Instrumental Achievements

Improved *o*-Sulfophenylfluorone-Titanium(IV) Complex Method for Total Urinary Protein Assay

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We have been working toward the determination of proteins by using dye-metal ion complex systems¹⁻⁹, such as Pyrogallol Red-molybdenum(VI) complex (denoted by PR method). As these methods are characterized by better precision, sensitivity and practicability, interest has arisen concerning the use of dye-metal ion complex methods for determining urinary protein. Among the proposed methods, *o*-sulfophenylfluorone-titanium(IV) [denoted by SPF-Ti(IV)] complex method⁶ is sensitive and precise; it experiences little interference by co-existing substances and shows good contrast with a blank solution, in comparison with other methods. However, a major problem with the assay is the variation in response to different proteins, especially the albumin-to- γ -globulin variation. Incidentally, during the course of our investigations concerning determination of protein, we have suggested that the sensitivity and selectivity of the proposed methods are dependent on dye, metal ion and/or surfactant. This report discusses how to minimize albumin-to- γ -globulin variation and at the same time how to increase sensitivity of the assay, by re-examining analytical conditions of the SPF-Ti(IV) method.⁶

Experimental*Reagents and solutions*

Standard solutions (100 μ g/ml) of human serum albumin (HSA) and human γ -globulin (γ -G) were prepared by dissolving HSA (Sigma Chemical Co., essential fatty acid-free, Fraction V) in water and γ -G (Sigma Chemical Co., purified from Cohn Fractions II, III) in 0.2% sodium chloride solution, respectively. A Ti(IV) standard solution (1.0×10^{-3} M, 1 M = 1 mol dm⁻³) was prepared from a stock solution (Wako Pure Chem., 1000 μ g/ml) by dilution with 0.1 M hydrochloric acid. A solution (1.0×10^{-3} M) of SPF, which had been synthesized according to the method described in the literature¹⁰, was prepared in 1.0×10^{-3} M methanol solution containing 1 cm³ of a 1 M sodium hydroxide

solution. A surfactant solution was obtained by dissolving 2.5 g of poly(*N*-vinylpyrrolidone) (PVP, K-90) and 1.25 g polyethylene glycol *p*-nonylphenylether (Triton N-101) in water to 100 ml. A 0.05 M citric acid-sodium citrate buffer solution was used for the pH adjustments. All other reagents and materials were of analytical grade, and were used without further purification.

Apparatus

A Shimadzu Model UV-160 recording spectrophotometer with 1.0-cm silica cells was used for conventional measurements. The pH measurements were made with a Horiba F-11 pH meter combining with a calomel electrode.

Standard procedure for total urinary protein assay

The following components were mixed in a 10-cm³ volumetric flask: 0.5 cm³ of a 1.0×10^{-3} M Ti(IV) solution, 2.0 cm³ of the surfactant solution, 3.0 cm³ of the buffer solution (pH 2.2), 0.5 cm³ of a 1.0×10^{-3} M SPF solution and a solution containing 5–200 μ g of protein. The mixture was diluted to 10 cm³ with water, transferred into a test tube, mixed well and kept at 70°C for 30 min. After the solution had been cooled for 5 min in water, the absorbance of the resultant solution was measured at 590 nm against a reagent blank prepared in the same way.

Results and Discussion*Effects of metal ions and optimal pH*

The effectiveness of different metal ions at various pH's was compared by measuring the absorbance of a SPF-metal ion-HSA solution against an SPF-metal ion solution. We re-confirmed that Ti(IV) was superior to the other metal ions tested with regard to sensitivity, width of optimal pH range and low absorbance of a reagent blank: the metal ions used were Ti(IV), molybdenum(VI), tungsten(VI), tantalum(V), vanadium(V), tin(IV), bismuth(III), antimony(III) and indium(III).

Fig. 1 Effect of metal ions at various pH's. [HSA]=50 μ g; [SPF]= 5.0×10^{-5} M; [metal ion]= 5.0×10^{-5} M; reference, each SPF-metal ion solution.

Table 1 Effect of surfactants on color intensity of HSA and γ -G

Surfactant	Absorbance at 590 nm	
	HSA	γ -G (%) ^a
PVP+Triton N-101	0.330	0.324 (98.2)
PVP+Triton X-405	0.307	0.209 (68.1)
PVP+PVA ^b	0.284	0.201 (70.8)
Triton N-101	0.308	0.196 (63.6)
Triton X-405	0.313	0.193 (61.6)
PVP (K-90)	0.321	0.232 (72.3)
PVA (<i>n</i> : 500)	0.314	0.205 (65.3)
Tween 80	0.274	0.188 (68.6)
Brij 58	0.299	0.192 (64.2)

HSA and γ -G taken, 50 μ g; SPF, 5.0×10^{-5} M; Ti(IV), 5.0×10^{-5} M; surfactant, respective suitable concentrations; pH, 2.2; reference, SPF-Ti(IV) solution.

a. Percent with respect to HSA.

b. Polyvinyl alcohol.

The results are shown in Fig. 1.

Choice of surfactant

The effect of different surfactants was examined by measuring the absorbances of an SPF-Ti(IV)-HSA and an SPF-Ti(IV)- γ -G solutions against an SPF-Ti(IV) solution. Among the surfactants tested, the use of a combination of PVP and Triton N-101 was superior to the others as regards sensitivity to HSA and γ -G and equality of color development of both. It seems likely that the advantage of the use of a combination of PVP and Triton N-101 correlates with the interaction between the surfactants and SPF-Ti(IV) complex or proteins or both. But further investigation is necessary to confirm this. The results are summarized in Table 1.

