A Micro-Immunochemical Procedure for the Measurement of Total Protein in Cerebrospinal Fluid*

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ABSTRACT

A procedure has been developed for the measurement of total protein in cerebrospinal fluid (CSF) based on light scattering of antigen-antibody complexes formed with anti-whole human serum. Twenty-five microliters of CSF are required. Precision of the method is 6.3 percent (R.S.D.) and close correlations are obtained with the trichloroacetic acid turbidimetric procedure. The essential condition of antibody excess has been shown to exist up to 390 mg per dl of total protein.

Introduction

Several methods for the measurement of total protein in CSF have been proposed and, at the present time, the relatively simple turbidimetric procedures are most commonly employed using either trichloroacetic acid or sulfosalicylic acid as a means of producing turbidity. These methods are technically straightforward and are adaptable to emergency use but suffer from a lack of sensitivity. The biuret reaction has been proposed with spectrophotometric measurement at 330 nm as a means of improving sensitivity. Folin and Ciocalteu reagent in conjunction with biuret has been adopted but is subject to some interferences. Ultraviolet spectrophotometric methods have been reported but require preliminary column separations to remove interfering substances before measurement at 280 nm. Ultraviolet fluorometry and the micro Kjeldhal technique have also been used.

An alternate approach to the measurement of proteins in aqueous solutions involves nephelometric measurements of antigen-antibody complexes formed in immunochemical reactions. Boyden et al in 1947 first showed that precipitin curves

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could be characterized by measuring the turbidity developed in antigen-antibody mixtures. Schultze and Schwick described the turbidimetric measurement of several plasma proteins. Ritchie developed methods for measuring albumin and total immunoglobulin in dilute solution and a similar procedure for measuring C'3 also has been described. A series of studies in our laboratory have led to the development of similar immunochemical methods using a fluorometer as a nephelometer for detecting antigen-antibody complexes. Continuous flow automation using these same principles has also been reported.

These nephelometric immunochemical procedures are sensitive, rapid and involve only simple technical manipulations. The present study was undertaken to evaluate a technique for the measurement of total protein in CSF with emphasis being placed on the development of a micro procedure possessing both accuracy and precision.

Materials and Methods

APPARATUS

A fluorometer equipped with a primary filter to give a peak transmission at 360 nm is used for the light scattering measurements. No secondary filter is required. The square micro flow cell of 0.2 ml volume is used for the light scattering measurements. A recorder attached to the fluorometer is used in the present study but is not essential to the procedure.

Reagents

Goat Anti-human Serum. Antiserum to human serum total proteins is obtained from a commercial source and is diluted 50 fold with 0.9 percent saline before the determinations are carried out. Unused portions of the antiserum can be stored in the refrigerator at 5° and are stable at least two weeks.

Saline Solution. Nine g of reagent grade sodium chloride are dissolved in one liter of distilled de-ionized water containing 0.5 ml per liter Triton X-100.

Human Serum Proteins

Total Protein Standard (650 mg per dl). To obtain the concentration of total protein, replicate determinations were performed on a pooled human serum standardized against bovine serum albumin using a biuret procedure. Appropriate dilutions of the standard were made to obtain total protein concentrations of 130, 93, 59, 41, 21, 13 and 7 mg per dl.

Serum Protein Fractions. The serum protein fractions are divided into two categories.

Human Serum Albumin (693 mg per dl). Human serum albumin obtained commercially was found to be electrophoretically homogenous demonstrating a single precipitin arc on immunoelectrophoresis.

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Concentrations of albumin 277, 231, 208, 187, 162, 139, 99 and 43 mg per dl were prepared from the stock albumin solution.

**Human Gamma Globulin (308 mg per dl).** Human serum gamma globulin|| shown by immunoelectrophoresis to contain mainly IgG with smaller amounts of IgA and IgM, was used in this study. The concentrations of gamma globulin prepared from the stock solution were 293, 270, 205, 154, 123, 116, 103, 98, 77 and 51 mg per dl.

**Procedure**

The diluted antiserum (1:50) is transferred volumetrically in 2.5 ml aliquots to $13 \times 100$ mm screw-cap tubes and 25 microliters of the CSF sample or standard are added to each tube and the contents are gently mixed ten times. The tubes are allowed to stand for 25 minutes at room temperature for development of the antigen-antibody complexes. The reaction mixtures are then introduced into the fluorometer (nephelometer) using a plastic syringe with a small tygon tubing extension. Diluted antiserum is first used to adjust the baseline to an arbitrary low setting on the fluorometer, and the light scattering from the immune complexes in the CSF samples and standards is measured. Rinsing with saline between readings of both the flow cell and syringe is essential to avoid sample interaction. Maximum recorder response should be set with the 130 mg per dl total protein standard to achieve optimal sensitivity. A blank correction for non-specific light scattering from CSF samples was found to be unnecessary. Concentration of total protein in the unknown samples is determined from the standard curve shown in figure 1.

