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Automated Turbidimetry of Rheumatoid Factor without Heat Inactivation of Serum

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Summary: We describe an improved turbidimetric procedure for rheumatoid factor measurement in human serum. Heat-aggregated human IgG is used as antigen. Interference produced by the complement component C1q, which required a previous heat pretreatment of sera, is avoided by the addition of the chemical inhibitor, poly(vinyl sulphonic acid). This inhibitor eliminates C1q interference without affecting the attachment of rheumatoid factor to the Fc part of IgG, thus permitting full automation of the assay.

After studying the reactivity of different heat-aggregated IgG preparations, we optimized the procedure for preparing the antigen in order to attain maximal reactivity: IgG was heated at 63 °C until the absorbance at 340 nm of a 1/10 dilution of the antigen lay between 0.65 and 0.95 absorbance units. The study of antigen stability showed a maximum aggregation two weeks after preparation. The antigen was then stable and could be used in the assay.

The standard curve extends from 30 to 500 × 10^3 IU/l. Intra- and inter-assay CV were less than 5.5%. Relative analytical sensitivity and specificity were 98.3% and 95.2%, respectively. The results agreed well with those obtained by the non-improved turbidimetric procedure ($r = 0.996$) and with nephelometry values on the Behring nephelometer analyser ($r = 0.964$). The correlation with an enzyme immunoassay was also good ($r = 0.923$).

Introduction

Rheumatoid factors are antibodies directed against the Fc portion of the IgG molecule. Rheumatoid factor can be detected in the serum of the majority of patients with rheumatoid arthritis (1). Rheumatoid factor measurement is important for the diagnosis and prognosis of rheumatoid arthritis, as those patients with high concentrations tend to suffer a more severe form of illness and to develop extra-joint complications more often (2).

Several methods for rheumatoid factor measurement (3, 4) have the disadvantage of requiring prior heating of serum at 56 °C for 30 minutes in order to prevent the binding of C1q to the Fc part of IgG, which would cause a positive interference (5).

To initiate activation of the classical pathway of complement, globular head regions of C1q are involved in ligand binding to the Fc parts of IgG of immune complexes (6), as well as a variety of non-antibody ligands, such as polyanions (7–8), polynucleotides (9) and several bacterial components (10). Therefore, these non-antibody agents could be used to eliminate the harmful effect of C1q in rheumatoid factor assays.

This work shows how the effect of complement is avoided by adding a chemical inhibitor. In this way, a previously published turbidimetric method (11) was improved, making it comparable in simplicity and analytical quality to the new automated nephelometric tests (12). The improved method was extensively evaluated and the results compared with those obtained with three other quantitative techniques.

Materials and Methods

Samples

Samples from our clinical laboratory and from the rheumatology unit of our hospital were used. Sera that were not measured immediately were stored in plastic containers at −30 °C until analysis. For method comparison studies, only one freeze/thaw cycle was used. Lipaemic samples were delipidated by the "Lipoclean" treatment.

Apparatus

A Cobas-Bio centrifugal analyser (Hoffmann-La Roche, Basel CH-4002, Switzerland) equipped with a DENS program to adjust non-linear standardization curves was used.

Reagents

Polyethylene glycol (PEG; M_r 5000−7000) was purchased from E. Merck, Darmstadt, W-6100, Germany; Poly(vinyl sulphonic acid, sodium salt), 317 g/l in water was from Aldrich Chemical Company, Milwaukee, WIS 53233, USA, Ref. 27,842-4; Heparin (calcium salt) and dextran sulphate (sodium salt) were from Sigma Chemical Co., St. Louis, MO 63178, USA, Refs. H-9768 and D-6001 respectively. Bilirubin was from Sigma, Ref. B-4126; Intralipid 20% was from Kabivitrum, Stockholm, Sweden; and "Lipoclean" clearing agent from Behring Institute (Behringwerke AG, Marburg, Germany). Purified human C1q was from Chemicon International, Los Angeles, USA. All other reagents were analytical grade.

Reagent 1

Borate buffer (50 mmol/l, pH 7.6) containing 0.2 mol of NaCl, 15 g of polyethylene glycol, and 15 mmol of NaN_3 per litre, with the pH adjusted to 7.6 with a 0.2 mol/l solution of NaOH. One part of polyvinyl sulphonate (PVS) is added to 9 parts of this buffer. Reagent 1 prepared in this way contains 31.7 g polyvinyl sulphonate per litre and was stable at least up to 8 months if kept at 4 °C.

