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A sequential injection fluorometric procedure for rapid determination of total protein in human serum

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Abstract

A sensitive procedure for the quantification of total protein bovine serum albumen (BSA) in human serum was presented with sequential injection sampling and fluorometric detection. A few microliters of sample and fluorescamine solutions were aspirated into the holding coil to facilitate the reaction of protein with fluorescamine by giving rise to a blue-green–fluorescent derivative. The derivative was afterwards excited by a 400 nm radiation from a UV radiator, and the emitted fluorescence was monitored at the wavelength of 470 nm. By loading 5.0 μl of sample and 4.0 μl of fluorescamine solution 0.075% (m/v), a linear calibration graph was obtained within 0.3–12.5 μg ml^{-1}, and a detection limit (3σ) of 0.1 μg ml^{-1} was achieved, along with a sampling frequency of 40 h^{-1} and a R.S.D. value of 2.1% at the 5.0 μg ml^{-1} levels. Protein contents in human serums were analyzed by using the present procedure, and reasonable agreements were obtained with those obtained by a documented spectrophotometric (Biuret) method.

Keywords: Protein; Sequential injection; Human serum; Fluorescamine; Fluorometry

1. Introduction

Protein is a kind of important biomaterial and the knowledge of protein concentration in aqueous medium especially in biological samples is a key reference for pathology and clinical medicine, therefore it has been kept as an interesting area of biochemical research. Analytical protocols frequently employed for protein assays including spectrophotometry [1,2], resonance light-scattering [3,4], fluorometry [5–7] as well as chemiluminescence methods [8,9]. Among these procedures, the ones with fluorometric detection have gained extensive attention due to their high sensitivities and superior tolerance to interferences or matrix components.

Generally, the commonly employed fluorescent reagents for protein assay give rise to very weak or even no self-fluorescence. While significant fluorescence could usually be obtained when they bind to proteins, attributed to either the energy transfer from proteins to the reagents or the formation of new complexes. Fluorescamine is frequently used as a fluorogenic reagent, it is intrinsically non-fluorescent but tends to yield a blue-green–fluorescent derivative after reacting with primary aliphatic amines in milliseconds, including peptides and proteins [7,10]. Excessive amount of the fluorescamine reagent is afterwards rapidly converted to a non-fluorescent product through a reaction with water [11], leaving no potential interferences for the assay and thus making it useful for protein analysis in solutions or fluids [12–14].

For biological assays, the cost of both sample and reagent are usually expensive, and the consumption of sample/reagent volume is large when employing batch mode operations. Thus, downscaling the consumption of expensive/rare sample and/or reagent is obviously one of the most effective approaches in order to reduce the analytical expenses. In addition, sample contamination is also regularly encountered which tends to cause further problems for trace level determinations. Therefore, on-line miniature systems with minimized sample and/or reagent consumption are highly demanded. At this juncture, sequential injection analysis has a great potential for on-line sample handling as well as measurements due to the simplicity and convenience with which sample manipulations, such as mixing, dilution, incubation and stop flow can be automated [15]. Furthermore, sequential injection manifold significantly econ-

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omizes sample/reagent consumption, and subsequently reduces waste production. In addition, the risk of sample contamination is effectively minimized, and labor-intensive sample pretreatment procedures are avoided. Thus, sequential injection is gaining wide applications in modern analytical chemistry especially in sample pretreatments [16–18], while miniaturized systems, i.e., the lab-on-valve mesofluidic system, offered promising approaches for minimizing sample consumption [19,20].

The aim of this communication is to provide a simple, robust, automatic and cost-effective analytical procedure for the assay of total protein in human serum with fluorescamine as the probe, by using sequential injection for sample handling and with fluorometric detection. As compared to the batch mode of the same reaction system [14], a significant improvement was achieved for the detection limit along with a remarkable reduction of the sample/reagent consumption.

2. Experimental

2.1. Reagents

All the reagents used were at least of analytical reagent grade unless specified otherwise and 18 MΩ ion-free water was used throughout.

A 1.0% (m/v) fluorescamine (Alfa Aesar, Ward Hill, MA, USA) stock solution was prepared by dissolving fluorescamine in acetonitrile (Shield Medicine Technology Co., Tianjin, China). Working solutions of different concentrations were obtained by step-wise dilution of the stock solution with acetonitrile.

Bovine serum albumen (BSA; Huamei Biological Engineering Co., Luoyang, China) stock solution was prepared by dissolving BSA in a 0.1 mol l^{-1} sodium borate (Shenyang Chemical Reagent Factory, Liaoning, China) buffer solution (pH 8.0) and stocked at 4 °C.

