Manual Immunochemical Nephelometric Assays for Serum Immunoglobulins IgG, IgA, and IgM

Introduction

Nephelometric methodology is a logical adaptation of the precipitin reaction to a solution-based system. Analytical methods involving measurements of light scattering or turbidity were described as early as 1914 (1), but results obtained with the instrumentation of that period caused nephelometry to fall into disrepute (2). Since about 1940 the use of light-scattering methods in immunochemistry has been revived and the number of procedures described has increased in the last 15 years. In 1967 several reports demonstrated the efficacy of this* form of measurement as applied to solution-based immunochemical reactions (3–6). Improved instrumentation has helped establish this approach as a valid analytical tool (7–10).

Principle

The reaction between antigen and antibody occurs in three stages:

\[
\begin{align*}
\text{Antigen (AG)} & + \text{antibody (AB)} \xrightarrow{k_1} AG_x AB_y + AB + AG \\
\text{AB} + \text{AG} & + AB_y AG_x \xrightarrow{k_2} AB_y AG_x + AB \\
& + AB_y AG_x \xrightarrow{k_2'} AB_y AG_x + AB \\
& + AB_y AG_x \xrightarrow{k_3} AB_y + AG_x' \xrightarrow{k_{-3}} AB_y + AG_x' (\text{precipitate})
\end{align*}
\]

In these stages \( k_1 \gg k_2 \gg k_3 \), and \( AB_y AG_x, AB_y AG_x', \text{and } AG_x - AB_y \) represent aggregates of increasing size.

The approach to equilibrium can be hastened by using linear polymers that have a high exclusion volume, such as polyethylene glycol (see next section).

Measurement of antigen involves mixing antigen and specific antibody in the appropriate proportion to produce a moderate excess of antibody. The reaction is allowed to proceed until a pseudo-equilibrium is reached. At this point, growth of the antigen–antibody complex is slow relative to the time required for a measurement. Light scattering from the immuno-complexes is measured for unknowns and calibrators. Relative light scattering of the unknowns is compared with that measured for the calibrators.

Materials and Methods

Reagents

**Diluent**: polyethylene glycol, \( M_r \) 6000–7500 (Carbowax 6000, cat. no. P-156, lot no. 763793; Fisher Scientific, Chemical Manufacturing Division, Fair Lawn, NJ 07410). Dissolve 40 g of polyethylene glycol in a sufficient volume of 0.15 mol/L sodium chloride to make 1 L of solution. Filter through a 0.45-μm filter. This solution is stable for several months.

**Working antiserum**: Anti-human IgG (γ-chain specific, code IGG, lot no. IGG-P038-N), anti-human IgA (α-chain specific, code IGA, lot no. IGA-P035-N), and anti-human IgM (μ-chain specific, code IGM, lot no. IGM-P034-N), all from Atlantic Antibodies, West Brook, ME 04092. All antisera are raised in goats, absorbed on solid-phase, and delipidated to provide transparent antiserum, known as “nephelometric grade” antiserum. Dilute all antiserum 80-fold in the polyethylene glycol diluent and let stand for 30 min. Then filter the antiserum through a 0.45-μm (av. pore size) disposable filter unit.

Other commercial sources of antiserum can also work well in these methods; however, most of our work has involved one supplier. We have noted that for some labile, multi-subunit proteins (e.g., C3, ceruloplasmin) the commercial source of antiserum can significantly affect the results (11).

**TWEEN 20** (Sigma Chemical Co., St. Louis, MO 63178) may be included in the antiserum solution. Some authors claim it improves performance for automated continuous-flow systems (3, 4). TWEEN 20 may also improve the precision of the flow-cell used in the transporter module of the Hyland Instrument. We have not evaluated this factor.

Apparatus

**Nephelometer**: We used a Hyland laser nephelometer PDQ (Hyland Laboratories, Costa Mesa, CA 92626). The laser emission line is 638 nm; the angle of measurement is 31° relative to the position of the sample. The instrument automatically corrects for background light scatter from the serum specimen. A description of other commercially available nephelometric instrumentation is presented elsewhere (12).

