

Adaptation of the Bicinchoninic Acid Protein Assay to a Continuous-Flow Autoanalyzer

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We have adapted the Pierce bicinchoninic acid protein assay method to a Technicon Auto-Analyzer. The automated assay's relative response to different proteins is comparable to the published manual procedure. The assay sensitivity can be easily increased or decreased by changing the reagent concentration, sample flow rate, or reaction time and temperature to accommodate various sample types and dilutions. At a sampling rate of 30 per hour, the within-day standard deviation ranged from 0.06 to 2.99% and the sample-to-sample carryover was less than 1%. The reagent cost was decreased to about one-half that of the commercially available kits by use of a lower concentration of the less purified bicinchoninic free acid rather than the more expensive sodium salt.

1. INTRODUCTION

The protein assay method using bicinchoninic acid (4,4'-dicarboxy-2,2'-biquinoline, BCA) was originally developed by Smith et al. in 1985 [1]. The assay is based upon the biuret reaction where Cu^{2+} is reduced by protein to Cu^{1+} . Complexation of the

Cu^{1+} by BCA leads to development of an intense purple color with an absorbance maximum at 562 nm. The assay is commercially available as a kit from Pierce Chemical Company (Rockford, IL). The BCA protein assay has about the same sensitivity and relative response to different proteins as the Lowry assay, but uses a much simpler procedure and has a more stable color and much less interference from detergents, denaturing agents, and biological buffers [1, 2]. The major known interfering substances in the BCA protein assay are reducing sugars [1], lipids [3], uric acid, and creatinine [4].

The BCA protein assay has been adapted to microtiter plates [5-7] that can be automated by the use of commercially available microtiter plate dilutor/dispensers and microtiter plate readers. The method has also been adapted to flow injection analyzers [8, 9] as well as to postcolumn reaction detection systems for use with high-performance liquid chromatography [10].

Although the published automated and semiautomated BCA protein assay methods are suitable for their intended applications, to our knowledge, the method has not previously been adapted for use with continuous-flow, segmented-stream chemistry analyzers of the type in use in many laboratories. In this article, we report the development and characterization of such a continuous-flow protein assay using the BCA chemistry. This automated method has a relative protein response comparable to the manual method, a lower reagent cost per sample, and a sensitivity that can be adjusted to suit the

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determination of protein in a variety of sample types and dilutions.

2. MATERIALS AND METHODS

2.1 Materials

The bicinchoninic free acid, bovine serum albumin (fraction V), chymotrypsinogen, insulin, ribonuclease A, and sodium lauryl sulfate were obtained from Sigma Chemical Company (St. Louis, MO). The gelatin protein standard was a commercial food grade product (Knox). All other chemicals used were reagent grade (J. T. Baker, Philipsburgh, NJ, or Mallinckrodt, Paris, KY).

2.2 Reagent A

This solution was similar to the Pierce Reagent A, except the concentrations were approximately four times lower and had the following composition: 5.84 mmol/L BCA, 40.8 mmol/L Na_2CO_3 , 28.6 mmol/L NaHCO_3 , 1.76 mmol/L sodium tartrate, adjusted to pH 11.25 with 10 mol/L NaOH.

2.3 Reagent B

A dilute version of the Pierce Reagent B had the following composition: 10 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 50 g/L sodium lauryl sulfate (w/v). The sodium lauryl sulfate was added to improve the bubble pattern in the segmented stream.

2.4 Working Reagent

One volume of Reagent B was added, with stirring, to 50 vol of Reagent A and stirring was continued until a clear solution was obtained (5 to 20 min).

2.5 Reagent Stability

Reagents A and B were stable indefinitely at room temperature. The working reagent was stable for at

least 2 days at room temperature, but could be used for longer periods if stored overnight at 4°C. When used for more than 2 days, the working reagent caused a slight increase in the baseline absorbance, but the slope of the standard curve did not change significantly for at least 7 days.

2.6 Sample Preparation

Human erythrocyte and plasma samples (stored at -70°C) had been obtained primarily for enzymatic analyses and were accordingly diluted 1:40 or 1:20, respectively, with 50 mmol/L Tris · HCl, 0.1 mmol/L disodium ethylenediaminetetraacetic acid, 5 g/L Triton X-100, pH 7.8. The bovine serum albumin and other protein standards were prepared in this same buffer. One-milliliter aliquots of a 50 mg/mL stock solution of bovine serum albumin were stored at -70°C then thawed at 4°C for preparation of the daily standard curves.