Antigen diluent

Borate buffer (50 mmol/l, pH 7.6), with 150 mmol of NaCl and 15 mmol of NaN_3 per litre, pH adjusted as above.

Preparation of heat-aggregated IgG

Human IgG was isolated from a large pool of normal human sera by precipitation with 45% saturated ammonium sulphate solution, followed by elution from diethylaminoethyl-cellulose with sodium phosphate buffer (25 mmol/l, pH 8.0). The IgG so collected was concentrated by precipitation with 140 g/l sodium sulphate, dialysed extensively against saline until the dialysates were sulphate-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 was formed. To IgG in saline (50 g/l) was added bovine albumin to give a final concentration of 5 g/l. This mixture was heated at 63 ± 1 °C until the absorbance at 340 nm of a ten-fold dilution in antigen diluent lay between 0.65 and 0.95 absorbance units.

Start reagent

Working antigen reagent was prepared just before use by diluting the heat-aggregated IgG 25-fold with antigen diluent.

Rheumatoid factor calibrator

The rheumatoid factor-positive calibrator used throughout this study was pooled from sera with concentrations of rheumatoid factor higher than 300 × 10^3 IU/l as determined by nephelometry. The rheumatoid factor value, following the procedure described below, was established with reference to the standard rheumatoid arthritis serum of the WHO (1st. International Reference Preparation. Centraal Laboratorium V. D. Bloedtransfusiedienst. Amsterdam. The Netherlands). Rheumatoid factor concentration was adjusted to 500 × 10^3 IU/l, and the calibrator was stored in aliquots at −30 °C. A calibration curve was prepared prior to analysis by serial two-fold dilutions of the rheumatoid factor calibrator using as diluent a heat-inactivated negative rheumatoid factor pool sera. The following points were obtained: 31.3, 62.5, 125, 250 and 500 × 10^3 IU/l.

Turbidimetric method in the Cobas-Bio (11)

The analysis is based upon the turbidity produced by the reaction between antigen (soluble heat-aggregated IgG) and the rheumatoid factor antibody in the sera. The formation of immune complexes is measured spectrophotometrically at 340 nm. To perform the assay, 30 µl of rheumatoid factor calibrators, controls or patient' sera is added automatically to the appropriate wells of the rotor, along with 100 µl of reagent 1 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 ("sample blank"). The instrument then 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 reagents. The analyser calculates the increase in absorbance, corrects for the reagent blank and estimates unknown concentrations (in 10^3 IU/l) by comparison with the concurrently analysed rheumatoid factor calibrators. The calibration curve is adjusted after a logit transformation by the program for the adjustment of non-linear curves from the Cobas-Bio. Samples were not heat-treated at 56 °C for 30 min when the chemical inhibitor was used.

Nephelometry

Serum samples were analysed in the Behring nephelometer analyser (BNA) (Behringwerke AG, Marburg, Germany) following the instructions of the manufacturer. The Behring NA-Latex-RF Reagent kit (Ref OUUA 10/11) for rheumatoid factor measurement was used.

Enzyme immunoassay

IgM-rheumatoid factor activity of sera was measured using the Rheumatoid Factor Microassay kit (Ref 783-22, Diamedix Corp. Miami, Fl., USA).

Results

Elimination of the complement interference

The usefulness of several polyanions such as dextran sulphate or heparin as C1q inhibitors in the rheumatoid factor test was evaluated. Although both of them inhibited the binding of C1q to IgG, unspecific precipitations were observed in the samples (results

not shown). We investigated polyvinyl sulphonate as inhibitor, in view of its chemical similarity with the above mentioned polyanions.

