Sensitive Spectrofluorometric Method for the Determination of Human Serum Albumin with Tetrakis(*p*-carboxyphenyl)porphine

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Dye-binding colorimetric methods are commonly used in the clinical assay of human serum albumin (HSA) because of their rapid and simple procedures. The Bromcresol Green (BCG) method¹⁻³, conventionally used in the clinical assay of HSA, is practically applicable only to HSA-rich samples, such as serum. Although methods using Bromcresol Purple⁴, Bromphenyl Blue⁵ or Chrome-azurol S aluminum chelate⁶ have been developed, the sensitivity has not been improved. We reported previously that the fluorometric method with Chrome-azurol S (CAS) could be applied to the determination of less than one-thirtieth of the HSA amount determined by the BCG method.⁷ In the present study, we focused on some porphine derivatives, some of which are well known to strongly fluoresce. It has been found that HSA enhances the fluorescence of tetrakis(*p*-carboxyphenyl)porphine (TCPP, Fig 1) at 660 nm; it can thus be applied to the determination of trace HSA.

Fig. 1 Structure of tetrakis(*p*-carboxyphenyl)porphine (TCPP).

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Experimental

Reagents and materials

Tetrakis(*p*-carboxyphenyl)porphine (TCPP) was purchased from Tokyo Kasei, Japan. Human serum albumin (Fraction V, Sigma, USA) and human γ -globulin (Cohn fraction II, Sigma) were used without further purification. Normal control serum (serum I) was obtained from Hyland Diagnostics, USA. A clinical kit, Albumin B-test Wako (Wako Junyaku, Japan), was used as the control BCG method. All of the other reagents were either of reagent or analytical grade. The reagent solution was a 2 : 3 mixture of TCPP (1×10^{-6} M) and pH 3.0 citrate buffer (0.1 M) solutions.

Apparatus

The fluorescence spectra and relative intensities were measured on a Shimadzu RF-500 spectrofluorophotometer equipped with an R-928 photomultiplier (Hamamatsu photonics, Japan), one of the most sensitive photomultiplier in the long-wavelength region. Quartz cells (10 mm) were used for the measurements.

Standard procedure

The reagent solution (5.0 ml) was added to a sample solution (1.0 ml) containing 5 – 20 μ g HSA, and the mixture was permitted to stand at 35°C for 20 min. The relative fluorescence intensity was measured against the standard TCPP neutral solution (5×10^{-8} M) at 660 nm with excitation at 412 nm. The bandwidths at the entrance-slit and at the exit-slit were 20 and 40 nm, respectively.

Results and Discussion

Ultraviolet and visible spectra

Ultraviolet and visible spectra of three water soluble porphines were checked in the presence of HSA. The

Fig. 2 Excitation (Ex) and emission (Em) spectra of TCPP with HSA. B, reagent blank; S, fluorescent solution (10 μ g HSA).

presence of a large amount of HSA induced a larger red-shift of the Soret band of TCPP than those of tetrakis(sulfophenyl)porphine (TSPP) and tetrakis(4-methylpyridinium 4-yl)porphine (TMPyP). Thus, TCPP was mainly used in the following investigation.

Fluorescence spectra

The emission and excitation spectra obtained by the standard procedure are shown in Fig. 2. The fluorescent solution exhibited emission and excitation maxima at 660 and 412 nm, respectively. Fluorescence of the HSA/TCPP complex may be concluded to reflect the binding TCPP being in a planar mode, whereas the phenyl rings of TCPP are supposed to be a propeller-like conformation in aqueous solution. It should be noted that the fluorescence of the reagent blank was extremely weaker than that of the sample solution. HSA induced a small change in the fluorescence spectra of TSPP and TMPyP, although a dramatic spectral change similar to that shown in Fig. 2 was not observed in the pH range tested (pH 2–11).

Selection of the standard conditions

Suitable conditions for the procedure were examined mainly by using 1.0 ml of HSA solutions (10 and 20 μ g/ml).

Emission and excitation wavelengths. We selected with confidence 660 and 412 nm as the emission and excitation wavelengths.

Effect of pH. The effect of the pH on the intensities is shown in Fig. 3. The maximum intensity of the fluorescent solutions was observed at around pH 3.0–3.5 in 0.1 M citrate buffer; the minimum intensity of the reagent blank was observed at around pH 3.0. As can be seen in Fig. 3, the linearity of the calibration curve seems to depend upon the pH. In view of the sensitivity and the linearity of the calibration curve, we selected a pH of 3.0 for the standard condition.

Fig. 3 Effect of pH on relative intensity. B, reagent blank; S_{10} , fluorescent solution (10 μ g HSA); S_{20} , fluorescent solution (20 μ g HSA).

