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Particle Counting Immunoassay (PACIA) of Ferritin[1])

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Summary: Particle Counting ImmunoAssay (PACIA) has been adapted to the determination of serum ferritin. Polystyrene particles (0.8 μm) were coated with anti-ferritin antibodies and the concentration of ferritin determined by the agglutinating activity of this protein. The agglutination was measured by the reduction of the number of non-agglutinated particles counted in an optical blood cell counter. Non-specific agglutination and inhibition of agglutination were prevented in 99% of clinical samples by the use of $F(ab')_2$ fragments of the antiserum IgG to coat the particles and by using slightly dissociating conditions (pH 9.2, ammonium thiocyanate, EDTA). The system was automated with a sampling rate of 50/h and an incubation time of 25 min. The standard curve ranged from 1 to 100 μg/l; recoveries were between 93.6 to 100.2%; the correlation coefficients of PACIA with radioimmunoassays calculated respectively on 99 and 91 samples were 0.974 and 0.984; maximal within- and between assay CV were 11.2% and 7.7%.

Particle Counting Immunoassay (PACIA) für Ferritin

Zusammenfassung: Das Prinzip des Particle Counting Immunoassay (PACIA) wurde auf die Bestimmung von Ferritin angewandt. Polystyrolteilchen (0,8 μm) wurden mit Antikörper gegen Ferritin beschichtet und Ferritin durch Agglutination bestimmt. Die Agglutination wurde aus der Verminderung der Anzahl nicht agglutinierter Teilchen mit einem optischen Blutzell-Zählgerät ermittelt.

Unspezifische Agglutination und Agglutinationshemmung wurden durch F(ab')-Fragmente von IgG des zur Beschichtung der Teilchen benutzten Antiserums sowie Bedingungen, bei denen die Dissoziation gering ist (pH 9,2; Ammoniumthiocyanat, EDTA), in 99% der klinischen Proben vermieden.

Das System wurde mit einer Probenrate von 50/h und einer Inkubationszeit von 25 min mechanisiert; die Standardkurve reichte von 1–100 μg/l; die Wiederfindung betrug 93,6–100,2%; die Korrelationskoeffizienten mit dem Radioimmunassay mit 99 bzw. 91 Proben waren 0,974 bzw. 0,984; die höchsten Variationskoeffizienten in der Serie und von Tag zu Tag wurden zu 11,2 bzw. 7,7% errechnet.

Introduction

Ferritin is generally determined in serum to assess the iron stores of the organism and as an index of tissue necrosis or progress of certain tumours (1). The low concentration of this protein, which normally ranges from 12 to 250 μg/l (1), requires sensitive techniques such as radioimmunoassay (RIA), immunoradiometry, and enzymeimmunoassay.

We describe here the application of a novel technique, called Particle Counting ImmunoAssay (PACIA), to the assay of ferritin. The principle of the technique is the agglutination of polystyrene particles of 0.8 μm dia-

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[2]) Aspirant at "Fonds National de la Recherche Scientifique".

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meter (latex), the extent of the reaction being measured by counting the residual non-agglutinated particles by a device designed to count blood cells.

The advantages of PACIA are:

(i) versatility, the technique being suitable for the determination of antigens (2, 3, 4), haptens (2), antibodies (2, 5), and immune complexes (6, 7);

(ii) sensitivity (5 μg/l) for ferritin despite a short incubation time of 30 min;

(iii) lack of interference by non-specific agglutinating or inhibiting factors (8, 4);

(iv) automation, which ensures precision, speed and facility.

Materials and Methods

Buffers and diluents

Glycine buffered saline contained 0.17 mol/l glycine, 0.1 mol/l NaCl and 0.04 g/l sodium azide, the pH being adjusted to 9.2 with NaOH. Glycine buffered saline-bovine serum albumin was containing 10 g/l bovine serum albumin (Biograde from Calbiochem, San Diego, CA). The serum and standard diluent was a mixture of 1 vol of normal rabbit serum and 9 vol of glycine buffered saline-bovine serum albumin. The solution called "additive" was glycine buffered saline supplemented with 50 mmol/l ethylene diamine tetraacetic acid, 3 mol/l thiocyanate and 60 g/l polyethylene glycol 6000 (Merck, Darmstadt, FRG).

