Automated Immunochemical Procedures for Measurement of Immunoglobulins IgG, IgA, and IgM in Human Serum

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Automated continuous-flow procedures have been developed for micro-determination of immunoglobulins IgG, IgA, and IgM in human serum. The three immunoglobulins are measured by reactions with specific commercial antisera, and standardized by use of pooled human serum containing known concentrations of IgG, IgA, and IgM. Values determined for the immunoglobulins by the automated procedures correlate with values obtained by radial immunodiffusion. The precision of replicate determinations for the IgG, IgA, and IgM methods is 3.64, 5.11, and 6.00% (CV), respectively.

Additional Keyphrases nephelometry • AutoAnalyzer • radial immunodiffusion, correlation

Materials and Methods

Apparatus

Automated systems. A manifold and flow system for the determination of IgG assembled for use with modules of the AutoAnalyzer (Technicon Instrument Corp., Tarrytown, N. Y., 10591) are shown in Figure 1 and a manifold and flow system for the determination of either IgA or IgM are shown in Figure 2.

The primary filter used with the fluorometer is a narrow band pass filter (G. K. Turner Assoc., Palo Alto, Calif. 94303; no. 110-811) that gives a peak transmission at 360 nm. We adapted a square quartz micro flow cell with three polished sides and a volume of about 0.2 ml (Hellma Cells, Inc., Jamaica, N. Y.; no. 176F-QS) for use with the fluorometer so as to decrease spurious light scattering and provide optimal optical characteristics for nephelometry (in comparison with results obtained with a tubular flow cell). No secondary filter is used. The fluorometer light source is a general purpose lamp that gives a peak emission at 350 nm (Turner, no. 110-850).

Reagents

Goat antihuman IgG, IgA, and IgM. Specific antiserum for each of the immunoglobulins IgG, IgA, and IgM was obtained commercially (Meloy

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Laboratories, Falls Church, Va. 22046); each antiserum produced only a single precipitin band on immunoelectrophoresis with use of whole human serum. The goat antihuman IgG antiserum was assayed at 3.8 mg of antibody per milliliter (batch no. GHG04); goat antihuman IgA at 3.9 mg Ab/ml (batch no. GHA05); and goat antihuman IgM at 2.5 mg Ab/ml (batch no. GHM05). All antisera were diluted 25-fold with saline solution (9 g/liter) immediately before use in the continuous-flow system. The original antisera were stored at 5°C, and their activity was not measurably changed after as much as two months of storage under these conditions.

**Saline solution.** Dissolve 9.0 g of reagent grade sodium chloride and fifteen drops of Triton X-100 (Harleco, Philadelphia, Pa. 19143) in one liter of distilled, de-ionized water.

**Human serum immunoglobulin standards.** Pooled human serum from 100 hospital patients was analyzed for IgG, IgA, and IgM by a commercial laboratory (Meloy) with use of an international reference standard prepared by the World Health Organization. This pooled serum was then diluted to provide IgG standards of 1410, 705, and 357.5 mg/dl; IgA standards of 180, 90, and 45 mg/dl; and IgM standards of 220, 110, and 55 mg/dl. Sodium azide solution (10 ml of a 1 g/l soln per 990 ml pooled serum) was the preservative. The standards were then apportioned into 1-ml aliquots and stored at −60°C. They were stable for at least one year under these conditions.

**Methods**

**Automated immunoglobulin G.** As shown in Figure 1, human serum is aspirated on the continuous-flow manifold at a rate of 0.03 ml/min and diluted with saline (6.00 ml/min). An aliquot of this dilution is then resampled and rediluted with saline (1.20 ml/min). This serum, which has now been diluted by a factor of 760, is again resampled (0.42 ml/min), and mixed with goat antihuman IgG (0.42 ml/min). This reaction mixture is then passed through a single mixing coil and the light-scattering owing to the antigen–antibody complex is measured by use of the fluorometer. Concentrations of IgG in the serum samples are determined from a standard curve (recorder peak height vs. concentration of IgG in the standard solutions).