We checked complement interference directly by spiking heat-inactivated negative and positive sera with purified human C1q. A dilution series, up to a spiked C1q concentration of 250 mg/l, was measured for rheumatoid factor concentration both before and after heat inactivation, and with or without the addition of the chemical C1q inhibitor polyvinyl sulphonate, using the turbidimetric method. Also, we tested the C1q interference in the BNA analyser. Results are expressed in figure 1. We found that, in the absence of polyvinyl sulphonate, there was an increase in rheumatoid factor values proportional to the C1q added, in both positive and negative sera. However, there was no significant variation of rheumatoid factor values after heat inactivation of sera or when the C1q inhibitor was in the reaction medium, indicating that polyvinyl sulphonate eliminates the effect of C1q in the turbidimetric test. In addition, we did not find any effect of C1q in the BNA system, using the latex nephelometric immunoassay for rheumatoid factor quantitation.

In order to select the optimum polyvinyl sulphonate concentration, twenty-two rheumatoid factor nephelometry-negative sera were analysed, using increased quantities of polyvinyl sulphonate in reagent 1. Sera were not heat inactivated. Figure 2 shows the mean

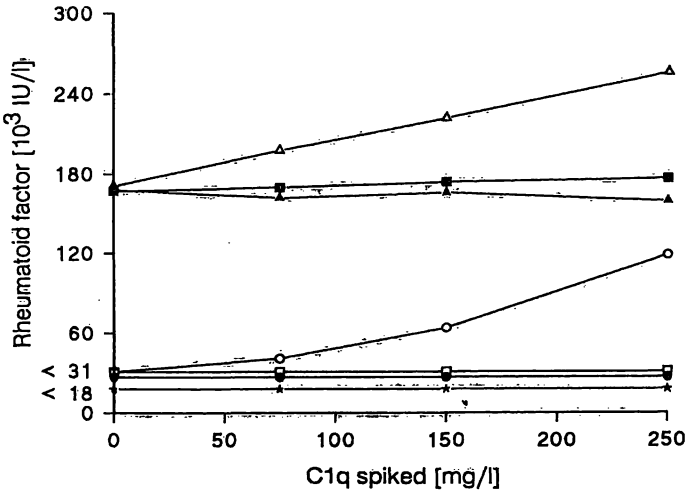

Fig. 1. Effect of added C1q on different rheumatoid factor assays. Heat-inactivated positive and negative rheumatoid factor samples were spiked with purified C1q from 0 to 250 mg/l and analysed by:
△—△ ○—○ old turbidimetric method without heat-inactivation;
▲—▲ ●—● old turbidimetric method with heat-inactivation;
■—■ □—□ improved turbidimetric method;
★—★ nephelometric method.

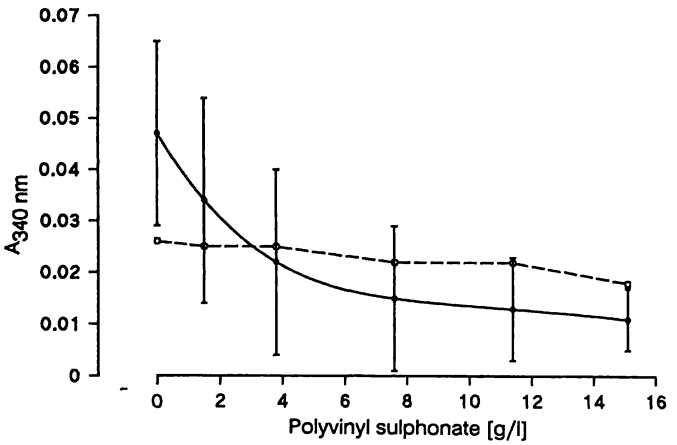

Fig. 2. Inhibition of the complement effect.
Reactivity (mean ± 2 S.D.) of 22 rheumatoid factor-negative sera versus the inhibitor concentration in the reaction cuvette.
Reactivity of standard 1: □—□ (31.3 × 10^3 IU/l).

± 2 standard deviations of the absorbance of these sera measured with the turbidimetric rheumatoid factor assay versus the polyvinyl sulphonate concentration in the reaction cuvette. Complement interference in negative rheumatoid factor samples decreased with the increase in the quantity of polyvinyl sulphonate. We chose a polyvinyl sulphonate concentration of 15.1 g/l in the reaction cuvette. This concentration of polyvinyl sulphonate produced no decrease in rheumatoid factor reactivity, and at this polyvinyl sulphonate concentration all normal serum samples tested gave rheumatoid factor values lower than the cut-off level of the assay. Moreover, unspecific precipitations were not observed in 60 serum samples in the turbidimetric rheumatoid factor test to which no heat aggregated IgG had been added (sample blank).

