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Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 232–238
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Rapid determination of ultra trace amounts of residual protein in penicillin based on its enhancement effects of Rayleigh light scattering on trimethoxyphenlyfluorone (TM-PF)–Mo(VI) complex

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Received 18 August 2004; received in revised form 23 December 2004; accepted 24 December 2004
Available online 12 February 2005

Abstract

This is a report on the ultra trace amount determination of protein in penicillin based on the interaction with trimethoxyphenlyfluorone (TM-PF)–molybdenum (Mo)(VI) complex in the presence of Triton X-100 microemulsion by Rayleigh light scattering (RLS). At pH 2.70, the weak RLS of TM-PF–Mo(VI) complex was enhanced greatly by the addition of proteins. Based on this, the interaction of TM-PF–Mo(VI) complex with proteins was studied and a new quantitative determination method for proteins was developed. This method is extremely sensitive (the linear range of the calibration curve was 0–80 ng mL^{-1} for bovine serum albumin (BSA). The detection limit was 0.47 ng mL^{-1}), simple, steady and free from interference of coexisting substances. TM-PF–Mo(VI) complex as a spectral probe can be used to the determination of the residual protein in penicillin samples successfully.

Keywords: Protein; Rayleigh light scattering; TM-PF–Mo(VI); Penicillin; Microemulsion; Spectral probe

1. Introduction

Penicillin, a kind of antibiotics derived from the penicillium mold, is widely used in the treatment of bacterial infections in humans. But the application of penicillin is limited because it causes allergic sensitivity in many individuals, including skin reactions and allergic shock. This may be due to blue mold thiazole albumen, penicillin polymer and polymer albumen combo in its impurities, which are the antigenicity substances and give rise to allergic reaction if they are in excess. On the other hand, as far as product itself is concerned, the excess of residual protein leads to many problems such as high viscosity, a lot of foam, difficulties in filter, time-consuming and the like. The yield of penicillin will also be affected consequently. Therefore, residual protein content in penicillin must be controlled and it is considerably essential for quantitative determination of residual protein in the process of penicillin production. To our knowledge, the determination of the residual protein in penicillin using spectrophotometry method has not been reported previously, which may be due to the interaction between the probe and cardinal body-penicillium. Based on a large amount of experiments, a novel, rapid and accurate method for the determination of residual protein in penicillin by Rayleigh light scattering has been developed with trimethoxyphenlyfluorone (TM-PF)–molybdenum (Mo)(VI) complex as a spectral probe.

The quantitative assay of protein is considerably important in pharmacy, biochemistry and clinical medicine. Up to date, most of the widely used protein methods are spectrophotometeric, such as the Lowry et al. [1], Coomassie brilliant blue (CBB) [2,3], bromophenol blue [4] and bromocresol green

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doi:10.1016/j.jpba.2004.12.030

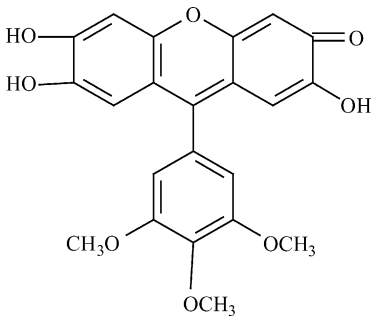

Fig. 1. Structure of TM-PF.

[5]. However, they have some limitations in terms of sensitivity, stability and simplicity. In recent years, a dye-binding method has been widely used for protein determination by spectrophotometry and fluorescence methods. Recently, sensitive methods for analysis of proteins have been developed on the basis of Rayleigh light scattering [6–16] and resonance light scattering [17–32].