Human serum samples were obtained from the Hospital of Northeastern University. The samples were diluted with 0.1 mol l^{-1} sodium borate buffer (pH 8.0) before analysis.

2.2. Instrumentation

Experiments were carried out by employing a FIAlab 3000 sequential injection system (FIAlab Instruments Inc., Bellevueo, WA, USA) equipped with a 500 μl syringe (CAVRO Scientific Instruments Inc., San Jose, CA, USA) and along with a six-position selection valve. The flow manifold setup of the sequential injection system is illustrated in Fig. 1. All the fluid channels used were made from FEP Teflon tubing (i.d. = 0.5 mm; Upchurch Scientific, Oak Harbor, WA, USA). The lengths of the holding coil and the reaction coil were 150 and 15 cm, respectively, as illustrated in Fig. 1.

A PMT-FL fluorometer (OceanOptics Inc., Dunedin, FL, USA) was used to monitor the fluorescence intensity at the wavelength of 470 nm with the excitation wavelength set at 400 nm. The entire system, including the sequential injection unit, the PMT-FL fluorometer and the data acquisition system, was controlled with a personal computer by running the FIAlab software for Windows.

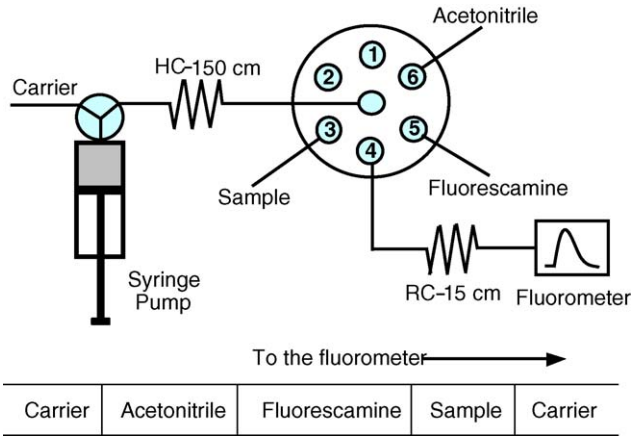

Fig. 1. Flow manifold of the sequential injection system for protein assay with detection by fluorometry.

2.3. Operating procedure

Four hundred microliters of deionized water was first aspirated into the syringe pump, which was afterwards used as carrier for delivering sample and reagent zones into the flow cell of the PMT-FL for measuring fluorescence intensity and/or subsequently directed to waste. Consequently, 5.0 μl of acetonitrile, 4.0 μl of fluorescamine and 5.0 μl of BSA sample solutions were sequentially aspirated into the holding coil at a flow rate of 5.0 μl s^{-1}. Thereafter, the syringe pump is immediately set to propel the stacked reagent/sample zones forward into the flow cell at a flow rate of 30 μl s^{-1}. Considering that the fluorescamine is prone to hydrolysis, the aforementioned small acetonitrile zone was therefore aspirated and inserted between the fluorescamine and the carrier zone, in order to prevent them from contacting [11]. The fluorescence intensity was monitored with the PMT-FL fluorometer at 470 nm. A calibration graph was derived on the basis of the relationship between the net enhancement of fluorescence intensity ΔI and the concentration of BSA.

3. Results and discussion

3.1. The fluorescence spectra of fluorescamine–protein derivative

A blue-green–fluorescent derivative is formed when fluorescamine reacts with proteins [7,10]. The fluorescence spectra of fluorescamine–BSA derivative are illustrated in Fig. 2, showing that the maximum wavelengths of excitation and emission in this case are 400 and 470 nm, respectively. These wavelengths were thus selected throughout the present study. Fig. 2 also demonstrates that a significant enhancement of fluorescence intensity was observed with an increase of the protein concentration, which established the basis of protein quantification.