Reactivity of heat aggregated IgG of different degrees of aggregation

The aggregation process of IgG was studied by heat-treatment at 63 °C of seven identical aliquots of a solution containing 50 g/l IgG and 5 g/l bovine serum albumin in saline, using heating times between 5 and 45 minutes. The degree of aggregation is expressed as the absorbance at 340 nm of a 1/10 dilution in antigen diluent of the different heat aggregated IgG preparations. Figure 3 shows the reactivity of these heat aggregated IgG preparations. This experiment was carried out 2 weeks after heating. Maximum reactivity was obtained when the degree of aggregation was between 0.9 and 1.5 absorbance units, which corresponds to approximately 20 minutes of thermal treatment.

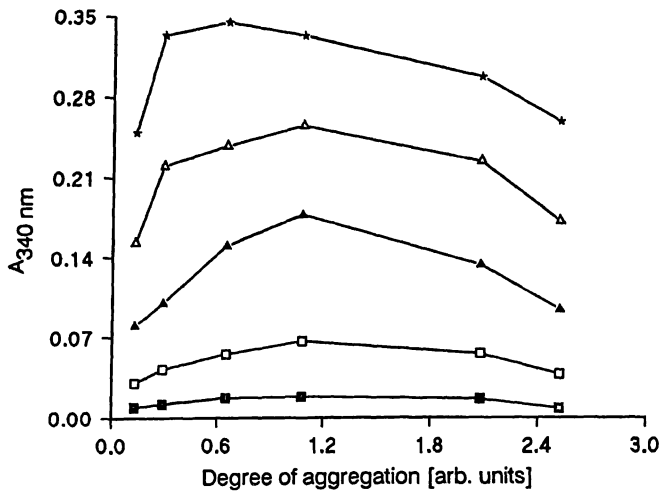

Fig. 3. Reactivity of standards with different degrees of antigen aggregation.
Degree of aggregation is expressed as the absorbance at 340 nm of 1/10 dilutions in antigen diluent of the different aggregated-IgG preparations.
rheumatoid factor, 10^3 IU/l:
▫—▫ 31.3; □—□ 62.5; ▲—▲ 125; △—△ 250; ★—★ 500.

Stability of the aggregation in time

The stability of heat aggregated IgG preparations obtained in the previous experiment was studied. The antigens were kept at 4 °C and their absorbance at 340 nm were measured at different times after the initial heating (15 min, 24 h, 7 days, 14 days, 21 days, 42 days and 4 months). Figure 4 shows that, after 2 weeks, no increase in the degree of aggregation of the IgG was observed. However, the heat aggregated IgG which was kept 45 min at 63 °C became unstable, resulting in precipitation after 48 hours.

Heat aggregated IgG used afterwards had a degree of aggregation of 0.7, as measured immediately after heating, and 1.1 absorbance units after 2 weeks of stabilization.

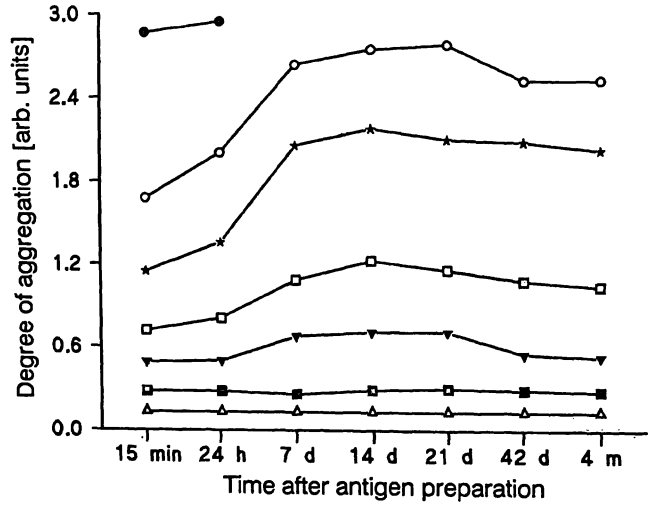

Fig. 4. Stability of aggregated-IgG preparations in time.
The increase of aggregation in time is expressed as the "degree of aggregation" (absorbance at 340 nm of 1/10 dilutions in antigen diluent of the different antigens).
The different aggregated-IgG preparations are expressed by the heating times (in minutes) employed in their preparation.
△—△ 5; ▫—▫ 10; ▼—▼ 15; □—□ 20; ★—★ 25; ○—○ 30; ●—● 45.

