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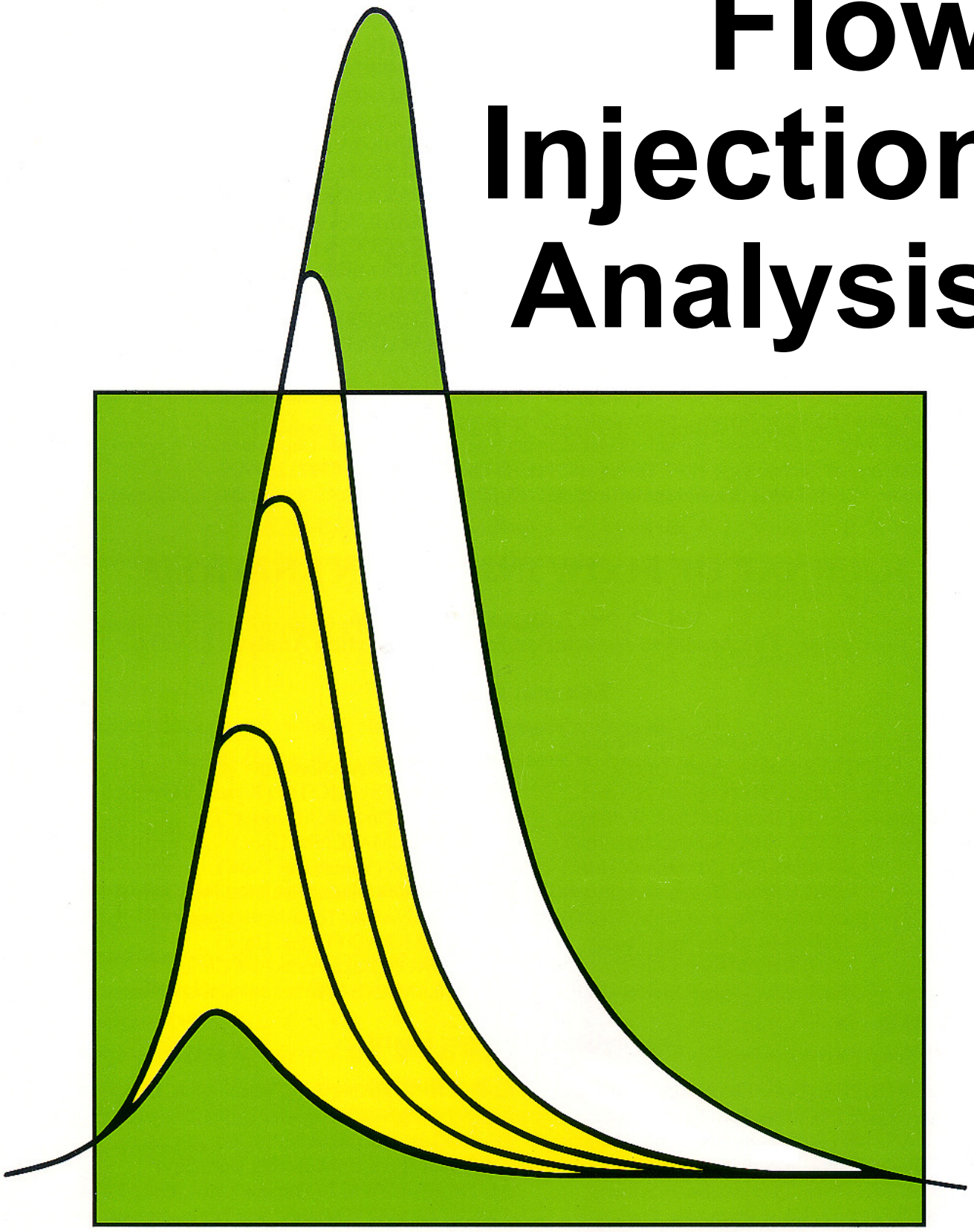

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Spectrophotometric Flow Injection Analysis of Protein in Urine Using Tetrabromophenolphthalein Ethyl Ester and Triton X-100

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Abstract

A sensitive, rapid and accurate determination of protein in patient urine was carried out by flow injection analysis (FIA) using tetrabromophenolphthalein ethyl ester (TBPE·H) and Triton X-100 at pH 3.0. The detection system was based on the ion association formation between human serum albumin (HSA) and TBPE·H in the micelle formed by Triton X-100. The calibration graph was linear in the range of 0.15 – 12 mg/dL HSA with R^2 = 0.998. The 3σ limit of detection of the proposed FIA method was 0.05 mg/dL at 610 nm. The relative standard deviation (n = 10) of 3.0 mg/dL HSA was 1.2% and the sample throughput was 30 h^{-1}.

