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Determination of proteins in serum by fluorescence quenching of Rose Bengal using the stopped-flow mixing technique

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The stopped-flow mixing technique was used to develop a very fast, sensitive and accurate method for determining total proteins. The method is based on the lower fluorescence of Rose Bengal caused by binding of the dye to the proteins. The decrease in the fluorescence intensity, measured at 572 nm with excitation 555 nm, was linearly related to protein concentration from 1.3 to 24.5 μg ml^{-1}. The detection limit was 0.3 μg ml^{-1}. The method was satisfactorily applied to the determination of total proteins in different serum samples.

Introduction

Serum protein levels are specific indicators of protein deficiencies caused by malnutrition or liver disease. Increases in total serum protein levels are seen during dehydration or as the result of an increase in immunoglobulins; the latter situation is often seen in patients with monoclonal gammopathies.[1,2]

The most frequently used approaches for determining total proteins in serum are: the biuret reaction,[2] Lowry method,[3] ultraviolet absorption,[1] chemiluminescence methods[4,5] and dye binding methods.[1] The last approach is based on the ability of proteins to bind dyes such as Amido Black 10B and Coomassie Brillant Blue. This property is invoked to stain protein bands after electrophoresis;[6] the dyes have also been used in spectrophotometric methods for total proteins.[6–9]

To date, few fluorophores such as dyes and metal chelates[10,11] have been developed for the fluorimetric detection of proteins, although measuring light emission is theoretically more sensitive than colorimetric detection.

There is still a need for a sensitive yet simple and rapid method for the analysis of total proteins which is capable of being used with a large number of samples (possibly adaptable to automation). The stopped-flow mixing technique is highly suitable for this purpose as it allows sample and reagent solutions to be mixed automatically and rapidly, and also measurements to be made shortly after mixing.[12–14].

The above methods for total proteins do not completely satisfy these criteria. Therefore, we have developed a stopped-flow procedure based on the fluorescence quenching of xanthene dyes by proteins. Our results showed that Rose Bengal offered the best possibilities in the stopped-flow mixing technique. The proposed method offers significant advantages as regards sensitivity, reproducibility and low protein-to-protein variability. This method appears to be suitable for the routine determination of total proteins.

Experimental

Apparatus

An SLM-Aminco Bowman (Urbana, IL, USA) Series 2 luminescence spectrometer equipped with a 150 W xenon lamp was used. The instrument was furnished with an SLM-Aminco Bowman Milliflow stopped-flow module. This module consists of two fill syringes, two drive syringes, an observation cell (pathlength 2 mm), a stop syringe, a stop block, and exhaust and fill valve levers. Hamilton gassing syringes of 2.5 ml were used to contain the two reactant solutions. The syringes are made from controlled inner diameter borosilicate glass with precision machined Teflon plunger tips (the pistons are simultaneously driven by air-operated plungers). The solutions in the stopped-flow module can be kept at a controlled temperature by circulating water from a thermostated tank.

Reagents

All experiments were performed with analytical-reagent grade chemicals and Milli-Q purified water.

Bovine serum albumin (fraction V) (BSA), human serum albumin (HSA), human immunoglobulin G (IgG), hemoglobin, transferrin and fibrinogen were obtained from Sigma (St. Louis, MO, USA). All standard solutions were prepared by weighing suitable amounts of the reagent grade material and diluting with the stock working buffer. The protein concentrations were accurately measured spectroscopically using the ε_{280} values as follows: BSA, 6.6; HSA, 5.3; human IgG, 13.8; fibrinogen, 15.1; transferrin, 11.2 and haemoglobin, 694.4 at 280 nm.[15]

Stock standard solutions of the dyes (1.0×10^{-3} mol l^{-1}), Rose Bengal (CI 45440), fluorescein (CI 45350), dichlorofluorescein (CI 45365), Eosin B (CI 45400), Eosin Y (CI 45380), Erythrosine B (CI 45430) and floxin (CI 45410) were prepared by dissolving the appropriate amount of the acid form of the dye (Sigma) in 0.02 mol l^{-1} sodium hydroxide solution and diluting with water to 250 ml. These solutions were kept in amber-coloured bottles in the dark.

