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A Solid Phase Radioimmunoassay Method for Ferritin in Serum using ^{125}I-labelled Ferritin

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ABSTRACT

A solid phase radioimmunoassay method for ferritin in serum was developed using ferritin conjugated with an ^{125}I-labelled ester. The method was based upon competitive inhibition utilizing the free antigen-binding sites of antibodies to ferritin bound to a ferritin which had been chemically coupled to insoluble polysaccharide particles. The method was evaluated with regard to specificity, sensitivity, precision and practicability and was found to be a robust method suitable for the assay of ferritin in human serum in clinical studies. There was a significant ($P<0.001$) difference between the ferritin levels in healthy men and women, mean values 100 and 66 arb U/l respectively. Male blood donors had significantly ($P<0.002$) lower ferritin levels in serum (mean 68.5 arb U/l) than other healthy men. Patients with iron overload or hepatic damage had high values, as expected, and the serum levels of ferritin reflected changes in iron deposits during phlebotomy and iron treatment in 3 healthy men.

INTRODUCTION

In 1972 an immunoradiometric assay for ferritin in serum was described by Addison et al. (1). Since then several laboratories have used this method (8, 9, 11, 14) for which a production of an immunosorbent for ferritin antibody, labelling of the antibody and subsequent elution of the labelled antibody is necessary. Even if not all workers find the immunoradiometric assay for ferritin as "exasperatingly difficult" to handle as Crosby (5), there is a need for a simpler method. Radioimmunoassays employing labelled antigens instead of labelled antibodies are commonly used in many laboratories for the assay of different antigens. Ferritin has been labelled with ^{125}I by the chloramine-T technique but only to a very low specific activity (12). Attempts to label ferritin by that method to high specific activity were unsuccessful in our laboratory. The present work presents a method for measuring ferritin in serum by using ferritin labelled with a *p*-hydroxyphenylpropionic acid, *N*-hydroxysuccinimid ester, which had been iodinated according to Bolton & Hunter (3), in a radioimmunoassay system with antibodies coupled to a solid phase.

MATERIALS AND METHODS

Ferritin preparation. Ferritin was purified from human spleen by a modification of the method described by Drysdale & Munro (7), including a final repeated chromatography on Sepharose 6B. The preparation was kindly supplied by Dr Tor Olofsson, University Hospital, Lund, Sweden. This preparation was used for immunization, for labelling with ^{125}I and as a provisional reference standard preparation.

Antisera to ferritin. Three different antisera were compared. Two antisera were raised in two rabbits by weekly injections for 4 weeks with 0.25 mg of the ferritin preparation in Freund's adjuvant. The animals were bled 3 weeks after the last injection. The third antiserum was a commercial product from Behringwerke, West Germany.

Labelling of ferritin. To 400 mg of *p*-hydroxyphenylpropionic acid *N*-hydroxysuccinimide ester was added 2 mCi Na^{125}I for labelling of the ester according to the technique of Bolton & Hunter (3). The iodinated ester was conjugated to 15 μg of the ferritin preparation by incubation at 0°C for 40 min. The labelled product was purified by chromatography on a Sephadex G 150 column. Two peaks with labelled material (apart from the free iodine) were obtained. The first peak was eluated with the void volume and contained almost all immunoreactive material. The second peak followed immediately after the first and had a very low immunoreactivity with the antiferritin antibodies. The radioactivity bound to immunoreactive material comprised less than 30% of the total proteinbound radioactivity.

Solid phase coupling of anti-ferritin. For direct coupling of antibodies 5 to 10 μl of the antisera were coupled to 100 mg of CNBr-activated ultrafine Sephadex® or microcrystalline cellulose as previously described (17). Antibodies were also indirectly coupled (19), i.e. ferritin (200 μg) was first coupled to the CNBr-activated particles (100 mg) and antiferritin-antibodies were then bound to the coupled ferritin. The particles were suspended in assay buffer solution.

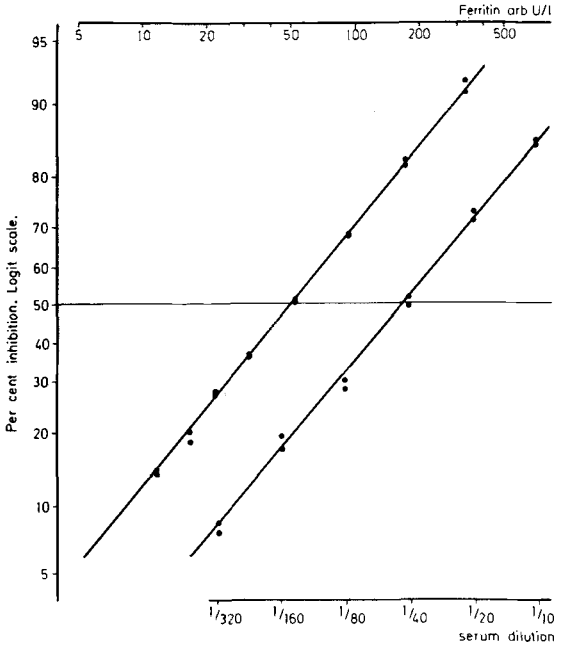

Fig. 1. Logit representation of the dilution curve for the reference standard and for serum sample with high ferritin content.

Assay buffer solution. 0.05 M phosphate buffer, pH 7.4, containing 0.01 M EDTA, 0.2% bovine serum albumin, 0.02% NaN_3, 0.08 M NaCl and 0.5% Tween® 20. The pH of the final solution was adjusted to 7.4.

