Turbidimetry of Rheumatoid Factor in Serum with a Centrifugal Analyzer

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We describe the simple, rapid turbidimetry of IgM rheumatoid factor in human serum by use of the Cobas-Bio centrifugal analyzer. Heat-aggregated human IgG is used as the antigen. The immunoturbidimetric reaction is monitored at 340 nm for 300 s, and the changes in absorbance after the antigen is added are used to prepare the standard curve. Test results are calculated from the stored curve and reported in int. units/mL, based on comparison with the WHO reference serum for rheumatoid factor. There is no interference from bilirubin (up to 340 μmol/L) or hemoglobin (up to 5600 mg/dL). Serum samples with a triglyceride concentration >2.20 mmol/L must be cleared of lipids before analysis. The standard curve is linear from 30 to 500 int. units/mL. Precision, accuracy, linearity, and sensitivity are quite acceptable. The CV was generally <5% for different concentrations of rheumatoid factor. Results agree well with those by a rate-nephelometric procedure on the Beckman ICS system (r2 = 0.932). However, both correlate poorly with a modified classical Waaler-Rose test. Of 47 patients with rheumatoid arthritis, 34 had IgM rheumatoid factor in their serum, but the measured value did not reflect the activity of the disease.

Additional Keyphrases: immunoglobulin · rheumatoid arthritis · immunoassay

The most consistent serological feature of rheumatoid arthritis is the increased concentration of autoantibodies directed against antigenic sites in the Fc region of human and animal IgG, namely rheumatoid factors (RFs) in the blood and joint fluid (1-2). The potential role of these factors in the pathogenesis of this disease has been studied extensively (3-5), with the finding that both environmental and genetic factors affect production of RFs, which are composed of a heterogeneous population of immunoglobulins having a wide spectrum of biological properties (6-8). RF determinations are clinically important for diagnosis (9), prognosis (10), and assessment of therapeutic efficacy (11) of rheumatoid arthritis. Although RFs may be found in all immunoglobulin classes (12, 13), the RF most frequently detected in the laboratory is IgM type, present in about 75% of adult patients with rheumatoid arthritis (14) but in about 10% of children with juvenile rheumatoid arthritis (15).

The conventional tests used in routine estimation of RF are based on its ability to agglutinate erythrocytes, latex, or similar passive particles attached to IgG (16-18). These assays have important disadvantages in routine clinical use, however, being difficult to quantify (at best being only semiquantitative), and yielding large variations in titers for the same serum samples—not only among different laboratories but also within laboratories (19, 20). In an effort to overcome these problems, several techniques have been developed—including quantitative immunodiffusion (21), radioimmunoassay (22, 23), enzyme immunoassay (24, 25), and nephelometry (26, 27)—that offer the advantages of having standardized reagents and being reproducible and quantitative. However, because these procedures generally require specialized reagents and equipment, long reaction times, and multiple incubation and washing steps, and because they are not easily automated, they are seldom used in routine practice. With the introduction of automated nephelometers in the last few years, some of the above shortcomings have been overcome (28, 29) and quantification of RF has become more widespread.

Here we describe the use of the Cobas-Bio centrifugal analyzer for turbidimetric measurement of IgM RF as a simple alternative to the automated nephelometric procedure. The method is fast and reliable, it takes advantage of this analyzer's sensitive detection system and its wide applicability in routine clinical chemistry, and it obviates the need for expensive special apparatus.

Materials and Methods

Apparatus

We used a Cobas-Bio centrifugal analyzer (Hoffmann-La Roche, Basel CH-4002, Switzerland) equipped with a BENS (Data Reduction for Non-linear Standard Curves) program (version 8326).

