SPECTROPHOTOMETRIC DETERMINATION OF URINARY PROTEIN WITH *o*-SULFOPHENYLFLUORONE-TITANIUM(IV) COMPLEX

YOSHIKAZU FUJITA, ITSUO MORI and MINAKO TOYODA

Osaka University of Pharmaceutical Sciences,
2-10-65, Kawai, Matsubara, Osaka 580, Japan

Abstract - A spectrophotometric method has been established for the determination of urinary protein with *o*-sulfophenylfluorone (SPF)-Ti(IV) complex. This method can be used to determine up to about 300 μg /10 ml of human serum albumin (HSA). Results obtained by the present method and the pyrogallol red-Mo(VI) method agree with each other in urinary proteins. The binding process between SPF-Ti(IV) complex and HSA is studied by determining the binding parameters, the thermodynamic parameters and so on.

Key words urinary protein, human serum albumin, spectrophotometry, *o*-sulfophenylfluorone-titanium(IV) complex

We have reported several methods for the determination of a small amount of protein by using dye(R)-metal ion(M) complex systems, such as pyrogallol red (PR)-Mo(VI) [1], pyrocatechol violet-Mo(VI) [2] and 3',4',5',6'-tetrachlorogalein-Mo(VI) [3] complexes. In a series of the studies on protein assay with (R)-(M) complex, we have confirmed that the sensitivity and selectivity of the proposed methods are dependent on (R), (M) and/or a surfactant. In a continuation of those investigations, we found that the response of *o*-sulfophenylfluorone (SPF)-Ti(IV) complex to protein was considerably sensitive and selective. An SPF belongs to 9-R-2,3,7-trihydroxy-6-fluorones and has a sulfonate group on the molecule, as shown in Fig. 1.

Fig. 1 Structure of SPF

EXPERIMENTAL

Apparatus A Shimadzu Model UV-160 recording spectrophotometer with 1.0-cm silica cells was used for both absorption spectra and absorbance measurements.

Reagents and Solutions A standard solution (200 $\mu\text{g}/\text{ml}$) of human serum albumin (HSA) was prepared by dissolving HSA (Sigma Chemical Co., essential fatty acid-free, Fraction V)₃ in water. An SPF solution (1.0×10^{-3} M) and a Ti(IV) solution (1.0×10^{-3} M) were prepared in methanol and deionized water, respectively, as described in the previous report [4]. A surfactant solution was obtained by dissolving 2.5 g of polyvinyl alcohol (PVA) and 2.0 g of Triton X-405 in water to 100 ml. A 0.1 M hydrochloric acid-sodium citrate buffer solution was used for the pH adjustments. All other reagents and materials used were of analytical grade.

RESULTS AND DISCUSSION

In order to examine the utility of a method for the determination of protein (as HSA) by using SPF-Ti(IV) complex, color reactions between SPF and (M) were studied by comparing the situations in the presence of HSA. The (M) materials used were Ti(IV), Mo(VI), W(VI), Ta(V), V(V), Sn(IV), Bi(III), Sb(III) and In(III). Ti(IV) was superior to the others with regard to

sensitivity, wide optimal pH range, influence of other substances and so on. A colored SPF-Ti(IV)-HSA complex solution without a surfactant formed a precipitate. The use of a combination of PVA and Triton X-405 was the best. Thus, the fundamental conditions for the spectrophotometric determination of urinary protein(as HSA) were established by using SPF-Ti(IV) complex in a PVA + Triton X-405 mixed micellar medium.

The recommended procedure for the determination of HSA is as follows – the following components were mixed in a 10-ml volumetric flask: 1.0 ml of the surfactant solution, 3.0 ml of the buffer solution(pH 2.3, optimal pH range between 1.7 and 2.7), 0.5 ml of a 1.0×10^{-3} M Ti(IV) solution, 0.5 ml of a 1.0×10^{-3} M SPF solution and a solution containing up to about 300 μ g of HSA. The mixture was diluted to 10 ml with water, transferred into a test tube, mixed well and kept at 45°C for 30 min. After the solution had been cooled for 10 min in water, the absorbance of the resultant solution was measured at 585 nm against a reagent blank prepared in the same way.

Figure 2 shows the absorption spectra of SPF and SPF-Ti(IV) solutions in the presence and absence of HSA under the standard procedure. The sensitivity of the present method is about 2 times greater than that of the PR-Mo(VI) and the coomassie brilliant blue(CBB) methods. The relative standard deviation was 0.45 % (n = 8) for 100 μ g of HSA.

