Simple and Sensitive Spectrophotometric Determination of Albumin with *o*-Sulfophenylfluorone–Uranium(VI) Complex, and Binding Study[1)]

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A spectrophotometric method for the determination of albumin was established by using *o*-sulfophenylfluorone (SPF)–uranium(VI) (U(VI)) complex. This method can be used to determine 20—500 μg/10 ml of human serum albumin (HSA), and is affected very little by the presence of globulin (as γ-globulin). Results obtained by the Bromocresol green (BCG) method and this method agree well with each other in artificial serum samples containing HSA and γ-globulin, with the regression equation being Y (this method) = 0.943X (BCG method) + 1.20 (in mg HSA/dl, $n = 18$, $r = 0.996$). The proposed method is 6 times more sensitive than the BCG method, and has an advantage that deviation of responses of the reagent to albumins derived from the different animal species is small in comparison with that of Bromocresol purple. The binding parameters (n and K) were calculated from dual double-reciprocal plots. From the thermodynamic parameters (ΔG, ΔH and ΔS) obtained from a van't Hoff plot, the binding between HSA and SPF–U(VI) complex or SPF was presumed to be a kind of hydrophobic interaction.

Keywords albumin; spectrophotometry; *o*-sulfophenylfluorone–uranium(VI) complex; binding parameter; thermodynamic parameter; hydrophobic interaction

Assay of individual proteins, in particular albumin, has recently become increasingly important in the field of clinical chemistry; *e.g.*, urinary albumin concentration is a good indicator for early detection of patients at risk of developing diabetic nephropathy.[2)] The methods available for albumin assay include radioimmunoassay (RIA),[3)] enzyme immunoassay (EIA),[3)] dye-binding methods and so on. RIA and EIA are specific and sensitive, but expensive. Dye-binding methods such as the Bromocresol green (BCG) method[4)] and the Bromocresol purple (BCP) method[5)] are not sensitive enough, in spite of being simple, rapid and inexpensive.

In a series[6)] of studies on protein assay with dye–metal complexes in a micellar medium, we have confirmed that the sensitivity and selectivity of the proposed methods are dependent on the dye, the metal ion and/or the surfactant. In the previous paper,[6c)] it was suggested that the response of *o*-hydroxyhydroquinonephthalein (Qnph)–uranium(VI) (U(VI)) complex to albumin was considerably selective. In a continuation of those investigations, we found that *o*-sulfophenylfluorone (SPF, Chart 1)–U(VI) complex was more selective and sensitive than Qnph–U(VI) complex.

This paper presents a simple and sensitive spectrophotometric method for albumin assay with SPF–U(VI) complex in a micellar medium. In addition, a comparative study between the present method and the dye-binding methods was carried out. The binding parameters of SPF–U(VI) to human urine albumin (HSA) and the thermodynamic parameters of binding between SPF–U(VI) and HSA were studied by using dual double-reciprocal plots and a van't Hoff plot, respectively.

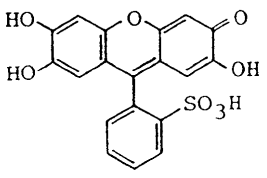

Chart 1. Structure of SPF

Experimental

Materials and Reagents Standard solutions (500 μg/ml) of albumin and γ-globulin were prepared by dissolving human serum albumin (HSA, essential fatty acid-free, fraction V) in water and human γ-globulin (γ-G, purified from Cohn fractions II, III) in 0.2% sodium chloride. HSA, γ-G and other proteins were purchased from Sigma Chemical Co. A 1.0×10^{-3} M (M = mol dm^{-3}) solution of U(VI) was prepared by uranyl nitrate (Merck) in water. A solution of SPF [2,3,7-trihydroxy-9-(2′-sulfophenyl)fluorone)], which had been synthesized as described in the literature,[7)] was prepared by dissolving 40 mg of SPF in 2 ml of 1.0 M sodium acetate solution, and diluted to 100 ml with water. A 10% aqueous solution of Triton N-101 (Nacarai Tesque Inc.) was prepared. A buffer solution (pH 3.5) was made by mixing 0.2 M sodium acetate and 0.2 M hydrochloric acid solutions. All other reagents and materials used were of analytical reagent grade unless otherwise stated. Deionized water was used in the preparation of all solutions.