**Diluter**: We used a digital dispenser (Hamilton Co., Reno, NV 89501) to make the initial 10-fold or 100-fold dilution of the specimen and to deliver an appropriate aliquot of the sample flushed with either blank diluent or antibody diluent.
Filters: Filter all solutions with Millex filter units that have an average pore size of 0.45 μm (cat. no. SLHA02505, lot no. 18302; Millipore Corp., Bedford, MA 01730).

Invertor rack: Use a self-sealing rack (Hyland Laboratories) for mixing the contents of several tubes simultaneously.

Collection and Handling of Specimens

Collect blood from venous or skin puncture and centrifuge at 3000 x g for 10 min to obtain serum. Moderate hemolysis or lipemia will not affect the results.

Immunoglobulins (IgG, IgA, and IgM) are stable in sera for at least two weeks when stored at 4 °C and indefinitely when stored at −20 °C in the absence of any preservatives. Avoid repeated freezing and thawing of serum.

Standardization

An assayed human serum pool (Atlantic Antibodies) is used as a calibrator for the standardization of the assays described here. Calibrator values were originally obtained by isolating and purifying the appropriate proteins and subsequently assigning appropriate values to a serum pool. Calibration ranges are given in Table 1. The stock calibrator is diluted with filtered 0.15 mol/L saline.

Typical calibration curves for IgG, IgA, and IgM are shown in Figures 1–3. We believe that the combination of the angle of measurement and a judicious choice of antigen and antibody concentration is the reason for the observed linearity in the assay. In theory, the assays could be calibrated with a single standard, but at this time we do not believe that the instrument and its cuvets allow sufficiently precise measurement to justify this approach.

Quality Control

The performance of the methods is evaluated by means of high- and normal-range controls obtained from Atlantic Antibodies. One high and one normal control are run at the beginning and end of each assay sequence. The duplicate values should agree within 10% to validate acceptable performances.

Procedure

The following steps describe the procedure for the analysis of serum immunoglobulins. The specific volume of diluted serum for each test is as follows: IgG, 10 μL; IgA, 15 μL; and IgM, 50 μL.

1. In 12 × 75 mm disposable tubes, make a 100-fold or 10-fold dilution of standards, control, and patient’s serum with a 0.15 mol/L solution of sodium chloride. The dilution ratio depends on the patient’s age. Generally, a specimen from a child less than three months old will require a 10-fold dilution;
a specimen from a child older than three months will require a 100-fold dilution.

2. Label 10 × 75 mm disposable tubes, one blank and one test for each standard, control, and patient’s specimen. Also label one diluent blank and one antiserum blank tube. Place the tubes in the inverting rack.

3. Into all blank tubes except antiserum blank, pipette 1 mL of the filtered polyethylene glycol diluent solution.

4. Into all unknowns and into the antiserum blank tube, pipette 1 mL of a filtered working antiserum.

5. Add diluted standards, control, and patient’s serum to respective test tubes and blank tubes; add an equal volume of sodium chloride diluent to tubes for the patient’s serum blank and for the antiserum blank.

6. Cover tubes with a sheet of Parafilm and place the rack cover on top of the Parafilm, checking that all tubes are covered. Mix by inversion. Incubate at ambient temperature for 40 min.

7. Measure light scattering from each tube with the nephelometer described under Apparatus.

Studies of the methods used in our laboratory show that antibody excess extends over a very broad range, compared with that in other immunochemical methods (Figures 4–6). These broad ranges were observed with three different commercial sources of antisera (Hyland, Meloy, and Atlantic Antibodies). However, because of the restricted clonal expression observed in plasma cell dyscrasias, there remains the potential of not detecting a very high concentration of a monoclonal immunoglobulin. We recommend the following as a means of evaluating antigen excess if protein electrophoresis is not concurrent to quantitation of immunoglobulins.