Ferritin

Pieces of a human liver obtained at autopsy (231 g) were homogenized in 1.6 l of ice cold 0.25 mol/l acetate buffer, pH 4.9. The soluble fraction was incubated with 500 g of carboxymethylcellulose in the same buffer for 1 h (9). The non-adsorbed proteins were precipitated at 0 °C by $(NH_4)_2SO_4$ at a final concentration of 350 g/l. The precipitate dissolved in water was dialysed against 1 mol/l NaCl and passed through a column (2.5 × 100 cm) of Ultrogel AcA 2.2 (LKB, Bromma, Sweden).

Ferritin was then further purified by isopycnic ultracentrifugation at 50,000 min^{-1} for 76 h in a gradient of CsCl starting at a concentration of 1.478 kg/l (SW 65 Ti rotor from Beckman Instruments, Inc., Palo Alto, CA). To remove trace contaminants the ferritin preparation was centrifuged again in an isokinetic sucrose gradient (50 to 200 g/l) with the Beckman SW27 rotor (10).

Immunoelectrophoresis of the ferritin solution (6 g/l) did not show any precipitin line with antisera against total serum proteins, hemoglobin albumin or IgG.

The concentration of ferritin was estimated by amino acid analysis (11).

Anti-ferritin sera

Three new Zealand white rabbits were immunized by intradermal multisite injections of 100 μg of ferritin in complete *Freund*'s adjuvant every two weeks. The animals were bled 6 weeks after the first injection and 10 days after each new injection. These antisera did not develop any precipitin line in immunoelectrophoresis of a normal human serum.

Preparation of $F(ab')_2$-fragments

IgG of the rabbit anti-ferritin sera were purified by $(NH_4)_2SO_4$ precipitation followed by diethylaminoethyl cellulose chromatography in 0.1 mol/l (hydroxymethyl) methylamine-HCl buffer, pH 8.5. IgG were digested in 0.1 mol/l acetate buffer, pH 4.5, for 24 h with 2 mg/l pepsin (twice crystallized, Sigma Chemicals Co., St. Louis, Mo.) for 100 mg of protein. After a tenfold dilution in distilled water, the $F(ab')_2$-fragments were separated from Fc fragments and non-digested IgG by chromatography on carboxymethyl-cellulose in 0.01 mol/l acetate buffer pH 4.5 with a linear NaCl gradient. After concentration and dialysis against physiological saline, the $F(ab')_2$-fragments were stored at − 20 °C with 0.2 g/l sodium azide.

Coupling of $F(ab')_2$-fragments to latex with carbodiimide

Carboxylated latex of 0.8 μm diameter (Estapor K150, batch No. 314, suspension at 100 g/l) was a gift from Dr. *J. C. Daniel*, Rhône-Poulenc, Courbevoie, France. The particles in 1 ml of the starting suspension were washed with 10 ml of 0.02 mol/l borate-buffered saline, pH 8.1, resuspended in 2 ml of the same buffer and incubated with 50 mg of 1-ethyl-3 (3-dimethylamino-propyl)-carbodiimide HCl (Sigma Chemical Co., St. Louis, Mo.), at room temperature, for 40 min with vortex mixing. After centrifugation the carbodiimide-activated latex was resuspended in 2 ml of borate buffered saline and mixed with 1.5 mg of $F(ab')_2$-fragments also in 2 ml of borate buffered saline. After overnight incubation at 4 °C on a rotating mixer 1 ml of glycine buffered saline-bovine serum albumin was added; then the particles were washed once with glycine buffered saline and twice with glycine buffered saline-bovine serum albumin. After resuspension in 20 ml glycine buffered saline-bovine serum albumin, agglutinates were dissociated by brief sonication (10 s) with the Branson Sonifier B12 (Danburry, CT). $F(ab')_2$-latex was stored lyophilized or frozen in 200 μl aliquots. Before use the lyophilized latex resuspended in 200 μl of water or the thawed aliquot were diluted 8-fold with glycine buffered saline-bovine serum albumin and sonicated. Each aliquot was sufficient for 40 tests.