Effect of buffer. Tartrate and lactate buffer solutions gave intensities comparable to that in citrate buffer. However, the intensities for 5 and 10 μ g HSA in phthalate or glycinate buffer were about 50 and 70% of that in citrate buffer, respectively, though the reagent blank was not affected by the kind of buffer solutions. In the present study, a 0.1 M citrate buffer solution, a relatively conventional buffer, was selected for the standard procedure.

Effect of standing time and temperature. The effect of the standing time on the intensity was examined. The intensity of the fluorescent solution was nearly constant for at least between 5 and 20 min. The intensity-reproducibility of the fluorescent solution left standing for 20 min was better than that for 10 min. Thus, the fluorescent solution was permitted to stand for 20 min. The effect of the temperature on the intensity was examined between at 25 and 45°C at intervals of 5°C. The intensities of both the fluorescent solution and the reagent blank decreased with temperature. Since the intensity of the reagent blank was closed to the lowest value in the range tested and the calibration curve seemed to be linear, we selected 35°C as the condition.

Effect of the concentration of TCPP. The effect of the TCPP concentration on the intensities is shown in Fig. 4, where the abscissa is the logarithmic scale of the TCPP concentration. The maximum intensities of the fluorescent solutions containing 10 and 20 μ g HSA were observed at 1×10^{-6} M TCPP solution. The fluorescence of the sample solution was weakened by concentration-extinction in the range of the TCPP concentration above 1×10^{-6} M. In the present study, a mixture of 2.0 ml of 1×10^{-6} M TCPP solution and 3.0 ml of the buffer solution was selected as the reagent solution in the standard procedure.

Calibration curve and sensitivity

The calibration curve was linear in the range 2–20 μ g of HSA ($r=0.9999$, 5 points). The relative standard deviation ($n=10$), 1.89% for 20 μ g of HSA, is very

Fig. 4 Effect of TCPP concentration on relative intensity. B, reagent blank; S₁₀, fluorescent solution (10 µg HSA); S₂₀, fluorescent solution (20 µg HSA).

satisfactory and is comparable to that of the BCG method. The intensity ratio of the fluorescent solution (5 µg HSA) to the reagent solution was 5.1 in the present TCPP method. This value is much larger than the corresponding value (1.3–1.6) of the CAS method.⁷ As described above, it is one of the advantages of the present method that the reagent blank is extremely small. This advantage contributes to the sensitivity of the present method, which requires a smaller amount of HSA than that required by the CAS method.

Interference

Under the conditions of the recommended procedure, various substances and ions were examined for interference. The results are summarized in Table 1. Among the inorganic ions examined, a serious decrease in the intensity was caused by iron(III), which may form a chelate with TCPP, similarly to the case of the CAS method. It is of interest that the interference of aluminum(III) was not so serious as that in the CAS method. Cetylpyridinium chloride, a cationic detergent and a model compound of HSA, gave a positive error of about 50%. In addition, human γ -globulin also gave a positive error of 19.7%. Although this error, more than twice those in both the BCG and CAS methods, is a demerit of the present method, it should be overcome by future examinations.

Application

The TCPP method was applied to the determination of HSA in a control serum. The emission spectra obtained from the serum by the standard procedure coincided completely with that from a standard HSA solution, indicating that other components in the serum did not affect the spectrum under the standard procedure. The HSA value (4.25 g/dl) obtained by the TCPP method

Table 1 Effect of foreign substances

Substance	Amount/µg	Error ^a , %
Cl ⁻	200	-2.7
Br ⁻	200	1.8
PO ₄ ³⁻	200	-2.4
Ca ²⁺	200	-5.5
Al ³⁺	200	-16.3
Fe ³⁺	200	-78.1
Fe ³⁺	20	-57.6
CePyCl ^b	20	-49.7
γ -Globulin	20	19.7

a. Error(%) = $100 \times (\text{HSA}_{\text{found}} - \text{HSA}_{\text{added}}) / \text{HSA}_{\text{added}}$.

b. CePyCl: cetylpyridinium chloride.

was comparable with that (4.20 g/dl) by the control BCG method. In the determinations, the present method required only 1/100–1/200 of the serum required by the BCG method. These results indicate that the present method is extremely sensitive and is compatible with the BCG method. In addition, the present method was not disturbed by any serum-components, including γ -globulin, which was expected to disturb the determination. This can be explained by the fact that a normal serum usually contains γ -globulin equal to about one third of HSA,⁸ thus γ -globulin did not give any serious error.

In conclusion, the present method provides a convenient and extremely sensitive method for determining HSA. This method may make it of value for a practical assay of HSA in samples containing low levels of albumin.

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