Procedure

For the preparation of the calibration graph, one of the two 5 ml reservoir syringes of the stopped-flow module was filled with a solution containing the protein (BSA, HSA, IgG, hemoglobin, transferrin or fibronogen) at a final concentration of 1.3–24.5 μg ml^{-1} and acetate buffer of pH 4.0 (0.02 mol l^{-1}). The other syringe was filled with a solution containing Rose Bengal (1.0×10^{-5} mol l^{-1}) and acetate buffer of pH 4.0 (0.02 mol l^{-1}). After the two 2 ml drive syringes had been filled, 0.04 ml of each solution was mixed at a flow rate of 20 ml s^{-1} in the mixing chamber in each run. The decrease in the fluorescence intensity with time throughout the reaction was monitored using the following instrumental conditions: λ_{ex} = 555 nm, λ_{em} = 572 nm, bandpass = 8 nm and detector voltage = 600 V. The kinetic curve was scanned up to 20 s, with a resolution of 0.1 s.

All measurements were made at 25 °C. The data were processed by a computer.

Determination of total protein in serum

Serum samples (25 μl) were diluted to 250 ml with 0.02 mol l^{-1} acetate buffer of pH 4.0. One of the reservoir syringes was filled with this solution, and total protein was determined by the recommended procedure using the standard additions method with BSA as standard.

Results and discussion

As indicated above, some organic dyes bind to protonated amine groups of amino acid residues in the polypeptide chain, and the bound species of the dye show an increase in molar absorptivity and a bathochromic shift of the absorption maximum compared with the dye alone. In the presence of fluorescent dyes, the binding to proteins generally involves a decrease in the fluorescence of the dye. This quenching effect can be used for the determination of proteins. We selected the xanthene dyes fluorescein, dichlorofluorescein, Eosin B, Eosin Y, Erythrosin B, Rose Bengal and floxin as fluorescent probes for the protein assay. The fluorescence of these dyes was quenched by the presence of BSA. The degree of quenching decreased as the degree of halogenation and the atomic number of the halogen substituent decreased. The order of quenching was fluorescein < dichlorofluorescein < Eosin B < Rose Bengal < Eosin Y < floxin. However, by placing the dye in one of the syringes of the stopped-flow module and BSA in the other, the greatest quenching effect was obtained with Rose Bengal, and this dye was selected for further studies.

Study of the BSA–Rose Bengal system

Rose Bengal binds to BSA to give a protein–dye complex which shows an absorption maximum centred around 558 nm [Fig. 1(A)]. The formation of the Rose Bengal complex is very fast and the colour of the complex is stable for a long period (at least 3 h). The increase in absorbance, measured at 558 nm, is proportional to the protein concentration but the sensitivity of the assay is not very high.

Taking into account that Rose Bengal is a strong fluorophore and BSA–Rose Bengal is a non-fluorescent complex, the fluorescence decrease of Rose Bengal in the presence of BSA provided a very sensitive assay for the protein [Fig. 1(B)]. This process is very fast so that the stopped-flow mixing technique is a very appropriate approach for developing a sensitive method for determining proteins. Fig. 2 shows the kinetic curves obtained by using different concentrations of BSA. Both the slope and amplitude of the kinetic curves were found to be directly proportional to the BSA concentration.

The initial rate of the reaction between BSA and Rose Bengal was very high, so that the time constant of the instrument did not allow this parameter to be measured with good resolution. Therefore, the amplitude signal was selected to study this system. We opted for the stopped-flow technique because it allows the rapid handling of reagents for routine determinations.

Effect of reaction variables

All concentrations given are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each result was the mean of three measurements.