Reagents

Bovine serum albumin (Cohn Fraction V) was obtained commercially (J.T. Baker Chemical B.V., Deventer, The Netherlands); polyethylene glycol (PEG; M, 5000-7000) was purchased from E. Merck, Darmstadt, D-6100 F.R.G.; NAD-dithiochretol from Sigma Chemical Co., St. Louis, MO 63178; and "Lipoclean" clearing agent from Behring Institute (Behringwerke AG, Marburg, F.R.G.). All other reagents were analytical grade.

All solutions were prepared in distilled water, and filtered through 0.45-μm (av. pore diameter) filters (Millipore Corp., Bedford, MA 01730). They were stable for at least six months when stored in well-stopped flasks at 4 °C.

Cobas-Bio buffer. Borate buffer (50 mmol/L, pH 7.6) containing 0.20 mol of NaCl, 15 g of PEG, and 15 mmol of Na3PO4 per liter, with the pH adjusted to 7.6 with a 0.2 mol/L solution of NaOH.

Antigen diluent. Borate buffer (50 mmol/L pH 7.6) containing 0.15 mol of NaCl and 15 mmol of Na3PO4 per liter, pH adjusted as above.

Cobas-Bio "start" reagent. The working antigen reagent was prepared just before use by diluting the heat-aggregated IgG (HA-IgG, see below) 25-fold with antigen diluent.

Isotonic saline. NaCl, 0.15 mol/L (8.7 g/L), plus Na3PO4, 1 g/L.

RF calibrator. The RF-positive control serum used throughout this study was pooled from 20 sera with titers greater than 1/160 as determined by the modified Waaler-Rose test (30). Its potency (in international units per milliliter) was established by the turbidimetric procedure outlined below, with reference to World Health Organization (WHO) 1st International Reference Preparation (IRP) of human rheumatoid arthritis serum as standard. The RF content of the control serum was adjusted to 500 int. units/mL with the saline and stored at -70 °C.

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1 Nonstandard abbreviations: Ig, immunoglobulin; RF, rheumatoid factor; HA-IgG, heat-aggregated IgG; PEG, polyethylene glycol.

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**Methods**

**Collection.** Blood was sampled into red-top Vacutainer Tubes (Becton Dickinson, Rutherford, NJ), allowed to clot, and the serum was removed for immediate assay or for storage (at −20 °C) if analysis was to be done later.

**Isolation and aggregation of IgM.** Human IgG was isolated from a large pool of normal human sera by precipitation with 45% saturated ammonium sulfate solution, followed by elution from diethylaminoethyl-cellulose with sodium phosphate buffer (25 mM/L, pH 8.0). The IgG so collected was concentrated by precipitation with 140 g/L sodium sulfate, diazylized extensively against saline until the diazylates were sulfate free, and stored in aliquots at −70 °C. Preparations of IgG were tested for purity by immunoelectrophoresis against antisera to whole human serum; only a single precipitation line formed. Turbidimetry (31) of these preparations in all cases yielded values for IgG exceeding 98%.

To IgG in saline, 50 g/L, was added bovine albumin to give a final concentration of 5 g/L. This mixture was heated for 30 min at 63 ± 1 °C in a water bath. This heat-aggregated preparation (HA-IgG) was stable for at least three months when stored at 4 °C.

**Turbidimetry.** The present assay is based upon the turbidity formed from the reaction between the antigen (soluble HA-IgG), and the multivalent IgM-RF antibody in the sera. The rapid generation of large complexes so formed is detected spectrophotometrically at 340 nm. To accelerate the formation of the complexes we find it convenient to add a small amount of PEG to the reaction mixture. Complement is inactivated by heating sample aliquots in a water bath at 56 ± 1 °C for 30 min. Lipemic sera are delipidated by treatment with "Lipoclean."

Table 1 lists the instrument settings for the Cobas-Bio. The use of five standards extends the useful working range for the assay from 30 to 500 units/mL. Samples with more RF than this must be diluted eightfold in saline and re-run. The notation "Type of analysis 6" corresponds to an endpoint method involving two reagents. Introducing this procedure into the present setup as a 7.6 code allows data reduction by the DENS program, in accord with the chosen printout mode "2": logit transformation.

