A New Spectrophotometric Method for Determination of Urinary Protein Using Erythrosin B

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A new sensitive spectrophotometric method for human serum albumin (HSA), the main protein in urine, has been developed using Erythrosin B. The proposed method is not based on conventional spectral shift but on an enhanced absorbance of the protein–bound dye and suppression of reagent blank in the presence of Triton X-100. The detection limit for HSA is 0.06 mg L^{-1} (S/N = 3), which is superior to conventional methods by a factor of 50–100. This is the first ever spectrophotometric method applicable for urinary protein even in healthy subjects.

Elevated levels of urinary protein imply renal failure, which is one of the world's biggest public health problems. In addition, a number of recommendations have been published on the need to prevent diabetic nephropathy and have referred to microalbuminuria as an early indicator.[1–3]

To date, several spectrophotometric methods based on either dye-binding reactions or the Lowry method have been used for the detection of urinary protein. A comparison of a number of different techniques has been made by Sperlingova et al.[4] However, the application of these methods is limited owing to insufficient sensitivity. Like most other dye-binding assays, a significant drawback of using test strips impregnated with a pH indicator dye is the lack of sensitivity. Depending on the type of strip used, the detection threshold is 100–200 mg L^{-1} for human serum albumin (HSA), while the protein concentrations in normal urine are less than 30 mg L^{-1}.

This work provides a new spectrophotometric method for HSA using Erythrosin B and Triton X-100 under acidic conditions. Earlier work by Soedjiak showed that Erythrosin B is excellent for the spectrophotometric determination of protein, offering high sensitivity and reproducibility.[5] However, in Soedjiak's method, the applicable range was 2–14 mg L^{-1} using a mixture of Erythrosin B and pH 3.0 sodium citrate–citric acid as a reagent solution. The excess dye was eliminated as a precipitate by heating the sample solutions to 90–95 °C for 1.5–2 min prior to spectrophotometric determination. A fluorescent quenching method was also studied for the determination of HSA using Erythrosin B.[6] However, its application to trace HSA is limited because of the lack of sensitivity. The noteworthy point of the proposed method is that rapid yet sensitive detection of trace HSA at levels as low as 0.06 mg L^{-1} is possible at room temperature despite the simple and easy operations involved.

Solutions of HSA (SIGMA Co.) were prepared by dissolving the protein in water. A solution (1×10^{-4} mol L^{-1}) of Erythrosin B (Wako Pure Chemical Industries Ltd.) was prepared by dissolving the dye in water. Triton X-100 solution (0.4%) was prepared by dissolving the reagent (Nacalai Tesque, Inc.) in water. All other reagents used were of guaranteed reagent grade.

Spectrophotometric measurements were made using a JASCO V-530 spectrophotometer. A model 680 microplate reader (Bio-Rad Laboratories, Inc.) was used for microtiter plate measurement.

A typical procedure is as follows; a sample solution containing less than 2 mg L^{-1} HSA is put in a 10 mL volumetric flask. 200 μL of 1×10^{-4} mol L^{-1} Erythrosin B, 400 μL of 0.5 mol L^{-1} citric acid (pH 2.3) and 250 μL of 0.4% Triton X-100 are added to the sample. Water is added to the solution mixture so that the total volume is 10 mL. After letting the solution stand for 1 min for the dye-binding reaction to take place, spectrophotometric measurements were carried out at 547 nm using a 1 cm cell.

The absorption spectra for Erythrosin B in aqueous solutions are shown in Figure 1. Erythrosin B (HA^-) shows an absorption maximum at 535 nm in aqueous solution. The acid dissociation constant of Erythrosin B was determined as 3.20 for pK_{a1} from the spectrophotometric data (Figure is not shown). In solutions with pH values below 3, protonation takes place, yielding the precipitation of the neutral dye, H_2A, at higher concentrations than 2×10^{-6} mol L^{-1}. When bound to HSA under acidic conditions, the color of the Erythrosin B solution changes from faint pinkish-orange to bright red with an enhanced absorption maximum at 547 nm caused by the conversion of the dye species from H_2A to HA^-.

The absorbance of the dye–protein solution is strongly dependent on pH. At citric acid concentrations lower than 0.02 mol L^{-1}, the blank value is high. At citric acid concentrations higher than 0.02 mol L^{-1}, the color development for HSA is poor, indicating that the dye does not associate with the protein under strongly acidic conditions. On the basis of these data, a citric acid concentration of 0.02 mol L^{-1} (pH 2.3) was adopted. The nonionic surfactant, Triton X-100 was employed because of its effectiveness as a sensitizer. Gum Arabic,[8] Triton X-405,[8] Triton X-100,[9] and poly(vinyl alcohol)[10] are widely used as

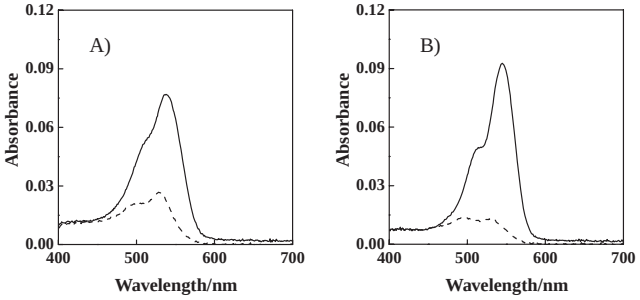

Figure 1. Absorption spectra of Erythrosin B. Erythrosin B: 2×10^{-6} mol L^{-1}; citric acid: 0.02 mol L^{-1} (pH 2.3); Temperature: 25 °C; dotted line: reagent blank; solid line: 1.5 mg L^{-1} HSA; A) Without Triton X-100; B) Triton X-100: 0.01%.