The effect of pH on the system was studied by filling one syringe with the buffer solution (with and without BSA) and the other syringe with a solution of Rose Bengal in the same buffer solution. As can be seen in Fig. 3, both the fluorescence of Rose Bengal and the formation of the protein–dye complex were affected by pH. The maximum difference in the fluorescence

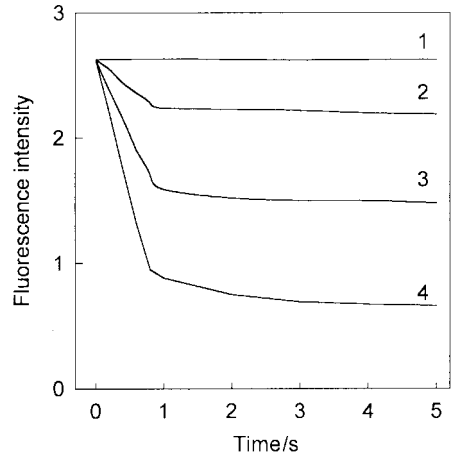

Fig. 2 Kinetic curves obtained at different concentrations of BSA: (1) 0; (2) 6.80; (3) 13.6; (4) 20.4 (μg ml^{-1}). Conditions: 1.0×10^{-5} mol l^{-1} Rose Bengal; acetate buffer of pH 4.0, 0.02 mol l^{-1}.

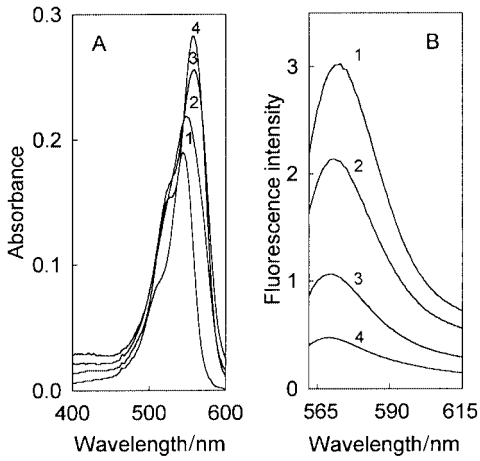

Fig. 1 (A) Absorption and (B) emision (λ_{ex} = 555 nm) of the BSA–Rose Bengal system. Rose Bengal, 1.0 x 10^{-5} mol l^{-1}; acetate buffer of pH 4.0, 0.02 mol l^{-1}. Curves 1-4, BSA concentration: (1) 0; (2) 5.44; (3) 10.88; (4) 16.32 μg ml^{-1}.

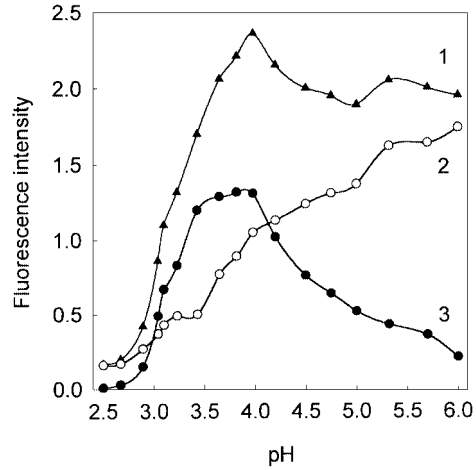

Fig. 3 Effect of pH on the BSA–Rose Bengal system. Curves (1) Rose Bengal 1.0×10^{-5} mol l^{-1}); (2) (1) plus BSA (13.6 μg ml^{-1}); (3) difference between (1) and (2).

intensity in the presence and absence of BSA was obtained at pH 3.8–4.0. Various buffer solutions (phosphate, citrate, acetate) were assayed to adjust the pH; the greatest difference in the fluorescence intensity between the sample and the blank was obtained with a 0.02 mol l^{-1} acetate buffer of pH 4.0, and this buffer was selected for further studies.

The influence of Rose Bengal concentration is shown in Fig. 4. The difference in fluorescence intensity corresponding to the sample and blank was maximum and constant at Rose Bengal concentrations higher than 1.0×10^{-5} mol l^{-1}.

Varying the temperature from 20 to 40 °C had no effect on the difference between the signals corresponding to the blank and sample.

