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Flow Injection Determination of Urinary Protein Using Fluorescence Quenching of Tetraiodofluorescein

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Abstract

A flow injection analysis system with spectrofluorimetric detection has been developed for trace protein in urine. The protein assay is based on the ion-association of protein and tetraiodofluorescein (TIF) in the presence of Triton X-100 at pH 3.2. Although the TIF-protein ion associate produces red color compounds, fluorescence quenching caused by bonding with protein was measured at 560 nm (excitation: 313 nm). The quenching is applied to the protein determination. Linearity range was up to 10 mg/L bovine serum albumin and the correlation coefficient was 0.999. The detection limit (S/N = 3) was 0.6 mg/L with RSD 0.134% (n = 4). The FI system is available for rapid screening in diabetic diagnosis.

Keywords Urinary protein, tetraiodofluorescein, fluorescence quenching, flow injection

1. Introduction

Proteins synthesized in the liver are the building blocks for all body parts. Usually, most proteins are too big to pass through the kidneys' filters into the urine unless the kidneys are damaged. Protein is excreted as a renal proteinuria in nephropathy and diabetic nephropathy. As a result, measurement of proteins excreted in urine is an important and necessary to the diabetes diagnosis. In the clinical laboratory, the first line test paper impregnating the dyestuff is commonly used for the rapid decision of renal disease. The visible detecting technique using the first line test paper is simple and convenient in handling, however, the detection range 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. The color development on the test paper relates to amount of abnormal products in the urine. However, it does not offer accurate analytical value with two or more significant figures. Yoshimoto *et al.* have proposed the visual, simple and sensitive analytical method for the protein detection [1,2] and Sakai *et al.* have reported a simple and visible method for urinary protein [3].

On the other hand, simple and accurate spectrophotometric determinations of urinary albumin using bromophenol blue [4,5] have been reported. And, Fujita *et al.* have reported highly sensitive spectrophotometric methods for urinary protein with dye-metal complexes in the micelle media [6–9]. Bromocresol green and bromocresol purple were used for the serum albumin determination [10] and bromothymol blue has been proposed for the determination of serum albumin [11]. Recently, spot test of urinary protein using erythrosine B and a membrane film was proposed for very sensitive detection method [12]. However, these methods mentioned above have been performed by the batchwise technique and the procedure needs much time and larger reagents consumption.

Flow injection analysis (FIA) developed by Ruzicka and Hansen is a versatile and practical technique [13]. 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 [14] and also, high-sensitivity flow method with micro-flow plunger pumps [15] was demonstrated for the serum albumin determination. However, the sample throughput was only 15/h.

In a previous paper, we reported two channel FIA system with TBPE·H (tetrabromophenolphthalein ethyl ester)-protein associate (λ_{max} 610 nm) formed at pH 3.8. The calibration graph was linear in the range of 1.5 – 12 mg/dL albumin and the sample throughput was 30/h [16]. A flow injection system is a semi-automation method when compared with a completely automatic sequential injection (SI) proposed in 1990 [17]. Moreover, the consumption of reagents and sample in SIA is usually smaller than that in FIA.

An accurate and automated SI method has been proposed for stepwise determination of protein and glucose in urine [18] because it was necessary to determine protein and glucose for the diagnosis in the clinical laboratory. Human serum albumin (HSA) was determined using an ion association reaction of HSA with TBPE·H, and glucose was indirectly determined by measuring hydrogen peroxide generated by the oxidation of glucose in the presence of glucose oxidase using iron-catalyzed oxidation reaction of *p*-anisidine by H_2O_2. As a result, the linear ranges were up to 10 mg/dL HSA and 12.5 mg/dL glucose. The sample throughput was 6/h.

The American Diabetes Association (ADA) recommends that "the measurement of urinary albumin excretion is suitable for a 24-h urine collection" [19]. However, this method is extremely time-consuming. To avoid this tediousness, the albumin/creatinine ratio (ACR) for a random urine specimen was proposed as a more convenient detection of micro-albuminuria [20]. We proposed an automated SIA system for successive determinations of albumin and creatinine and their ratio in urinary samples based on eosin Y binding and Jaffe's reaction, respectively [21]. The calibration graph was linear up to 20 mg/L for albumin. The relative standard deviation (n = 10) was 2.49 % for 20 mg/L albumin. The sample throughput was 20/h. However, these procedures are not rapid and sensitive.