Optimization of conditions

As shown in Fig. 1, the best pH range was from 1.7 to 2.7. Further experiments were carried out at pH 2.2

Fig. 2 Absorption spectra of SPF-Ti(IV)-HSA (= γ -G) and SPF-Ti(IV) solutions. [HSA or γ -G]=200 μ g (curve 1), 100 μ g (curve 2); curve 1 and 2, SPF-Ti(IV)-HSA (= γ -G) solution; curve 3, SPF-Ti(IV) solution; reference, water.

using 3.0 cm³ of 0.05 M citric acid-sodium citrate buffer solution. The effects of the PVP and Triton N-101 concentrations were examined. The use of an approximate 0.75% PVP and 0.375% Triton X-101 in the final concentration was the most suitable in terms of sensitivity and stability of coloration.

The effect of the amount of SPF was examined, maintaining a fixed final concentration of Ti(IV) (5.0×10^{-5} M). The use of 5.0×10^{-5} M SPF in the final concentration was the best, namely the ratio of SPF to Ti(IV) was 1 : 1.

The color formation in this reaction system did not occur instantaneously at room temperature, more than 180 min being required for completion of the color reaction. The effects of incubation temperature and time were examined at 40, 50, 60, 70 and 80°C. Experiments proved that maximum and constant absorbance was obtained on heating at 70°C for 30 min, following by cooling to room temperature. The absorbance remained constant for at least 3 h after the solution had been cooled to room temperature.

The same absorbance was obtained even when the order in the addition of the reagents was varied.

Figure 2 shows the absorption spectra of SPF-Ti(IV)-HSA (=SPF-Ti(IV)- γ -G) and SPF-Ti(IV) solutions under the standard procedure. The maximum absorption of the sample solution by the improved method showed a smaller bathochromic shift than that produced by the previous method.⁶

Calibration curve

Beer's law was obeyed at 590 nm, in the range 5–200 μ g of HSA (= γ -G) in the final volume of 10 cm³. The sensitivity of the present modified SPF-Ti(IV) method is about 3 times and 1.3 times greater than the PR method¹ and the previous SPF-Ti(IV) method⁶, respectively. The difference of the reactivity of HSA and γ -G was small by comparison to values for other

Table 2 Effect of foreign substances on assay of HSA

Substance	Added/ μg	Recovery, %
Mg ²⁺	2.4×10^3	100.0
Ca ²⁺	4.0×10^3	100.0
Fe ³⁺	2.8×10^2	105.2
Cu ²⁺	6.5×10^2	100.0
NaCl	5.8×10^3	100.0
KH ₂ PO ₄	2.7×10^3	96.3
NaF	8.4×10	98.1
Urea	6.0×10^3	100.0
Glycine	7.5×10^3	100.0
Taurine	1.3×10^3	100.0
Glucose	3.6×10^3	100.0
Oxalic acid	1.8×10^2	97.3
Citric acid	3.8×10^3	100.0
Tartaric acid	3.0×10^3	100.0
Ascorbic acid	1.8×10^3	100.0
Bilirubin	5.8×10^3	100.0
Uric acid	8.4×10^2	100.0
Allantoin	2.1×10^3	100.0
Creatinine	2.3×10^3	100.0
Hippuric acid	1.8×10^3	100.0
Sodium oleate	2.0×10^2	100.0
Caffeine	3.9×10^3	100.0

HSA taken, 50 μg ; SPF, 5.0×10^{-5} M; Ti(IV), 5.0×10^{-5} M; surfactant, 3.0 $\text{cm}^3/10 \text{ cm}^3$; pH, 2.2; reference, SPF-Ti(IV) solution.

methods.^{1-5,7-9} The relative standard deviation is 0.17% ($n=5$) for 50 μg of HSA. There was a remarkable rise in the precision.

Effects of foreign substances

For an assessment of its usefulness in urinary protein assays, the influence of various foreign substances on the determination of HSA was examined. Among foreign substances, the presence of iron(III) ion resulted in a slight increase in measurement. A small amount of fluoride ion and large amounts of phosphate and oxalate ions caused minor decreases in absorbance. Absorbance values were hardly affected at their usual concentrations in the sample solution. Other substances exerted practically no effect on absorbance. The results are summarized in Table 2.

Correlation with the PR method

The present method and the PR method (commercially available total protein assay kit, micro TP-test Wako, Wako Pure Chem.) were compared for human urine samples. A regression line was estimated using the least squares method. The present method correlated well with the PR method ($r=0.956$; $y=1.217x-8.264$; $n=30$), as shown in Fig. 3. The value ($\bar{y}=91.85$) determined by the present method was somewhat large in comparison with that ($\bar{x}=82.52$) obtained by the PR method. The PR method is reported to be more sensitive to HSA than γ -G.¹¹⁻¹⁴ Compensation for differences in sensitivity is achieved by this improved SPF-Ti(IV) method.

Fig. 3 Comparison of the present method and the PR method for urinary protein assay in humans.

In conclusion, the advantages of this procedure developed for proteins can be summarized as follows:

- (1) This method achieved similar sensitivities to HSA and γ -G.
- (2) This method was more sensitive than the previous SPF-Ti(IV) method.⁶
- (3) There was a remarkable improvement in the precision by this method.

Though the study of the response of other proteins, for example Tamm-Horsfall protein, is necessary, the improved SPF-Ti(IV) method will be available for total urinary protein assays.

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