Calibration graph

Under the instrumental and chemical operating conditions outlined above, a method to determine BSA based on its quenching effect on the fluorescence of Rose Bengal is proposed. The calibration graph was constructed with three replicates per point (three injections each). The plot of ΔI_F (obtained by subtracting the amplitude of the kinetic curve corresponding to the samples from that of the blank) against BSA concentration was linear in the range 1.3–24.5 μg ml^{-1}. The linear fit was $\Delta I_F = (-0.08 \pm 0.02) + (0.100 \pm 0.001)C$, where C is the BSA concentration in μg ml^{-1}, with a correlation coefficient of 0.9994 for $n = 12$. The limit of detection, as defined by IUPAC,[16] was 0.3 μg ml^{-1}. The precision of the method was evaluated at two BSA concentrations, 5.4 and 17.6 μg ml^{-1}, and the relative standard deviations ($n = 10$ for each level) were 0.79 and 0.20%, respectively.

Protein-to-protein variability

The likely mechanism of BSA–Rose Bengal complex formation is an electrostatic interaction between the dye anion and $-NH_3^+$ groups of protein at acidic pH. Similar charge-dependent interactions have been reported with other dyes.[1,6–11] Because the number of free $-NH_2$ groups differs from protein to protein, the decrease in the fluorescence intensity of Rose Bengal for the same concentration of different proteins in the proposed method is not the same as in any other dye binding method. However, there were no significant differences between the signals yielded by HSA, human globulin, human IgGs, transferrin, fibronogen and BSA, and so it is possible to determine total proteins in serum.

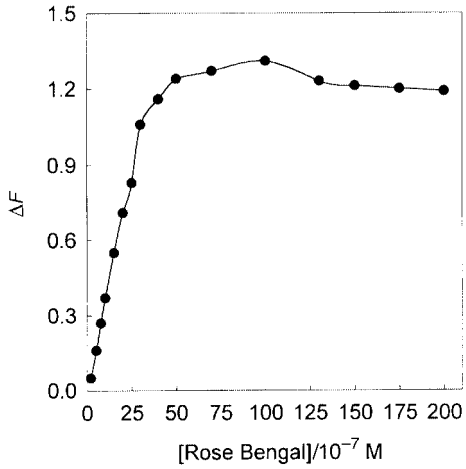

Fig. 4 Influence of Rose Bengal concentration. Conditions: 13.6 μg ml^{-1} BSA; 0.02 mol l^{-1} acetate buffer of pH 4.0. The values on the ordinate represent the difference of the amplitude of the kinetic curves corresponding to the blank and BSA.

Interferences

In order to apply the proposed method to the determination of total proteins in serum, the interference due to salts, reducing agents and neutral detergents which are commonly used to solubilize the proteins was examined. Solutions of BSA and each foreign species were mixed prior to the assay. Table 1 shows that ethanol, urea, glucose and ascorbic acid did not interfere; amino acids, except for L-Tyr, did not affect the assay. Neutral detergents were tolerated up to 0.01%, whereas cationic and anionic detergents were interferents because they hindered the interaction between BSA and Rose Bengal. Alkali metal salts and divalent metal ions did not interfere but trivalent metal ions were not tolerated; however, the tolerable level for all metal ions could be increased to 20 mmol l^{-1} by retaining them on a Dowex 50W–X8 (Na^+) (Fluka, Buchs, Switzerland) cation exchange minicolumn when the sample was passed through the column prior to filling the syringe of the stopped-flow.

Application to serum analysis

Human, bovine and rat serum samples were measured after having been diluted because the present method was too sensitive to determine proteins in the original serum samples. The samples were diluted 1000-fold with acetate buffer of pH 4.0 and the total proteins were determined by the standard additions method using BSA as standard. The results in Table 2 are in good agreement with those obtained by the biuret method.[2]

The standardization of the present method was also carried out using a suitable dilution of serum with a normal albumin-to-globulin ratio, obtained from a healthy subject, and quantified for total protein with the biuret assay. This choice was dictated by the intent to provide a mixture of proteins in the standard whose dye-binding behaviour is similar to that of proteins in the sample to be analysed. The values obtained for human serum (data not included) were not significantly different from those shown in Table 2.