To perform the assay, 30 μL of heat-inactivated RF calibrators, controls, or patients' sera is added automatically to the appropriate wells of the rotor, along with 100 μL of reagent buffer and 10 μL of water diluent. The contents of cuvettes on the rotor are mixed by centrifugation and incubated for 10 s at 25 °C, after which the first absorbance reading at 340 nm is taken, the "sample blank." Then, the instrument adds 50 μL of start reagent and 20 μL of water diluent and monitors the reaction via the absorbance at 340 nm. The final reading is made 300 s after mixing. Each run includes a cuvette containing only the reagent. The analyzer calculates the increase in absorbance, corrects for the reagent blank, and estimates unknown concentrations (in int. units/mL) by comparison with the concurrently analyzed RF calibrators.

**Rate-nephelometry.** For comparison, we assayed the sample by an automated rate-nephelometric assay for RF quantitation (auto ICS-II; Beckman Instruments, Inc., Fullerton, CA 92634), following the instructions of the manufacturer. The nephelometer measures the changes in lightscatter when serum containing IgM RF is added to partly aggregated human IgG. The rate of formation of the complexes is estimated by reference to a precalibrated standard curve, and the value is displayed as int. units/mL. Results <60 int. units/mL were considered negative.

**Waaler–Rose test.** We also performed a modified Waaler–Rose test procedure ("Rheuniaton"; Wampole Laboratories, Cranbury, NJ 8512) as described by the manufacturer. Agglutination of sheep erythrocytes that have been sensitized with rabbit gamma-globulin, when mixed with the 10-fold diluted serum, was considered positive.

**Analytical Variables**

**Assay optimization.** We prepared a series of standard curves by twofold serial dilution of the RF calibrator in saline to give RF concentrations from 30 to 500 int. units/mL. The instrument was programmed to acquire absorbance readings at 30-s intervals from 0 to 900 s.

To investigate the optimized conditions for the assay we varied several analytical factors. We evaluated the effect of pH by using 60 mM/L phosphate buffers (pH ranging from 5.7 to 7.8) and borate buffers (pH ranging from 7.4 to 9.0), each containing 0.20 mol of NaCl per liter. The PEG concentration in the reagent buffer was varied from 0 to 30 g/L. To establish the effect of antigen concentration in the start reagent, we prepared HA-IgG solutions in diluent buffer, at concentrations of 0.75, 1.5, 2.0, 3.0, and 6.0 mg/L. The effect of temperature on the assay was examined at 25.0, 30.0, and 37.0 °C.

**Interfering substances.** We assessed the effect of bilirubin and hemoglobin by adding known amounts of these substances to a serum pool containing 250 int. units of RF per milliliter. Interference from endogenous triglycerides (triacylglycerols) was assessed by adding to the same pool various amounts of a serumnegative lipemic serum and evaluating RF recovery. The triglyceride concentration was determined by a standard enzymatic colorimetric method.

**Detection of RF excess.** The prozone phenomenon was studied by analyzing five patients' samples for which the Waaler–Rose titers ranged from 1/1280 to 1/5120.

**Precision.** Three serum pools with different RF contents were assayed repeatedly. Within-run precision was assessed with a single rotor load of 20 replicates of each pool. Day-to-day precision was evaluated by analyzing one replicate of each concentration daily over a 10-day period.

**Limit of detection.** The sensitivity of the turbidimetric assay was taken as the lowest RF concentration for which the ratio of absorbance to noise, in terms of the CV, for 100 sera analyzed without added antigen was 2 or greater.

