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A Sensitive Spectrophotometric Method for the Determination of Human Serum Albumin with Chrome-azurol S Aluminium Chelate[1)]

YUTAKA SAITO,[a] ATSUKO KANETSUNA,[a,2)] HIDEKI OKUDA,[b] MASAKI MIFUNE,[a] JUNICHI ODO,[a] and YOSHIMASA TANAKA*,[a]

Faculty of Phamaceutical Sciences, Okayama University,[a] 1–1–1, Tsushima-Naka, Okayama 700, Japan and Shionogi & Co., Ltd.,[b] 192 Imafuku, Amagasaki 660, Japan

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A simple and highly sensitive method for the determination of serum albumin based on the formation of a ternary complex from chrome-azurol S aluminium chelate was developed. A nonionic detergent, polyethylene glycol-*p*-nonylphenyl ether $n=10$, acted as a stabilizer of the ternary complex in the present system. The formation of the ternary complex caused a large increase in absorbance at 630 nm, and the calibration curve was linear between 10 and 200 μg of albumin in the final colored solution (8.0 ml). The sensitivity of the present method was about eight times that of a bromcresol green control method. Heparin and bilirubin did not interfere with the determination.

Keywords——chrome-azurol S; aluminium chelate; ternary complex; determination of albumin; serum albumin; color reaction; dye binding method

The sulfonphthalein dye-binding methods represented by the bromcresol green (BCG) method[3)] have been commonly used in the clinical assay of human serum albumin (HSA).[4)] Although these methods are sensitive enough for albumin-rich samples such as serum, they do not have enough sensitivity for assaying the samples which contain small amounts of HSA, such as spinal fluid. Previously, it has been demonstrated that chrome-azurol S aluminium chelate (CAS-Al) is a sensitive reagent for the determination of cationic detergents by formation of a ternary complex.[5)] We considered that HSA might behave similarly to cationic detergents in dye-binding systems, and attempted to develop a new method for the determination of HSA by the application of CAS-Al.

Experimental

Reagents and Materials——Chrome-azurol S (CAS) purchased from Merck AG was reprecipitated from concentrated hydrochloric acid and purified by a method similar to that reported previously.[6)] Human and bovine serum albumins (HSA and BSA, fraction V, Sigma Chemical Co.), and nonionic detergents, polyethylene glycol *p*-nonylphenyl ether (PGNP) $n=5$, 10 and 20 (Tokyo Kasei), polyethylene glycol 400, polyethylene glycol monostearate $n=10$ and 55 (Ishizu Seiyaku), Span 20, 40 and 60 (Wako Junyaku or Tokyo Kasei) and Tween 20 and 60 (Kao-Astra), were used without any purification. Control sera and spinal fluid were purchased from Kekken or Hyland Diagnostics. A clinical kit based on the BCG method was used as a control (Albumin B test Wako; Wako Junyaku). All the other reagents were of reagent or analytical grade.

Apparatus——Absorption spectra and absorbances were measured on a Shimadzu UV-180 double-beam spectrophotometer by the use of 1.0 mm quartz cells. A Komatsu-Yamato CTR120-CTE 120 thermostat was used to control the incubation.

Recommended Procedure——The reagent solution consisted of a 1:1:2:2 (by volume) mixture of CAS

(1.6×10^{-4} M), $K_2Al_2(SO_4)_4 \cdot 10H_2O$ (1.6×10^{-4} M Al^{3+}), PGNP $n=10$ (7.5×10^{-4} M) and 0.2 M acetate buffer (pH 3.7). The reagent solution (6.0 ml) was added to the sample solution (2.0 ml of diluted serum or spinal fluid; 10—200 μg of HSA), and the mixture was permitted to stand at around 27.5 °C for 30 min. The absorbance at 630 nm was measured against the reagent blank.

Results and Discussion

Absorption Spectra

Absorption spectra of the reagent blank and the colored solution obtained by the recommended procedure are shown in Fig. 1. The presence of a small amount of HSA caused a new band to appear at around 630 nm, and the absorbance of the band changed in proportion to the amount of HSA. A large amount of HSA caused a red-shift of the absorption maximum by about 60 nm, as can be seen in Fig. 1. The ternary complex solution may be colloidal, because the colored solution showed a small absorbance even at 700 nm, probably caused by scattering.

Selection of Standard Conditions

The conditions for the procedure were examined mainly by the use of 100 μg of HSA in 8.0 ml of the final solution.

Effects of pH and Buffer——The effect of pH on the absorbance in 0.2 M acetate buffer (pH 2.7—5.6) is shown in Fig. 2. The reagent blank value increased with increase of pH, whereas the colored solution showed maximum absorbance around pH 3.7, as shown in Fig. 2. Although the concentration of acetate buffer had a small effect on the absorbance, the absorbance difference was nearly maximum when 0.2 M acetate buffer was used. Citrate and phthalate buffers (0.2 M, pH 3.7) were found to give 2.2% and 86.0% of the absorbance obtained with acetate buffer, respectively. We selected 0.2 M acetate buffer (pH 3.7).