Analytical performances

Calibration curve: Figure 5 shows the mean ± 2 standard deviations of 10 calibration curves obtained on 10 different days. The increase in absorbance from 30—500 × 10^3 IU/l was higher than 0.3 absorbance units.

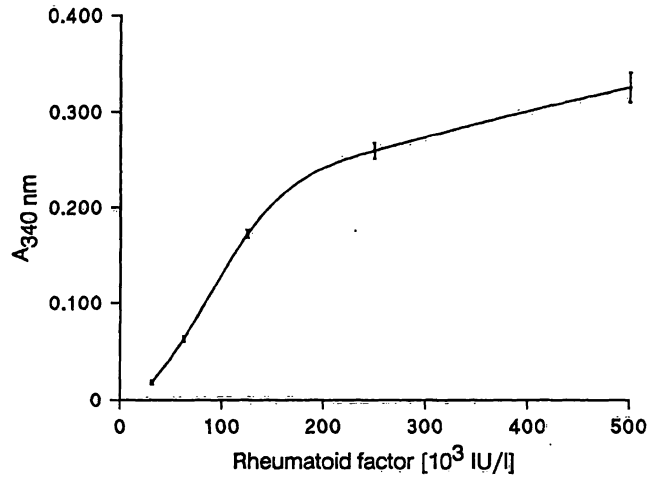

Fig. 5. Rheumatoid factor calibration curve for the improved method. Absorbance (mean ± 2 S. D. of 10 calibrations) versus standard concentrations.

Precision: Three pools of sera with different rheumatoid factor values were obtained. Intra-assay precision was determined with a single rotor charged with 12 aliquots of each pool. Inter-assay precision was evaluated by analysing one aliquot of each concentration in 12 different runs with a new calibration curve each time. Precision data are given in table 1. For interference studies we followed the procedure of *Glick* (13). We assessed the effect of bilirubin and haemoglobin by adding known amounts of these substances to a baseline serum pool containing 165 × 10^3 IU/l of rheumatoid factor. Interference from lipaemia was assessed by adding various amounts of a 20% Intralipid solution to the same pool. Figure 6 shows interferences associated with addition of haemoglobin, bilirubin and Intralipid. No effects were seen with levels up to 340 µmol/l for bilirubin and 2300 mg/l for haemoglobin. A negative interference was found when Intralipid was added. After treatment of the Intralipid spiked samples with "Lipoclean" clearing agent, the rheumatoid factor activity was completely recovered ($x = 101.1 \pm 1.8\%$; $n = 4$).

Tab. 1. Precision data

	Rheumatoid factor (10^3 IU/l)		
	Mean	S.D.	CV (%)
Intra-assay[a]			
Sample I	50	1.6	3.1
Sample II	123	2.5	2.0
Sample III	225	4.5	2.0
Inter-assay[b]			
Sample I	54	2.9	5.3
Sample II	127	4.6	3.6
Sample III	223	6.7	3.0

[a] n = 12 [b] n = 12

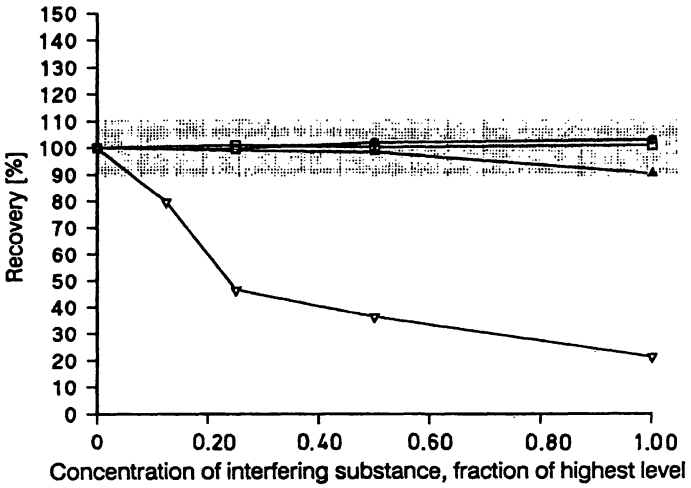

Fig. 6. Interference studies in the improved turbidimetric test. On the abscissa are represented the concentrations of interfering substances, expressed as % of highest level: ▽—▽ Intralipid 0.5%; ▲—▲ haemoglobin 2300 mg/l; □—□ bilirubin 340 µmol/l; ●—● Intralipid-spiked series treated with "Lipoclean" clearing agent.