Apparatus Absorption spectra and absorbance were measured with a Shimadzu model UV-160 spectrophotometer using 10-mm quartz cells. A Hitachi-Horiba model F7 AD pH meter equipped with a combined glass electrode was used for the pH measurements.

Standard Procedure The following components were mixed in a 10-ml volumetric flask: 1.0 ml of 10% Triton N-101 solution, 3.0 ml of the buffer solution, 0.5 ml of 1.0×10^{-3} M U(VI) solution, 1.0 ml of 1.0×10^{-3} M SPF solution, and a solution containing 20—500 μg of HSA. The mixture was diluted to 10 ml with water, and kept at room temperature for 15 min. The absorbance was measured at 555 nm against the reagent blank without HSA.

Results and Discussion

Choice of Dye, Metal Ion and Surfactant The effect of different dyes was examined by measuring the absorbance of a dye–U(VI)–HSA solution against a dye–U(VI) solution. SPF was superior to the other dyes tested in terms of sensitivity; the dyes (1.0×10^{-4} M) used were SPF, Qnph, phenylfluorone,[7)] *o*-nitrophenylfluorone,[7)] *o*-chlorophenylfluorone,[7)] pyrogallol red, pyrocatechol violet, gallein, chrome azurol S, eriochrome cyanine R, xylenol orange, arsenazo III and 4-(2-pyridylazo)resorcinol. The effect of 20 kinds of metal ions was examined. Among the metal ions tested, U(VI) was superior to the others as regards sensitivity to HSA, the large difference in color reaction between HSA and γ-G, and the influence of other substances. The results are summarized in Table I.

In the absence of surfactant, a colored SPF–U(VI)–HSA complex was precipitated from the solution immediately. In

TABLE I. Effect of Metal Ions

Metal ion	Absorbance at λ_{max}		
	HSA	γ-G (%)[a]	(nm)
U(VI)	0.347	0.023 (6.6)	555
Mo(VI)	0.170	0.102 (60.0)	545
Fe(III)	0.240[b]	0.105 (43.8)	640
Al(III)	0.209	0.049 (23.4)	510
Sc(III)	0.454[b]	0.175 (38.5)	530
In(III)	0.422[b]	0.113 (26.8)	545
Ta(V)	0.345	0.194 (56.2)	540
Zr(IV)	0.098	0.015 (15.3)	495
Cu(II)	0.135	0.020 (14.8)	520
Pd(II)	0.174	0.044 (25.3)	530
Mn(II)	0.065	0.017 (26.2)	510

HSA and γ-G, 200 μg/10 ml; metal ion, 5.0×10^{-5} M; SPF, 1.0×10^{-4} M; Triton N-101, 1%; pH, 3.5; reference, SPF–metal ion solution. *a*) Percent with respect to HSA. *b*) Turbidity.

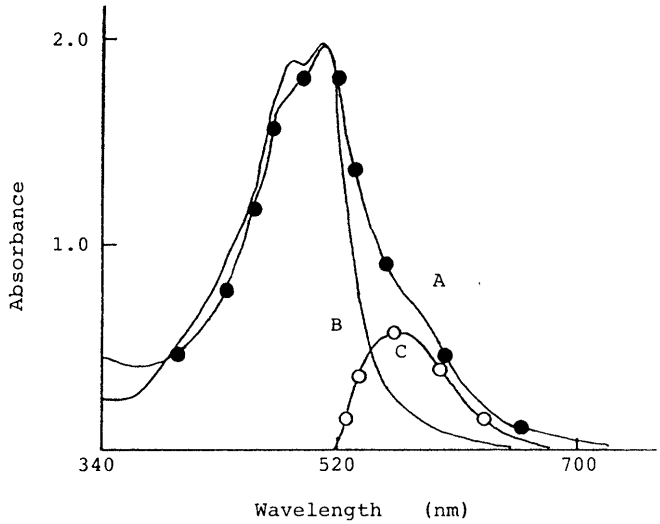

Fig. 1. Absorption Spectra Obtained under the Standard Procedure

HSA taken, 400 μg; curve A (—●—), sample *vs.* water; curve B (——), reagent blank *vs.* water; curve C (—○—), sample *vs.* reagent blank. Sample, SPF–U(VI)–HSA solution. Reagent blank, SPF–U(VI) solution.

