A novel immunoradiometric assay for human liver ferritin

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SUMMARY Rivanol, the cationic salt of an acridine base, has been used as a novel separation procedure in an immunoradiometric assay for human liver ferritin. The separation step is based on the differences in charge and molecular weight between the labelled antibody-ferritin complex and free labelled immunoglobulins. The resultant assay is simple, reproducible and sufficiently sensitive to determine serum concentrations of ferritin.

All immunoradiometric assays (IRMA) are based on the use of specific antibodies labelled with a radionuclide and offer several advantages as compared with radioimmunoassays (RIA), which employ isotopically labelled antigen.^{1,2} In a conventional IRMA, of the type first described by Miles and Hales,^{3,4} sample (or standard) is incubated with an excess of the labelled antibodies and, once equilibrium has been reached, it is necessary to separate the remaining free labelled antibodies from those bound to the antigen. To date this has always been achieved by the addition of an immunoabsorbent comprising antigen covalently coupled to a solid phase.

Such a separation step is wasteful of purified antigen and prohibits the use of conventional IRMA for analytes that are either expensive or in short supply. This disadvantage is avoided in IRMA of the "two-site sandwich" type in which unlabelled antibodies coupled or adsorbed to a solid phase are used to bind all the antigen present in the sample (or standard) and the labelled specific antibodies to determine how much has been bound.⁵ However, this approach is limited to analytes having more than one antigenic determinant and is wasteful of antisera. As an alternative, it seemed likely that differences in charge and/or molecular weight between the antigen-labelled antibody complex and free labelled antibodies in some conventional IRMA might enable use of a simpler separation technique.

Horejsi and Smetana⁶ have shown that it is possible to precipitate all the main plasma proteins, other than the gamma globulins, by addition of a carefully chosen amount of rivanol at neutral or

slightly alkaline pH. The present paper describes a simple conventional IRMA for the iron storage protein, ferritin, in which rivanol is used to precipitate the ferritin-antibody complex while leaving the free labelled antibodies in the supernatant.

Material and methods

Rivanol, the cationic salt of the acridine base, 2-ethoxy 6,9-diaminoacridine lactate monohydrate, was supplied as a yellow powder of more than 97% purity by Koch-Light (Colnbrook, Berkshire); Sepharose 6B was obtained from Pharmacia (Hounslow, Middlesex); bovine serum albumin (BSA) and glutaraldehyde from Sigma (Poole, Dorset); potassium thiocyanate and potassium ferrocyanide from BDH (Poole, Dorset); human serum albumin (HSA) from Kabi (Stockholm, Sweden); Coomassie brilliant blue (R250) from Biorad (Richmond, USA); iodogen from Pierce Chemical (Rockford, USA); and carrier free Na¹²⁵I from Amersham International (Amersham, Buckinghamshire).

PREPARATION OF HUMAN LIVER FERRITIN

Ferritin was extracted and purified from human liver by a slight modification of the method of Aherne and Worwood.⁷ Thus the product from this method was chromatographed on a Sepharose 6B column (1.6 × 100 cm) equilibrated and eluted with 0.008 M Na₂HPO₄/0.002 M NaH₂PO₄ adjusted to pH 7.5 with 1 M NaOH. The protein content of the final product was determined by the method of Lowry and his colleagues⁸ using HSA as a standard. Its purity was determined by 4% (wt/vol) polyacrylamide gel electrophoresis using glycine Tris buffer, pH 8.5 as described in a commercial instruction

manual (Shandon Southern Instruments, Runcorn, Cheshire). The gels were run in duplicate and one was stained for ferric iron using potassium ferrocyanide⁹ and the other for protein, with Coomassie brilliant blue. There was one main band representing the ferritin monomer and a smaller band due to ferritin polymer. The highly purified human liver ferritin was used as the immunogen, to prepare standards and as the immunoabsorbant.

PREPARATION OF SHEEP ANTIFERRITIN IMMUNOGLOBULINS

Three Border-Leicester ewes were each injected intramuscularly at several sites with human ferritin (200 µg in 2 ml of water) emulsified with complete Freund's adjuvant (6 ml). The sheep were reimmunised at seven week intervals, each with 100 µg of ferritin in incomplete Freund's adjuvant, and bled 10 days after each reimmunisation. All produced high titre antisera and the third bleed from one sheep (S-457-3) was used for all subsequent studies.