Nonionic Detergent——The ternary complex precipitated in the absence of nonionic detergent. The resulting blue precipitate could not be isolated, since it disappeared completely at the time of the separation. In order to dissolve and stabilize the ternary complex, the addition of nonionic detergent or water-miscible organic solvent was examined. Acetone, ethanol, dioxane and isopropanol accelerated the precipitation of the ternary complex, whereas all the examined detergents except for Span 20 and 40 seemed to dissolve the ternary complex to some degree. As can be seen in Table I, PGNP $n=10$ was found to develop the strongest color among the detergents examined.

A concentration of PGNP $n=10$ above the critical micelle concentration had no serious effect on the absorbance but the linearity of the calibration curve depended upon the concentration as shown in Fig. 3. In the present study, PGNP $n=10$ (7.5×10^{-4} M, 2.0 ml),

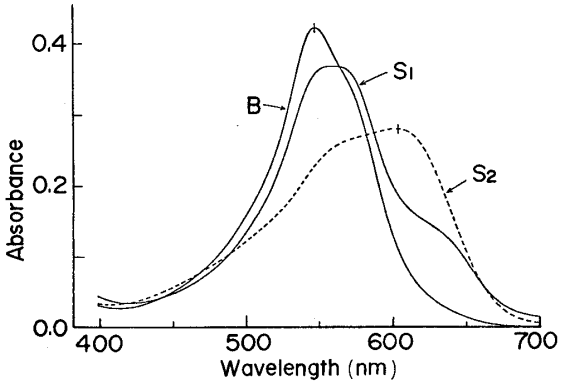

Fig. 1. Absorption Spectra

S_1, colored solution (100 μg of HSA/8 ml) *vs.* water; S_2, (1 mg of HSA/8 ml); B, reagent blank.

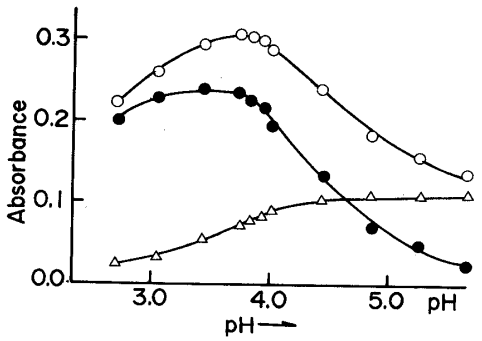

Fig. 2. Effect of pH of 0.2 M Acetate Buffer

○, colored solution; △, reagent blank; ●, absorbance difference.

Table I. Effect of Nonionic Detergents

Nonionic detergent		Difference (%)[a]
PGNP	$n=10$	0.246 (100)
	$n=5$	0.236 (97)
	$n=20$	0.204 (83)
Span 20		0.218 (88)
PEGS[b]	$n=55$	0.188 (76)
Tween	60	0.177 (72)
	20	0.154 (63)
Polyethylene glycol 400		0.102 (42)

a) Difference in absorbances between the colored solution and the reagent blank (percent with respect to PGNP $n=10$). *b*) Polyethylene glycol monostearate.

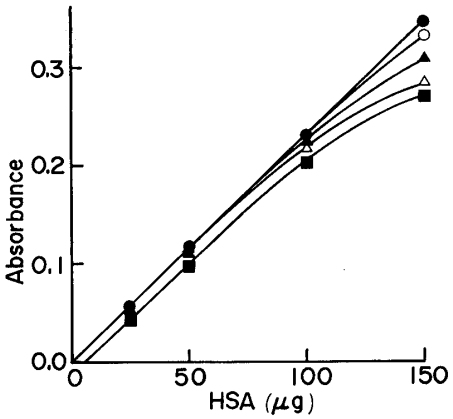

Fig. 3. Effect of Concentration of PGNP $n=10$ on the Calibration Curve

●, 5.0×10^{-4} M PGNP $n=10$ was added; ○, 1.0×10^{-3} M; ▲, 1.5×10^{-3} M; △, 2.0×10^{-3} M; ■, 2.5×10^{-3} M.

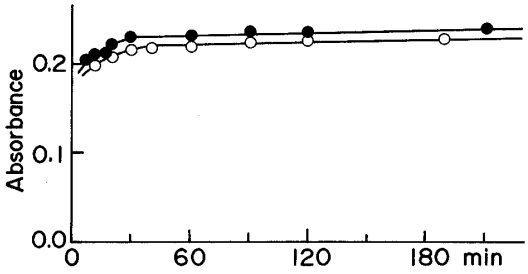

Fig. 4. Effect of Standing Time on the Absorbance Difference

○, at 25 °C; ●, at 27.5 °C.

which gave a linear plot, was added.

Effect of Incubating Temperature——The effect of the incubating temperature on the absorbance was examined between 20 and 35 °C. The absorbance of the reagent blank decreased very slowly with increase of the temperature. However, the absorbance difference was hardly affected by the temperature except between 25 and 27.5 °C, where the difference changed by about 5%. As the standard incubating temperature, we selected the vicinity of 27.5 °C in the present study.

