Highly Sensitive Spectrophotometric Determination of Human Serum Albumin with 3′,4′,5′,6′-Tetrachlorogallein–Molybdenum(VI) Complex[1)]

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A highly sensitive spectrophotometric determination of human serum albumin (HSA) with 3′,4′,5′,6′-tetrachlorogallein (T.Cl.Gall)–Mo(VI) complex in a Triton X-100 + polyvinyl alcohol micellar medium is proposed. This method can be used to determine up to *ca.* 150 μg/10 ml of HSA from the optical absorbance at 640 nm, and is superior in sensitivity to the other extremely sensitive spectrophotometric methods. The great sensitivity of this method results from the use of third-derivative spectrophotometry. The binding parameters of T.Cl.Gall–Mo(VI) complex to HSA are $n = 77.3$ and $K = 1.05 \times 10^4\ \mathrm{M}^{-1}$ as determined from dual double-reciprocal plots. It is suggested that the colored complex in this system may be the association complex between $[\mathrm{HSA}]^{m+}$ and $[\mathrm{Mo^{VI}(T.Cl.Gall)_2}]^{n-}$ involving hydrophobic interaction between HSA and T.Cl.Gall. The proposed method should also be useful for the detection and determination of some peptides (*e.g.* low molecular weight peptides containing basic amino acids), as well as proteins.

Keywords human serum albumin; spectrophotometry; third-derivative spectrophotometry; 3′,4′,5′,6′-tetrachlorogallein–molybdenum(VI) complex; association complex; binding parameter

The authors have already reported spectrophotometric methods for the determination of a small amount of protein by using metal ion–dye complex systems, *i.e.* Pyrogallol red (PR)–Mo(VI)[2)] and Pyrocatechol violet (PV)–Mo(VI).[3)] These methods are superior to the conventional methods using dye alone (such as Bromophenol blue and Comassie brilliant blue) in terms of sensitivity, reproducibility, influence of foreign substances, calibration range, *etc.* As a continuation of our investigations of protein analysis, we considered that a superior method for the determination of protein might be developed by selecting a suitable combination of dye, metal ion and surfactant, and that a method using 3′,4′,5′,6′-tetrachlorogallein (T.Cl.Gall)–Mo(VI) complex in Triton X-100 + polyvinyl alcohol (PVA) micelles was recommended.[4)]

The paper presents details of a spectrophotometric method for the determination of human serum albumin (HSA) with T.Cl.Gall–Mo(VI) complex, with the introduction of derivative spectrophotometry for the purpose of enhancing of determination. In addition, binding parameters of T.Cl.Gall–Mo(VI) complex to HSA and the nature of the colored complex formed in the reaction system were studied.

Experimental

Reagents and Materials A standard solution (200 μg/ml) of HSA was prepared by dissolving human serum albumin (Sigma Chemical Co., essentially fatty acid-free, fraction V) in water. Other proteins and peptides used were purchased from Sigma Chemical Co., and Peptide Institute, Inc., Osaka. A stock solution (1.0×10^{-2} M) of Mo(VI) was prepared by dissolving sodium molybdate in water. The working solution (1.0×10^{-3} M) was prepared by dilution of this stock solution as required. A T.Cl.Gall solution was prepared by the published procedure[5)] in methanol to give a 2.0×10^{-3} M solution. A surfactant solution was obtained by dissolving 1.0 g of Triton X-405 and 3.0 g of PVA ($n = 500$) in water to 100 ml. A buffer of pH 2.8 was prepared by mixing 2.5 M hydrochloric acid and 2.5 M sodium acetate solutions. All other chemicals used were of analytical reagent grade unless otherwise stated. Deionized water was used in the preparation of all solutions.

Apparatus Absorption spectra, absorbance and derivative spectra 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. A Shimadzu model RF-540 spectrofluorometer with 1.0-cm quartz cells was also used.

Standard Procedure for the Spectrophotometry The following components were mixed in a 10-ml volumetric flask; 1.0 ml of the surfactant solution, 2.5 ml of the buffer solution, 0.75 ml of 1.0×10^{-3} M Mo(VI) solution, 0.75 ml of 2.0×10^{-3} M T.Cl.Gall solution and a solution containing up to 150 μg of HSA. The mixture was diluted to 10 ml with water and kept at 50 °C for 10 min, and then cooled for 10 min in water. The absorbance of the resultant solution (solution A) was measured at 640 nm against a reagent blank (solution B) prepared under the same conditions.

Procedure for Third-Derivative Spectrophotometry When the HSA content of the colored solution prepared by the standard procedure was too low to give a measurable absorbance, the ordinary spectrum of solution A was taken from 800 to 500 nm against solution B as a reference. The third-derivative spectrum was then recorded by using a third-order differentiation circuit of $\Delta\lambda = 31.5$ nm with a scan speed of 1500 nm min^{-1}, and the third-derivative value (the vertical distance from peak to trough) was measured.

