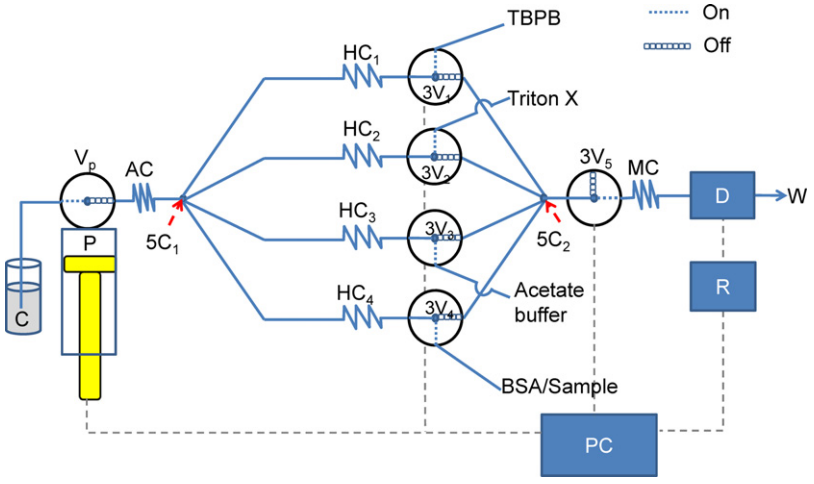

Fig. 1. Schematic flow diagram of the SIEMA system for albumin determination. C, water; P, syringe pump (5 mL); V_p, 2-way solenoid valve atop P, AC, auxiliary coil (2 mm i.d., 65 cm long); $5C_1$ and $5C_2$, 5-way cross connectors; HC_1, HC_2, HC_3 and HC_4, holding coils (100 cm, 0.8 mm i.d.); $3V_1$, $3V_2$, $3V_3$, $3V_4$ and $3V_5$, 3-way solenoid valves; MC, mixing coil (0.8 mm i.d., 50 cm long); D, spectrophotometer (625 nm); R, recorder; PC, computer; W, waste.

Table 1
Operation sequence of the SIEMA system.

Step[a]	SP (↓↑)[b] flow rate[c]	V_p	$3V_1$	$3V_2$	$3V_3$	$3V_4$	$3V_5$	Function
W1	(↓)100	OFF	OFF	ON	ON	OFF	OFF	Simultaneous aspiration of 750 μL of Triton X-100 and buffer into HC_2 and HC_3, respectively
A1	(↓)100	OFF	ON	ON	ON	ON	OFF	Simultaneous aspiration of 200 μL of standard solution/sample and reagents into each HC, respectively
A2	(↑)300	ON	OFF	OFF	OFF	OFF	OFF	SP plunger moves to the zero position
A3	(↓)300	ON	OFF	OFF	OFF	OFF	OFF	Aspiration of 5000 μL carrier solution into glass syringe
A4	(↑)100	OFF	OFF	OFF	OFF	OFF	ON	Dispensing all aspirated zones to detector simultaneously

[a] Cycle time (steps W1–A4) = 97 s.
[b] (↓) forward flow direction, (↑) reverse flow direction.
[c] Flow rate in μL/s.

In order to eliminate this surface adsorption, the procedure was modified to include an extra wash step (W1 in Table 1) of an aspiration of 750 μL Triton X-100 and 750 μL acetate buffer. Fig. 2 (bottom) shows data for the system operating with the original and the modified procedures. The addition of the washing plug eliminated adsorption of the TBPB–BSA complex. Results obtained from employment of the modified procedure (Fig. 2b) clearly demonstrate that the washing step is necessary. A constant baseline and well defined blank signals were now obtained.

3.2. System optimization

3.2.1. Chemical parameters

TBPB was used as the dye binding reagent in this work. The effect of TBPB concentration on the response was studied from 1.0 to 5.0×10^{-5} mol/L in increments of 1.0×10^{-5} mol/L. The results are shown in Fig. 3. The net signal increased linearly with TBPB concentration, but the blank signal also slightly increased (data not shown). TBPB concentration at 2.0×10^{-5} mol/L was chosen as a compromise between large analytical signal and low reagent consumption.

Triton X-100 is a non-ionic surfactant and is often used for solubilization of non-polar compounds, such as the TBPB–BSA complex. The concentration of Triton X-100 was varied from 0 to 0.4% (w/v) for constant TBPB concentration at 2×10^{-5} mol/L. The results in Fig. 3 show that the response increased with increasing Triton X-100 in the range 0–0.1% (w/v). However at concentration greater than 0.1% (w/v), the response decreased sharply. However, a concentration lower than 0.2% (w/v) was not sufficient for complete removal of the adsorbed blue complex from the tubing, as elevation of baseline was observed. Therefore, the concentration of 0.2% (w/v) was chosen.