Fig. 2 Absorption spectra of SPF-Ti(IV)-HSA, SPF-Ti(IV), SPF-HSA and SPF solutions. [HSA] = 150 μ g; [Ti(IV)] = 5.0×10^{-3} M; [SPF] = 5.0×10^{-3} M; [surfactant] = 1.0 ml of (2.5 % PVA + 2.0 % Triton X-405)/10 ml; pH = 2.2; Curve A(—●—), SPF-Ti(IV)-HSA; curve B(—○—), SPF-Ti(IV); curve C(—) SPF-HSA; curve D(---), SPF; reference, water.

The calibration curves for other proteins as well as HSA passed through the origin. Though albumin globulin ratio(A/G) in the present method was somewhat greater than that in the PR-Mo(VI) method, the deviation of reactivity among various proteins was relatively small. The results are summarized in Table 1. The influence of various ions and substances on the determination of HSA was investigated. Among substances tested, large amounts of chlorpromazine and Fe(III) caused a slight increase in the absorbance at 585 nm. A small amount of fluoride ion and large amounts of oxalate and phosphate ions caused a minor decrease in absorbance. The influence of other substances, especially citrate and tartrate ions, was extremely little in comparison with other methods. The human urine samples were measured by the present method and by the PR-Mo(VI) method(a clinical kit, micro TP-test Wako, Wako Pure Chem.) The

values determined by the present method(Y) correlated well with those obtained by the PR-Mo(VI) method(X) as shown in Fig. 3.

TABLE 1 Reaction between several proteins and SPF-Ti(IV) complex

Protein*	Absorbance at 585 nm (%)**	
HSA	0.510	(100.0)
BSA	0.515	(101.0)
γ -Globulin (Human)	0.323	(63.3)
β -Globulin (Human)	0.321	(62.9)
Hemoglobin (Human)	0.516	(101.2)
Cytochrome c (Horse)	0.529	(103.7)
Transferrin (Human)	0.484	(94.9)
Haptoglobin (Human)	0.525	(102.9)
α -Chymotrypsinogen-A (Bovine)	0.529	(103.7)
BSA, Glycated	0.542	(106.3)
BSA, Methylated	0.522	(102.4)

* Protein taken, 100 $\mu\text{g}/10\text{ ml}$ (uncorrected).

** Percent with respect to HSA.

Fig. 3 Correlation between results for urinary protein assays, as obtained by the PR-Mo(VI) method(X) and the present method(Y)

The binding parameters of Ti(IV)-SPF complex to HSA were obtained from dual double-reciprocal plots[5, 6] by using the least-squares method. The number of binding site(n) and the association constant(K) at 18°C were 54.8 and $5.80 \times 10^5\text{ M}^{-1}$, respectively. The thermodynamic parameters, obtained by the least-squares method from a van't Hoff plot, were determined to be: $\Delta G = -7.68\text{ kcal mol}^{-1}$, $\Delta H = 13.47\text{ kcal mol}^{-1}$, $\Delta S = 72.64\text{ cal mol}^{-1}\text{ K}^{-1}$. In addition, the color reactions between some polyamino acids and SPF-Ti(IV) complex were examined. The polyamino acids used were poly-L-arginine, poly-D-lysine, poly-L-aspartic acid, poly-L-alanine, poly-L-phenylalanine, poly-DL-tryptophan and poly-D-tyrosine. Poly-L-arginine and poly-D-lysine produced colored complexes at 575 nm and 580 nm, respectively, whereas other polyamino acids were scarcely colored.

Generally, hydrophobic bonding[7] 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 . On the other hand, as the iso-electric point(pI)[8] of HSA is 4.8, HSA exists as a cationic species, $[HSA]^{m+}$ at pH 2.2. The strong color response of polymers of the basic amino acids indicates that the SPF-Ti(IV)-HSA complex may be the association complex; an ionic interaction between $[HSA]^{m+}$ and SPF-Ti(IV) complex(probably SPF). Thus, the binding between SPF-Ti(IV) complex and HSA was presumed as the overall interactions ~ ionic, hydrophobic and so on, but further investigation is necessary to confirm this. In conclusion, application of the present method to biological samples should be feasible, as with the PR-Mo(VI) method.

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