**Linearity.** To evaluate assay linearity, we made serial twofold dilutions with saline of 20 patients' samples for

| Table 1. Cobas-Bio Settings for the Determination of IgM RF in Serum |
|-------------------------|------------------|
| 1 Units | int. units/mL |
| 2 Calculation factor | 1000 |
| 3 Std I concn | 31.3 |
| 4 Std II concn | 62.5 |
| 5 Std III concn | 125 |
| 6 Std IV concn | 250 |
| 7 Std V concn | 500 |
| 8 Limit | 500 |
| 9 Temp, °C | 25.0 |
| 10 Type of analysis | 7.6 |
| 11 Wavelength, nm | 340 |
| 12 Sample (undiluted) vol, μL | 30 |
| 13 Diluent (water) vol, μL | 10 |
| 14 Reagent vol, μL | 100 |
| 15 Incubation time, s | 50 |
| 16 Time of reading, s | 1.0 |
| 17 Interval, s | 300 |
| 18 No. of readings | 4 |
| 19 Blanking mode | 1 |
| 20 Printout mode | 2 |

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which RF content exceeded 100 int. units/mL, then analyzed these in duplicate. Values exceeding the limit of detection were multiplied by the appropriate dilution factor and compared with the expected value.

Clinical Studies

The study population consisted of nine men and 38 women diagnosed by members of the rheumatology staff as having rheumatoid arthritis according to criteria of The American Rheumatism Association (32). Their average age was 56 years (range 27–75), and the average duration of the disease was 10.5 years (range 1–36). Rheumatoid arthritis activity was assessed and an index of disease activity was calculated based on a multivariate analysis (33) of two subjective factors (morning stiffness and pain scale); two semi-objective factors (grip strength and articular index), and two objective determinations; hemoglobin concentration and erythrocyte sedimentation rate, both measured by standard methods.

The control group was 280 apparently healthy adults.

Results and Discussion

Analytical Variables

Effect of sodium chloride concentration. The ionic strength of the reaction medium and the charge density of the ions present significantly affect the antigen–antibody reaction (34). Because the concentration of sodium chloride can profoundly affect the reaction rate (35), its effect should be investigated in each antigen–antibody system. We have observed that sodium chloride affects the solubility of the antigen; consequently, a low concentration of sodium chloride in the reagent buffer will correspond to increased absorbance in the reagent blank. This effect is negligible when the sodium chloride concentration in the reaction mixture exceeds 0.15 mol/L. However, higher concentrations of sodium chloride have a marked chaotropic effect (34–36) on the reaction rate; therefore, we chose a concentration of 0.20 mol/L for NaCl in the reagent buffer, to compensate for the water added as diluent.

Effect of pH and temperature. The immunoprecipitin reaction is relatively independent of temperature and pH, within reasonable limits (34, 36, 37). We found the most sensitive pH range to be pH 6.5 to 8.5, with the maximum absorbance response at pH 7.6. Outside this pH range, the reaction was appreciably inhibited. We adopted borate buffer for all further work, because the turbidimetric response was slightly superior to that with the phosphate buffers at pH 7.6.

There were no substantial differences in the reaction rate at the three reaction temperatures we examined, although we noted a slight decrease in absorbance at higher temperatures. We therefore assayed at 25 °C.

Effect of PEG 6000. Polymer enhancement of immunochromal reactions was described by Hellsgang (38), who found PEG 6000 to be the most suitable. As Figure 1 shows, increased concentrations of PEG in the reagent buffer markedly enhance the reaction rate and increase the absorbance changes, which improves assay sensitivity and shortens analysis time. However, mixing antigen or serum specimens with buffers containing PEG may cause these solutions to become turbid, because PEG decreases the solubility of serum proteins and HA-IgG. Thus, in choosing the optimum working concentration of PEG, we want to use the maximum PEG concentration that does not lead to nonspecific precipitations. To determine this concentration, we tested the effect of various concentrations of PEG in the reagent buffer on the solubility of the antigen and on 25 serum samples analyzed under routine conditions but without the addition of the antigen. A 15 g/L PEG concentration did not produce high absorbances for reagent blanks, and nonspecific precipitation in serum samples was avoided; at higher concentrations there was substantial precipitation of antigen. We therefore used a final concentration of PEG in the reagent buffer of 15 g/L.