Ferritin measurement

PACIA

The Technicon PACIA system (Technicon International Division, Geneva, Switzerland), as marketed for the measurement of immune complexes, was used for this assay. Briefly, the equipment, which is described in detail elsewhere (4, 12) consists of four modules;

(i) the DIAS (Diluter-Incubator-Agitator-Sampler) which aspirates the sample (50 to 60 per h), mixes it with additive and latex and dilutes the suspension as a first step to reduce the count to 3000–4000 particles per second;

(ii) the continuous flow manifold into which the reacted sample is introduced by the peristaltic Technicon pump III;

(iii) the optical cell counter (AutoCounter);

(iv) the Technicon Slimline recorder indicating the concentration of particles flowing through the cell by peak height.

The temperature of the water bath was set at 37 °C and the stations of DIAS positioned to permit an incubation time of 25 min. The total throughput time was 30 min.

After centrifugation for 5 min at 5000 min^{-1}, unmeasured amounts (50–200 μl) of the serum samples diluted tenfold with glycine buffered saline-bovine serum albumin-normal rabbit serum were pipetted into sample cups and placed in the inner row of the sampler tray.

Reference assays

The accuracy of the results of PACIA was evaluated by reference to conventional (double antibody) radioimmunoassays using the commercial reagent kits of Clinical Assays (Cambridge, Mass., 02139) and CIS (CEA, BP21, F-91190 Gif-sur-Yvette).

Results

Calibration curves

A plot of peak height (number of non-agglutinated particles) vs. log ferritin concentration formed a sigmoidal curve extending from 1 to 100 μg/l (fig. 1). Calibra-

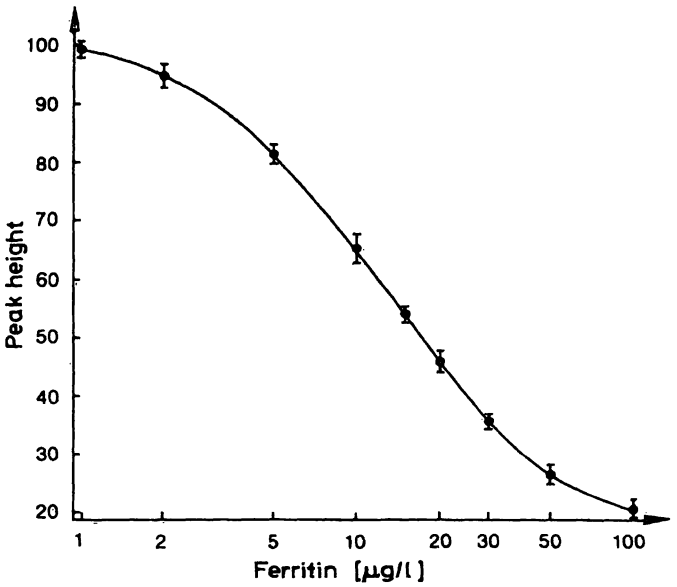

Fig. 1. Standard curve for the determination of ferritin by PACIA. This is a composite of 7 curves run on the same day. Vertical bars = 1 SD. Peak height is proportional to the concentration of non-agglutinated particles flowing through the AutoCounter.

tion curves were established not only with liver ferritin but also with spleen ferritin (a gift from Dr. *M. Worwood*, Cardiff, U.K.). Standard curves with both types of ferritin were similar. Because of the high sensitivity most patients' sera had to be diluted 10 or 20 times to fit the calibration curve. Excess of agglutinator can result in a decrease of agglutination. This so-called prozone effect can be observed in PACIA (2). With the present latex preparation a significant prozone effect was observed at a concentration of ferritin of about 50,000 µg/l. The corresponding peak height indicated a concentration of 50 µg/l on the calibration curve.

Non-specific agglutination

The agglutinating activity of 246 sera, diluted 10-fold, was estimated with a latex coated with $F(ab')_2$-fragments from a non-immunized rabbit. The same concentration of $F(ab')_2$-fragments was used as for the preparation of anti-ferritin latex. The samples were from patients with various disorders which could affect the physical and immunological properties of the sera, i.e. presence of rheumatoid factor, chylomicrons, monoclonal immunoglobulins and haemoglobin. The coefficient of variation for the mean concentration of non-agglutinated particles expressed as peak heights, was 2.5%, corresponding in this region of calibration curve to a theoretical ferritin level of about 5 µg/l, taking into account the 10-fold dilution of the sample. These results indicated that non-specific agglutination was negligible.

