[Chem. Pharm. Bull. 34(7)2880—2884(1986)]

# A Sensitive Spectrofluorimetric Method for the Determination of Human Serum Albumin with Chrome-azurol S[1)]

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(Received November 21, 1985)

An extremely sensitive spectrofluorimetric method for assay of human serum albumin (HSA) was developed, based on binding of chrome-azurol S(CAS) by HSA. The CAS–HSA complex shows a fluorescence at 616 nm, whereas CAS alone exhibits only a weak fluorescence at this wavelength. A nonionic detergent, polyethylene glycol-*p*-nonylphenyl ether $n=10$, acts as a stabilizer of the CAS–HSA complex. The calibration curve was linear in the range from 5 to 80 $\mu$g of HSA in the final solution. Relative standard deviation was 1.3% ($n=10$). The method requires less than one-thirtieth of the amount of HSA required by the control bromcresol green method. HSA values in control sera and spinal fluid assayed by the new method were in agreement with those obtained by the control bromcresol green method.

**Keywords**——chrome-azurol S; dye binding method; albumin sensitive determination; albumin spectrofluorimetry; albumin chrome-azurol S complex; spinal fluid albumin

Dye-binding methods are commonly used in the clinical assay of human serum albumin (HSA), because of their rapidity and simplicity. At present, the bromcresol green (BCG) method[4)] is used most widely in the clinical assay of HSA, but the BCG method is practically applicable only to HSA-rich samples such as serum, and not to spinal fluid. In order to improve the sensitivity, methods using a sulfonphthalein acid-base indicator such as bromcresol purple[5)] or bromphenol blue[6)] have been developed, but the sensitivity was not improved much. Previously, we reported that the method with chrome-azurol S aluminum chelate (CAS-Al) was about eight times more sensitive than the BCG method.[7)] However, the CAS-Al method is not sensitive enough in practice for the assay of HSA in spinal fluid, and is probably disturbed by globulins.

It has been demonstrated briefly that chrome-azurol S (CAS) bound to HSA fluoresces at 616 nm, and one-thirtieth of the HSA required by the BCG method can be practically determined by measurement of this flurorescence.[7b)] This paper presents full details of that work, and describes a spectrofluorimetric method for HSA determination with CAS, which is not an acid-base indicator but a metal-indicator.

## Experimental

**Reagents and Materials**——Chrome-azurol S purchased from Merck AG was purified by the reprecipitation method reported previously.[7c)] Human and bovine serum albumins (Fraction V), and human $\gamma$-globulins (Cohn fraction II) were obtained from Sigma Chemical Co. Nonionic detergents, Brij 35 (30% solution, Fujisawa-Technicon), Triton X-100 (Ishizu Seiyaku) and polyethylene glycol *p*-nonylphenylether $n=5$, $n=10$ and $n=20$ (PGNP 5, 10 and 20, Tokyo Kasei Co.) were used without further purification. Normal and abnormal control sera

(sera I and II), control sera elevated by bililubin and by lipids, and control spinal fluid wre purchased from Hyland Diagnostics. A clinical kit, Albumin B test *Wako* (Wako Junyaku), was used as the control BCG method. All the other reagents were of reagent or analytical grade.

The reagent solution A was a 2:2:1 (by volume) mixture of CAS ($5.0 \times 10^{-6}$ M), PGNP 10 ($2.5 \times 10^{-3}$ M) and pH 3.0 citrate buffer (0.1 M). The reagent solution B used for the selection of the assay conditions was a 2:1:1 mixture of CAS, PGNP 10 ($5 \times 10^{-3}$ M) and citrate buffer.

**Apparatus**——Fluorescence spectra and relative fluorescence intensities were measured on a Shimadzu RF-500 spectrofluorophotometer equipped with photomultiplier tube (R928, Hamamatsu Photonics). Quartz cells (10 mm) were used for the measurements.

**Standard Procedure**——The reagent solution (5.0 ml) was added to a sample solution (20 or 100 $\mu$l) containing 5—80 $\mu$g of HSA, and the mixture was permitted to stand at around 27.5 °C for 30 min. The relative fluorescence intensity was measured against the reagent blank at 616 nm with excitation at 493 nm. Bandwidths at the entrance-slit and at exit-slit were 20 and 40 nm, respectively. The reagent solution B (4.0 ml) and the HSA solution (1.0 ml) were used similarly for the selection of the assay conditions.