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In this study, we found that tetraiodofluorescein (TIF) reacts with albumin to show quenching and the negative intensity is in proportion to the albumin concentration. The proposed FIA system using TIF-BSA binding method is more rapid and sensitive compared with previous results by FIA and SIA methods and the method is applicable to real samples.

2. Experimental

2.1. Reagents

All chemicals and reagents were analytical reagent grade and were used without further purification. The deionized water used throughout the experiments was purified by an Advance GSH-210 apparatus. A standard bovine serum albumin (BSA) (1000 mg/L) was prepared by dissolving 0.0250 g of BSA (Wako Pure Chemical Co., Japan) in 25 mL of water. The BSA standard solutions should be prepared freshly every time. Working standard solutions were prepared by accurate dilution of the stock solution with 0.03 M dihydrogenphosphate. TIF (5×10^{-3} M) was prepared by dissolving 0.22 g of TIF (Tokyo Kasei Kogyo, Japan) in 50 mL of water. The working concentrations of the dye were prepared by suitable dilution. A Triton X-100 solution (0.5%) was prepared by dissolving 0.5 g of *t*-octylphenoxypolyethanol (Sigma Chemicals Japan) in 100 mL of water. Buffer solution (pH 3.2) was prepared by mixing 0.4 M sodium acetate and 0.4 M acetic acid.

2.2. Appratus

JASCO, Model FP-6200 spectrofluorimeter (Japan) was used for measuring fluorescence intensity. The manifold of the flow injection system is shown in Fig. 1. The dual micro-pump (FIA Instruments, Model 201) was used to deliver carrier (CS) and reagent solution (RS). The flow lines were of Teflon tubing (0.5 mm inner diameter). CS is 0.4 M acetate buffer and RS is the mixture of 2.0×10^{-5} M TIF and 0.08% Triton X-100. An aliquot (200 μL) sample solution is injected into the carrier stream by a six-way injection volume. The flow rate is 1.0 mL/min. Excitation wavelength is 313 nm and emission wavelength is 560 nm for measuring fluorescence intensity.

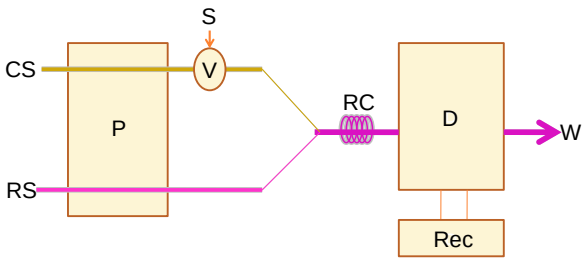

Fig. 1 Schematic diagram of the flow system for the determination of albumin.
CS: 0.2 M acetate buffer (pH3.2)
RS: A mixed solution of 1.0×10^{-5} M TIF and 0.04% TX-100
P: 1.0 mL min^{-1} (0.5 mL min^{-1} each line)
S: BSA (0 – 10 ppm) or sample
V: Six-way injection valve
RC: Reaction coil (0.3 m, 0.5 mm i.d.)
D: Fluorescence detector (λ_{ex} = 313 nm; λ_{em} = 560 nm)
Rec: Recorder
W: Waste

2.3. Preparation of urine samples

The urine samples supplied from diabetic patients were kept at –20°C. Before analysis, the urine samples were filtered to remove small particles. Then, the filtered solution was diluted at 400-fold with 0.4 M acetate buffer solution.

3. Results and discussion

3.1. Fluorescent spectra

Fluorescent spectra were measured for 5 mg/L BSA standard solution with 0.4 M buffer (pH 3.2), 2.0×10^{-5} M TIF and 0.048% Triton X-100 by the batchwise procedure. We found the excitation wavelength was at 313 nm and emission wavelength was at 560 nm. The fluorescent intensity with formation of the ion associate, BSA-TIF, decreased in proposition to the concentration of BSA.