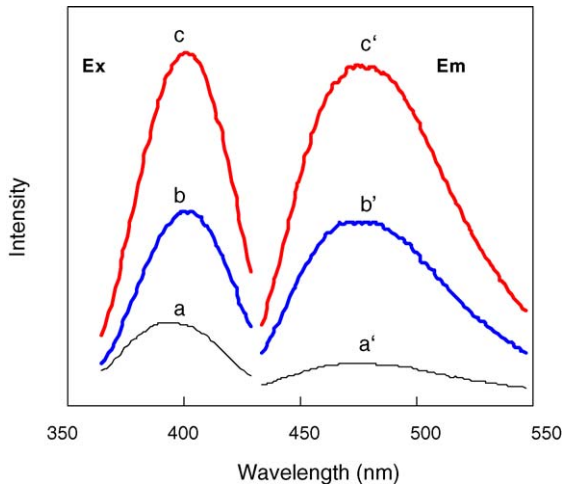

Fig. 2. The fluorescence spectra of fluorescamine in the absence/presence of BSA. BSA concentration ($\mu g\,ml^{-1}$): a (a′), 0.0; b (b′), 5.0; c (c′), 10.0.

3.2. The effect of buffer pH

It had been indicated by Udenfriend et al. [7] that the most favorable conditions for primary aliphatic amines to react with fluorescamine or for protein assays via the reaction with fluorescamine including the adoption of a pH $\geq$ 7, along with sodium borate buffer to maintain a constant pH of the reaction medium [21]. In the present system, sodium borate solution was thus selected as buffer without further investigation. The effect of the buffer pH was studied and the observed results are shown in Fig. 3. It is obvious that the net enhancement of the fluorescence intensity reached maximum at a pH 8, while afterwards a plateau was maintained until pH 9 (no higher pH values were tested). For further experiments, sodium borate buffer of pH 8.0 was employed.

3.3. Effect of fluorescamine concentration and volume

The effect of fluorescamine concentration was studied in the range of 0.025–0.15% (m/v) with the concentration and volume of BSA fixed at 5.0 $\mu g\,ml^{-1}$ and 5.0 μl, respectively. The experimental results showed that a steady increment of the net enhancement of fluorescence intensity was recorded with the increase of fluorescamine concentration up to 0.075%, which afterwards kept virtually unchanged with further increase of fluorescamine concentration until 0.15%. The reason for this observation might be attributed to the fact that at a lower concentration of fluorescamine, e.g., less than 0.075% (m/v), the molar ratio of fluorescamine/protein did not reach the stoichiometric point. Therefore, within the range of 0.025–0.075%, an increase of fluorescamine concentration gave rise to a significant improvement of the net enhancement of fluorescence intensity. On the other hand, once the stoichiometric point was reached, a further increase of the fluorescamine concentration contributed virtually nothing to the reaction, and a plateau for the net fluorescence intensity was observed. In the present case, a fluorescamine concentration of 0.075% was adopted for the ensuing experiments.

By fixing the fluorescamine concentration, the effect of its sampling volume was also investigated and the obtained results are illustrated in Fig. 4. It is obvious that the recorded net fluorescence intensity reached a maximum with a sampling volume of 4.0 μl, while afterwards the curve was leveled off with further increase of its sampling volume. This phenomenon could apparently be explained by the programmed flow pattern in the sequential injection system, that is, the two adjacent fluidic zones inside the flow channel tends to disperse into each other and thus promoted their reaction. Taken into account the narrow-bore of the channel, effective dispersion or diffusion between the adjacent fluid zones can only penetrate a certain length within a limited flow or reaction time, which consequently restricted the length of the fluid zones get involved into the actual reaction, and the contribution of excessive amount of either zone is therefore neglected. It is conceivable that within a tube of 0.5 mm inner diameter, a sample/reagent zone of 4.0 μl is appropriate to disperse into the adjacent one. For further experiments, a fluo-

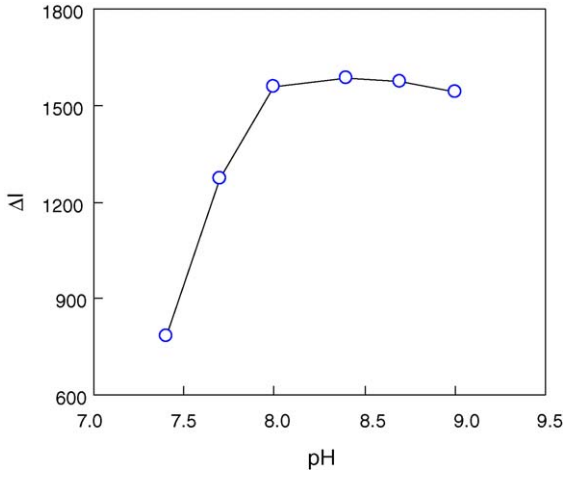

Fig. 3. The effect of pH on the net enhancement of fluorescence intensity. The concentration and volume of fluorescamine: 0.075% (m/v) and 4.0 μl; the concentration and volume of BSA: 5.0 $\mu g\,ml^{-1}$ and 5 μl; flow rate: 30 $\mu l\,s^{-1}$.