## Results and Discussion

### Fluorescence Spectra

Emission and excitation spectra obtained by the standard procedure are shown in Fig. 1. The fluorescent solution exhibited emission and excitation maxima at 616 and around 485 nm, respectively. The maxima of the reagent blank were observed at 604 and 471 nm. The fluorescence of the complex may be concluded to reflect binding of CAS to HSA in a planar mode. Since the intensity at 616 nm rose proportionally to HSA concentration, as seen in Fig. 1, the fluorescence of the HSA/CAS complex should be useful for the determination of HSA.

### Selection of the Standard Conditions

Suitable conditions for the procedure were examined mainly by use of 1.0 ml of HSA solution (40 $\mu$g/ml) and 4.0 ml of the reagent solution B.

**Emission and Excitation Wavelengths**——We selected 616 and 493 nm as the emission and the excitation wavelengths, respectively, since the reagent blank was then relatively small.

**Effect of pH**——The effect of pH on the intensity is shown in Fig. 2. The maximum intensity was obtained at pH 3.0 in 0.1 M citrate buffer. Although the reagent blank increased with increase of pH, the ratio of the intensities of the fluorescent solution to the reagent blank was the maximum at pH 3.0. We selected pH 3.0 as the standard condition.

**Effect of Buffer**——Lactate, glycinate, tartrate and phthalate buffers gave intensities

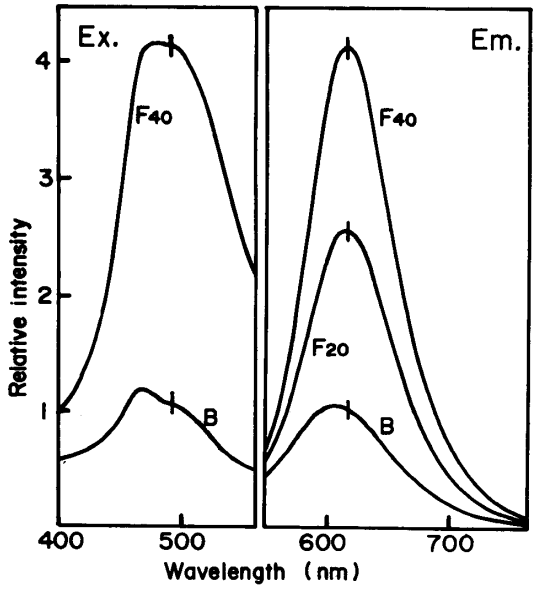


Fig. 1. Excitation (Ex.) and Emission (Em.) Spectra

B, reagent blank; $F_{20}$, fluorescent solution (20 $\mu$g of HSA/5.0 ml); $F_{40}$, (40 $\mu$g of HSA/5.0 ml).

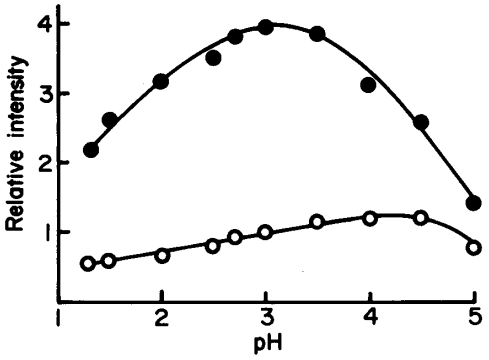


Fig. 2. Effect of pH of 0.1 M Citrate Buffer
—●—, fluorescent solution; —○—, reagent blank.

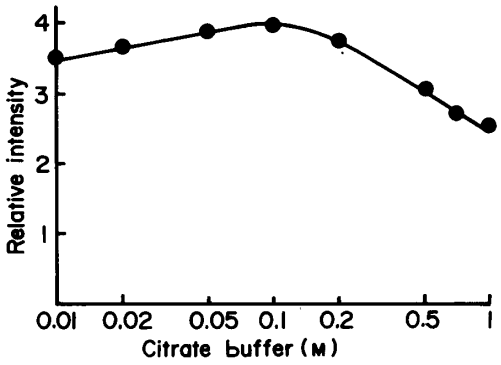


Fig. 3. Effect of the Concentration of Citrate Buffer on the Fluorescence Intensity of the Solution

comparable to that in citrate buffer, whereas acetate buffer gave less than one-half of the intensity.