Keywords Protein determination, Urine, Tetrabromophenolphthalein ethyl ester, Flow injection analysis; Ion association formation

1. Introduction

Of proteins, albumin is very important as an active substance in our body and is synthesized in the liver. In the liver disease, the amount of albumin in the blood decreases. And also, protein is excluded as a renal proteinuria in nephropathy and diabetic nephropathy. Especially, the measurement of protein amount excluded in the urine is an important indicator to the diabetes diagnosis. In the clinical laboratory, the test paper impregnating the dyestuff is commonly used for the rapid examination of renal disease. The visible detecting technique using the test paper is simple and easy to deal with, however, the detection of protein is semi-quantitative and the measurable concentrations are classified as follows; (–) < 15 mg/dL, (±) 15 – 30 mg/dL, (+) > 30 mg/dL, (++) > 100 mg/dL, (+++) > 300 mg/dL.

Accordingly, simple and accurate spectrophotometric determinations of urinary albumin and micro-albuminuria assay using bromophenol blue [1,2] have been reported. And, Fujita et al. have reported highly sensitive spectrophotometric methods for urinary protein with dye-metal complexes and micelle media [3–7]. Huang et al. have reported the determination of albumin and globulin with α-, β-, γ-, δ-tetrakis(4-sulfophenyl)porphine [8]. Bromocresol green and bromocresol purple were used for the serum albumin determination [9] and bromothymol blue has been proposed the determination of serum albumin [10]. And also, the chemical equilibrium of protein-dye binding for protein error was studied [11,12] and the interactions of dye and/or complexes with protein were reported [13,14]. On the other hand, Yoshimoto et al. have proposed the visual, simple and sensitive analytical method for the protein detection [15,16]. Recently, simultaneous determination of human albumin, globulin and glucose by near-infrared spectroscopy [17] and fluorescence quenching method [18] of bovine serum albumin by CdS nanoparticles have been reported. And, Hashimoto et al. reported the protein assay with tetrabromophenol blue [19]. However, these methods mentioned above have been performed by the batchwise technique and the procedure needs much time and large reagent consumption.

In the clinical laboratory, the routine and accurate quantification of albumin in urine is very important for a clinical evaluation of patients with renal disease. Flow injection analysis (FIA) developed by Ruzicka and Hansen is a versatile and practical technique [20]. The technology has been widely used in environmental, clinical and pharmaceutical analyses because the method permits automatic, rapid and sensitive analysis compared with the batchwise method. FIA with Coomassie brilliant blue G-250 was used to determine rapidly protein in urine [21] and also, high-sensitivity flow method with micro-flow plunger pumps [22] and capillary stream sensor [23] were demonstrated for the serum albumin determination. However, the sample throughput was only 15 h^{-1}. The albumin in biological fluids was analyzed using FIA-CL technology, however, the derivatization reaction was carried out off-line [24].

We found that an insoluble TPBE·H formed around at pH 3.8 was dissolved in the presence of Triton X-100. In this paper, a simple two channel FIA system using TBPE·H with Triton X-100 is proposed as a rapid and precious sensor of urinary protein.

2. Experimental

2.1. Reagents

All reagents used were of analytical-reagent grade, and deionized water purified by an Advantec GSH-210 apparatus was used throughout.

A TBPE stock solution (1.0×10^{-3} M) was prepared by dissolving 0.07 g of tetrabromophenolphthalein ethyl ester potassium salt (MW: 700, Tokyo Kasei) in 100 mL of ethanol.

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

A human serum albumin standard (HSA, 100 mg/dL) was prepared by 0.1 g of human serum albumin (MW: 66,000, Seikagaku Kogyo) in 100 mL of water.

