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Spectrophotometric Determination of Micro Amounts of Albumin Using Zirconium(IV) and Xylenol Orange

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A highly sensitive and selective spectrophotometric method is developed to measure micro amounts of albumin. The proposed method is based on the fact that zirconium(IV)–Xylenol Orange complex fades in the presence of albumin. In the concentration range of 0.1–0.9 $\mu g\,cm^{-3}$, human serum albumin obeys Beer's law and shows an effective molar absorptivity of $8.4 \times 10^7\,dm^3\,mol^{-1}\,cm^{-1}$ at 605 nm.

Although urine does not normally contain any protein, small amounts of albumin may leak into the urine because of kidney damage.[1] This condition of leakage of albumin from the kidney is called microalbuminuria, and its most common cause is kidney damage due to diabetes. However, many other conditions can also lead to kidney damage, such as high blood pressure, cardiovascular events, cirrhosis, or systemic lupus erythematosus (SLE). Therefore, a microalbumin urine test is very important in clinical diagnosis, and early detection of this condition may enable treatment to preserve kidney function to the greatest possible extent.[2] Nowadays, assays such as the radioimmune assay (RIA)[3] and enzyme-linked immunosorbent assay (ELISA)[4] are widely used for microalbuminuria. However, these methods suffer from several drawbacks, i.e., inconvenience, low sensitivity, high cost, etc.

In our previous series of studies conducted on protein assay using dye–metal complexes, we developed the Pyrogallol Red–molybdenum(VI) method[5–7] (PR method). This method is used for 90% or more of the urine protein assays conducted in Japan and has various advantages. However, a significant drawback of this method is that its reactivity differs among proteins.

From continual investigations, we developed a method that is based on the fact that zirconium(IV)–Xylenol Orange ({Zr(IV)}–XO) complex fades in the presence of albumin. This method would serve as a very sensitive measurement method for albumin because it is not influenced by other proteins such as γ-globulin. In this article, we report a spectrophotometric method for measuring micro amounts of albumin by monitoring the fading of the Zr(IV)–XO complex.

Standard solutions (5 $\mu g\,cm^{-3}$) of human serum albumin (HSA) and human γ-globulin (γ-G) were prepared by dissolving HSA and γ-G obtained from Wako Pure Chem. Co., Ltd. in a 0.9% sodium chloride solution. A working solution (1.0×10^{-3} $mol\,dm^{-3}$) of Zr(IV) was prepared from a stock solution (Wako Pure Chem. Co., Ltd., 1000 $\mu g\,cm^{-3}$) by dilution with water. A solution ($1.0 \times 10^{-3}\,mol\,dm^{-3}$) of Xylenol Orange (Wako Pure Chem. Co., Ltd.) was prepared in water. A surfactant solution was obtained by dissolving 0.8 g of cetyltrimethylammonium chloride (CTAC) and 0.2 g polyoxyethylene sorbitan monolaurate (Tween 20) in 100 mL of water. A 0.2 M hydrochloric acid/0.2 M sodium acetate buffer solution was used for pH adjustments. All other reagents and materials were of analytical grade and were used without further purification. A Shimadzu spectrophotometer (Model UV-1700) with 1.0-cm matched silica cells was used to measure absorbance. A Horiba (F-11) pH meter in combination with a calomel glass electrode was used to measure pH.

We preliminary examined the utility by using dye–metal complex by comparisons with the color reactions between dyes and metal ions in the presence of HSA or γ-G. The dyes used were XO, Methylthymol Blue, Pyrocatechol Violet, chromazurol S, gallein, and Pyrogallol Red. The metal ions used were zirconium(IV), titanium(IV), tantalum(V), niobium(V), cobalt(II), manganese(II), copper(II), palladium(II), gallium(III), and molybdenum(VI). From the comparison, we found that micro amounts of HSA severely interfered with the color reaction between XO and Zr(IV), and the decrease in the absorbance of XO–Zr(IV) complex increased in proportion to the HSA concentration. On the other hand, even large amounts of γ-G did not cause any decrease or increase in the absorbance of the XO–Zr(IV) complex. Further, in the absence of Zr(IV), the absorption spectra of XO and HSA–XO solutions did not show any difference.