Effect of antigen concentration. Figure 2 shows the influence of varying the antigen concentration. Two opposite effects are produced when antigen concentration is increased: an important increase in absorbance for the high RF values, thus extending the assay range, and a marked decrease in absorbance for the low RF values, thus decreasing sensitivity. As a compromise between these effects we used an antigen concentration of 2 mg/mL in the start reagent.

Method Performance

The turbidimetric method performance is demonstrated in several areas:

Kinetics of the immunocomplex formation. The turbidimetric curves of absorbance vs time (Figure 3) all show a similar shape: an initial rapid increase in absorbance when antigen is added, then a leveling off. There was little increase in absorbance after 300 s, and the fit of the standard curve was the best for values taken at this time; therefore we used this reaction time in all further work, which allowed analysis of more than 100 specimens per hour.

![Graph](https://via.placeholder.com/150)

Fig. 1. Effect of PEG 6000 on the reaction rate of the RF assay
PEG concentration (g/L): 0, 15; V, 10; A, 5; M, 0

![Graph](https://via.placeholder.com/150)

Fig. 2. Effect of antigen concentration on absorbance changes for three RF calibrators: 500 (○), 250 (▼), and 62.5 int. units/mL (△)
Standard curve. Figure 4 illustrates a typical standard curve for RF determination. The range covered by this curve suffices to provide an excellent signal-to-noise ratio for accurate determination of RF. The change in the response from 30 to 500 int. units/mL is more than 0.300 A; an absorbance change (ΔA) of 0.015 is equivalent to 30 int. units/mL.

Interferences. Hemoglobin (up to 5600 mg/L), bilirubin (up to 340 μmol/L), and tryglycerides (up to 2.20 mmol/L) do not interfere with either immunocomplex formation or the turbidimetric reading. Icteric and hemolyzed samples assayed by this method gave results similar to those obtained by the rate-nephelometric procedure compared. However, lipemic samples gave different results by each method.

Table 2 summarizes the results we obtained for five lipemic sera before and after delipidation. Both the turbidimetric and the nephelometric method are affected by lipemia, but the effect on the turbidimetric procedure was statistically significant (r = 0.900, p <0.001). We conclude that all samples with a triglyceride concentration exceeding 2.20 mmol/L should be clarified before assaying.

Detection of RF excess. The problem of "antigen excess" is a difficult one in immunoprecipitation. In any immunoprecipitin method one should be able to distinguish when antigen or antibody is in excess, to facilitate the accurate measurement of the amount of precipitin formed. Figure 5 illustrates the response curve of a specimen with a Waaler-Rose test of 1/5120 and an estimated RF content about 30 000 int. units/mL. Similar results were obtained for four other high-RF sera analyzed.

The course of the precipitation pattern in the RF excess zone does not follow the classical Heidelberger curve. Very high RF concentrations do not produce a decrease in measured absorbances; on the contrary, there is a tendency to plateau, at least up to the concentration assayed. Thus, the risk of misinterpreting a high value of RF is very small indeed. This is in contrast to the fluid-phase immunoprecipitin reaction of proteins, but it has been observed by others in the nephelometry of RF (39).

Precision. Table 3 shows the precision of the method at various RF concentrations. The precision and reproducibility of the turbidimetric assay are excellent, with CVs of less than 5%, even for relatively low concentrations of RF.