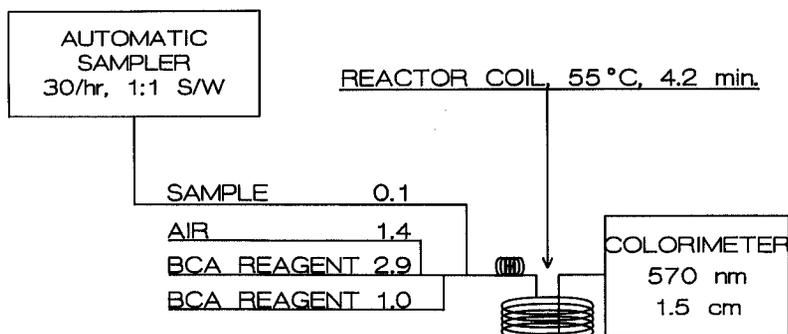
2.7 Continuous-Flow Analytical System.

The analytical system, shown in Figure 1, was constructed from standard Technicon Auto-Analyzer™ components (Technicon Industrial Systems, Tarrytown, NY). The transmission tubing, mixing coil, and reaction coil were all glass with an internal diameter of 2.4 mm. The heating bath oil was replaced with water to improve the temperature stability ($55 \pm 0.05^\circ\text{C}$). Data were collected with a DP-1000 computerized data system (Labtronics, Guelph, Canada), and results were expressed as net peak height over baseline.

3. RESULTS

Preliminary experiments with a 4.2-min reaction at room temperature or 37°C showed considerably less

Figure 1. Schematic diagram of the continuous-flow, segmented-stream analytical system. Numbers above the reagent pump lines represent the nominal flow rates in milliliters per minute. The BCA reagent was added at two points in the flow system to avoid disruption of the bubble pattern due to turbulence. S/W, sample-to-wash ratio.



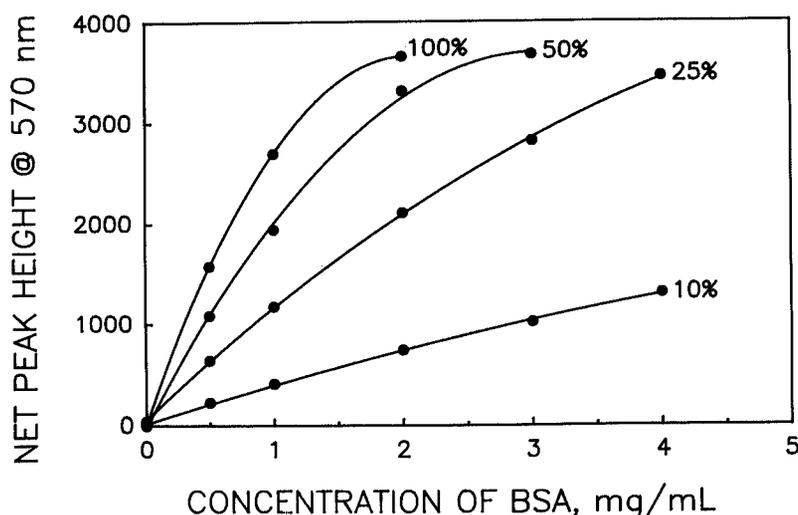


Figure 2. Effect of reagent concentration on assay sensitivity with bovine serum albumin. Reagents A and B were made up at four times the concentrations shown under Materials and Methods (except sodium lauryl sulfate), and the working reagent was prepared as described. The working reagent concentration was adjusted by dilution in 1 g/L sodium lauryl sulfate. The percentage concentrations shown are relative to the four times concentrated reagent (standard working reagent = 25%). The colorimeter sensitivity was 1.2 A. Peak heights are in arbitrary units as assigned by data system, where 4000 represents approximately 1.2 A. BSA, bovine serum albumin.

sensitivity than reaction at 55°C (data not shown). The higher sensitivity at 55°C was desirable to avoid interference from intensely colored samples and was sufficient for the intended applications. Figure 2 shows the effect of working reagent concentration on the sensitivity of the assay with bovine serum albumin. Because the assay cost was a major concern in our application, the 25% working reagent (as described under Materials and Methods) was chosen as the best compromise between sensitivity and cost. At the sampling rate of 30 per hour (1:1 sample-to-wash ratio), the sample-to-sample carryover was measured at less than 1% and was therefore not included in the calculations.

The automated assay showed approximately the same relative sensitivities to the five protein standards tested (Figure 3) as was reported for the manual assay [1]. The response to gelatin was about half as great as it was to bovine serum albumin in

both the manual and automated procedures. The responses to ribonuclease, chymotrypsinogen, and insulin were greater than the response to bovine serum albumin in both assays. However, the automated assay had the greatest response to insulin, whereas the manual method responded most strongly to ribonuclease. These small differences in relative protein responses between the two methods are most likely due to the different reaction rates of BCA with various proteins [1] and, from a practical standpoint, are not significant. The curvature of the standard calibration graph for the automated assay (Figure 2, 25% curve) appeared to be slightly greater than with the manual assay [1]. However, acceptable results were obtained both by linear regression over an abbreviated range as in the manual assay or by second-order regression over the entire range of protein concentrations. We recommend the second-order calibration for those data systems ca-