The effect of pH on the reaction was examined, and the maximum and almost constant absorbance was observed over a pH range of 2.3–3.0 by using 2.5 cm^3 of the buffer solution in a final volume of 10 cm^3.

To stabilize color development and enhance the sensitivity of reactants, the effect of different surfactants was examined by measuring the difference in absorbance (ΔA) between XO–Zr(IV) and XO–Zr(IV)–HSA solutions. Among the surfactants tested, a combination of CTAC (cationic surfactant) and Tween 20 (nonionic surfactant) showed a red shift for the XO–Zr(IV) complex solution and was superior to other surfactants in terms of sensitivity to HSA. These results can probably be attributed to the interaction between the surfactant and the XO–Zr(IV) complex, proteins, or both. However, further investigation is necessary to confirm this.

The effects of the concentrations of XO and Zr(IV) on ΔA were examined by maintaining a constant final concentration of HSA (0.5 $\mu g\,cm^{-3}$). The maximum ΔA was obtained when the molar ratio of XO to Zr(IV) was 1:1; therefore, all subsequent work related to the absorbance of the XO–Zr(IV) complex

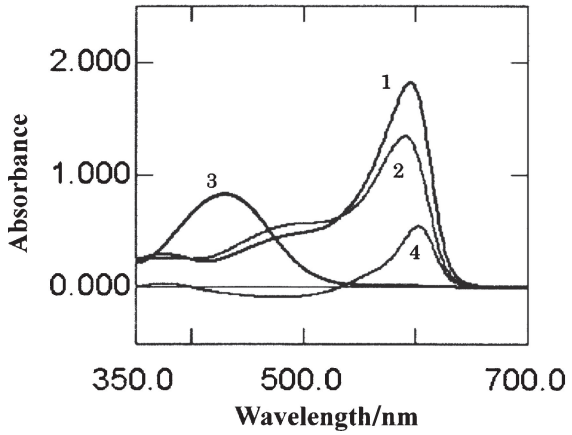

Figure 1. Absorption spectra obtained by the standard procedure. Curve 1 indicates the XO–Zr(IV) and XO–Zr(IV)–γ-G (20 $\mu g\,cm^{-3}$) solutions; curve 2 indicates the XO–Zr(IV)–HSA (0.5 $\mu g\,cm^{-3}$) solution; curve 3 indicates the XO and XO–HSA (20 $\mu g\,cm^{-3}$) solutions; curve 4 is expressed as curve 1 minus curve 2 taking water as reference.

was carried out using 4.0×10^{-5} mol dm^{-3} of XO and Zr(IV) solutions.

Next, we studied the effect of the addition order of the reagents on ΔA and discovered the most suitable order: HSA, surfactant, buffer, Zr(IV), and XO. The effects of the incubation temperature and incubation times were assessed by heating the mixtures for 10–60 min at 40 and 50 °C. The maximum ΔA was obtained when the mixtures were incubated at 40 °C for 10 min and subsequently cooled in water for 5 min. ΔA remained constant for at least 60 min after the solution had been cooled to room temperature.

The standard procedure used for the measurement of HSA amount is as follows. The following components were mixed in a 10-cm^3 volumetric flask: a solution containing HSA, 1.5 cm^3 of the surfactant solution, 2.5 cm^3 of the buffer solution (pH 2.5), 0.4 cm^3 of a 1.0×10^{-3} mol dm^{-3} Zr(IV) solution, and 0.4 cm^3 of a 1.0×10^{-3} mol dm^{-3} XO solution. The mixture was diluted to 10 cm^3 with water, transferred into a test tube, mixed well, and kept at 40 °C for 10 min. After the solution had been cooled in water for 5 min, ΔA between the resultant solution and a reagent blank solution prepared under the same conditions was measured at 605 nm against water. Figure 1 shows the absorption spectra of XO–Zr(IV)–HSA, XO–Zr(IV) (=XO–Zr(IV)–γ-G), and XO (=XO–HSA) solutions measured using the standard procedure.