As a check for antigen excess, add an additional aliquot of a mid-range calibrator (see Standardization) to each unknown tube and re-incubate at room temperature for about 15 min. After the incubation period check the relative light scattering of each tube. If total light scattering is increased, there is still an antibody excess. If light scattering does not change or if it decreases, the specimen should be re-assayed at a higher dilution. Ideally, all specimens assayed for immunoglobulins should be screened by protein electrophoresis so that the presence or absence of monoclonal or oligoclonal banding can be evaluated. Because of the limitations of antiserum specificity, protein electrophoresis with densitometric scanning probably provides the more nearly accurate quantitative method for measuring monoclonal proteins.

Calculations

Blank corrected light-scatter readings are plotted vs. the assigned calibrator concentration. The calibration curve is used to reduce the blank-corrected light-scatter readings of the unknowns to concentration in grams per liter. The con-

![Fig. 4. Antibody excess range for assay of IgG](image)

![Fig. 5. Antibody excess range for assay of IgA](image)

![Fig. 6. Antibody excess range for assay of IgM](image)

### Table 2. Precision of Measurement

<table>
<thead>
<tr>
<th></th>
<th>Mean g/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>14.53</td>
<td>0.41</td>
<td>2.8</td>
</tr>
<tr>
<td>IgA</td>
<td>4.03</td>
<td>0.11</td>
<td>2.7</td>
</tr>
<tr>
<td>IgM</td>
<td>2.17</td>
<td>0.08</td>
<td>3.7</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>8.01</td>
<td>0.66</td>
<td>8.2</td>
</tr>
<tr>
<td>IgA</td>
<td>1.68</td>
<td>0.12</td>
<td>7.1</td>
</tr>
<tr>
<td>IgM</td>
<td>0.89</td>
<td>0.08</td>
<td>9.0</td>
</tr>
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</table>
centrations of unknown specimens that were initially diluted 10-fold are divided by 10 to compensate for their difference from the initial 100-fold dilution of the calibrator.

Precision

Within-assay and inter-assay variations are given in Table 2. We determined within-assay variation by measuring 20 replicates of a pooled serum within a single assay. Inter-assay variation was estimated by repeated analysis of a pooled serum on 31 days over a four-month period. These data are representative of data collected in the routine laboratory, by different technologists who were assigned to this station. Standard deviation and coefficient of variation are for a one-standard-deviation limit. Much of the error in the measurement is caused by the inherent variation in the reading of light scattering in this particular instrument. We estimated the precision of the light-scattering measurement by using a single fluid with a relative scattering of 93 units. The readings were found to vary by 1.2% when the sample was read 20 times in the same tube without changing the position of the tube. Measurements made on the same liquids varied by 1.7% and 1.8%, respectively, when a single tube was rotated and read 20 times and when 20 light-scattering measurements were made on the same liquid in 20 different tubes. This form of imprecision is unique to a manual approach to nephelometric assays. Use of a well-designed flow cell would significantly improve precision. A reduction in within-assay variation to 3 to 4% would make single-point calibration a realistic possibility.

Reference Values

Because of the inherent difficulty of collecting pediatric reference values, we have used literature estimates of reference limits for radial immunodiffusion methods (13, 14).

Discussion

Variation in serum concentration of immunoglobulins in pathological states is well described (13, 14). The assessment of immunoglobulin concentration is commonly used to evaluate fetal infection in utero (15), humoral immunodeficiency (13, 14), liver disease, collagen vascular disease, and chronic infection (13, 14). The increase or decrease in immunoglobulins in these cases is usually polyclonal in nature. Expression of a monoclonal immunoglobulin is most often associated with a plasma cell dyscrasia. In these cases the increase in a single Ig class can amount to several grams of protein per liter. A more in-depth discussion of the efficacy of immunoglobulin measurement in clinical diagnosis is beyond the scope of this article. Interested readers are referred to more appropriate sources (13, 14).

References


Editor's note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. They are methods that seem durable and generally useful, and that have been checked by several evaluators. As stated elsewhere (Clin. Chem. 19, 1207 [1973]), these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume. Selected Methods of Clinical Chemistry. The last such volume was published by the Association in 1977.

No reprints of these papers will be available, because they are not regarded as necessarily being final versions.