The Rayleigh light scattering has been used as a tool of analytical chemistry in recent years. The present work is aimed at developing a method based on Rayleigh light scattering. According to Pasternack et al. [33], a particle, assumed to be spherical, absorbs and scatters light depending on its size, shape and refractive index relation to the surrounding medium. The intensity of RLS was calculated by Anglister to be:

$$R(90^\circ) = \left(\frac{4000\pi^2 n^2 c}{\lambda^4 N_A}\right)\left[\left(\frac{d_n}{d_c}\right)^2 + \left(\frac{d_k}{d_c}\right)\right] \quad (1)$$

where $R(90^\circ)$ is the Rayleigh ration for the incident beam and total scattered light at 90°; n is the refractive index of the medium; λ is the wavelength of incident light; N_A is the Avogadro constant, c is the molarity of the scattering particle, while d_n/d_c and d_k/d_c are the increments in real and imaginary components, respectively, of the refractive index of the solution due to the scattering particles. So when analytical conditions such as wavelength, pH and dye concentration are fixed, the RLS intensity is only proportional to the concentration of scattering particles.

Trimethoxyphenlyfluorone (TM-PF) is selected in this assay, which is one kind of acidic triphenylmethane dyes. Its structure is presented in Fig. 1. In the previous investigation, microemulsion was used in kinetic spectrophotometric method [34], flame atomic absorption spectroscopy [35] and spectrophotometric method [36–38]. The introduction of microemulsion makes the RLS intensity steadier and more sensitive. In this report, the excitation and emission wavelengths were set in 373 nm, which is corresponding to the absorption valley of the TM-PF–molybdenum (Mo)(VI) complex. At pH 2.70, in the presence of Triton X-100 microemulsion, a regression equation between the intensity and the concentration of protein has been gained by spectroscopic data. A new quantitative determination method for proteins has been developed. The method developed in this report is more sensitive than most of the reported dye and dye–metal complex probe methods and was used to the determination of ultra trace amounts of residual protein in penicillin with satisfactory results (Table 1).

2. Experimental

2.1. Reagents

Unless otherwise mentioned, all chemicals were of analytical reagents grade and doubly distilled water was used throughout this experiment.

Standard stock solutions of proteins were prepared by dissolving commercial products in doubly distilled water and stored at 0–4 °C. Proteins used in this study include bovine serum albumin (BSA), human serum albumin (HSA), γ-globulin (γ-G) and lysozyme (Lys), which were purchased from Sigma. The concentrations of their working solutions were 1.00 μg mL^{-1}.

TM-PF solution (1.50×10^{-4} mol L^{-1}) was prepared by dissolving 0.00620 g of TM-PF in ethanol containing several drops of 1:1 sulfuric acid and brought to 100 mL in a volumetric flask with pure ethanol. The working solution (1.50×10^{-5} mol L^{-1}) was made by dilution.

Triton X-100 microemulsion was prepared according to the mass ratio given below:

Triton X-100 : n-butanol : n-heptane : H_2O

$= 5.0 : 3.3 : 0.8 : 90.9$

A Mo(VI) stock solution of 0.100 mol L^{-1} was prepared by dissolving 1.4394 g of spectroscopically pure MoO_3 in 20 mL of concentrated HCl, under heating. After cooling, the solution was diluted to 100 mL and stored. The Mo(VI) working solutions were prepared from stock as needed.

A pH 2.70 Clark–Lubs buffer was prepared by mixing 0.2 mol L^{-1} $C_8H_5KO_4$ and 0.2 mol L^{-1} HCl at appropriate ratio. The accurate value of the Clark–Lubs buffer was measured by a Model PHS-3B pH meter.

2.2. Apparatus

Intensity and spectra of RLS were made with a Perkin-Elmer Model LS-55 Luminescence Spectrometer using 1.0 cm quartz cells. A Model PHS-3B pH meter was employed for pH measurements.

2.3. Method

In a 10-mL color comparison tube, 1.00 mL of Clark–Lubs buffer (pH 2.70), 0.05 mL of Triton X-100 microemulsion, 1.00 mL of 1.50×10^{-5} mol L^{-1} TM-PF solution, 1.00 mL of 1.50×10^{-5} mol L^{-1} Mo(VI) and an appropriate volume of protein or sample working solution were added in order,