3.2.2. Physical parameters

As described above, solutions of BSA standard/sample and reagents were simultaneously aspirated into each holding coil and so identical volumes of each solution were introduced into the system. Thus, the volume in Fig. 3 refers to the volume of each solution. The effect of increasing the aspirated volume from 100 to 400 μL was examined. It was observed that the response greatly increased when the aspirated volume was increased to 200 μL. However, further increase in the volume did not give rise to relatively larger response. In this work, the volume of 200 μL was selected for each solution as a compromise between consumption volume and signal height.

Length of the mixing coil can also be a parameter affecting sensitivity. In this work, lengths between 15 and 150 cm were investigated. It was found that the response remained almost constant over the studied range. Similar results were found for the mixing flow rate between 30 and 120 μL/s. Thus, the ion associ-

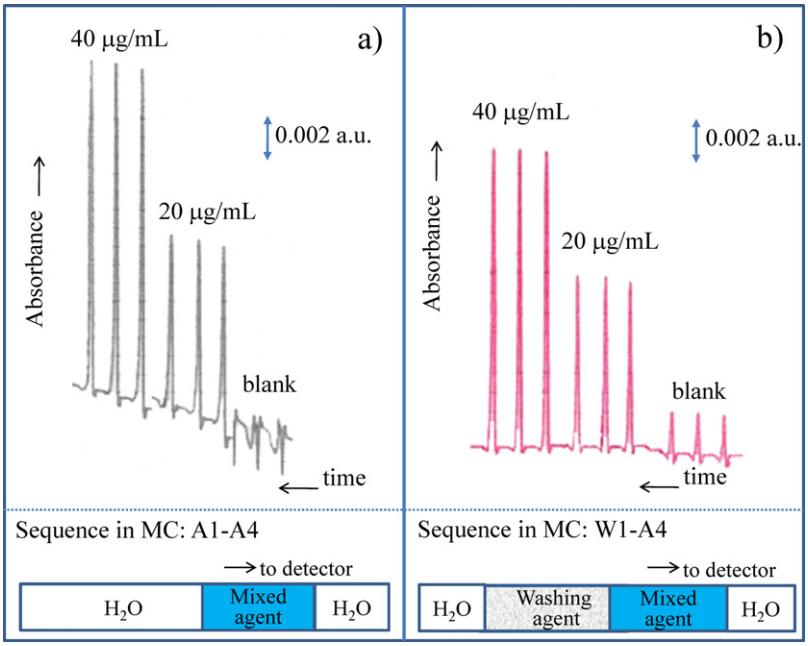

Fig. 2. Comparison of signal profiles obtained from the normal operation (a) and the operation with washing agent (b). Sequences of solutions in mixing coil (MC in Fig. 1) are also attached below. Difference in baseline's behavior was clearly observed.

Table 2
Tolerance limits of various components on BSA determination.

Chemical species	Tolerance limit (mg/dL)
SO_4^{2-}	20
Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+, Cl^-	100
Ascorbic acid, creatinine, urea, glucose	1000

ation reaction between BSA and TBPB is rapid and mixing by the four-channel SIEMA system is highly effective. A mixing coil of 50 cm and dispensing flow of 100 μL/s were chosen.

3.3. Analytical features

Using the optimized parameters, the calibration curve was always linear for the concentration range 0–50 μg/mL: $A = (3.50 \times 10^{-4} \pm 6.55 \times 10^{-6})C_{BSA} - (4.49 \times 10^{-4} \pm 1.72 \times 10^{-4})$ with $r^2 = 0.998$, where A is absorbance and C_{BSA} is the concentration of BSA in μg/mL. This system provides rapid analysis with sample throughput of 37/h. Moreover, the system uses small volume of TBPB dye of 200 μL at 4×10^{-5} mol/L per analysis. The percent relative standard deviation at 50 mg/L BSA ($n = 9$), was 0.59%. The detection limit (defined as S/N = 3σ, where σ is the standard deviation of the blank ($n = 10$)) was 0.53 μg/mL and the limit of quantitation (S/N = 10σ) was 1.75 μg/mL.