Results and Discussion

Optimization of Experimental Conditions for Spectrophotometry The maximum difference of absorbance between solutions A and B was observed at about 640 nm, which was selected for the determination of HSA.

The influence of pH on the color development was investigated. It was found that the coloration was pH- and ionic strength-dependent and maximal absorbance was observed at 640 nm in the pH range from 2.2 to 3.5 by using 2.5—3.5 ml of 2.5 M hydrochloric acid/2.5 M sodium acetate solution for pH adjustments; the absorbance using 2.5 ml of 2.5 M buffer solution was approximately the same as that using 2.5 ml of 0.2 M hydrochloric acid–sodium acetate buffer solution and 2.5 M sodium chloride solution.

Solution A without a surfactant formed a precipitate. Among various nonionic surfactants examined, a combination of Triton X-405 and PVA was most effective with respect to stability and sensitivity.

The effects of concentrations of T.Cl.Gall and Mo(VI) solutions were examined by varying the molar ratio of T.Cl.Gall to Mo(VI), the amount of HSA being kept constant. The maximum absorbance was obtained when the molar ratio of Mo(VI) to T.Cl.Gall was approximately 1 : 2. Furthermore, the molar ratio of Mo(VI) to T.Cl.Gall in the

complex was found to be 1 : 2 in the presence or absence of HSA, as determined by the continuous variation method. All further work was thus carried out with 7.5×10^{-5} M Mo(VI) and 1.5×10^{-4} M T.Cl.Gall in the final volume of 10 ml. The same absorbance was obtained even when the order of addition of the reagents was modified.

The color formation in this reaction system did not occur instantaneously at room temperature, more than 60 min being required for completion of the color reaction. The effect of temperature was examined between 40 and 60 °C. Experiments proved that maximum and constant absorbance was obtained on heating at 50 °C for 10 min. The absorbance remained constant for at least 1 h after the solution had been cooled to room temperature.

Figure 1 shows the absorption spectra of solutions A and B, and their difference absorbance (curve C) under the standard procedure.

Calibration Curve A calibration curve for HSA was constructed by the standard procedure. Beer's law held over the range up to *ca.* 150 μg of HSA in the final volume of 10 ml. The sensitivity of this procedure is about 2.5—3 times greater than that of the PR–Mo(VI),[2] PV–Mo(VI)[3] and Chromazurol S (CAS)–Al(III)[6] methods. The relative standard deviation (R.S.D.) was 0.8% for five replicate determinations of 100 μg of HSA. The calibration curves for several proteins as well as HSA passed through the origin, and the sensitivities are summarized in Table I.

Influence of Miscellaneous Substances The influence of diverse substances on the determination of HSA was investigated under the optimal conditions. Among the substances examined, large amounts of Fe(III), bilirubin, uric acid, *etc.* caused an increase in absorbance at 640 nm. The interference with Fe(III) could be overcome by adding L-ascorbic acid (1.0×10^{-3} M) solution. The presence of large amounts of organic acids such as tartaric acid and citric acid caused a decrease in absorbance. The presence of amino acids, glucose, creatinine, urea, Mg(II), Ca(II), sodium chloride and phosphate ion in large amounts did not interfere. Among various drugs tested, large amounts of some organic bases such as thiamine and diphenhydramine, which form association complexes with T.Cl.Gall–Mo(VI) complex, caused an increase, but other drugs such as caffeine, cephalexin, *etc.* did not interfere. The results are summarized in Table II.

Recovery Test of HSA Added to Human Urine and Saliva The recovery of HSA added to human urine and saliva was examined by the standard procedure. In the case of human urine, urine containing the same ingredients without HSA was used as a reference. The results are given in Table III.

Derivative Spectrophotometry Derivative spectrophotometry[7] for the determination of HSA was also investigated for the purpose of enhancing the sensitivity of determination.

The conditions established for derivative spectrophotometry are as follows: the ordinary spectrum of solution A was

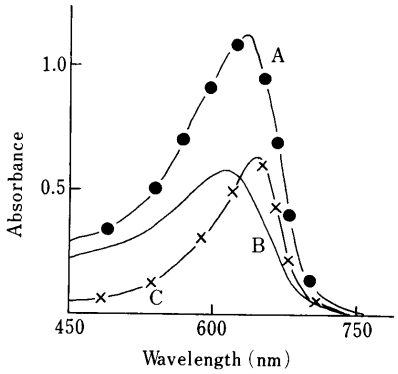

Fig. 1. Absorption Spectra Obtained by Employing the Standard Procedure

HSA taken, 100 μg; curve A, sample *vs.* water; curve B, reagent blank *vs.* water; curve C, sample *vs.* reagent blank. Sample, T.Cl.Gall–Mo(VI)–HSA solution. Reagent blank, T.Cl.Gall–Mo(VI) solution.