Table 1
Comparison of some methods for the determination of proteins

Reagent	Determination technique	pH	λ (nm)	Linear range (μg mL^{-1})	Detection limit (ng mL^{-1})	Determination sample	Reference
Arsenazo-DBN	RLS	4.10	420	0.085–34.62	44.8	Human serum,urine samples	[8]
Arsenazo-DBS	RLS	4.10	410	0.9–31.0(BSA), 0.8–33.4(HSA)	77 (BSA), 74(HSA)	Human serum sample	[12]
3-[(2-Arsonophenyl)azo]-6-[(2,6-dibromo-4-nitrophenyl)azo]-4,5-dihydroxy-2,7-naphthalenedisulfonicacid–Al(III)	RLS	5.3–7.0	470	0.34–41.71	103	Human serum samples	[15]
Fast green FCF	RLS	4.10	279.0	0.02–2.0	1.86	Human urine serum sample	[18]
Dibromohydroxyphenylfluorone–molybdenum(VI) complex	RLS	2.8	586	0.05–0.75 (BSA, HSA)	13(BSA), 15(HSA)	Human serum sample	[22]
Pyrocatechol violet (PV)	RLS	1.80	399	0–8.0(BSA), 0–9.0(HSA)	0.052(BSA), 0.069(HSA)	Human serum, urine and saliva samples	[25]
Morin-cetyltrimethylammonium bromide	RDLS	7.20–7.60	ex:305.0 em:610.0	0.075–10.0	66.0(BSA), 23.0(HSA)	Synthetic samples	[27]
m-Carboxychloro-phosphonazo (CPA-mK)	RLS	4.10	410	0.5–35.0	104	Human serum albumin	[29]
Fuchsine acid (FSA)	RLS	4.10	277.0	0–3.8	0.47	Human urine samples	[30]
TM-PF–Mo(VI)	RLS	2.70	373	0–0.080	0.47	Penicillin	This method

then diluted to 10 mL with water and stirred thoroughly. After laying aside for 20 min at room temperature, the RLS spectra were scanned synchronously with the same wavelength of excitation and emission by spectrofluorometer with 1.0 cm quartz cells in the range of 250–800 nm at 10 nm slit-width. The enhancement of the RLS intensity was taken as the intensity difference between sample and blank under the same conditions. For RLS intensity measurements, the excitation and emission wavelengths were kept at 373 nm. ΔI_{RLS} was defined as follows: $\Delta I_{RLS} = I_{RLS} - I_{RLS_0}$, where I_{RLS} and I_{RLS_0} were the RLS intensity of the sample and blank at the wavelength of 373 nm.

3. Results and discussion

3.1. Reaction and spectra

The RLS spectra and absorption spectrum of TM-PF–Mo(VI)–BSA, TM-PF, and TM-PF–Mo(VI) compound were shown in Figs. 2 and 3. The spectra of the system of TM-PF and TM-PF–BSA were almost the same, which showed that TM-PF could not combine with BSA directly. But when Mo(VI) (1.50×10^{-5} mol L^{-1}) was added into the system of TM-PF, the RLS intensity at 373 nm was reduced, which showed a new compound was formed. When BSA was added, the peak was almost at the same wavelength, while the intensity was enhanced and the increased intensity was proportional to the concentration of BSA. Different from the resonance light scattering spectra that peaks were situated at the absorption bands envelope, the peaks of this light scattering spectra of the complex were located at the minimum absorption, while the valleys of the light scattering spectra were located at the peaks of the absorption spectra. According to the theory about resonance light scattering, the intensity increase dues to the increase of the refractive index of the solution in the optical absorption region. Usually, this increase is masked by the absorption. However, when dye aggregates are formed this effect can be strongly enhanced as the resonance light scattering intensity is proportional to the square of the scattering particle volume. So, the resonance light scattering bands are expected for large aggregates with the wavelength where the molar absorption coefficient is large. As shown in the absorption spectrum, the light scattering is not the resonance light scattering but an ordinary Rayleigh light scattering. The peaks and valleys of the light scattering spectra are produced because the complex absorbs the RLS intensity.