3.4. Interference study

Various amounts of compounds were added to 50 μg/mL BSA to determine their interference on the analysis. The results are given in Table 2. The tolerance limit was defined as the concentration that yielded a relative error less than or equal to 5% when compared to the response obtained for the standard solution. Major components of urine, such as chloride ion, creatinine and urea, were selected. Their mean concentrations in urine are 4780 mg/L, 1960 mg/L and 18,200 mg/L, respectively [19]. Although these values may vary from the sample to sample, it was verified that there was no significant interference from chloride, creatinine and urea when the samples were diluted 10-fold before analysis. Other minor constituents of urine, such as sulfate, glucose and ascorbic acid also did not interfere with the BSA analysis.

3.5. Dilution effect and recovery

Since urine contains highly variable matrix that may influence the urinary albumin assay, dilution was included in the design and evaluation of the assay. Various dilution ratios of a sample urine, containing added 20 μg/mL BSA, were studied for recovery. The results (Table 3) indicate that dilution of at least 10-fold gave rise to good recovery. These results agreed with those described in Section 3.4, which also indicated that 10-fold dilution could avoid the effect of major components of chloride ion, creatinine and urea in urine.

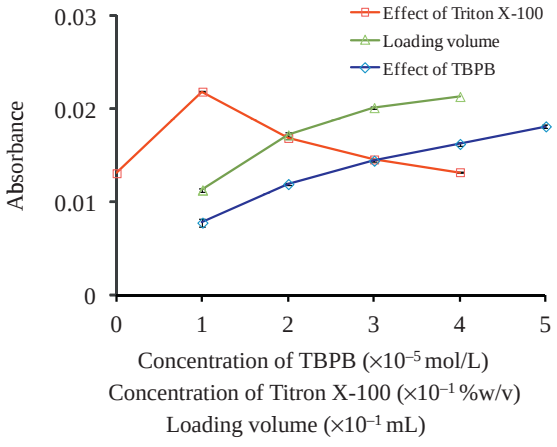

Fig. 3. Effects of concentrations of TPBP and Triton X-100 and loading volume on the signal. Measurement was carried out in triplicate injections of 50 μg/mL BSA.

Table 3
Recoveries of the 20 μg/mL BSA in urine sample at various dilution factors ($n = 3$).

Dilution (fold)	% Recovery
0	58.1 ± 0.7
10	94.9 ± 0.6
25	97.7 ± 0.7
50	103.7 ± 0.3
100	103.7 ± 0.3
200	103.7 ± 0.1

Table 4
Recovery studies of BSA added to urine samples ($n = 3$).

Sample	Sample conc. (μg/mL)	Added (μg/mL)	Found (μg/mL)	Percentage recovery
1	nd	20	18.7 ± 0.4	94 ± 0.7
		40	38.9 ± 0.1	97 ± 0.5
2	nd	20	19.8 ± 0.1	99 ± 0.3
		40	38.9 ± 0.1	97 ± 1.5
3	nd	20	20.0 ± 0.3	100 ± 0.7
		40	39.8 ± 0.4	99 ± 0.1
4	nd	20	21.0 ± 0.2	105 ± 2.2
		40	40.1 ± 0.6	100 ± 0.5
5	nd	20	20.9 ± 0.3	104 ± 1.6
		40	39.8 ± 0.1	99 ± 0.9

In addition, further investigation was carried out on five different BSA-free urine samples. Recoveries of added 20 μg/mL and 40 μg/mL BSA standards are given in Table 4. Good recoveries for all samples were found.

3.6. Application to urine samples and validation of method

The proposed SIEMA system was applied to 16 urine samples obtained from one diabetic patient (10 samples) and 6 healthy subjects (one sample per subject). The range of protein concentrations, as determined by the reference Brandford's assay, was 13–36 μg/mL and 40–640 μg/mL for normal subjects and diabetic patient, respectively. Validation of the developed system was carried out using linear regression test. The protein contents by the proposed SIEMA system agree well with the results obtained by the conventional batchwise Brandford's method, with the linear equation: $y = (1.004 \pm 0.012)x + (0.708 \pm 2.42)$, $r^2 = 0.998$ ($n = 16$). The intercept and a slope did not differ significantly from the ideal values of 0 and 1, respectively, at 95% significance level. Thus there is no evidence for systematic differences between the two sets of results.

4. Conclusion

The simple automated SIEMA system for determination of protein was successfully achieved using ion associate formation of the protein with TBPB in the presence of Triton X-100. The system has the advantage of highly efficient mixing of the reagents, low reagent consumption and rapid analysis. In addition, sharp signal peaks with constant baseline were obtained when a washing step was included. The assay of protein in urine using this system was in good agreement with the Brandford's method. Therefore, we propose that this SIEMA system can be an alternative method for routine diabetic screening.

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