Analytical recovery

Ferritin was assayed by PACIA in a first group of 30 patients' sera. They contained between 24 and 320 µg/l of ferritin. Each serum was then supplemented with 176 µg/l of ferritin and reassayed at a 20-fold dilution. The mean concentration of ferritin recovered was 170.6 µg/l (SD = 15.4) corresponding to a recovery of 96.7% (CV = 5.6%). The test was repeated in another group of 25 patients' sera with ferritin concentrations ranging from 20 µg/l to 478 µg/l, the concentration of ferritin added being 388 µg/l. The mean concentration recovered was 386 µg/l (SD = 22) corresponding to a recovery of 99.5% (CV = 5.6%).

Recovery was also assessed on 5 sera supplemented with 10 µg/l, 100 µg/l, and 1,000 µg/l of ferritin, and analyzed at a 10-fold dilution (tab. 1). Considering the results obtained in each serum supplemented with the three different concentrations of ferritin we did not observe any systematic increase or decrease of the recovery, indicating that these five sera were devoid of non-specific agglutinators or inhibitors. The recovery (93.6%) after addition of 1,000 µg/l ferritin was relatively low, probably because this concentration is at the upper limit of a flattening calibration curve.

Tab. 1. Analytical recovery of ferritin by PACIA. Ferritin concentration in patients' sera.

	Ferritin recovered (µg/l) after addition of		
(µg/l)	10 µg/l	100 µg/l	1,000 µg/l
92.3	11.6	114.0	1002
13.3	10.5	94.2	924
< 10	ND	83.0	948
14.5	11.0	87.3	905
24.5	7.0	100.0	901
Mean:	10.02	95.7	936
SD:	2.06	12.1	41.3
Recovery:	100.2% ± 20.6	95.7% ± 12.1	93.6% ± 4.1

ND = not determined

Precision

The PACIA precision was studied on four sera with ferritin concentrations ranging from 25 to 420 µg/l (tab. 2). The assays were repeated 20 times during the same day and once daily during 20 days. Maximal with-

Tab. 2. Precision of PACIA in its application to the determination of ferritin.

Within assay*		Between assay**	
Concentration (µg/l ± SD)	CV (%)	Concentration (µg/l)	CV (%)
25 ± 1	4.0	28 ± 2	6.0
78 ± 2	2.6	55 ± 3	5.8
260 ± 10	3.8	175 ± 13	7.3
420 ± 50	11.9	338 ± 26	7.7

* Twenty assays repeated on the 4 samples within a day with one standard curve.
** Assay repeated during 20 days with a new latex suspension taken every day from the same lyophilized batch.

in-assay and between-assay CVs were 11.9% and 7.7%. (The large within-assay CV arose from a single analysis of the 420 µg/l sample which was recorded as 640 µg/l. When this single value was rejected, the CV was then reduced to 7.5%). In the assay of α_1-fetoprotein (4) we used a special protocol, which permitted evaluation of carry-over and drift. The carry-over was minimal, as it did not exceed 1.3%, but the drift was sometimes significant.

Correlations

Sera containing 10 to about 10,000 µg/l of ferritin were assayed by RIA, using kits from Clinical Assays for 99 of them, and kits from CIS for 91 of the same sera. The samples identified only by code were then assayed by PACIA. The correlation of PACIA results with Clinical Assays and CIS results (figs. 2 & 3) were respectively r = 0.974 (y = - 18.1 + 0.97 x) and r = 0.984 (y = - 93.8 + 1.07 x). We were concerned at the high intercept (- 93.8) of the correlation curve PACIA vs. RIA CIS. The most logical explanation was one of dilution error for samples above the ranges of PACIA and RIA. Accordingly we recalculated the correlation and regression coefficients for samples not requiring extra dilution (4–500 µg/l) and those which were subsequently diluted (> 500 µg/l). The recalculated values did indeed indicate two curves: the "undiluted" curve with r = 0.90 and y = 7.21 + 1.1 x and the "diluted" curve with r = 0.99 and y = - 521 + 1.16 x. These values agreed with the correlation between CIS and Clinical Assays r = 0.99 and y = 18.1 + 0.99 x and PACIA vs. Clinical Assays r = 0.97 and y = - 18 + 0.97 x (fig. 4).