3.2. Effect of acidity

The RLS intensity of the system was seriously affected by acidity. It reached a maximum within pH 2.56–2.96. Therefore, pH 2.70 Clark–Lubs buffer solution was chosen for this assy. BSA appearing in cationic forms reacted with negatively charged TM-PF–Mo(VI) complex mainly by electrostatic interaction. As the pH increased, the more negative

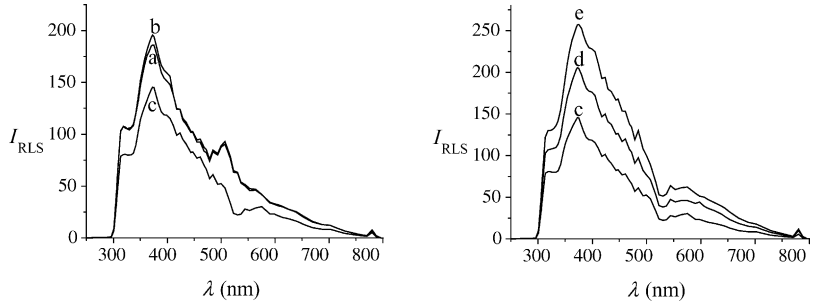

Fig. 2. RLS spectra at pH 2.70: (a) TM-PF, (b) TM-PF–BSA (0.02 μg mL^{-1}), (c) TM-PF–Mo(VI), (d) TM-PF–Mo(VI)–BSA(0.01 μg mL^{-1}), (e) TM-PF–Mo(VI)–BSA (0.02 μg mL^{-1}); concentrations: TM-PF, 1.50×10^{-6} mol L^{-1}; Mo(VI), 1.50×10^{-6} mol L^{-1}.

charge on TM-PF–Mo(VI) complex probably enhanced the interaction between BSA and the complex. On the other hand, the increase in pH also caused an increase of negative charges on the protein, which would weaken the binding of the complex with BSA. These two opposite effects of pH resulted in strongest binding and maximum RLS intensity enhancement at pH 2.70. The dosage of buffer solution was studied under the optimization acidity. A 1.00 mL pH 2.70 of Clark–Lubs buffer solution was chosen for this assay, which also could change the micro-conditions of the system.

3.3. Selection of medium

In the experiment, Triton X-100, sodium dodecyl sulfate (SDS), cetypyridinium bromide (CPB), cetytrimethyl ammonium bromide (CTAB) and emulsifier OP (OP) microemulsion and each corresponding micelle solution were chosen for investigating the effect of medium on ΔI_{RLS}.

It was found that each microemulsion was more sensible than its corresponding micelle. As far as microemulsion media were concerned, the effect of Triton X-100 microemulsion was the most remarkable. So, 0.05 mL Triton X-100 microemulsion was chosen for this assay.

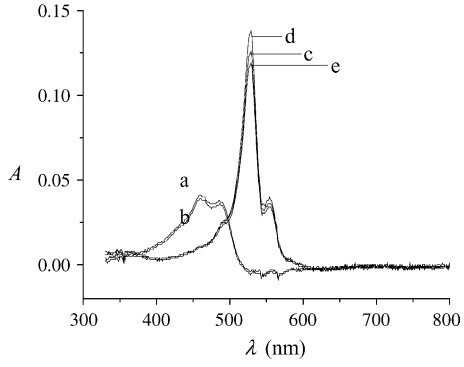

Fig. 3. Absorption spectrum at pH 2.70; (a) TM-PF, (b) TM-PF–BSA (0.01 μg mL^{-1}), (c) TM-PF–Mo(VI), (d) TM-PF–Mo(VI)–BSA (0.01 μg mL^{-1}), (e) TM-PF–Mo(VI)–BSA (0.02 μg mL^{-1}); concentrations: TM-PF, 1.50×10^{-6} mol L^{-1}; Mo(VI), 1.50×10^{-6} mol L^{-1}.

3.4. Effect of molar ratio of Mo(VI) to TM-PF

The influence of molar ratio of Mo(VI) to TM-PF on the RLS intensity enhancement of protein was examined by changing the concentration of TM-PF and retaining those of Mo(VI) and BSA at 1.50×10^{-6} mol L^{-1} and 0.02 μg mL^{-1}, respectively. When the molar ratio is 1:1, the RLS intensity of the system reaches its maximum, and remains at the maximum within 0.80–1.20. Hence, the 1:1 molar ratio of Mo(VI) to TM-PF is beneficial for the enhancement effect of protein on RLS intensity of the system. On the other hand, the RLS intensity reaches its maximum within 0.75–1.25 molar ratio of TM-PF and Mo(VI). Hence, the 1:1 molar ratio of Mo(VI) to TM-PF was selected in this assay.