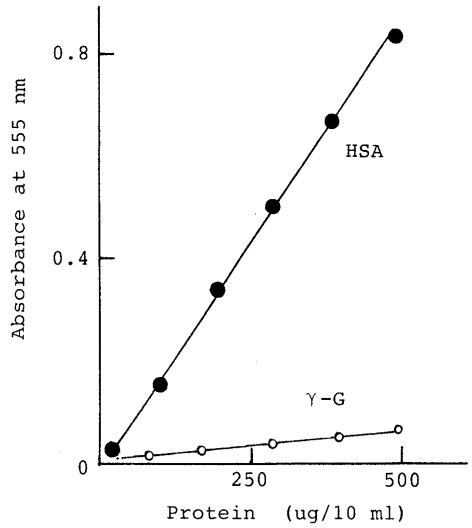

Fig. 2. Calibration Curve for HSA and γ-G According to the Standard Procedure

TABLE II. Influence of Foreign Substances

Substance	Added (μg/10 ml)	Absorbance at 555 nm	Recovery (%)
—	—	0.347	100.0
Fe(III) (alum)	1.1	0.387	111.5
Ca(II) (chloride)	8.0×10^2	0.347	100.0
$H_2PO_4^-$ (potassium)	1.9×10	0.339	97.7
Citric acid	9.6×10	0.331	95.4
Uric acid	8.4×10^2	0.337	97.1
Creatinine	1.1×10^3	0.358	103.2
Bilirubin	2.9×10^2	0.368	106.1
Hemoglobin	5.0×10	0.405	116.7
Ascorbic acid	8.8×10^3	0.325	93.7
Urea	6.0×10^3	0.347	100.0
Glycine	7.5×10^3	0.347	100.0
Glucose	1.8×10^3	0.347	100.0
Caffeine	3.9×10^3	0.347	100.0
Salicylic acid	1.4×10^2	0.347	100.0

HSA taken, 200 μg/10 ml; U(VI), 5.0×10^{-5} M; SPF, 1.0×10^{-4} M: Triton N-101, 1.0%; pH, 3.5; reference, SPF–U(VI) solution.

order to solubilize the colored complex, various non-ionic surfactants[6] were examined; Triton N-101, Triton X-100, Tween 20, Brij 35, gum arabic, polyvinyl alcohol, polyvinyl pyrrolidone and methyl cellulose. Triton N-101 was found to be the best.

Figure 1 shows the absorption spectra of the sample and the reagent blank, and their difference spectrum under the standard procedure. The absorbance of the difference spectrum at 555 nm was proportional to the concentration of HSA.

Analytical Variables The absorbance at 555 nm of SPF–U(VI)–HSA solution against the SPF–U(VI) solution was maximum when the pH was between 3.2 and 4.2. However, the absorbance of γ-G–reagent mixture exhibited the maximum between pH 3.8 and 4.3. We selected pH 3.5 as the optimal pH, and used acetate buffer (Walpole buffer, 0.2 M sodium acetate/0.2 M hydrochloric acid) solution. Maximum and constant absorbance was observed upon the addition of more than 0.2 ml of 10% Triton N-101 solution to the final volume of 10 ml.

When the concentration of SPF in the SPF–U(VI) reagent was fixed at 1.0×10^{-4} M, this method was most sensitive for the determination of HSA in the range of U(VI) concentrations higher than 4.0×10^{-5} M. On the other hand, the U(VI)-to-SPF ratio determined by Job's method of continuous variation was 1 : 2 in the presence or absence of HSA. In the succeeding experiments, we decided on 5.0×10^{-5} M U(VI) and 1.0×10^{-4} M SPF in the final volume of 10 ml.