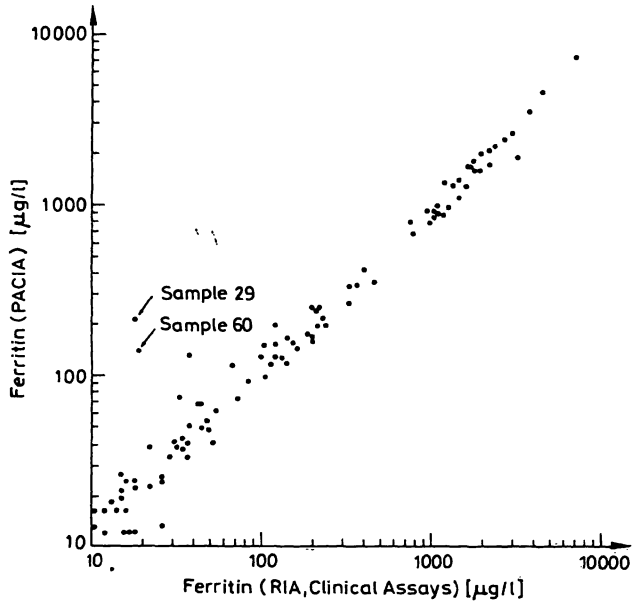

Fig. 2. Correlation between the PACIA and RIA (Clinical Assays) results for ferritin.
N = 99; r = 0.974; slope = 0.97; intercept = - 18.1.

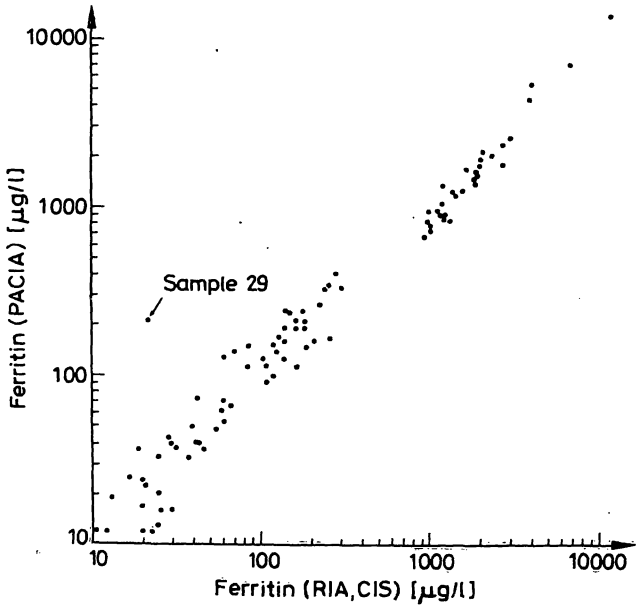

Fig. 3. Correlation between the PACIA and RIA (CIS) results for ferritin.
N = 91; r = 0.984; slope = 1.07; intercept = 93.8.

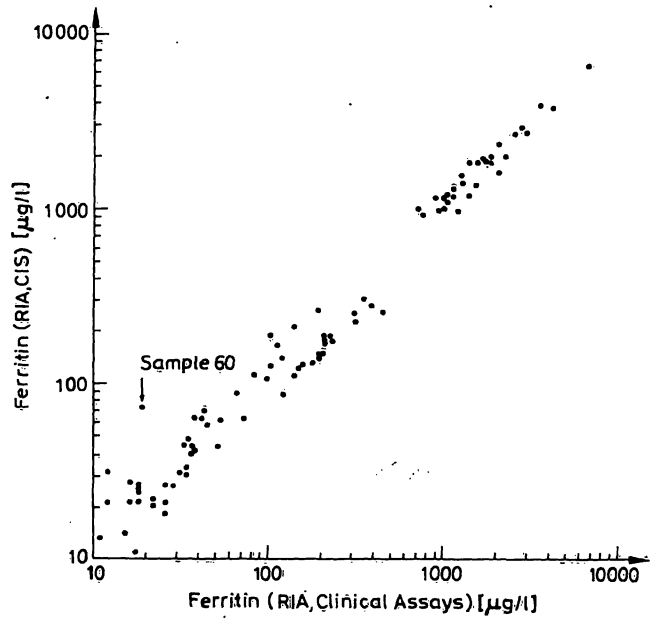

Fig. 4. Correlation between the RIA results obtained with kits from either Clinical Assays or CIS for ferritin.
N = 90; r = 0.990; slope = 0.99; intercept = 18.0.