In summary, the RLS intensity of the system was significantly effected by the molar ratio and the volume of TM-PF to Mo(VI), and it reached the maximum at the ratio 1:1 when the concentration of TM-PF and Mo(VI) were 1.50×10^{-6} mol L^{-1}.

3.5. Impact of the addition order of reagents

The addition sequence of reagents affects the intensity of the system. Two kinds of addition sequence were investigated. It was found that mixing buffer solution and Triton X-100 microemulsion first can give a higher RLS intensity compared to mixing BSA, TM-PF and Mo(VI) first. This can show that the electronic coupling makes TM-PF, Mo(VI) bind to protein. After being mixed with buffer (pH 2.70), TM-PF was negatively charged, so that it would be easy to bind to positively charged BSA (p*I* 4.8–4.9). If TM-PF and Mo(VI) were mixed with BSA first at neutral condition (pH ∼7), the negatively charged dye would be hard to bind with the negatively charged BSA.

3.6. Stability

Experiments showed that the RLS intensity reached its maximum 20 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.

Table 2
Calibration graphs of different proteins

Protein	Isoelectric point	Regression equation ρ ($\mu g\,mL^{-1}$)	Linear range ($ng\,mL^{-1}$)	r	Detection limit ($ng\,mL^{-1}$)
BSA	4.8–4.9	$\Delta I_{RLS} = 1.895 + 5342\rho$	0–80	0.9994	0.47
HSA	4.7	$\Delta I_{RLS} = -4.112 + 5090\rho$	0–60	0.9998	0.49
γ-G	5.8–6.6	$\Delta I_{RLS} = -4.743 + 5129\rho$	0–70	0.9986	0.49
Lys	11.0–11.2	$\Delta I_{RLS} = -6.747 + 24150\rho$	0–25	0.9997	0.10

3.7. Effect of ethanol concentration

To examine the effect of ethanol concentration, various volumes of absolute ethanol were added after the addition of Mo(VI). Additional ethanol, has little effect on the system up to 0.40 mL. Ethanol within 0.60–0.80 mL, increases the RLS intensity of system. This maybe due to the denaturalization of the BSA and the loosen structure caused by the adding of ethanol, therefore BSA can combine with more TM-PF–Mo(VI) which makes the volume of the TM-PF–Mo(VI)–BSA aggregate and the light scattering intensity increase; whilst above 0.80 mL of ethanol a rapid decrease of the RLS intensity was noted. This may due to the different refractive index of ethanol and water. The refractive indexes of ethanol and water are $n_{ethanol,20\,^{\circ}C} = 1.36049$, $n_{water,20\,^{\circ}C} = 1.33299$, respectively, therefore, adding of ethanol makes the refractive index increase. According to the theory of light scattering, the increased refractive index of the medium makes the signal decrease.

3.8. Effect of protein denaturation

BSA solution was heated by water bath for 30 and 60 min, respectively, at a temperature of 100 °C, then cooled to room temperature. Under the optimal conditions selected above, the RLS intensity was studied.

The RLS intensity of the BSA heated about 30 min increased approximately 25.54% (from 113.62 to 142.64), which suggested that the loosen structure caused by the denaturalization of BSA made more hydrophobe radical expose to the outside which made more TM-PF–Mo(VI) combine with BSA and the volume much bigger. Therefore, the RLS intensity increased.

It was found that the intensity of the BSA, heated about 60 min, fell significantly, which suggested that the secondary and the tertiary structure of BSA also contributed partly to the binding of TM-PF–Mo(VI) and BSA.