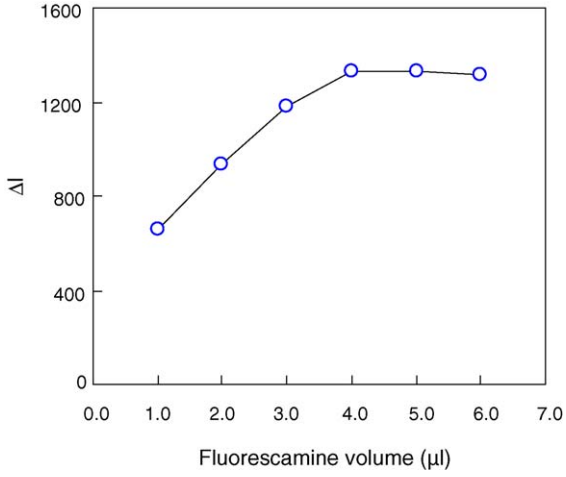

Fig. 4. The effect of fluorescamine sampling volume on the net enhancement of fluorescence intensity. The concentration of fluorescamine: 0.075% (m/v); the concentration and volume of BSA: 5.0 $\mu g\,ml^{-1}$ and 5 μl; flow rate: 30 $\mu l\,s^{-1}$.

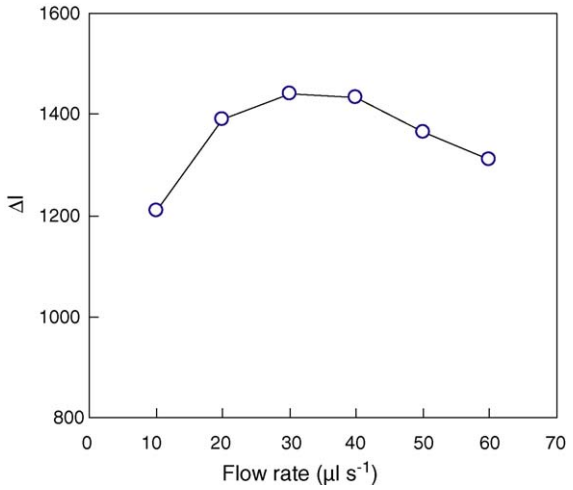

Fig. 5. The effect of flow rate on the net enhancement of fluorescence intensity. The concentration and volume of fluorescamine: 0.075% (m/v) and 4.0 μl; the concentration and volume of BSA: 5.0 μg ml^{-1} and 5 μl.

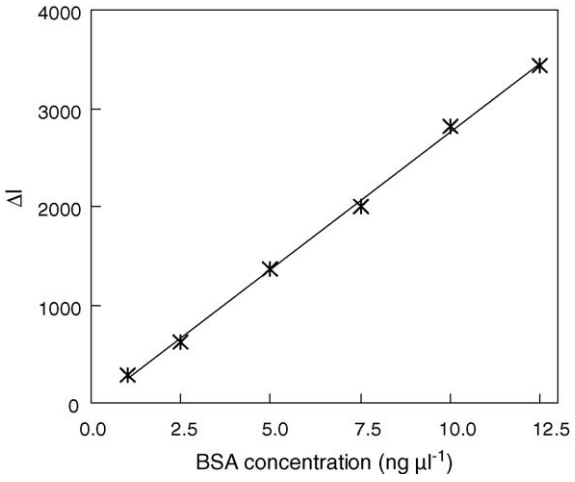

Fig. 6. The calibration curve for BSA determintion. The concentration and volume of fluorescamine: 0.075% (m/v) and 4.0 μl; the volume of BSA: 5 μl (0.3–12.5 μg ml^{-1}).

rescamine volume of 4.0 μl was employed along with a sample volume of 5.0 μl.

3.4. The flow rate and stopped flow time

Fig. 5 illustrates the trends of the net enhancement of fluorescence intensity with changes of dispensing flow rates of the various zones previously aspirated into the holding coil, within the range of 10–60 μl s^{-1}. It is obvious that a steady improvement of the recorded signal was observed with the increase of flow rates up to 30 μl s^{-1}, while afterwards a decline was encountered when even higher flow rates were employed. As discussed previously, for the adjacent sample/reagent zones in the flow channel, the extent of dispersion is among the most important issues governing the reaction and thus the sensitivity of detection. When lower flow rates were adopted, although longer reaction time gave rise to more products, while at the same time a larger dispersion of the sample zone into the carrier stream caused a decrease of the concentration of the detectable product. Similarly, when too high a flow rate was employed, the reaction time was not sufficient and the concentration of the product was limited. Thus, only an appropriate flow rate ensures optimal sensitivity. For the ensuing experiments, a flow rate of 30 μl s^{-1} was thus selected.