In the PACIA vs. Clinical Assays correlation two sera (samples 29 and 60) gave discrepant results. The result of Clinical Assays was confirmed for sample 29 (18 µg/l) by the result of CIS (24 µg/l), the PACIA result being 178 µg/l. However, for sample 60, the CIS value (71 µg/l) was closer to that of PACIA (117 µg/l) than to that of Clinical Assays (19 µg/l).

It should be noted that our standards differed from those of Clinical Assays and CIS; the standards of these two kits contained, according to our own preparation, 20% less ferritin than expected. Therefore, we calculated the correlation coefficients on values corrected for this difference, assuming our standard to be correct.

A further collaborative study was undertaken on 103 samples kindly provided by Dr. *M. Woorwood*, Welsh National School of Medicine, Department of Haematology, Cardiff, Wales, Great Britain, and identified only by code. The sample had been assayed and stored at - 20 °C for not more than 2 months before our assay. Ferritin had been determined by the two-site immunoradiometric assay (IRMA) of *Miles* (13) as modified by *Dawkins* (14). Standards and immunogens were human spleen ferritin prepared according to *Worwood* (15).

Above 6,000 µg/l our agreement with the immunoradiometric assay was poor (r = 0.50 for 10 samples). However, in the range between 4 µg/l to 5,000 µg/l, the linear regression equation was: y = 32.4 + 1.054 x and r = 0.95 (N = 93).

One result was grossly discrepant (2,080 PACIA vs. 5,100 IRMA) which was checked by serial dilution on PACIA and showed no evidence of non-specific agglutination. The omission of this one aberrant result yields a linear regression equation for 92 samples: y = 16.7 + 1.01 x, r = 0.98, which is in close agreement to the values obtained with Clinical Assays and CIS.

Discussion

The weakness of most agglutination tests is the interference from various factors, i.e. non specific agglutinators such as IgM rheumatoid factor and C1q factor of complement, inhibitors to agglutination such as IgG rheumatoid factor and C3b factor of complement. Minor interference can also arise from protein-protein interaction. The present work confirms that the use of $F(ab')_2$ fragments rather than the whole immunoglobulin and the selection of slightly dissociating conditions, pH 9.2, chelation of bivalent cations by EDTA and the presence of a chaotropic agent such as ammonium thiocyanate, eliminated the interferences. However, in the correlation study one particular sample (No. 29) had, according to PACIA, a much higher concentration than that found by the two RIAs. Since this sample was unable to agglutinate latex coated with the $F(ab')_2$ fragments of the IgG of a non-immunized rabbit, a reaction between some putative antibodies in the serum and rabbit proteins present on latex was unlikely, inasmuch as the reaction medium contained 10% rabbit serum. A possible explanation would be the presence in our rabbit anti-ferritin serum of antibodies against an antigen other than ferritin and only present in few sera. Such an antigen could be a viral antigen (hepatitis Bs) or could originate from bacteria contaminating the samples. It should be remembered that in PACIA no competitive antigen is used. Therefore, the specificity of the reaction relies entirely on the antibodies coupled to latex. A practical consequence is that a correlation with RIA or a recovery study is an absolute necessity for each new batch of antiserum. The high sensitivity of PACIA requires specificity checks that are much more sensitive than gel precipitation tests.

The detection limit set at three times the noise level (16) was 13 µg/l (see Results) corresponding to 10 µg/l using Clinical Assays or CIA standards.

In addition to interferences by various serum factors the second possible cause of error in agglutination tests is the prozone effect. However, we showed that the accuracy of our assay could not have been affected, since a prozone was observed only at a very high concentration of ferritin (50 mg/l).

In conclusion, the two main causes of errors in agglutination tests have been overcome, so that PACIA provides a useful and practical assay for ferritin:

(i) radioisotopes and competitive antigens are not required,

(ii) the absence of a separation step combined with short incubation time makes the assay simple, and

(iii) the ease with which production lots of stable reagents (more than 1 year) may be prepared should reduce reagent costs to below those for radio- or enzymeimmunoassays.

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