Relative analytical specificity of the improved rheumatoid factor test

Rheumatoid factor activity of 130 routine samples from our laboratory was analysed by the nephelometric and by the improved and old turbidimetric methods. A serum was considered rheumatoid factor-positive or rheumatoid factor-negative whenever at least two out of three test results were, respectively, positive or negative. The relative specificities obtained, defined as the ratio of true negatives to the sum of true negatives and false positives for each method, are shown in table 2.

Method comparison and relative analytical sensitivity

A wide range of rheumatoid factor concentrations in sera was measured by the turbidimetric method with chemical inhibitor, and by the other three methods.

Tab. 2. Relative sensitivities and specificities of the several rheumatoid factor methods

	Specificity (%)	Sensitivity (%)
Improved turbidimetry	95.2	98.3
Turbidimetry with heat inactivation	97.6	96.7
Nephelometry	96.8	98.3
Enzyme-immunoassay	–	96.7

Tab. 3. Method comparison (correlation coefficients)

	Improved turbidimetry	Turbidimetry with heat inactivation	Nephelometry
Enzyme-immunoassay	0.923	0.917	0.926
Nephelometry	0.964	0.962	
Turbidimetry with heat inactivation	0.996		

Correlations between all the assays are shown in table 3. It can be seen that the coefficients of correlation between methods were all higher than 0.90. In figure 7, the serum values obtained with the improved method are plotted against the three other tests.

Values obtained from the previous comparison studies were used to calculate relative analytical sensitivities. A serum was considered rheumatoid factor-positive or rheumatoid factor-negative when it was, respectively, positive or negative in at least 3 out of 4 tests. The analytical relative sensitivity (tab. 2) is defined as the ratio of true positives to the sum of true positives and false negatives for each method.

Discussion

Several authors have described the binding of polyanions to the C1q fraction of complement (15—17). Although dextran sulphate and heparin inhibited C1q binding to IgG, unspecific precipitations occurred in the samples, disqualifying the use of these inhibitors in the turbidimetric assay of rheumatoid factor. We researched the possibility of using polyvinyl sulphonate as C1q inhibitor, in view of its chemical similarity with the above mentioned polyanions.

Complement interference, which required previous inactivation of samples at 56 °C for 30 minutes, was avoided by adding polyvinyl sulphonate to the reaction medium. Unspecific precipitations were not observed with this polyanion. The harmful effect of heat-pretreatment of samples on endogenous rheumatoid factor has been described (5, 18). Elimination of this step, therefore enabled us to avoid this inconvenience