The concentration of citrate buffer had a marked effect on the intensity, as shown in Fig. 3, though the reagent blank was not affected by the concentration. The maximum intensity was obtained at around 0.1 M. We selected 0.1 M citrate buffer (pH 3.0) for the standard procedure.

**Effect of Nonionic Detergent**——Previously, it has been reported that the CAS–HSA complex forms a red precipitate in a simple aqueous system, and the complex can be dissolved by a nonionic detergent, PGNP 10.[7c] PGNP 10 was also selected as a solubilizer of the complex for the present assay, and the effect of the concentration of PGNP 10 on the intensity was examined. The result (shown in Fig. 4) suggested that as low a concentration as possible should be used. However, solubilization of not only the complex but also the components of serum requires a sufficient concentration of PGNP 10. Thus, we selected $5 \times 10^{-2}$ M PGNP 10. It was comfirmed by nephelometry that the complex and the components were completely dissolved by the addition of $5 \times 10^{-2}$ M PGNP 10.

Triton-X 100 and PGNP 20 gave intensities comparable to that obtained with PGNP 10, while Brij 35 and PGNP 5 gave smaller intensities by about 10% and 60%, respectively.

**Effects of Time and Temperature**——The effect of standing time on the intensity was examined. The intensity of the fluorescent solution decreased with standing time up to 60 min and was constant at least between 60 and 240 min. However, the intensity at 30 min was close to the constant value. We therefore chose 30 min as a standing time for experimental convenience.

The effect of temperature on the intensity was examined between 20 and 35 °C at intervals of 2.5 °C. The intensity of the fluorescent solution decreased by about 5.0% when the temperature was raised above 22.5 °C. In the present study, we used a temperature of around 27.5 °C, the same as that used for the CAS-Al method[7a] was used.

**Calibration Curves and Sensitivity**

The range of linearity of the calibration curve depended upon the concentration of CAS as shown in Fig. 5. The range increased over with increase of the concentraion of CAS. In the present study, $5 \times 10^{-6}$ M CAS, which gave good linearity in the range from 5 to 80 $\mu$g of HSA, was selected as the standard condition. The coefficient of variation was 1.3% for 40 $\mu$g of HSA ($n = 10$), and this is very satisfactory.

Since the new method involves fluorimetry whereas the BCG method involves photometry, it is rather difficult to compare directly the sensitivities of two methods. However, it is noteworthy that the new method can determine an amount of HSA less than one-thirtieth of

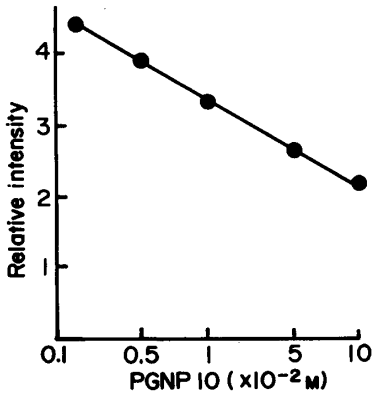


Fig. 4. Effect of the Concentration of PGNP 10 on the Fluorescence Intensity of the Solution

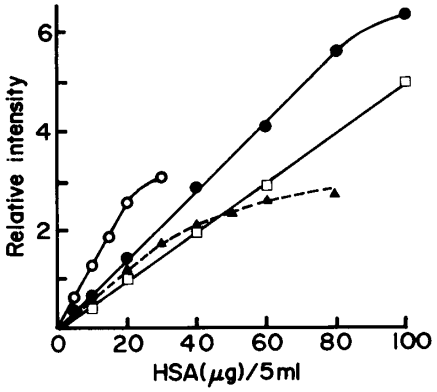


Fig. 5. Calibration Curves

—○—, $2.5\times10^{-6}$ M CAS for HSA; —●—, $5\times10^{-6}$ M CAS; —□—, $1\times10^{-5}$ M CAS; ---▲---, $5\times10^{-6}$ M CAS for BSA; Standard, the corresponding reagent blank.