3.9. Calibration graphs and sensitivity

The calibration graph for BSA was constructed under the conditions given above, and it follows the linear regression equation in the range 0–80 $ng\,mL^{-1}$:

$$\Delta I_{RLS} = 1.895 + 5342\rho, \quad r = 0.9994$$

where r represents the coefficient of correlation. The 3σ limit of detection is 0.47 $ng\,mL^{-1}$ (here σ represents the standard deviation of 11 replicate blank measurements). Other proteins such as HSA, γ-G and Lys were also studied, and the analytical parameters were summarized in Table 2. Different proteins have different isoelectric points, at the same time, the weight, size and shape of molecules are also different. So the RLS signals for various proteins were different.

3.10. Effect of coexisting substances

Under the optimum conditions, a number of coexisting substances were examined for interference. As shown in Table 3, glucose, amino acid had relatively large tolerances. Metal ions in the range 0.100 to 0.500 $\mu g\,mL^{-1}$ causes a relative error $\leq \pm 10\%$. Therefore, no special preparation was needed to perform before sample determination.

3.11. Application to sample analysis

The method was applied to the determination of residual protein in penicillin samples including penicillin sodium salt

Table 3
Effects of coexisting substances (BSA 0.02 $\mu g\,mL^{-1}$)

Substances	Added ($\mu g\,mL^{-1}$)	Change of RLS intensity (%)	Substances	Added ($\mu g\,mL^{-1}$)	Change of RLS intensity (%)
L-Arginine	2.00	−1.4	Ca^{2+}	0.250	3.3
DL-Valine	2.00	−4.9	Zn^{2+}	0.100	2.2
L-Histidine	2.00	−0.92	Cu^{2+}	0.200	−6.1
Glycolamine	2.00	−8.6	Cr^{3+}	0.200	3.3
L-Methionine	2.00	−4.8	Al^{3+}	0.500	1.7
DL-α-Alanine	2.00	9.3	Ni^{2+}	0.500	3.4
L-Lysine	2.00	−5.3	Cd^{2+}	0.250	−3.2
L-Glutamic acid	2.00	−6.4	Pb^{2+}	0.250	2.5
L-Tyrosine	2.00	−2.0	Mn^{2+}	0.250	−0.29
Glucose	2.00	2.0			

Table 4
The results for the determination of protein in penicillin

Sample[a]	Found ($\mu g\,g^{-1}$)	Mean[b] ($\mu g\,g^{-1}$)	R.S.D. (%)	Added ($\mu g\,g^{-1}$)	Recovery (%)
A_1	147.9, 144.8, 146.9, 146.9, 150.0	147.3 ± 2.3	1.9	50.00	94.5
A_2	171.0, 174.0, 175.5, 169.4, 177.8	173.5 ± 4.2	3.4	50.00	95.8
B_1	15.3, 14.6, 14.4, 15.8, 15.4	15.1 ± 0.7	0.6	7.00	101
B_2	15.7, 16.9, 16.1, 15.8, 17.9	16.5 ± 1.1	0.9	7.00	102

[a] A_1 and A_2 were penicillin G potassium salt samples; B_1 and B_2 were penicillin sodium salt samples.
[b] Results expressed as: $\bar{x} \pm st/\sqrt{n}$ ($n = 5$) where $\bar{x}$ 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: $t = 2.78$.

and penicillin G potassium salt. A 0.2400 g of the sample was accurately weighted and diluted to the mask in a 25.00-mL calibrated flask. An appropriate amount of the solution was transferred to 10 mL color comparison tube and determined as described above. The results were displayed in Table 4.

As can be seen from Table 4, the recovery of protein was 94.5–102% and the relative standard deviations were in all instances less than 3.4%. All these presented sufficient precision and high accuracy.

4. Conclusion

A new spectral probe utilizing the Rayleigh light scattering technique has been developed based on the interaction of protein, TM-PF and Mo(VI). It is selective, accurate and stable and relative unaffected by foreign interference. Furthermore, this method is more sensitive, especially after Triton X-100 microemulsion was introduced to the system. It has been used in the determination of protein in penicillin yielding satisfactory results.

Acknowledgements

This work was supported by the Natural Science Foundations of Shandong Province(Y2004B11), Shandong science and technology program (03C05) and Doctorial Startup Foundations of Jinan University (B0405) and all the authors express their deep thanks.

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