Table 1 Effect of foreign substances on the determination of BSA

Substance	ΔI_F value[a]	BSA found[b]/μg ml^{-1}
None	0.61	7.0
1% ethanol	0.62	7.1
2500 μg ml^{-1} glucose	0.58	6.7
50 mM urea	0.59	6.8
100 mM NaCl	0.62	7.1
100 mM KCl	0.59	6.8
50 mM $NaNO_3$	0.58	6.7
25 mM Na_2SO_4	0.58	6.7
2 mM Mg(II)	0.59	6.8
2 mM Ca (II)	0.66	7.5
2 mM Zn (II)	0.60	6.9
2 mM Cd(II)	0.64	7.3
2 mM Ni(II)	0.64	7.3
20 mM Al(III)[c]	0.61	7.0
20 mM Cr(III)[c]	0.63	7.2
20 mM Fe(III)[c]	0.59	6.8
2 mM ascorbic acid	0.64	7.3
100 μg ml^{-1} L-Tyr	0.64	7.3
5000 μg ml^{-1} L-Arg	0.63	7.2
5000 μg ml^{-1} L-Cys	0.59	6.8
5000 μg ml^{-1} L-Glu	0.60	6.9
5000 μg ml^{-1} L-His	0.63	7.2
5000 μg ml^{-1} L-Leu	0.62	7.1
5000 μg ml^{-1} L-Lys	0.61	7.0
5000 μg ml^{-1} L-Phen	0.59	6.8

[a] Arbitrary units. [b] Average value of three measurements. [c] Passing the solution through a cation exchange minicolumn.

Conclusions

The results clearly demonstrate that the fluorescence quenching of Rose Bengal by proteins provides a means for the sensitive and reproducible determination of proteins. The stopped-flow mixing technique was used in order to allow sample and reagents to be mixed automatically and rapidly and measurements to be made shortly after mixing. The method is simple, highly sensitive and rapid. Interferences of salts, reducing substances and neutral detergents in the formation of the protein–dye complex is minimal. These interferences can be overcome by dilution.

The advantages of the present method mean that it can be applied to routine total protein determinations in serum because there is no significant difference in the response of the principal component proteins of this body fluid.

Table 2 Determination of total proteins in serum

Sample	No. of samples	Proteins found ± *s*/mg ml^{-1}	
		Proposed method	Biuret method
Bovine serum—			
Sample 1	3	38.6 ± 0.8	40.9 ± 1.0
Sample 2	3	39.1 ± 0.9	41.2 ± 1.0
Sample 3	3	38.9 ± 0.8	41.0 ± 1.2
Sample 4	4	42.5 ± 1.1	42.7 ± 1.4
Sample 5	5	47.3 ± 1.3	49.1 ± 1.3
Sample 6[a]	5	21.5 ± 0.6	21.8 ± 0.7
Sample 7[b]	5	16.1 ± 0.5	16.7 ± 0.6
Sample 8	4	36.2 ± 0.7	37.0 ± 0.9
Rat serum—			
Sample 1	3	54.7 ± 1.4	58.1 ± 1.4
Sample 2	3	57.1 ± 1.4	60.0 ± 2.0
Sample 3	3	55.7 ± 2.1	57.9 ± 1.7
Sample 4	5	60.3 ± 2.3	62.1 ± 1.9
Sample 5	5	63.5 ± 1.8	65.6 ± 2.4
Sample 6[a]	5	29.7 ± 1.2	30.8 ± 1.2
Sample 7[b]	5	21.6 ± 1.1	22.9 ± 1.3
Human serum—			
Sample 1	3	71.32 ± 2.34	73.25 ± 2.53
Sample 2	3	75.62 ± 2.68	78.04 ± 3.01
Sample 3	3	73.15 ± 2.87	75.41 ± 2.23

[a] Two fold dilution of sample 4. [b] Threefold dilution of sample 5.

Compared with the manual method based on the fluorescence quenching of Erythrosine B by BSA and other proteins,[10] the approach presented here has some advantageous features which are probably due to the use of the stopped-flow technique. For example, interferences are minimized and the precision and sampling rate are increased.

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