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Microdetermination of Residual Protein in Penicillin by Resonance Light Scattering Technique with *m*-Nitrophenylfluorone-Mo(VI) Complex

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Abstract: Based on the enhancement of resonance light scattering (RLS) of *m*-nitrophenylfluorone -Mo(VI) complex by protein, a novel method for protein microdetermination in Tween 20 microemulsion has been developed. Under optimum condition, the linear ranges of bovine serum albumin are 0 ~ 0.03 μg·mL^{-1} with detection limits of 3.10 ng·mL^{-1}. Most of amino acids and metal ions do not interfere. The method can be applied to determination of residual protein in penicillin Na salt and penicillin G potassium salt samples with satisfactory results.

Keywords: Protein, microemulsion, *m*-NPF-Mo(VI) complex, penicillin, resonance light scattering.

Use of penicillin may cause allergic sensitivity in many individuals. The content of residual proteins in penicillin may be the antigenicity substances that may cause allergic reaction in case of excess. On the other hand, an excess of residual protein leads to many problems in technology such as high viscosity, difficulties in filteration, a lot of foam, time-consuming *etc*. And the yield of penicillin is affected consequently. Therefore, content of residual protein in penicillin must be controlled and it is necessary to seek accurate method for quantitative determination of residual protein in process of penicillin production.

Since Pasternack *et al*.[1] established resonance light scattering (RLS) technique to investigate the aggregation of porphyrins on DNA, RLS has become a new interesting method because it can be performed by an ordinary fluorescence spectrometer and the detection limit of proteins can be quite low. This method has been widely used in inorganic ions, biomacromolecule determination and so on[2,3]. In Tween 20 micro-emulsion, the reaction between *m*-NPF-Mo(VI) complex and protein has never been reported by RLS method. As indicated by our previous study[4,5], microemulsion has increased the sensitivity of many systems. This assay is much more sensitive than most reported methods (**Table 1**). This method also has the advantages of selectivity and rapidity. Especially, it can solve the problems about ultra trace amounts of residual protein detection in penicillin and will become a valuable tool for the guidance to penicillin industrial production.

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Table 1 Comparison of resonance light scattering methods for protein determination

Reagent	pH	λ (nm)	Linear range ($\mu g \cdot mL^{-1}$)	Detection limit($ng \cdot mL^{-1}$)	Determination samples
Thorin[6]	3.50~3.95	345	1.8~14.7	52.0	Human plasma
Fuchsine acid[7]	4.10	277	0~3.8	0.47	Human urine
Resorcinol yellow[8]	2.35	340	0.02~4.0	10.4	Human plasma
Orange G[9]	0.6~2.0	548	0~5.0	2.6	Synthetic sample
Arsenazo-DBC–Al^{3+} [10]	5.60	410	2.5~50	123.4	Human serum
4-azochromotropic acid phenylfluorone[11]	0.5~1.8	337	0.2~4.0	68	Human serum, Urine
Pyrocatechol violet[12]	1.80	399	0~8.0	0.052	Serum, Urine, Saliva

Experimental

Intensity and spectra of RLS were made with a Perkin-Elmer Model LS-55 luminescence spectrometer using 1.0 cm quartz cells. UV-3101PC was employed in absorption spectra recordings. A Model PHS-3B pH meter was employed for pH measurements.

The concentrations of bovine serum albumin (BSA), human serum albumin (HSA), lysozyme (Lys), γ-globulin (γ-G) and pepsin (Pep) were 0.5 $\mu g \cdot mL^{-1}$. Tween 20 microemulsion was prepared with Tween 20 : *n*-butanol : *n*-heptane : water =5.0 : 3.3 : 0.8 : 90.9 (mass ratio). *m*-NPF ethanol solution: 1.5×10^{-5} $mol \cdot L^{-1}$. The Mo(VI) working solutions were made by suitable dilution of stock solution as required. pH 2.80 buffer was used to control the acidity of tested solution. Unless otherwise mentioned, all chemicals were of analytical grade and doubly distilled water was used throughout this experiment.

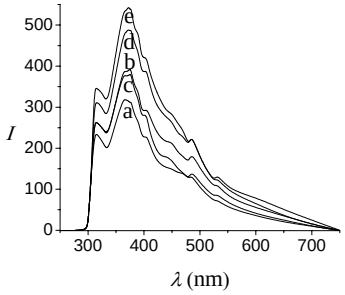
In a 10 mL color comparison tube, 1.0 mL pH 2.80 buffer, 50 μL of Tween 20 microemulsion, 0.3 mL of 1.5×10^{-5} $mol \cdot L^{-1}$ *m*-NPF ethanolic solution, 0.3 mL of 1.5×10^{-5} $mol \cdot L^{-1}$ Mo(VI) and an appropriate volume of protein or sample working solution were added in turn. Then the mixture was diluted to 10 mL with water and kept static for 20 minutes at room temperature. Finally, the RLS spectra were measured by scanning synchronously at the same excitation and emission wavelengths. The intensity of RLS against the blank was obtained with the excitation and emission wavelengths at 363 nm. Both excitation and emission slits were 5 nm.