Table 2. Results for RF in Five Patients' Lipemic Samples before (L) and after (DL) Delipidation

<table>
<thead>
<tr>
<th>Triglyceride in serum, mmol/L</th>
<th>Turbidimetry</th>
<th>Rate-nephelometry (ICS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>DL</td>
</tr>
<tr>
<td>2.22</td>
<td>179</td>
<td>270</td>
</tr>
<tr>
<td>2.26</td>
<td>126</td>
<td>142</td>
</tr>
<tr>
<td>2.43</td>
<td>130</td>
<td>184</td>
</tr>
<tr>
<td>3.55</td>
<td>61</td>
<td>2019</td>
</tr>
<tr>
<td>3.80</td>
<td>828</td>
<td>2018</td>
</tr>
</tbody>
</table>

Sera were delipidated by treating 1.5 mL of sera with 1.0 mL of Lipoclean.

Table 3. Precision Data

<table>
<thead>
<tr>
<th>Rheumatoid factor, int. units/mL</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample I</td>
<td>69</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Sample II</td>
<td>132</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Sample III</td>
<td>261</td>
<td>3.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Day-to-day*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample I</td>
<td>81</td>
<td>2.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Sample II</td>
<td>126</td>
<td>4.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Sample III</td>
<td>250</td>
<td>6.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*n = 20. *n = 10.
error of this method is comparable with that of other quantitative techniques for RF quantification (25, 28).

Specificity for pentameric IgM RF. This method appears to be specific for pentameric IgM RF. Treatment of RF-positive samples with equal volumes of a 10 mmol/L dithiothreitol solution for 30 min completely abolished their RF activity as detected by the turbidimetric assay (data not shown).

Linearity. Most samples diluted serially showed some nonlinearity at the RF values measured. The difference between the extreme values for each sample was always less than 40%. The most severe departure from linearity normally occurred with undiluted sample. This effect can not be attributed to endogenous interference from lipids, because delipidating the same samples before diluting them gave similar nonlinear results. This lack of linearity is probably related to interference from endogenous IgG (40), a phenomenon also noted by others for the ICS RF assay (28).

Method comparison. Sera from 296 patients, submitted from the rheumatology unit, were analyzed for RF activity by the Waaler–Rose test, the RF ICS rate nephelometry, and the turbidimetric method. Analytical sensitivity and specificity obtained are summarized in Table 4. We considered as true positive or true negative those specimens that were positive or negative for at least two of the three assays. The frequency of "false-positive" results appears to be the same for all three methods, but the Waaler–Rose test showed the highest incidence of "false-negative" results. The sensitivity of the other two methods was excellent and similar to each other.

The concentrations of IgM RF in pathological sera as measured by the turbidimetric assay were compared with those obtained by the other two methods. Correlations were determined by calculating Spearman's rank correlation coefficients ($r_s$). The overall correlation coefficient between the turbidimetric assay and the ICS method was excellent ($r_s = 0.932$) and better than others reported between quantitative assays (25). However, as Figure 6 shows, nephelometry overestimates RF values that exceed 400 int. units/mL. The poor linearity of the nephelometric method (28) may explain this phenomenon, because the ICS system requires higher dilutions of sera in the reaction mixture to measure RF exceeding 400 int. units/mL. Results by turbidimetry ($r_s = 0.601$) and nephelometry ($r_s = 0.522$) correlated poorly with those by the modified Waaler–Rose test, presumably because of the inherent subjectivity and lack of precision of slide-agglutination tests (29).

Clinical Studies

Serum samples were positive for RF in 34 of 47 patients (73%) who had definite or classical rheumatoid arthritis, but in only nine (3.4%) of 260 controls. Thus the clinical specificity and sensitivity of the turbidimetric assay is similar to that obtained with slide-agglutination or nephelometric techniques (25, 26, 29). However, there was no significant correlation between actual RF values and the individual activity index ($r_s = 0.283$). This confirms others' reports (11, 41, 42) that the concentration of serum IgM RF does not reflect the activity of rheumatoid arthritis.

We conclude that turbidimetry provides an attractive alternative to other methods of RF estimation. Although turbidimetry does not increase the diagnostic specificity or sensitivity of RF detection, it is easier to perform, less time consuming, very inexpensive, and more precise than previous tests.

References