TABLE I. Effect of Foreign Substances

| Substance | Amount (μg) | Error (%)[a] | Substance | Amount (μg) | Error (%)[a] |
|---|---|---|---|---|---|
| $NH_4^+$ | 500 | −1.2 | $Cl^-$ | 500 | 0.4 |
| $Cu^{2+}$ | 500 | −7.6 | $Br^-$ | 500 | 0.4 |
| | 50 | −3.3 | $SO_4^{2-}$ | 500 | 1.0 |
| $Ca^{2+}$ | 500 | −0.9 | $CO_3^{2-}$ | 1000 | 7.4 |
| $Al^{3+}$ | 500 | −36.9 | $PO_4^{3-}$ | 500 | 2.5 |
| | 50 | −3.9 | EDTA[b] | 10 | 3.1 |
| | 5 | 3.5 | Heparin | 1 | 4.5 |
| $Fe^{3+}$ | 500 | −60.8 | NaF | 100 | 3.0 |
| | 50 | −18.8 | $NaN_3$ | 5 | 6.6 |
| | 5 | 1.0 | γ-Globulin (human) | 50 | 7.4 |

*a*) Error (%) = $(HSA_{added} - HSA_{found})/HSA_{added} \times 100$; $HSA_{added} = 50\,\mu g$. *b*) EDTA = ethylenediaminetetraacetic acid.

that required by the BCG control method. This suggests that the new method is extremely sensitive. HSA in 20—100 μl of spinal fluid could be conveniently determined by the new method.

The calibration curve for bovine serum albumin (BSA) was not linear and was very different from that for HSA, as shown in Fig. 5. One reason for this may be difference of the binding sites. In any case, BSA cannot be used as a standard albumin instead of HSA for the new method, in contrast to the BCG method.

**Interference**

Under the conditions of the recommended procedure, various substances and ions were examined for interference. The results are summarized in Table I. Among the inorganic ions examined, a large decrease in the intensity was caused by the presence of 500 μg of $Al^{3+}$ or $Fe^{3+}$, which formed chelates with CAS, but the interference was negligible when the amount was less than 5 μg. Usual doses of common anticoagulant did not interfere with the determination. However, human γ-globulins gave a positive error of 7.4%. The γ-globulins used in the present study also caused a 7.0% positive error in the BCG control method. Thus, the error caused by γ-globulins was essentially the same in both methods, although it is not clear whether this error was caused by γ-globulins themselves, or by HSA contaminating in the γ-globulins.

TABLE II. Determination of HSA in Sera and Spinal Fluid

| Sample | BCG method | Present method (C.V.)[a] |
|---|---|---|
| Serum I (normal) | 4.72 g/dl[b] | 4.73 g/dl (2.18%)[c] |
| Serum II (abnormal) | 4.61 g/dl[b] | 4.56 g/dl (0.97%)[c] |
| Serum elevated by bilirubin | 4.83 g/dl[b] | 4.72 g/dl (0.86%)[c] |
| Serum elevated by lipids | 5.40 g/dl[b] | 5.15 g/dl (0.66%)[b] |
| Spinal fluid | 59.0 mg/dl[d] | 55.2 mg/dl (1.39%)[e] |

*a*) C.V.=coefficient of variation ($n=10$). *b*) Sample amount=20 $\mu$l of serum. *c*) 20 $\mu$l of 5% serum. *d*) 1.0 ml of spinal fluid. *e*) 100 $\mu$l of spinal fluid.

## Application

The new method was applied to the determination of HSA in control sera and spinal fluid, and the results are summarized in Table II. All the emission spectra obtained by the new method coincided completely with that of a standard HSA solution, indicating that other components in sera or spinal fluid did not affect the spectrum under the standard conditions. The HSA values obtained by the new method were comparable with those obtained by the BCG control method, as seen in Table II. It is of interest that the value in serum elevated by lipids was smaller by about 4% than that by the BCG control method. Since lipids give a positive error in the BCG method, this result suggested that the present method is probably less affected by lipids than the BCG method. The results described above indicated that the new method is compatible with the BCG method, and is not disturbed by any components, except for globulins.

## Conclusion

The present method is compatible with the BCG method and provides a convenient and extremely sensitive procedure for the determination of HSA. Interference by usual components of serum is negligible, though globulins may interfere with the determination to about the same degree as with the BCG method. The high sensitivity of the present method should make it of value for a practical assay of HSA in samples containing low levels of albumin, such as spinal fluid.

### References and Notes


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