Effect of Standing Time——As shown in Fig. 4, the absorbance of the colored solution reached a maximum and constant value after standing for 30 min at 25 °C as well as at 27.5 °C. The colored solution was stable for a few hours, but complete precipitation of the ternary complex was observed when it was allowed to stand overnight. However, this blue precipitate dissolved with vigorous shaking, and the resulting solution gave almost the same absorbance as that of the initial solution. This observation indicates that the interference of the precipitate could be removed by shaking just before the measurement, even when the precipitate had developed over a long period of time.

Effect of the Amount of Al^{3+} Ion——The effect of the ratio of CAS to Al^{3+} on the absorbance is shown in Fig. 5. When the ratio was less than 1.0, the absorbance difference

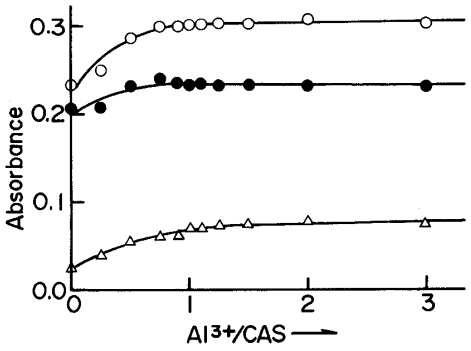

Fig. 5. Effect of Amount of Al^{3+} Ion

○, colored solution; △, reagent blank; ●, absorbance difference.

TABLE II. Effect of Foreign Substances and Ions

Substance and ion	Amount (μg)	Error (%)[a]
Fe^{3+}	290	202.2
	4.3	−1.7
Cu^{2+}	1000	−5.2
Ca^{2+}	1000	3.5
K^{+}	1000	−1.3
I^{-}	1000	2.2
PO_4^{3-}	1000	−7.4
SO_4^{2-}	1000	−6.6
Zephiramine	25	44.1
NaF	1000	−92.8
	40	−90.7
Bilirubin	0.3	0.4
Heparin-Na	10	−51.1
	0.4	−0.4
γ-Globulin (human)	100	25.3
Citric acid	500	−48.9
	20	−1.1
Oxalic acid	200	−68.6
	8	−5.5
EDTA	100	−55.1
	4	−0.4
Ammonium potassium oxalate	80	−15.4
	8	−1.4

a) Error (%)$=(HSA_{added}-HSA_{found})100/HSA_{added}$, $HSA_{added}=100\,\mu g$.

increased with increase in the ratio, whereas it was nearly constant when the ratio was in the range from 1.0 to 4.0. This result indicates that the 1 : 1 complex formed between CAS and Al^{3+} combines with HSA.

Calibration Curves and Sensitivity

The calibration curves for HSA and BSA obtained by the recommended procedure follow Beer's law with a coefficient of variation ($n=10$) of 1.3% at 100 μg of HSA. The absorption coefficient was found to be about 1.2×10^6 for both HSA and BSA. It is noteworthy that the sensitivity of the present method reached about 8 and 4 times those of the BCG control and the CAS methods,[6] respectively. The sensitivity of the present method is comparable with that of the extremely sensitive methods for the determination of proteins, such as the coomassie brilliant G[7] and the pyrocatechol violet-molybdenum[8] methods.

Table III. Determination of HSA in Sera and Spinal Fluid

Sample	Present method	BCG method
Serum I (Hyland)	3.78 g/dl (CV = 0.91%)[a]	3.10 g/dl[b]
Serum (Kekken)	5.02 g/dl[a]	4.62 g/dl[b]
Spinal fluid (Hyland)	48.0 mg/dl (CV = 0.73%)[c]	40.7 mg/dl[d]

a) Sample amount = 2 μl of serum. *b*) 0.02 ml of serum. *c*) 0.1 ml of spinal fluid. *d*) 1.0 ml of spinal fluid.

Interference

Under the conditions of the recommended procedure, various substances and ions were examined for interference. The results are summarized in Table II. A large increase in absorbance was caused by the presence of 290 μg of Fe^{3+} among the inorganic ions examined, but the increase was negligible when the amount was less than 4.2 μg (the amount contained in 0.02 ml of serum). The interference by Fe^{3+} was probably caused by the replacement reaction of Al^{3+} with Fe^{3+} ion. A large amount of anticoagulant caused a decrease in absorbance but the usual dose did not interfere with the determination. In connection with similarlity of proteins to cationic detergents in the dye-binding system, it is of interest that a cationic detergent, zephiramine, gave a large positive error. A disadvantage of the system is that globulins caused an increase in the absorbance.

Application

The present method was applied to the determination of HSA in one-tenth of the amount of serum and spinal fluid used for the BCG control method. The absorption spectra obtained by the recommended procedure coincided with that obtained by the use of HSA solution. The obtained HSA values shown in Table III were larger than those obtained by the BCG control method. It deserves an attention that HSA in 0.1 ml of spinal fluid is determined with a coefficient of variation of less than 1%, whereas the BCG method requires 1.0 ml of spinal fluid.

Conclusion

The present method is a highly sensitive and convenient method for the determination of a small amount of HSA, and may be practically applicable to the determination of HSA in spinal fluid, since the sensitivity reaches about eight times that of the control BCG method.

References and Notes

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