Working solutions were prepared by suitable dilution of the stock solutions with water.

Buffer solution was prepared by mixing 0.1 M potassium hydrogen phthalate and 0.1 M hydrochloric acid.

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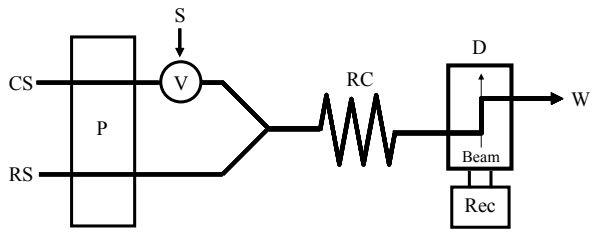

Fig. 1 Schematic diagram of the flow system. CS, water; RS, TBPE·H (1.2×10^{-5} M) + TritonX-100 (0.02%) + buffer (potassium hydrogen phthalate-hydrochloric acid, pH 3); P, pump (0.9 mL/min); V, valve; S, sample (200 μL); RC, reaction coil (ϕ 0.25 mm × 5 m); D, detector (610 nm); Rec, recorder; W, waste.

2.2. Apparatus

A JASCO, Model V-550, double beam spectrophotometer with 10 mm cell and a Horiba, Model F-22, pH meter were used for the batchwise method.

The manifold of the flow injection system is shown in Fig. 1. The dual micro-pump (F·I·A Instruments, Model PD-2000) was used to deliver carrier (CS) and reagent solution (RS). An LED visible spectrophotometer with an interfering filter (610 nm) (F·I·A Instruments, Model KCM-0306) equipped with a micro flow cell was used for the measurement of protein associate.

2.3. Procedures

2.3.1. Batchwise method

To the HSA standard solution, 5 mL of 1.0×10^{-4} M TBPE solution, 5 mL of 0.1% Triton X-100 and 2 mL of buffer solution (pH 3.1) were added in a 25 mL volumetric flask and the mixture was diluted to the mark with water. After mixing, absorbance was measured at 610 nm against water.

2.3.2. FIA method

The two channel flow system as shown in Fig. 1 is assembled. CS (water) and RS (a mixture of 1.2×10^{-5} M TBPE, 0.02% TritonX-100 and buffer (pH3.0)) were pumped at the rate of 0.9 mL/min. An aliquot (200 μl) sample solution is injected into the CS stream by a six-way injection valve. After mixing in the 5 m reaction tube (inner diameter 0.25 mm), absorbance was monitored at 610 nm.

3. Results and discussion

3.1. Batchwise study

3.1.1. Effects of TBPE and Triton X-100 concentrations

TBPE concentration was varied from 1.0×10^{-5} M to 8.0×10^{-5} M and Triton X-100 concentration was varied in the range of 0.005 – 0.1% in the absence of HSA. TBPE (blue) dissolved in ethanol was converted to yellow TBPE·H molecule in an aqueous media below pH 3.5 because the dissociation constant, $pK_{a,TBPE}$, was 4.2 [25]. A yellow TBPE·H was insoluble in water, however, TBPE·H was soluble in the presence of Triton X-100. Absorbance of the reagent blank increased with increase of TBPE concentration. This could be due to that TBPE·H in the micelle produced a dissociated TBPE ion slightly. In this study, 0.02% Triton X-100 and 2.0×10^{-5} M TBPE were chosen.

3.1.2. Effect of pH for ion association formation

The effect of pH on the formation of TBPE-protein associate was investigated. pH was varied from pH 2.9 to 4.2. The 2.0×10^{-5} M TBPE and 0.02% Triton X-100 solutions were used for 2.4 mg/dL HSA. With increasing pH, absorbance of the associate increased, however, absorbance of the reagent blank increased gradually. However, the calibration curve at pH 3.4 gave $y = 0.21x + 0.05$, $R^2 = 0.956$ (y = absorbance, x = HSA concentration in mg/dL, R^2 = correlation coefficient) and that at pH 3.1 gave $y = 0.17x + 0.03$, $R^2 = 0.980$ in the range of 0 – 3.0 mg/dL HSA. Consequently, pH 3.1 was chosen in this study.