The specific antiferritin immunoglobulins from 1 ml of antiserum were extracted with 8 mg of immunosorbant, comprising human ferritin that had been polymerised using glutaraldehyde at a glutaraldehyde to ferritin ratio of 1:10 (wt/wt).¹⁰ After washing, the specific antibodies were eluted from the immunoabsorbant by use of 3 M potassium thiocyanate in 0.05 M NaH₂PO₄ adjusted to pH 6.0 with 1 M NaOH.¹¹

RADIOIODINATION OF SPECIFIC ANTIFERRITIN IMMUNOGLOBULINS

The purified sheep antiferritin immunoglobulins were radioiodinated by the iodogen technique.¹² Briefly, 50 µl of iodogen solution (40 µg/ml in dichloromethane) were allowed to evaporate to dryness in the bottom of a polypropylene tube. Iodine-125 (¹²⁵I) (ImCi, 0.5 nmol) was added followed by 38 µg (0.25 nmol) of purified antibody in 38 µl of 0.1 M sodium phosphate buffer, pH 7.5. The reaction was terminated after 10 min by transferring the reaction mixture to 500 µl of 0.05 M sodium phosphate buffer, pH 7.5. After a further 10 min the iodination mixture was chromatographed on a Sephadex G200 column (0.9 × 60 cm) previously equilibrated with 0.04 M Na₂HPO₄/0.01 M NaH₂PO₄ adjusted to pH 7.5 with 1 M NaOH, containing BSA (0.5% wt/vol) and sodium azide (0.2% wt/vol). The fractions from the peak comprising the ¹²⁵I labelled immunoglobulins were pooled and stored at -20 °C.

In a series of 10 radioiodinations, the mean (±SD) incorporation of ¹²⁵I was 70.5 ± 3.5% and the specific activity of the labelled product ranged from 17.6 to 19.5 µCi/µg of IgG. The labelled product was stable for at least 10 wk at -20 °C and

more than 65% bound to an excess of the immunoabsorbant.

SAMPLES AND STANDARDS

Serum samples from 91 patients were provided by Dr S Woodhead of the Welsh School of Medicine. These had all been assayed by a "two-site" immunoradiometric automated technique (S Woodhead, personal communication, 1982).

Ferritin-free serum was obtained by incubating pooled normal human serum with sheep antiferritin serum covalently coupled to magnetisable particles as described previously.¹³ After overnight rotatory mixing, the particles were sedimented on a magnetic block and the serum supernatant demonstrated to contain no measurable ferritin. Sodium azide was added (0.2%) and 10 ml aliquots stored at -20 °C.

Highly purified ferritin standards were prepared in standard buffer (0.05 M sodium phosphate buffer, pH 7.5, containing BSA (4%)) at a concentration of 1000 µg/l and stored in 0.5 ml aliquots at -20 °C.

ASSAY PROTOCOL

Patients' samples (previously diluted 1/10 in standard buffer) or standards, ranging from 0.5 to 1000 µg/l (100 µl) were incubated with 150 µl of ¹²⁵I-labelled specific antiferritin antibodies (sufficient to give 100 000 cpm) in assay buffer (0.5 M sodium phosphate buffer, pH 7.5 containing 0.1% BSA) at room temperature.

After equilibrium had been reached the rivanol solution in 0.05 M sodium phosphate buffer pH 7.5 was added and, after a short further incubation period, the tubes were centrifuged, the supernates decanted and the precipitates (containing the ferritin-anti-ferritin-complex) counted for 60 s.

Results

STUDIES OF THE RIVANOL SEPARATION STEP

Horejsi and Smetana⁶ have demonstrated the critical importance of pH and of the volume and concentration of rivanol in the differential precipitation of plasma proteins. Therefore, studies were undertaken of the rivanol separation step by employing tubes containing the top standard (1000 µg/l) and excess labelled specific antibodies and, in others, labelled antibodies in the absence of human liver ferritin. A concentration of 0.05% rivanol was chosen on the basis of initial experiments and from 50 to 1250 µl was added to each tube. Less than 5% of the labelled antibodies alone were precipitated but addition of 1 ml, or more, resulted in complete precipitation of the ferritin-antibody complex (Fig. 1). Next, 1 ml of 0.05% rivanol in a range of buffers

Fig. 1 Assessment of the effect of the volume of rivanol used to achieve separation of the bound and free fractions. Tubes contained excess labelled antiferritin antibodies both in the presence (●—●) and absence (○—○) of human liver ferritin.