3.2. FIA study for determination of albumin

3.2.1. Effect of reaction coil

For 5 mg/L BSA, 0.025 M buffer, 1×10^{-5} M TIF and 0.04% Triton X-100 solutions were used and the length of the reaction coil as shown in Fig.1 was varied in the range of 0.3 m – 2.0 m. The shortest 0.3 m length gave the strong intensity because of quick formation of the BSA-TIF associate. With increasing the length, the intensity decreased gradually. A 0.3 m-tubing was used in the system.

3.2.2. Effect of pH

Under the same condition described above, the effect of pH for the ion associate formation was investigated in the range of 2.8 – 4.2. Above pH 3.1, the negative intensity increased, however, the intensity decreased over 3.4. In the system, pH 3.2 was chosen.

3.2.3. Effect of flow rate

The flow rate was varied from 1.0 mL/min to 3.5 mL/min. Over 2.5 mL/min, the peak height was decreased slightly because of dispersion. In this study, 1 mL/min was used.

3.2.4. Effects of TIF and TritonX-100 concentrations

For 5 mg/L BSA, TIF concentration was varied in the range of 6×10^{-6} M – 2.0×10^{-5} M. The negative intensity decreased up to 1.0×10^{-5} M. Over its concentration, the baseline was noisy. And also, when quenching of TIF was used, the higher concentration was not suitable because of larger blank signal. 1.0×10^{-5} M TIF was chosen in this study. On the other hand, the effect of Triton X-100 was studied in the range of 0.02% – 0.05%. At 0.02%, the intensity was small, however, the intensity increased with increasing Triton X-100 concentration. Over 0.03%, the optimum and constant peak height was obtained as shown in Fig. 2. In this study, 0.04% Triton X-100 was chosen.

3.3. Analytical characteristics

Calibration graph for BSA was established under the optimal conditions. Fig. 3 shows typical flow signals for BSA. The linearity was obtained in the range of 0 – 10 mg/L with r^2 0.999 ($y = 4.22x - 0.178$, y = fluorescent intensity, x = albumin concentration in mg/L). The detection of limit (S/N = 3) was 0.6 mg/L. When TBPEH was used for the dye-binding method, the linearity was obtained in the range of 1.5 – 120 mg/L in FIA

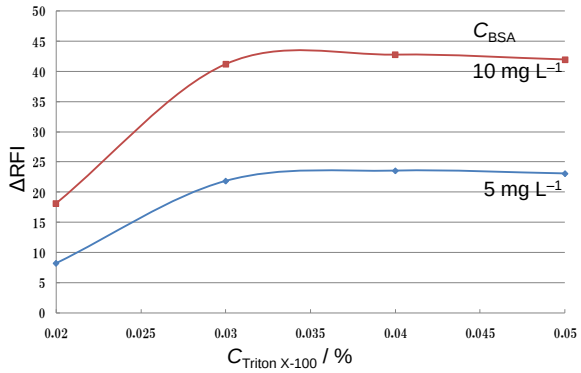

Fig. 2 Effect of Triton X-100 concentration.

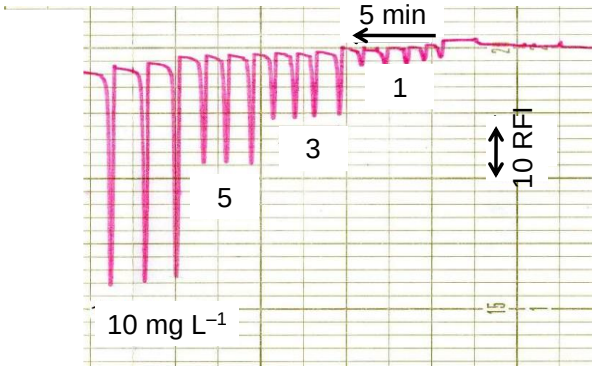

Fig. 3 Typical flow signals for BSA standards.