TABLE I. Reaction between Several Proteins and Mo(VI)–T.Cl. Gall Complex

Protein	Absorbance at 640 nm (%)[a]
HSA	0.610 (100)
γ-Globulin	0.382 (62.5)
Transferrin	0.502 (82.2)
Hemoglobin	0.574 (94.1)
Insulin	0.534 (87.5)
Xanthine oxidase	0.418 (68.5)
Immunoglobulin G	0.502 (82.5)
α-Chymotrypsinogen-A	0.372 (61.0)
Cytochrome c	0.544 (89.2)

a) Percent with respect to HSA. Protein taken, 100 μg/10 ml.

TABLE II. Influence of Foreign Substances

Substance	Added (μg/10 ml)	Absorbance at 640 nm	Recovery (%)
—	—	0.610	100.0
Fe(III) (sulfate)	5.6	0.649	106.4
Ca(II) (chloride)	4.0×10^2	0.610	100.0
$H_2PO_4^-$ (potassium)	3.4×10^3	0.610	100.0
Sodium chloride	5.8×10^3	0.610	100.0
Uric acid	3.4×10^2	0.677	111.0
Creatinine	1.1×10^3	0.610	100.0
Urea	3.0×10^4	0.610	100.0
Glucose	1.8×10^4	0.610	100.0
Glycine	1.5×10^3	0.610	100.0
Tartaric acid	7.5×10	0.550	90.2
Citric acid	1.9×10	0.569	93.3
Bilirubin	1.2×10^2	0.715	117.2
Glutathione	6.2×10^3	0.610	100.0
Caffeine	3.9×10^3	0.610	100.0
Cephalexin	1.7×10^3	0.610	100.0
Riboflavin	1.8×10^3	0.610	100.0
Thiamine	6.3×10^2	0.645	105.7
Diphenhydramine	5.1	0.702	117.2

HSA taken, 100 μg/10 ml; Mo(VI), 7.5×10^{-5} M; T. Cl. Gall, 1.5×10^{-4} M; surfactant solution, 1.0 ml/10 ml; reference, solution B (reagent blank without HSA).

TABLE III. Recovery Test of HSA Added to Human Urine (HU) and Human Saliva (HS)

Sample	Added (μg)	Found[b] (μg)	Recovery[b] (%)	R.S.D.[c] (%)
HU[a]	20	19.6	98.0	3.2
	50	50.8	101.6	2.3
HS[a]	20	19.3	96.5	2.9
	50	49.0	98.0	2.0

a) HU and HS (in 0.2 ml). *b*) Average of 5 determinations. *c*) R.S.D., Relative standard deviation.

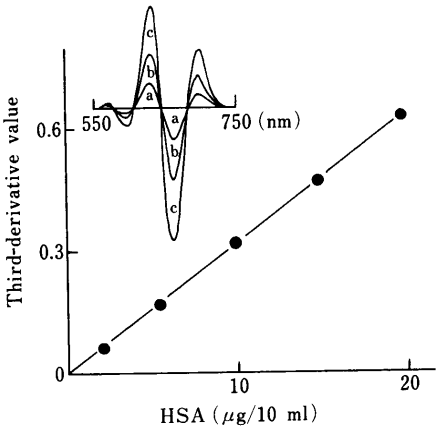

Fig. 2. Calibration Graph for HSA in Third-Derivative Spectrophotometry

HSA concentration: a, 5 μg; b, 10 μg; c, 20 μg. Reference, reagent blank without HSA.

taken from 800 to 500 nm against solution B as a reference, and the third-derivative spectrum was then recorded by using the third-order differentiation circuit of $\Delta\lambda = 31.5$ nm with a scan speed of 1500 nm^{-1}. The vertical distance from peak to trough in the third-derivative spectrum was measured.

The calibration graph prepared under the recommended conditions by plotting the third-derivative value *versus* the HSA concentration was linear and passed through the origin, as shown in Fig. 2. The sensitivity in the third-derivative spectrophotometry was about 5 times greater than that in ordinary spectrophotometry. The R.S.D. ($n=5$) was 3.8% in the case of 5 μg of HSA. HSA can easily be determined in amounts up to 20 μg in this manner.

Binding Parameters and Nature of Colored Complex Formed The binding parameters of T.Cl.Gall–Mo(VI) complex to HSA were obtained from dual double-reciprocal plots[8] by using the least-squares method. The results indicated that the number of binding sites (n) and the association constant (K) were 77.3 and 1.05×10^4 M^{-1}, respectively.