Fig. 2 shows absorption spectra at pH 3.1 and 3.8 for various HSA concentrations. At pH 3.1, the reagent blank gave a low absorbance at 610 nm and absorbance of the associate increased in proportion to the HSA concentration. And also, the isosbestic point appeared at 490 nm. The larger absorbance was obtained at pH 3.8, however, the absorbance of the reagent blank became larger and the absorbance of the associate was not proportional to the HSA concentration, because TBPE·H in the micelle dissociated into TBPE anion at higher pH, and the ion associate formation was not stoichiometric.

3.1.3. Binding ratio of protein-TBPE associate

The binding ratio of the protein-TBPE associate was investigated by the continuous variation plots. The result is represented in Fig. 3. According to the continuous variation plots, it can be assumed that the binding number of TBPE to HSA is over 18. And also, it was simulated to obtain a presumed binding number as given below. When 3.6×10^{-7} M HSA was added, the absorbance was 0.417 at 610 nm. Hence, the molar absorptivity

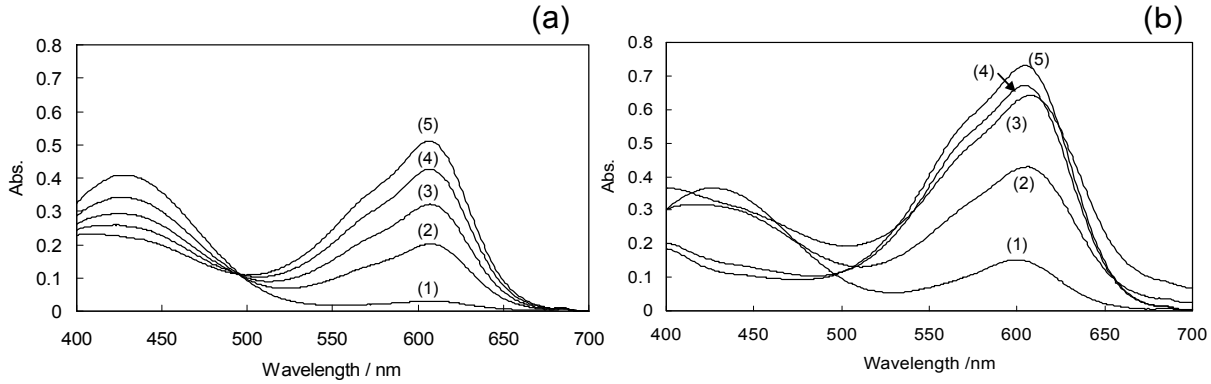

Fig. 2 Absorption spectra at different pH by batchwise method. (a), pH 3.1; (b), pH 3.8. HSA concentrations (mg/dL): (1), 0; (2), 0.8; (3), 1.6; (4), 2.4; (5), 3.2. TBPE·H, 2.0×10^{-5} M; Triton X-100, 0.02%.

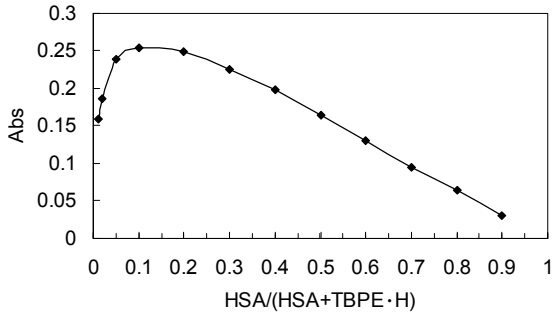

Fig. 3 Continuous variation plot of TBPE-HSA associate. $C_{HSA} + C_{TBPE} = 4\times10^{-5}$ M.