[16] with RSD 1.2% and/or in the range of 0 – 100 mg/L with SIA [18]. On the other hand, the calibration graph was obtained in the range of 0 – 20 mg/L with another SIA [21]. In this study, RSD for 10 mg/L albumin (n = 4) was 0.134%. The obtained RSD was small compared with other SIA, 2.7% in Ref. 18 and 2.5% in Ref. 21. Although the sample throughputs were 6/h in Ref. 18, 18/h in Ref. 21 and 30/h in Ref. 16, 60/h sample throughput in the proposed FI system was obtained. Consequently, the proposed method is rapid, sensitive and reproducible compared with other flow-based techniques.

3.4. Interference study

For the determination of albumin, various amounts of foreign compounds were added in the 5 mg/L BSA standard solution and their interference was examined. The results are shown in Table 1. The tolerance limit was defined as the interference that yielded a relative error less than 5% when compared the response obtained from the standard concentration. In the normal urine, the chloride and urea contents are worthy of remark and the average concentrations in the urine are 4751 for chloride ion and 18200 mg/L for urea, respectively [18]. Sodium chloride, urea and creatinine did not interfere in the protein determination up to 100 mg/L, 500 mg/L and 10 mg/L. Other foreign species (potassium, ammonium, magnesium, calcium ions, glucose) normally present at low concentrations in the real urine samples did not interfere.

3.5. Dilution effect

The influence of dilution effects was investigated to ensure accurate quantitation. Urine samples may be diluted with buffer solution to obtain the concentration within the examined range. The recovery tests were carried out to evaluate the effects for the determination of 5 mg/L BSA in a real urinary sample. The results are shown in Table 2. As a result, 200-fold dilution eliminated interferences. When distilled water was used for the dilution of sample, we found a small ghost peak. Consequently, we used 400-fold dilution with 0.025 M buffer solution for the real sample before analysis.

3.6. Application to real samples

The proposed method was applied to evaluate albumin in urinary samples from diabetic individuals. And also, the correlation between the proposed method and Bradford method [22] was investigated (Table 3). A paired t-test with 5 degrees of freedom was performed on the data obtained. The experimental t-value between two pairs of the methods was 1.357 for the 95% confidence interval (2.571) and it was significantly small. Accordingly, the measurements are reliable and acceptable.

Table 1 Tolerance limits of foreign compounds for the determination of 5 mg L^{-1} BSA

Compound	Tolerance limit / mg L^{-1}	Remarks
NaCl	100	Na^+ 39 mg L^{-1}; Cl^- 61 mg L^{-1}
KCl	50	K^+ 26.2 mg L^{-1}; Cl^- 23.8 mg L^{-1}
$MgCl_2$	500	Mg^{2+} 127 mg L^{-1}; Cl^- 373 mg L^{-1}
$CaCl_2$	100	Ca^{2+} 36 mg L^{-1}; Cl^- 64 mg L^{-1}
NH_4Cl	100	NH_4^+ 33.6 mg L^{-1}; Cl^- 66.4 mg L^{-1}
Urea	500	
Glucose	300	
Creatinine	10	

The average concentration levels in an ordinary person's urine: Na^+ 2940 mg L^{-1}; Cl^- 4780 mg L^{-1}; K^+ 2346 mg L^{-1}; Urea 18200 mg L^{-1}; Uric acid 420 mg L^{-1}; Creatinine 1960 mg L^{-1}.

Table 2 The recoveries of the determination of 5 mg L^{-1} BSA in a real urinary sample using different dilution factors

Dilution factor	Recovery / %
20	180
50	133
100	115
200	104
300	98.4
400	97.1
500	97.1
600	100

Table 3 Determination of albumin in urine taken from diabetic patients

Proposed method / mg dL^{-1}	Bradford method / mg dL^{-1}
95	110
110	117
145	125
171	168
324	383
437	590

Samples were diluted to 400-folds before measurements.

4. Conclusion

A simple, sensitive, reproducible and rapid flow injection system with TIF was developed for the determination of albumin. Rapid quenching of fluorescence based on formation of the ion associate was used in the system. The sample throughput and reproducibility were improved compared with other flow-based techniques. The system could be applied to the determination of albumin in real urinary sample with diabetics. This method is useful for prediction of health status and/or disease diagnosis.

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