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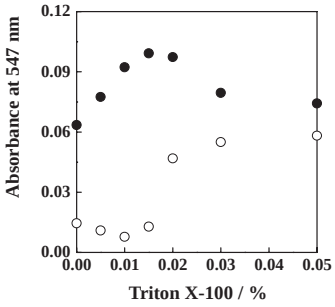

Figure 2. Effect of Triton X-100 on HSA detection. The other conditions are the same as in Figure 1B.; ●: 1.5 mg L^{-1} HSA; ○: reagent blank.

unfolding agents or stabilizers for protein analysis. In this study, it was found that Triton X-100 plays a significant role at lower concentration than CMC (0.019%) both in enhancing the absorbance for HSA and in eliminating the reagent blank (Figure 2). At higher concentrations than CMC, the blank value markedly increases.

The dye–protein reaction completes within 1 min at room temperature under analytical conditions. In Soedjiak's method,[5] at room temperature and pH 3.0, the excess dye provided for significant background absorbance, with the dye-binding reaction taking two hours to complete.

The calibration curve is linear over the concentration range up to 1.5 mg L^{-1}. The detection limit (S/N = 3) is 0.06 mg L^{-1} of HSA. The relative standard deviation is 1.6% ($n = 10$) for 1 mg L^{-1} of HSA. When 1 mL of urine is used to prepare 10 mL of the sample solution, the practical detection limit is 0.6 mg L^{-1} in urine using a 10× dilution factor. Our method is sufficiently sensitive to detect HSA in urine even for healthy subjects. Since the detection limits of conventional spectrophotometry based on other dye–binding reactions range from 30–50 mg L^{-1},[4] these methods are not applicable to healthy subjects or appropriate for early diagnosis.

The effect of foreign substances, such as creatinine, creatine, urea, uric acid, hippuric acid, and sodium chloride, on HSA detection was tested, with none of these substances showing any significant effect.

Table 1 shows the responses of this method to various proteins. The excessive excretion of these proteins implies either renal failure or other serious health problem requiring urgent medical attention.

Having established suitable conditions to detect HSA, the results using this method for increased concentration of HSA

Table 1. Protein-to-protein variability

Protein[a]	Responses/%[b]
Human serum albumin	100.0
Hemoglobin	90.1
Transferrin	89.3
Bence–Jones protein	32.2
Fibrinogen	21.3
α_1-Microglobulin	35.4
β_2-Microglobulin	47.8

[a]Protein taken: 1.5 mg L^{-1}. [b]Percent with respect to HSA.

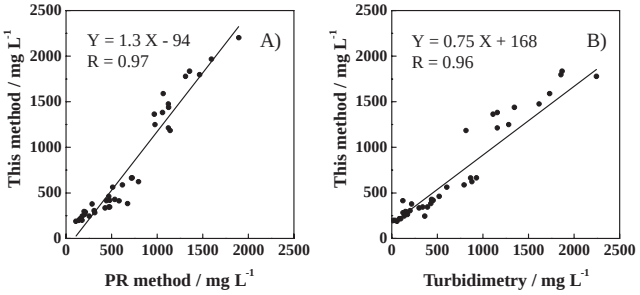

Figure 3. Correlation diagram between this method and conventional methods. This method: The urine samples were analyzed with dilution by a factor of 10–100 using 96-microplate reader.; A) PROTEIN ASSAY RAPID KIT (Wako Pure Chemical Industries Ltd.) was used for the PR method. B) Sulfosalicylic acid (1.0 w/v %) was used for the turbidimetry.

in 44 patients' urine were in agreement with the results by the Pyrogallol Red–molybdenum method (PR method) (Figure 3A). Fujita et al. developed the PR method in 1983.[7] Nowadays, their method accounts for more than 80% for urinary protein assay in Japan. Our method also strongly correlated with turbidimetry, which uses sulfosalicylic acid (Figure 3B). Cross validation was carried out only for those patients' urine because of the poor sensitivities of the conventional methods available. It is well understood that there are significant differences between the urinary protein values depending on the analytical method employed. Our future work will investigate the mechanism of the 1.3 and 0.75 slopes (as shown in Figure 3) in order to better understand the extent to which this technique can be applied to routine assay work.

Our method is simple, cost-effective, and clearly applicable to use as a daily diagnostic test, and as a mass-screening test for renal failure and diabetic nephropathy.

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