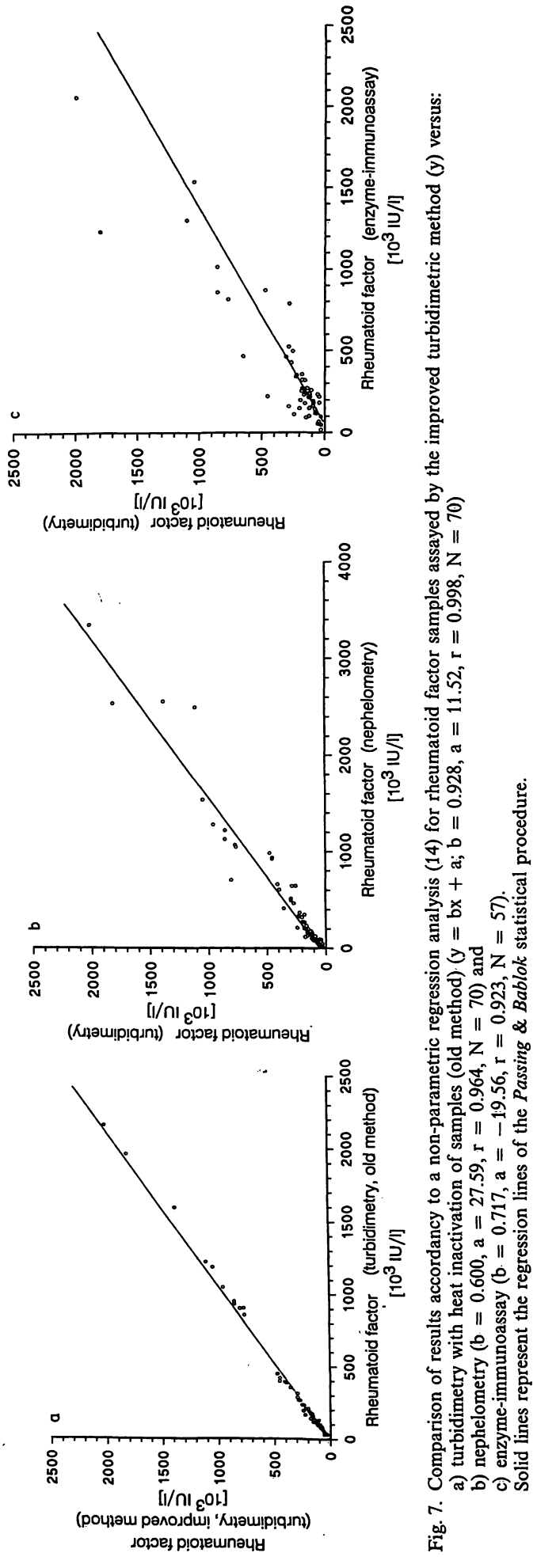

Fig. 7. Comparison of results accordancy to a non-parametric regression analysis (14) for rheumatoid factor samples assayed by the improved turbidimetric method (y) versus:
a) turbidimetry with heat inactivation of samples (old method) (y = bx + a; b = 0.928, a = 11.52, r = 0.998, N = 70)
b) nephelometry (b = 0.600, a = 27.59, r = 0.964, N = 70) and
c) enzyme-immunoassay (b = 0.717, a = −19.56, r = 0.923, N = 57).
Solid lines represent the regression lines of the *Passing & Bablok* statistical procedure.

and achieve complete automation of rheumatoid factor turbidimetry.

By determining reactivity for different degrees of aggregation, it was possible to standardize the preparation of heat-aggregated IgG. For this purpose, the 30 minute heat treatment at 63 °C (19) is replaced by more homogeneous and reproducible final conditions, giving maximal reactivity in the measurement of rheumatoid factor. The heat-aggregated IgG so obtained, stored at 4 °C, continues to aggregate for approximately 2 weeks, and cannot be used until then. It then remains stable for at least 4 months, in contrast to the aggregated-IgG obtained by other investigators (20).

This improved method shows intra- and inter-assay CVs similar to those of the original procedure (11). Thus, even for low rheumatoid factor concentrations, the CVs were less than 5.5%, values comparable or better than those of other quantitative techniques (3, 21–23). The test has excellent relative analytical specificity and sensitivity compared with other assays (24, 25). The correlation coefficient between the two turbidimetric procedures was exceptional (r = 0.996), probably due to the fact that both use the same antigen and the same apparatus. On the other hand, the discrepancy in bias might be explained by the effect of heat inactivation (5). Also, the improved turbidimetry procedure correlates well with the nephelometric assay (r = 0.964), but not so well with an enzyme-immunoassay (r = 0.923). Correlation of the enzyme-immunoassay values with those of the rest of the procedures was in every case worse than the other comparisons (tab. 3), a result already obtained by other authors (26, 27). Proportional differences between the present method and the nephelometric and enzyme-immunoassay tests were considerable. This indicates possible differences in the standardization of the procedures, although the calibrations in all cases were in accordance with the WHO standard; similar observations have been reported elsewhere (27).

In conclusion, addition of polyvinyl sulphonate to the reagent buffer of a turbidimetric procedure for rheumatoid factor quantification eliminates the time-consuming sample heat-inactivation and removes the error associated with this treatment on rheumatoid factor values (5). This improved method correlates well with other quantitative assays, showing the advantages of complete automation, simple operation and reduced costs. The analytical quality similar to that of the other methodologies. In addition, the procedure can be easily automated in other analytical systems.

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