The calibration curve passes through the origin and follows Beer's law from 0.1 to 0.9 $\mu g\,cm^{-3}$ of HSA, and the detection limit was 0.02 $\mu g\,cm^{-3}$. The effective molar absorptivity (ε) was 8.4×10^7 $dm^3\,mol^{-1}\,cm^{-3}$. The relative standard deviation (RSD) for five runs of 0.5 $\mu g\,cm^{-3}$ of HSA was 2.5%. The sensitivity of this method in measuring the HSA amount about 50 times that of the PR method and 400 times or more than that of the Bromocresol Green method.

The color reaction of various proteins with the XO–Zr(IV) complex solution was examined. As shown in Table 1, none of the proteins, except HSA, showed any difference in absorbance between the XO–Zr(IV) and XO–Zr(IV)–protein solutions. On the other hand, the reactivities of albumins derived from different animal species such as cow, goat, sheep, rabbit, and rat were considerably less than that of HSA, but the albumins were detected in the concentration range of 0.5–30 $\mu g\,cm^{-3}$. Further investigation of the difference in sensitivity between HSA and the albumins derived from different animal species is necessary.

Table 1. Reaction between various proteins and XO–Zr(IV) complex solution

Protein[a]	ΔA at 605 nm/%[b]	Molecular weight	pI[c]
HSA	0.536 (100)	6.9×10^4	4.8
γ-G	0 (0)	1.5×10^5	6.5
Hemoglobin	0 (0)	6.5×10^4	6.8
Myoglobin	0 (0)	1.7×10^4	6.8
β_2-Microglobulin	0 (0)	1.2×10^4	
Transferrin	0 (0)	7.5×10^4	
Fibrinogen	0 (0)	4.0×10^5	5.5
α_1-Acid Glycoprotein	0 (0)	4.4×10^4	2.7
HSA, glycated	0 (0)		
α_1-Antitrypsin	0 (0)	5.1×10^4	
Tamm-Horsfall Glycoprotein	0 (0)	7.1×10^6	
Cystatin C	0 (0)	1.3×10^4	9.3

[a]Uncorrected. HSA taken: 0.5 $\mu g\,cm^{-3}$; other proteins, except HSA, taken: 50 $\mu g\,cm^{-3}$. [b]Percentage with respect to HSA. [c]Isoelectric point.

Under standard conditions, the effect of various substances on the measurement of 0.25 $\mu g\,cm^{-3}$ of HSA was assessed. Foreign substances, such as sodium, potassium, calcium, magnesium, iron(III), cobalt(II), zinc(II), aluminum(III), copper(II), chloride, nitrate, sulfate, oxalate, ascorbic acid, glucose, glycine, urea, uric acid, thiamine, caffeine, creatinine, and bilirubin, did not noticeably affect the accuracy of the measurement of HSA amount, even when these substances were present in excess (20–100 $\mu g\,cm^{-3}$) of that of HSA. However, a high concentration of phosphate ion (20 $\mu g\,cm^{-3}$) interfered with the measurement of HSA. The interference of the phosphate ion solution was eliminated by passing it through an anion-exchange resin (Meta CEP IC-MA, GL Sciences Inc.) before the reaction.

The recovery of the HSA added to human urine was 98.5% (the average of five runs for 0.25 $\mu g\,cm^{-3}$ of HSA).

In conclusion, the HSA assay method described herein has remarkably high sensitivity and selectivity and can enable the rapid detection of microalbuminuria. Although further investigations are necessary, especially regarding the application of this method to real samples and the elucidation of the reaction mechanism, this method should prove useful and convenient for the assay of micro amounts of HSA in urine.

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