The effect of the reaction time was further investigated by using stopped flow. The experiments indicated that no significant gain was achieved by employing a stopped flow time up to 15 s. This indicated that the fluorescence of the fluorescamine–BSA derivative is relatively stable after the reaction has been completed, and this observation was in accordance with what had been reported [14]. Therefore, no stopped flow was employed for further experiments. Under these conditions, a sampling frequency of 40 h^{-1} was obtained.

3.5. Interfering effects

In order to evaluate the applicability of the present procedure for direct quantification of protein in body fluids, the potential effects of some species frequently encountered in biological samples were thus tested by analyzing a standard solution containing 5.0 μg ml^{-1} BSA and various amounts of coexisting species. Within a ±5% error range, no interferences were encountered in the presence of the following species: ethylenediamine tetraacetic acid (EDTA) (50 μmol l^{-1}), urea (50 mmol l^{-1}), cetyltrimethylammonium bromide (CTAB) (10 μmol l^{-1}), sodium dodecylsulfonate (SDS) (1.0 mmol l^{-1}), Triton X-100 (0.01%, m/v), guanidine hydrochloride (GuHCl) (0.04 mol l^{-1}), glucose (4.0 mmol l^{-1}), Fe^{3+} (1.0 μmol l^{-1}), Ca^{2+} (20 mmol l^{-1}), Mg^{2+} (40 mmol l^{-1}) and $H_2PO_4^-$ (10 mmol l^{-1}). For protein assay in body fluids, appropriate dilutions are always necessary and thus the concentration levels of these species in the final solutions are well-controlled within the tolerance limit, and no further treatment is required.

3.6. Performances of the present procedure

Under the aforementioned optimzed experimental conditions, the obtained calibration curve is illustrated in Fig. 6 and the characteristic performance data are summarized in Table 1.

Table 1
The characteristic performance data of the sequential injection fluorometric procedure for protein assay

SI mode (this procedure)	
Linear calibration range	0.3–12.5 μg ml^{-1}
Regression equation	$279.5C_{BSA}$ (μg ml^{-1}) − 38.78
Sampling frequency	40 h^{-1}
BSA consumption	5.0 μl (0.3–12.5 μg ml^{-1})
Fluorescamine consumption	4.0 μl (0.075%, m/v)
R.S.D. (5.0 μg ml^{-1}, n = 11)	2.1%
Detection limit	0.1 μg ml^{-1}
Batch mode of the same reaction system [14]	
BSA consumption	50 μl
Fluorescamine consumption	50 μl (0.03%, m/v)
Detection limit	10.0 μg ml^{-1}

Table 2
Analysis results of protein in human serums

Sample	Biuret method ($g\,l^{-1}$)	This method ($g\,l^{-1}$)	R.S.D. (%)
1	81.4	78.5	1.32
2	78.3	75.7	2.34
3	73.9	74.9	0.81
4	78.9	80.0	3.20
5	77.5	80.4	1.49

As a comparison to the documented batch mode procedure of the same reaction system, the sequential injection method provides a much better detection limit with an improvement of two orders of magnitude. Furthermore, sample and reagent consumption was also minimized, i.e., a reduction of one order of magnitude was achieved. The faster sampling frequency is, of course, an additional figure of merit of this procedure.

3.7. Sample analysis

The practical applicability of the proposed procedure was demonstrated and validated by analyzing protein contents in human serums. The samples were first diluted with sodium borate buffer, which were afterwards analyzed by using both the present method and the documented spectrophotometric procedure [22]. The obtained results are given in Table 2. It is obvious that reasonable agreements were achieved between the proposed procedure and the documented one.

4. Conclusions

The sequential injection procedure for protein assay offers the advantages of low detection limit, that is, two orders of magnitude improvement as compared to the documented spectrophotometric method. The sample and reagent consumption was also minimized with one order of magnitude reduction. Furthermore, the sampling frequency of this procedure provides one more virtue with respect to the batch mode protocol. In addition, the entire sampling process as well as the detection of the formed product were performed in the closed channel system, and thus avoided the potential of cross-contamination of samples.

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