Under the standard conditions, constant absorbance was obtained by keeping the solution at room temperature for longer than 10 min, and lasted for at least 90 min. Therefore, a standing time of 15 min at room temperature was selected for all measurements.

Calibration Curve A calibration curve for HSA was constructed by the standard procedure. Beer's law held over the range of 20—500 μg of HSA in the final volume of 10 ml, as shown in Fig. 2. The relative standard deviation (R.S.D.) was 0.82% ($n=8$) at 200 μg of HSA. A calibration curve for γ-G is also shown in Fig. 2. The effects of proteins other than HSA and γ-G should be studied in greater detail.

Influence of Foreign Substances The influence of various ions and substances on the determination of 200 μg of HSA was studied. Among the ions and substances tested,

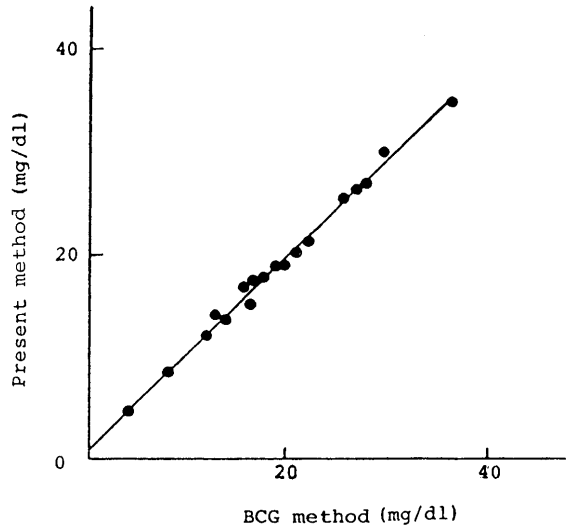

Fig. 3. Correlation between Results for HSA Assays, as Obtained by the BCG Method(X) and by the Present Method (Y)

$n=18$. $Y=0.943X+1.20$, $r=0.996$, $\bar{X}=18.96$, $\bar{Y}=19.08$.

Table III. Comparison in Reactivity of Albumin Derived from the Different Animal Species

Animal species	Present method (%)[a]	BCP method (%)[a]
Human	100.0	100.0
Bovine	100.2	41.9
Goat	97.3	52.4
Dog	94.3	48.4
Rat	80.1	—
Chicken	67.5	6.5

a) Percent with respect to human (HSA).

iron(III), bilirubin, hemoglobin and creatinine caused an increase in absorbance at 555 nm. The presence of phosphate, citrate, uric acid and ascorbic acid caused a decrease in absorbance. The presence of ions and substances such as glucose, amino acids, urea, caffeine, salicylic acid, nitrate, sulfate, chloride, Na(I), K(I), Ca(II) and Mg(II) in large amounts (100- to 1000-fold excess over HSA) scarcely interfered. The results are summarized in Table II.

Comparison with Other Dye-Binding Methods Artificial serum samples (albumin-globulin ratio set at 1.5—2.0) were measured by the present method and by the BCG method.[4b] The values determined by the present method (Y) correlated well with those obtained by the BCG method (X), as shown in Fig. 3. The sensitivity of the method was 6 times higher than that of the BCG method.

Furthermore, it is known that the conventional dye-binding methods such as BCG and BCP methods show large deviations with albumins derived from different animal species. Thus, a comparative study of the reactivity of different animal albumins was carried out. As shown in Table III, the present method exhibited little deviation in comparison with the BCP method.[5d]

Binding Parameters and Thermodynamic Parameters The acid dissociation constants of SPF[8] in the presence of Triton N-101 were determined spectrophotometrically[9] by the method of the previous paper.[6d] It was found that pK_{a0} and pK_{a1} were 2.05 and 6.58, respectively, and about

Table IV-1. Binding Parameters

Compound	K (M^{-1})	n
SPF–U(VI)	1.75×10^3	46.0
SPF	8.38×10^3	10.3

Table IV-2. Thermodynamic Parameters

Compound	$-\Delta G$ (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
SPF–U(VI)	4.42	16.02	68.57
SPF	5.39	4.89	34.49

96.6% of SPF was estimated[10] to be in the form H_4R at pH 3.5. Further, U(VI) at pH 3.5 exists as UO_2^{2+}. Thus, the composition of SPF–U(VI) complex at pH 3.5 may be expressed as $[UO_2^{2+}(SPF)_2]$.