Blank determinations are unnecessary, because the dilution of the original serum sample is so great that even lipemic samples provide negligible blanks.

**Automated immunoglobulin A and immunoglobulin M.** IgA and IgM are present in human serum at lower concentrations than IgG, which necessitated the construction of a different automated manifold with only one dilution circuit, as shown in Figure 2.

Human serum is aspirated at a rate of 0.05 ml/min and diluted with saline (6.00 ml/min). A constant amount of this diluted sample is then resampled (0.42 ml/min) and mixed with the specific antiserum (0.42 ml/min) before the stream passes through the fluorometer cell for light-scattering measurement.

Blank determinations for each sample assayed for IgA and IgM are made with saline solution being aspirated through the antiserum reagent line. After the blank corrections, concentrations of IgA and IgM are determined from their respective standard curve.

**Fig. 1. Flow diagram for the measurement of human serum IgG. SMC, single mixing coil**

**Fig. 2. Flow diagram for the measurement of either human serum IgA or IgM. SMC, single mixing coil**
Radial immunodiffusion of IgG, IgA, and IgM.

Commercial quantitative immunodiffusion plates (Meloy) were used for the immunodiffusion determinations; the manufacturer's instructions were followed for preparation of standards, filling of wells, incubation times and temperatures, and the reading of precipitin ring diameters.

Results and Discussion

Precipitin Curves

In order to characterize the precipitin curves for the immunoglobulin-anti-immunoglobulin complexes, serum from patients known to have a supranormal concentration of gamma globulin was assayed for IgG, IgA, and IgM by radial immunodiffusion. Appropriate dilutions of these sera were then made to provide ranges of concentration of 495–4950 mg/dl for IgG, 29–390 mg/dl for IgA, and 24–240 mg/dl for IgM. It will be noted that the highest of these values extended well above the upper limits of normal for each immunoglobulin. These samples were aspirated into the automated system and the recorder response from the antigen–antibody complex was compared with the known concentration of each immunoglobulin. Reaction conditions were optimized by varying the amount of dilution carried out in the initial dilution circuit to provide the precipitin curves shown in Figures 3–5; these same reaction conditions were used for the standard assay procedure. In present automated procedures for IgG, IgA, and IgM we use an excess of antibody, and working standard curves fall in the initial linear or almost linear portions of the precipitin curves. We observed that peaks resulting from concentrations of IgG outside the region of antibody excess had a characteristic blunted and asymmetrical shape, which could easily be detected and the serum sample diluted appropriately. A recorder tracing for IgG standards and unknown serum samples is shown in Figure 6, which also illustrates a peak with the characteristic blunted asymmetrical shape. The condition of antibody excess is essential if one is to derive meaningful results from an automated immunochemical procedure. Owing to the shape of precipitin curves, one could obtain the same peak height from a sample in antigen excess as with a sample in an appropriate region of antibody excess.

Sufficient dilution was carried out in the initial dilution circuit of the manifold assembled for the measurement of IgA and IgM to provide an excess of the specific antibody, even with the most concentrated IgA or IgM that might be expected in serum samples. The sole example of IgM outside the region of antibody excess occurred in a patient with Waldenstrom's macroglobulinemia, whose IgM concentration was measured after dilution of the original sample and found to be greater than 6000 mg/dl.

The reactions for the determination of IgG were made with serum diluted 760-fold, and those of IgA and IgM with serum diluted 120-fold prior to mixture with antiseraum. Light-scattering measurements at these dilutions were made adequately
sensitive by use of the square micro flow cell, which was a new innovation for automated immunochemical procedures.