Results and Discussion

From RLS spectra (**Figure 1**), it can be seen that the intensity of *m*-NPF RLS is enhanced to a certain degree by protein. And if suitable amounts of Mo(VI) was added into the system, the RLS will be greatly improved due to the formation of ternary system of *m*-NPF-Mo(VI)-BSA. So to the tested system was added some amount of Mo(IV). 363 nm was chosen as excitation and emission wavelengths of RLS spectrum.

The RLS intensity of the system was significantly affected by acidity. It reached the maximum value and remained constant in the pH range of 2.56~3.05. So the optimum pH was 2.80.

Figure 1 The RLS spectra



Conditions: a): *m*-NPF, b): *m*-NPF-BSA(0.005 μg·mL^{-1}), c): *m*-NPF-Mo(VI) d): *m*-NPF-Mo (VI)-BSA (0.005 μg·mL^{-1}), e): *m*-NPF-Mo(VI)-BSA(0.015 μg·mL^{-1}); *m*-NPF: 4.5×10^{-7}mol·L^{-1}; Mo(VI): 4.5×10^{-7} mol·L^{-1}.

In the experiment, Tween 20, SDS and CTAB microemulsion and each corresponding micelle solution were chosen for investigating the effect of medium on the intensity. Each microemulsion was more sensible than its corresponding micelle. For microemulsion medium, the effect of Tween 20 microemulsion was the most remarkable. And the suitable amount of microemulsion was 50 μL.

The influence of molar ratio of *m*-NPF and Mo(VI) was also tested. The results indicated that 1 : 1 ratio of *m*-NPF to Mo(VI) was suitable for the binding reaction of BSA. At the same time, maximum enhancement occurred when the concentration of *m*-NPF-Mo(VI) complex was 4.5×10^{-7} mol·L^{-1}.

The addition order of the reagents had a substantial effect on the enhanced RLS intensity. In this work, the addition order of the reagents was selected as follows: Buffer solution-Tween 20 microemulsion-*m*-NPF-Mo(VI)-protein.

The tests showed that the RLS intensity reached a maximum 15 min after all the reagents had been added and remained stable for about 24 h. In this study, 20 min was set as the standard for measurements.

The influence of ionic strength on the RLS intensity was studied by the addition of NaCl. The intensity was stable when the concentration of NaCl was lower than 0.1 mol·L^{-1} and decreased when it was higher than 0.1 mol·L^{-1}.

The method was applied to several proteins-BSA, HSA, Lys, *γ*-G and Pep. The results are presented in **Table 2**. Different proteins have different isoelectric points. The weight, size and shape of molecules are also different. So the RLS signals for various proteins are different. Pep could not react with *m*-NPF-Mo(VI) because Pep existed in anion form at pH 2.80 and there was electrostatic repulsion between them.

Under the recommended conditions, the effects of a number of foreign substances including metal ions, amino acids and glucose were examined. The results showed that amino acids, glucose and metal ions (except for Cu^{2+}, Hg^{2+}) did not interfere with this assay.

Table 2 Analytical parameters for proteins

Protein	Isoelectric point pI	Regression equation (ρ: μg·mL^{-1})	Linear range (ng·mL^{-1})	r	Detection limit (ng·mL^{-1})
BSA	4.8~4.9	ΔI=68.86+6402.00ρ	0~30	0.998	3.10
HSA	4.7	ΔI=69.44+6470.80ρ	0~30	0.998	3.16
γ–G	5.8~6.6	ΔI=71.85+6307.26ρ	0~30	0.998	3.14
Lys	11.0~11.2	ΔI=123.52+6093.09ρ	0~30	0.999	3.25
Pep	1.0	No RLS	------	------	------

Table 3 The results for the determination of protein in penicillin

Sample[a]	Found (μg·g^{-1})					Mean[b] (μg·g^{-1})	RSD (%)	Recovery (%)
A_1	157.3	168.1	160.8	149.8	155.7	158.3±8.4	4.26	104
A_2	162.7	166.3	170.1	159.5	168.9	165.5±5.5	2.65	98.5
B_1	15.33	15.69	14.97	15.12	16.20	15.46±0.61	3.19	94.8
B_2	14.83	15.16	14.33	15.57	14.29	14.84±0.68	3.69	97.3

[a] A_1 and A_2 were penicillin G potassium salt samples; B_1 and B_2 were penicillin Na salt samples.
[b] Results expressed as: $X \pm st/\sqrt{n}$ (n=5) where X is the mean of n observations of x, s is the standard deviation, t is distribution value chosen for the desired confidence level. Theoretical values at 95% confidence limit: t=2.78.

Sample determination

Penicillin Na salt and penicillin G potassium salt were analyzed and the results were given in **Table 3**. It could be seen that the satisfactory results were obtained.

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