The parameters for binding of SPF–U(VI) complex and SPF to HSA were obtained from dual double-reciprocal plots[11] by using the least-squares method. The results are shown in Table IV-1.

The thermodynamic parameters for the interactions between HSA and SPF–U(VI) complex can be obtained from Eq. 1 and 2, where ΔG, ΔH and ΔS are the Gibbs free energy change, enthalpy change and entropy change, respectively, and $\boldsymbol{R}$ and T have their customary definitions.

$$\Delta G=-\boldsymbol{R}T\ln K=\Delta H-T\Delta S \quad (1)$$

$$\ln K=-\Delta H/\boldsymbol{R}T+\Delta S/R \quad (2)$$

With a constant value (the intercept of the Y-axis) of ΔS, therefore, plots of $\ln K$ *versus* T^{-1} (van't Hoff plot) should be approximately linear with a slope of $-\Delta H/\boldsymbol{R}$, provided that ΔH is independent of temperature in the measured range. These thermodynamic data, obtained by the least-squares method from a van't Hoff plot, are listed in Table IV-2.

Generally, hydrophobic bonding[12] is entropy-driven; its formation is an endothermic process, and the negative sign for the ΔG indicates that the binding process is spontaneous. The binding process in this reaction system was endothermic (positive ΔH), and the large increase in entropy resulted in negative ΔG. As SPF–U(VI) complex and SPF are a positive molecule and a neutral molecule, respectively, under the experimental conditions, electrostatic forces can not play a major role in the binding process. From the obtained data, the binding in these molecular complexes was presumed to be a kind of hydrophobic interaction, the HSA interacting with SPF–U(VI) or SPF to form a hydrophobic compound that squeezes out the water molecules that originally surrounded these compounds in an ordered structure. When binding occurs between SPF–U(VI) or SPF and HSA, the ordered structure of water is partially destroyed and becomes less ordered. Presumably, this is the reason for the entropy change on complexation.

Conclusion

A simple and sensitive method for the spectrophotometric determination of albumin (as HSA) with SPF–U(VI) complex was established. The sensitivity of the present method was about 6 times better than that of the BCG

method for albumin assay. Compared with the BCP method, deviation of reactivity among various albumins derived from different animal species was small. In artificial serum samples, a good agreement between the present method and the BCG method was obtained. The proposed method should be useful for the simple and sensitive determination and detection of albumin in urine, spinal fluid and serum. From the binding parameters (n and K) and the thermodynamic parameters (ΔG, ΔH and ΔS) obtained, the binding between HSA ans SPF–U(VI) or SPF was presumed as a kind of hydrophobic interaction.

References and Notes

1) This paper is Part LXXX of a series entitled "Application of Xanthene Derivatives for Analytical Chemistry." This work was presented at the 38th Meeting of the Kinki Branch, Pharmaceutical Society of Japan, Osaka, November 1988.
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8) SPF exists in solution in the following forms, depending on pH:
$$H_5R^+ \overset{K_{a0}}{\rightleftharpoons} H_4R \overset{K_{a1}}{\rightleftharpoons} H_3R^- \overset{K_{a2}}{\rightleftharpoons} H_2R^{2-} \overset{K_{a3}}{\rightleftharpoons} HR^{3-} \overset{K_{a4}}{\rightleftharpoons} R^{4-}$$
where R denotes the undissociable part of SPF and $K_{a0}-K_{a4}$ are the acid dissociation constants.
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