Spectral Characteristics of the Complex

A fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn. 06852) was used to scan both the reaction and blank solutions from the automated system, to determine the optimal wavelength for the excitation light source. Figure 7 shows the scan of the reaction mixture corrected for the blank and antiserum baseline and reveals a broad spectrum of light-scattering from 325 to 500 nm with a small peak at 465 nm. The choice of the source was therefore somewhat arbitrary, since any excitation source between 325 and 500 nm would result in about the same degree of light-scattering from the antigen–antibody complex. The light source we used was the all-purpose lamp supplied with the fluorometer, which has a peak intensity at 350 nm.

Serum Immunoglobulin Measurements

With both reference standards and samples, sharp, uniform peaks were obtained because of the light-scattering from the antigen–antibody complexes for IgG, IgA, and IgM. The flow characteristics of the square micro flow cell required that two wash cups be placed between each sample, to eliminate carryover. With a 50/1:1 sample cam, this gave a sample rate of 17 samples per hour. This sample rate is much more rapid than radial immunodiffusion, the alternative manual method. For those investigators wishing to construct special cams, a 20-per-hour cam with a sample: wash ratio of 1:6 should give the same results. In our initial experiments, we used both cylindrical and square micro flow cells. Background light-scattering from the cylindrical flow cell made it necessary to close the slits of the fluorometer; sensitivity was so decreased by this that light-scattering from the antigen–antibody complex could not be detected. This background light-scattering was not observed with the square micro flow cell, and the resulting enhancement in sensitivity more than compensated for the sample rate of 17 per hour. Under the present conditions of analysis, 90% of steady state was attained by the sample peak.

Standard curves for the measurement of IgG, IgA, and IgM were either linear or nearly so.

Fig. 6. Recorder tracing for the continuous-flow immunochromical method for IgG

Fig. 7. Fluorescence spectrophotometer scan of net emission from antigen–antibody reaction
because the reactions were carried out in the linear section of the antibody-excess portion of the precipitin curve. As with most continuous-flow automated procedures, several standards were run with each group of tests.

The precision of the methods for IgG, IgA, and IgM, as determined from replicate analyses of pooled human serum, is given in Table 1. These data indicate that the precision of the present automated methods is much better than the semi-quantitative procedures commonly used (1-4).

Correlation Studies

IgG, IgA, and IgM concentrations of 30 sera from hospital patients were determined by both the present automated methods and manual radial immunodiffusion. The correlation of these two procedures for all three immunoglobulins is given in Table 2, and demonstrates the validity of the present automated method as compared with results from presently accepted reference procedures.

We conclude that development of the present method required the optimization of several parameters, including optical conditions for nephelometry, reaction conditions for the antigen-antibody complex, and cost. Here, all these factors were made optimal, with considerable improvement in time required, cost, and precision over the manual methods presently used to measure immunoglobulins IgG, IgA, and IgM in human serum.

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### Table 1. Precision of the Automated Immunoglobulin Methods

<table>
<thead>
<tr>
<th>Protein</th>
<th>n</th>
<th>Mean ± SD mg/dl</th>
<th>Range</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>30</td>
<td>1035 ± 37.64</td>
<td>1130-935</td>
<td>3.64</td>
</tr>
<tr>
<td>IgA</td>
<td>30</td>
<td>176 ± 9.00</td>
<td>190-164</td>
<td>5.11</td>
</tr>
<tr>
<td>IgM</td>
<td>40</td>
<td>113 ± 6.81</td>
<td>130-106</td>
<td>6.00</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of Methods for Measuring the Immunoglobulins IgG, IgA, and IgM

<table>
<thead>
<tr>
<th>Method</th>
<th>Slope of regression lines</th>
<th>Correlation coefficient</th>
<th>Standard error of estimate, mg per dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immuno-diffusion automated IgG</td>
<td>1.003</td>
<td>0.932</td>
<td>230.26</td>
</tr>
<tr>
<td>Immuno-diffusion automated IgA</td>
<td>1.180</td>
<td>0.960</td>
<td>29.58</td>
</tr>
<tr>
<td>Immuno-diffusion automated IgM</td>
<td>1.042</td>
<td>0.881</td>
<td>66.64</td>
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* Using the relationship \( Y = mX \).

### References