Next, the acid dissociation constants of T.Cl.Gall in the presence of nonionic surfactant were determined spectrophotometrically at an ionic strength of 0.1 at 20±0.1 °C and methanol : water = 1 : 4. T.Cl.Gall may exist 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 T.Cl.Gall and K_{a0}—K_{a4} are the acid dissociation constants. It was found that pK_{a0}, pK_{a1} and pK_{a2} in the presence of Tween 20 were −2.5, 5.67 and 6.58, respectively, and about 99.8% of T.Cl.Gall was estimated to be in the form of H_4R[9] at pH 2.8. Further, Mo(VI) at pH 2.8 exists as $(Mo_7O_{24})^{6-}$ or $(Mo_8O_{26})^{4-}$.[10] Thus, the composition of T.Cl.Gall–Mo(VI) complex at pH 2.8 may be expressed as $[Mo^{VI}(T.Cl.Gall)_2]^{n-}$. On the other hand, as the iso-electric point (pI)[11] of HSA is 4.8, HSA exists as a cationic species, $[HSA]^{m+}$ at pH 2.8.

In the second place, the color reactions between T.Cl.Gall–Mo(VI) complex and organic bases or some peptides were examined. As shown in Tables VI and V,

Table IV. Color Reaction between Some Organic Bases and Mo(VI)–T.Cl. Gall Complex

Organic base[a]	Absorbance at (λ_{max}, nm)
Streptomycin	0.496 (640)
Papaverine	0.954 (645)
Spartein	0.527 (645)
Diphenhydramine	0.174 (640)
Cetyltrimethylammonium	2.480 (675)
Quinine	1.414 (645)
Tubocurarine	1.631 (645)
Dibucaine	1.414 (650)
Thiamine	0.682 (640)
Chlorhexidine	1.562 (635)
Spermine	1.083 (635)
Spermidine	0.200 (640)

a) Organic base, 5.0×10^{-5} M.

Table V. Reaction of Several Peptides or Amino Acids

Peptide or amino acid	Added (M)	Absorbance at (λ_{max}, nm)	Amino acid sequence
Bradykinin	5.2×10^{-5}	1.600 (635)	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
Angiotensin III	5.7×10^{-5}	1.618 (635)	Arg–Val–Try–Ile–His–Pro–Phe
Tuftsin	1.2×10^{-4}	0.437 (635)	Thr–Lys–Pro–Arg
Liver-cell growth factor	1.7×10^{-4}	0.633 (635)	Gly–His–Lys
Peptide T	6.5×10^{-5}	0 (—)	Ala–Ser–Thr–Thr–Thr–Asp–Tyr–Thr
Leucine-enkephalin	9.7×10^{-5}	0.046 (630)	Tyr–Gly–Gly–Phe–Leu
Arginine	5.0×10^{-4}	0.038 (630)	
Histidine	5.0×10^{-4}	0.038 (630)	
Lysine	5.0×10^{-4}	0.034 (625)	
Glycine, tryptophan, proline, phenylalanine	5.0×10^{-4}	0 (—)	

substances which are apt to form association complexes produced the colored complexes, as well as HSA.

On the other hand, investigations of emission spectra indicated appreciable interaction between HSA and T.Cl.Gall. Further, the charge on HSA is delocalized.

From these results, it was suggested that the colored complex formed in this reaction system may be the association complex between HSA and $[Mo^{VI}(T.Cl.Gall)_2]^{n-}$ involving hydrophobic interaction and hydrogen bonding between HSA and T.Cl.Gall. However, further investigation may be necessary.

Conclusion

A highly sensitive method for the spectrophotometric determination of HSA with T.Cl.Gall–Mo(VI) complex was established. The sensitivity [0.61 absorbance corresponding to 100 μg of HSA] of the present method is about 2.5—3 times better than that of extremely sensitive methods for the determination of proteins, such as the PR–Mo(VI) [0.21] the PV–Mo(VI) [0.25] and the CAS–Al(III) [0.25] methods, and recoveries of HSA from human urine and saliva were good. Third-derivative spectrophotometry of HSA was also employed for the purpose of enhancing the sensitivity of determination. These proposed methods may be applicable to determine HSA in biological and clinical samples. In addition, the present method should also be useful for the detection and determination of some pep-

tides, (such as low-molecular-weight peptides containing basic amino acids), as well as proteins.

The colored complex formed in this reaction system may be regarded as the association complex between HSA and T.Cl.Gall–Mo(VI) complex, involving coulombic interaction between $[HSA]^{m+}$ and $[Mo^{VI}(T.Cl.Gall)_2]^{n-}$ and/or hydrophobic interaction and hydrogen bonding between HSA and T.Cl.Gall, but further investigation is necessary to confirm this.

References and Notes

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