This standard curve is non-linear and, therefore, several points on the curve must be determined with each group of tests.

||Parke-Davis and Co., Detroit, MI 48232.

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**Results**

**Time Delay**

To determine the optimum time for development of the antigen-antibody complexes, a standard of known concentration was mixed with antiserum and was injected immediately into the fluorometer. Increasing light scattering from the antigen-antibody aggregates, as they developed, could be followed. As shown in figure 2, a plateau was reached in approximately 15 minutes and remained stable for several hours. In the recommended procedure, 25 minutes was selected as the suitable delay period in development of the immune complexes.

![Figure 2. Light scattering from the antigen-antibody complexes as a function of time.](image)

**Precipitin curve for the total protein-anti total protein complexes.**

![Figure 3. Precipitin curve for the total protein-anti total protein complexes.](image)
Figure 4. Precipitin curves for albumin and gamma globulin.

Precipitin Curves

Total Proteins. Conditions for measuring proteins by immunochemical reactions require the presence of antibody excess. Erroneous results are obtained from samples in antigen excess owing to solubilization of antigen-antibody complexes giving falsely low light scattering readings. It is necessary, therefore, to construct a precipitin curve to assure conditions of antibody excess with each new batch of antiserum. Experience with different batches from a single commercial source have shown, although precipitin curves vary slightly, this variation has not necessitated altering the reaction conditions. A typical precipitin curve constructed from dilutions of the standard is shown in figure 3. Screening of samples in antigen excess can be carried out by using 10 μl of CSF in the reaction mixture and noting the light scattering response in comparison to the original test which requires 25 μl of CSF. Samples in antibody excess would exhibit a definite decrease in response whereas those in antigen excess most likely would demonstrate the opposite effect. The authors routinely screen for antigen excess on all samples.

Albumin and Globulin. Since total protein in CSF is composed of many different fractions, it is necessary to ascertain that...
the region of antibody excess exists in all instances. Although albumin constitutes the major fraction in CSF, pathologic samples particularly the IgG fraction of the gamma globulin, can demonstrate notable increases. It is therefore important to establish a albumin and gamma globulin. In figure 4 condition of antibody excess for both are shown two curves constructed for these fractions; it is seen that antibody excess is present up to 130 mg per dl for albumin and 103 mg per dl for gamma globulin. The light scattering response observed here for the respective precipitin curves is not indicative of absolute sensitivity derived from the immune complexes but is simply a reflection of the change in sensitivity setting on the fluorometer.

The total protein standard curve used in the measurement of CSF samples employs a 130 mg per dl standard for the highest concentration which certainly is well within the range of antibody excess that exists up to a concentration of 390 mg per dl.

**Correlation**

CSF samples from 50 hospital patients were analyzed by both the turbidimetric trichloracetic acid method of Henry et al and the present immunochemical technique. The results of this correlation are shown in figure 5.

**Precision**

In table 1 is shown the precision of the method as determined by performing 50 duplicate measurements of CSF samples encompassing a broad range of concentrations.

**Discussion**

The present procedure described for the measurement of total protein in CSF is a microtechnique using only 25 to 35 μl of sample. Other methods for performing this same determination require 0.5 to 1.0 ml.

<table>
<thead>
<tr>
<th>Number of Duplicate Determinations</th>
<th>Mean (mg/dl)</th>
<th>S.D.</th>
<th>Range (μg/μl)</th>
<th>Coefficient of Variations (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50.8 ±3.2</td>
<td></td>
<td>12-290</td>
<td>6.3</td>
</tr>
</tbody>
</table>

The real advantage, therefore, of the immunochemical technique is that larger quantities of unused CSF are available for additional studies such as the measurements of immunoglobulins and other specific proteins which can have importance in evaluating diseases of the central nervous system.

There are, however, inherent problems using the immunochemical approach for measuring mixtures of several proteins such as proposed in the present procedure. The precipitin curve is a composite of several curves and different proteins will obviously provide varying light scattering responses when bound to their specific antibodies. However, this problem of proteins responding dissimilarly is not unique to immunochemical techniques and a similar problem exists with both turbidimetric and ultraviolet absorbance approaches. For example, different proteins in CSF produce varying degrees of turbidity with TCA and also have differing molar absorptivities at 280 nm.

In the present procedure, errors due to the presence of several antigen-antibody reactions occurring in the reaction mixture are reduced by using a standard containing all of the CSF protein fractions in amounts similar to those encountered in CSF. Pooled human serum is used as this standard and dilutions are made to achieve protein levels in the range frequently encountered in CSF. Relative proportions of CSF proteins are changed in a few disease states but not enough to cause significant errors.
Summary

A method is proposed for measuring total protein in CSF using a nephelometric immunochemical technique. Only 25 to 35 μl of sample are required and measurements can be made 25 minutes after mixing sample and antiserum. The method is precise and correlates well with a conventional turbidimetric procedure.

References