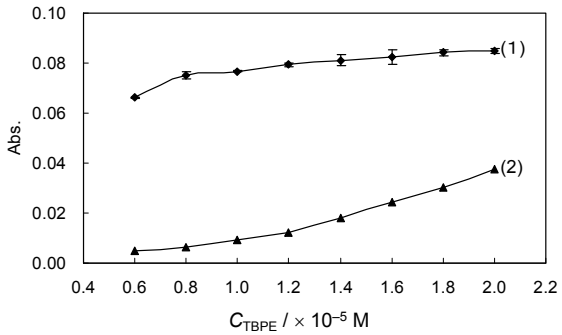

Fig. 4 Effects of TBPE concentration in FI method. (1) HSA (3 mg/dL) and (2) baseline absorbance against water. pH 3.0; Triton X-100, 0.02 %; RC: ϕ 0.25 mm×5 m; flow rate, 0.9 mL/min; sample loop, 200 μL. Error bar denotes standard deviation in tripricate.

for 3.6×10^{-7} M HSA, $\varepsilon_{HSA\cdot(TBPE)x}$ (x denotes the binding number of TBPE), was 1.2×10^{6} L mol^{-1} cm^{-1}. In this study, ε_{TBPE} in the presence of Triton X-100 was 5.7×10^{4} L mol^{-1} cm^{-1}. The ε_{TBPE} multiplied by 21 gives the same value of 1.2×10^{6} L mol^{-1} cm^{-1} as the $\varepsilon_{HSA\cdot(TBPE)x}$. Consequently, we estimated the binding ratio of TBPE to HSA was 21:1. Hence, the chemical equilibrium of the association reaction and its micelle extraction constant ($K_{ex(m)}$) can be expressed as:

$$21(TBPE\cdot H)_m + HSA^{21+} \rightleftharpoons (HSA^{21+}\cdot(TBPE^{-})_{21})_m + 21H^{+} \quad (1)$$

$$K_{ex(m)} = \frac{[HSA^{21+}\cdot(TBPE^{-})_{21}]_m[H^{+}]^{21}}{[TBPE\cdot H]_m^{21}[HSA^{21+}]} \quad (2)$$

where $[\ ]_m$ is the concentration of chemical species in the micelle. Motomizu et al. have reported micelle extraction constants of ion associate formed between quaternary ammonium salts (quaternary phosphonium salts) and TBPE·H in the presence of Triton X-100 [26,27]. However, in this study, it was difficult to determine the micelle extraction constant of TBPE-HSA because HSA is a large and hydrophilic molecule.

3.1.4. Calibration graph and interference study

A calibration graph with a good linear relationship (R^2 = 0.980) was obtained up to 3.0 mg/dL of HSA. The 3σ limit of detection (LOD) and 10σ limit of quantitation (LOQ) were 0.05 and 0.17 mg/dL, respectively.

Sodium chloride and creatinine concentrations in normal urine are 1000 mg/dL and 100 mg/dL, respectively. The interferences on sodium chloride and creatinine were studied. As the results, sodium chloride did not interfere up to 30 mg/dL and 18 mg/dL creatinine did not give the interference. Accordingly, the coexisting sodium chloride and creatinine in urine did not give the interference on the determination of HSA in this method because the urine sample was diluted with water to 62.5-fold before measurements.

3.2. FIA study

3.2.1. Effect of pH

The effect of pH for 3.0 mg/dL HSA was investigated in the range of pH 2.7 – 3.3. With increasing pH, absorbance increased gradually. However, over pH 3.1, absorbance of the baseline also increased. In this study, pH 3.0 was selected.

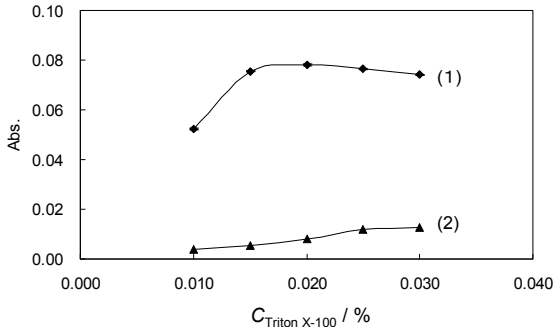

Fig. 5 Effects of Triton X-100 concentration in FI method. (1) HSA (3 mg/dL) against reagent blank and (2) baseline absorbance against water. TBPE, 1.2×10^{-5} M; pH 3.0; RC, ϕ 0.25 mm×5 m; flow rate, 0.9 mL min^{-1}; sample loop, 200 μL. Error bar denotes standard deviation in tripricate.