Fig. 2 Assessment of the effect of the pH of the rivanol solution used to achieve separation of the bound and free fractions. Tubes contained excess labelled antiferritin antibodies both in the presence (●—●) and absence (○—○) of human liver ferritin.

was added. Neither the free labelled antibodies nor the antigen-antibody complex were precipitated over the pH range 3–5. However, separation was achieved between pH 7.5 and 10 (Fig. 2) and phosphate buffer, pH 7.5 was chosen in order to minimise non-specific precipitation.

In further experiments it was shown necessary to leave the tubes 10 min after the addition of the rivanol before centrifugation and that separation was not improved by longer periods up to 24 h. Centrifugation for 10 min at 1000 rpm was sufficient to ensure maximum precipitation whether at 4°, 25°, or 37°C.

OPTIMISATION AND VALIDATION OF ASSAY

Standard curves were set up and incubated for from 10 min to 8 h. Equilibrium was attained in 120 min when the standard curve covered the clinically important range for diagnosing iron deficiency¹⁴ (Fig. 3). Use of four different amounts of the ¹²⁵I-specific antibodies was studied and that sufficient to give 100 000 cpm found most suitable. Use of 100 µl of undiluted ferritin-free serum affected the assay non-specifically but this was avoided by diluting all samples 1/10, or more, in phosphate buffer, pH 7.5, containing BSA (4%).

The sensitivity of the IRMA, as determined by the method of Rodbard,¹⁵ was 10 µg/l of serum and, by appropriate sample dilution, it was possible to assay all samples. Recovery, as determined by adding from 1 µg/l to 125 µg/l of human liver ferritin to three serum pools was in the range of 92.1 to 106.1%. Within-assay variation as assessed by assaying four individual samples 10 times in an assay ranged from 2.7 to 8.0%; the between-assay coefficient of variation for the same samples assayed in duplicate in 10 different assays ranged from 3.3 to 10.0% (Table 1).

Of the 91 patient samples, 25 had been recorded as having values either < 10 µg/l or > 6000 µg/l by the automated "two-site" IRMA. Our results were

Fig. 3 Assessment of the effect of incubation time, prior to the addition of rivanol, on the standard curve.

Table 1 Precision data

Sample No	Within-assay variation		Between-assay variation	
	Serum ferritin ($\mu\text{g/l}$) Mean $\pm$ SD (n = 10)	CV (%)	Serum ferritin ($\mu\text{g/l}$) Mean $\pm$ SD (n = 10)	CV (%)
1	249 $\pm$ 7	2.7	250 $\pm$ 8	3.3
2	156 $\pm$ 10	6.2	151 $\pm$ 7	5.5
3	59 $\pm$ 3	5.6	59 $\pm$ 4	6.7
4	16.7 $\pm$ 1.3	8.0	15.9 $\pm$ 1.6	10.0

Fig. 4 Correlation between the results obtained on 66 samples by the Cardiff assay and by our assay using rivanol separation.

in agreement with this. A comparison of the results obtained with the present assay for the remaining 66 samples is shown in Fig. 4.

Discussion

It has been assumed that the only means of separating excess free labelled specific antibodies from the antigen-labelled antibody complex in a conventional IRMA is by means of an immunosorbant which limits this analytical approach to analytes that are inexpensive and widely available. Ferritin is a much larger molecule than IgG and it seemed likely that simpler means could be devised for the separation step. Selective precipitation of the antigen-antibody complex seemed one obvious approach and, in the present study, rivanol has proved suitable for this purpose. The resultant assay is simple, rapid and sufficiently sensitive and precise for clinical purposes.

There is no doubt that other means of selectively precipitating the bound fraction could be devised

and chromatographic techniques would clearly separate labelled antibodies bound to ferritin and free labelled antibodies. Many compounds of clinical relevance are of a much smaller size than ferritin and the development of alternative separation techniques in conventional IRMA for such compounds would be more difficult. However, once it has been recognised that the use of immunosorbants does not represent the only separation technique available, it should not be difficult to devise suitable alternatives. For example, rheumatoid factor and Clq bind antibodies that have themselves bound their specific antigen, but do not bind free antibodies and several separation techniques based on such factors suggest themselves.

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