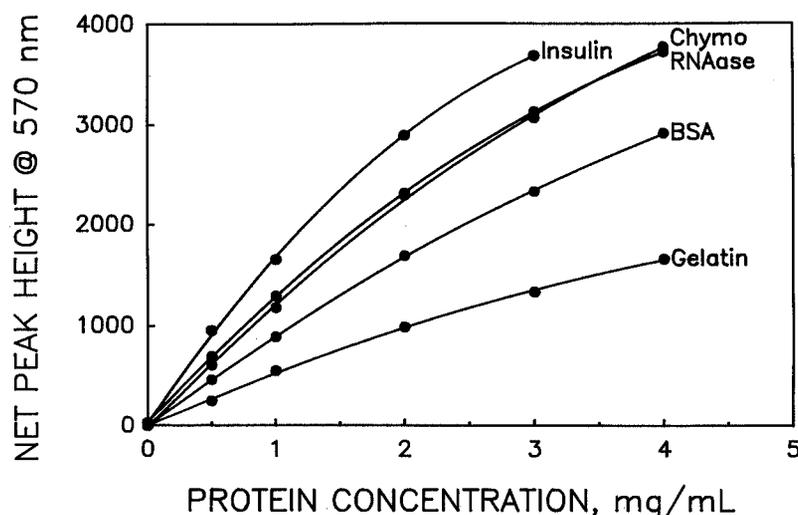


Figure 3. Relative response of the automated assay to various proteins. Standard working reagent concentration (25%), other conditions as in Figure 2. RNAase, ribonuclease A, Chymo, chymotrypsinogen.

TABLE 1. Within-Day and Between-Day Precision^a of the Continuous-Flow Bicinchoninic Acid Protein Assay

Sample Type	Mean mg/mL	Within-Day Standard Deviation		Pure Between-Day Standard Deviation ^b	
		mg/mL	%CV	mg/mL	%CV
Bovine serum albumin	0.609	0.018	2.99	0.086	14.1
Bovine serum albumin	2.283	0.024	1.04	0.099	4.33
Bovine serum albumin	5.000	0.003	0.06	0.290	5.79
Human erythrocytes, 1:40	3.987	0.028	0.71	0.353	8.84
Human plasma, 1:20	3.552	0.056	1.58	0.057	1.62

^aSets of four replicates were assayed on 4 separate days. Variances were separated and estimated by one-way analysis of variance [11].

^b"Pure" between-day standard deviation does not include the contribution from within-day sources of variance [12].

pable of it because of the increased dynamic range this provides.

It was possible to measure protein concentrations below 0.5 mg/mL by changing the colorimeter sensitivity from its normal setting of 1.2 absorbance units (A) full scale to 0.2 A full scale. At the 0.2 A full scale setting, the baseline remained free of excessive noise, but exhibited a slight drift toward higher absorbance, which was accurately corrected for by the data system. To estimate the limit of detection at the colorimeter sensitivity of 0.2 A, 15 replicate assays of a 0.2 mg/mL bovine serum albumin standard were performed in one run. The limit of detection (the smallest concentration that is significantly different from zero at the 99% confidence level) was estimated as three times the standard deviation of this set of assays [11], or 0.027 mg/mL.

The within-day repeatability and between-day reproducibility of the automated assay were evaluated by assaying four replicates of each sample type on each of 4 separate days. One-way analysis of variance was used to separate and estimate the contributions from within-day and between-day sources [11]. The between-day variance thus calculated is an estimate of the "pure" between-day variance and does not include the within-day variance [12]. The separated variances were used to calculate the standard deviation data shown in Table 1. The repeatability (0.06% to 2.99% CV, coefficient of variation, standard deviation/mean \times 100%) and reproducibility (1.62% to 14.1% CV) were found to be adequate for routine applications.

4. DISCUSSION

The automated assay using the 25% concentrated working reagent, as described under Materials and Methods, is approximately five-fold less sensitive

than the manual procedure, in terms of the absorbance increase per unit concentration of bovine serum albumin. This is a consequence of the lower reagent concentration (four-fold) and the shorter reaction time (three-fold) that were used in the automated procedure. The sensitivity obtained with the automated system is more than adequate for the diluted blood samples that the system was developed for. If greater sensitivity is required for a particular application, it can be achieved without increasing the reagent cost by increasing the length [1, 8, 10] or temperature [1, 10] of the reaction coil or by increasing the sample flow rate. If reagent cost is not a limitation, sensitivity can also be increased by use of a more concentrated working reagent (Figure 2) [10]. The ability to adjust the sensitivity of the automated method over a wide range without losing the advantageous features of the BCA method is particularly valuable because of the flexibility it offers to the analyst.

Using the 25% working reagent, the reagent and supply cost per sample of the automated assay (ca. \$0.06) is about half the cost of the commercially available kit assay. If needed, the cost could be further decreased by increasing the sampling rate or by decreasing the reagent flow rates and the internal diameters of the transmission tubing and reaction coil. If sensitivity is not a concern, further savings could also be realized by using a less concentrated working reagent.

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