3.2.2. Effects of TBPE and Triton X-100 concentrations

TBPE·H concentration was varied in the range from 0.6×10^{-5} to 2.0×10^{-5} M (Fig. 4). Over 1.2×10^{-5} M TBPE·H, absorbance increased slightly. However, over 1.4×10^{-5} M TBPE·H, absorbance of the baseline increased drastically. A higher TBPE·H concentration gave the large reagent blank in a similar manner as mentioned in the batchwise study. In this study, 1.2×10^{-5} M TBPE·H was used.

The effect of Triton X-100 concentration was varied from 0.01 to 0.03%. At the 0.01% solution, the absorbance of the associate was very low because the dissolution of TBPE·H into the micelle was not sufficient. Over 0.015%, the largest and constant absorbance was obtained as shown in Fig. 5. The 0.02% concentration was chosen.

3.2.3. Other variables

The reaction coil (tubing of 0.25mm ID) length was varied in the range of 0.5 to 5 m using 3 mg/dL HSA. The constant and largest absorbance was obtained even below 2 m. However, the correlation coefficient (R^2) of the calibration curve was 0.977 at 2 m and R^2 at 5m was 0.998 in the range of 4–8 mg/dL HSA. As a result, a 5-m tubing was chosen.

Table 1 Determination of protein in human patient urine

Sample No.	Test paper[a]	Batchwise method /mg/dL[b]	FIA method /mg/dL[b]
1	++	50.1±2.4	43.2±1.3
2	++	82.3±1.9	83.7±4.5
3	++	58.3±2.3	53.2±1.2
4	++	89.7±2.2	88.0±2.2
5	++	70.6±2.0	66.3±1.5
6	++	89.8±3.0	81.8±0.8
7	++	108.8±1.2	109.7±1.0

a. Semi-quantitation by Albustix purchased from Bayer Medical Ltd., The symbol (++) shows > 100 mg/dL protein
b. Dilution ratio for sample determined was 62.5.

The effect of flow rate was investigated in the range of 0.5 to 1.3 mL min^{-1}. Over 0.7 mL min^{-1}, the constant and largest absorbance was obtained and the baseline became lower.

The sample volume was varied from 50 μL to 300 μL. With increasing the volume, absorbance increased gradually. The 200 μL sample volume was used.

3.2.4. Calibration graph

The calibration graph was linear up to 12 mg/dL of HSA with $R^2 = 0.998$ and the graph passed through the origin: $y = 0.026x$ (y = absorbance, x = HSA concentration in mg/dL). The LOD and LOQ of the proposed FIA method were 0.05 and 0.15 mg/dL, respectively. The sample throughput was 30 h^{-1}. The relative standard deviation ($n = 10$) was 1.2% for 3 mg/dL.

3.2.5. Interferences

Interference by sodium chloride and creatinine was studied on the determination of 3 mg/dL HSA by the proposed FIA method. Over 60 mg/dL sodium chloride, absorbance decreased. However, sodium chloride less than 20 mg/dL did not interfere. And, 10 mg/dL creatinine did not give any interference. Since urine sample was diluted with water to 62.5-fold in this study, the influence from sodium chloride and creatinine could be negligible.

3.3. Application to urinary protein and serum protein analysis

The urinary protein contents in samples collected from nephropathy and diabetic nephropathy patients were determined by a paper test kit (commercially available Albustix by Bayer Medical), batchwise method and the proposed FIA method. Each urine sample was centrifuged at 3000 rpm and the supernatant was diluted with water to 62.5-fold. The results obtained by the three methods are summarized in Table 1. The concentrations by the test paper showed around 100 mg/dL for all samples because the judged concentration range was rough and not accurate as described above. The correlation coefficient (R^2) between batchwise method and FIA method was 0.954, and the slope was 1.03. The experimental t-value was 2.42 and the theoretical t-value was 2.447 for $n = 7$. Accordingly, the proposed FIA using TBPE·H and Triton X-100 was useful by offering some advantages on low reagent blank, rapidity, simplicity, accuracy and less reagent consumption.

In conclusion, two channel flow injection system using TBPE·H dissolved in Triton X-100 was proposed for the determination of urinary protein collected from patients. The calibration range was 0.15 – 12 mg/dL with RSD of 1.2%. The sample throughput was 30 h^{-1}. This could be an alternative method in the clinical laboratory.

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