Performance of assay. The assays were technically performed as previously described for the assay of gonadotropins (19). All samples were run in duplicate. 0.1 ml of test serum was incubated with 1 ml of the solid phase antibody suspension. The amount of polymer-coupled antibodies was chosen so that about 10% of added labelled ferritin was bound after incubation for about 20 hours. After 3–4 h of incubation at room temperature the labelled ferritin (40 000 cpm) was added in 0.1 ml of assay buffer solution and the mixture was further incubated for 20 hours with a slow vertical rotation of the test tubes. After washing three times with saline with 0.5% Tween 20 the remaining radioactivity was measured in a gamma-counter. The assays were calibrated using the ferritin preparation diluted in 25% serum containing a small amount of ferritin as a provisional reference standard. The values given are expressed in arbitrary units per litre, obtained by giving the geometric mean for a population of 25 normal young men (no blood donors), age 26–30 years, the value of 100. The results of the assay and different quality control parameters were calculated using a Compucorp 445 desk computer with a logit-log program (19).

Gel chromatography. Fractionation of 3 ml of a serum sample from a healthy young man was performed by gel chromatography on a 26×930 mm Sephadex G 200 column at +4°C in a buffer solution of pH 7.5 containing 0.1 M Tris-HCl, 0.2 M NaCl, 2 mM EDTA, 0.02% NaN_3. Flow rate was 9 ml/h and 3 ml fractions were collected.

Subjects. Serum samples were obtained from the following subjects: 25 male and 33 female medical students 26–30 years old. Blood donors were excluded as well as pregnant women. Thirty-seven male blood donors, randomly selected, were investigated. Two patients with excess iron due to repeated transfusions, one with pure red cell aplasia and one with anaemia of chronic renal failure, as well as 4 patients with well established alcoholic liver cirrhosis were selected. Three healthy young males were phlebotomized once weekly until they were anaemic and iron depleted, after which parenteral iron treatment was given in a dose not quite sufficient to restore the haemoglobin level to the initial values. From this experiment samples were selected before phlebotomy, at the time of lowest haemoglobin level and after iron treatment. All serum samples were kept at −20°C until analysed.

RESULTS

Using the conjugation method with iodinated ester, ferritin was labelled with ^{125}I to a specific activity of about 10 Ci/g. The labelled ferritin was found to be sufficiently stable, when stored at +4°C, to be used in the assays for at least 4 months. The antisera were tested for ferritin-binding capacity both after direct and indirect coupling. The three antisera could be used for 250 000–400 000 assays per ml with both variants of the coupling method. The estimated ferritin concentration in serum was not significantly different ($p>0.05$, paired t-test) when the three antisera were compared, testing serum samples from 10 different individuals. The Sephadex® particles gave a slightly better precision than microcrystalline cellulose. The slope of the standard line was steeper in assays with indirectly than with directly coupled antibodies (mean values: 1.22 versus 0.97). The ferritin concentration that gave a 50% inhibition to the assay was 50 arb. U/l with the indirect and 110 arb. U/l with the direct coupling. One of the antisera raised in our laboratory and indirectly coupled to Sephadex® particles was chosen for further experimental and clinical studies.

Quality control data were obtained from nine assays over a period of 4 months which included duplicate tests on a total of 793 serum samples. The average percentage of labelled ferritin bound to B° was 10.1±1.5 (S.D.). The mean ±S.D. values for the slope was 1.22±0.07 and for the 50% inhibition 50.2±5.8 arb. U/l. The intra-assay variation calculated as the average percentage standard error of the mean of duplicates giving an inhibition between 20 and 80% of total bound was 4.7. The inter+intra

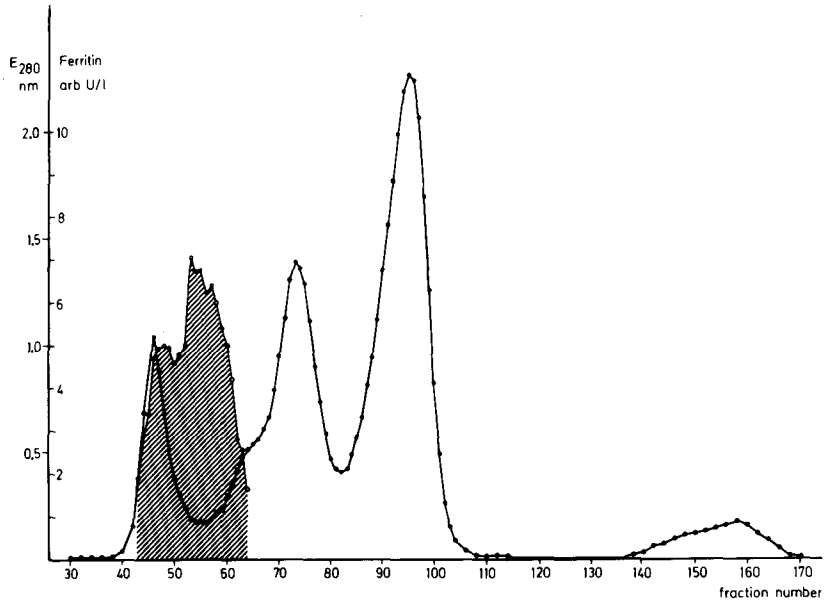

Fig. 2. Ferritin concentration in fractions from a Sephadex G-200 gel chromatography of a serum sample from a healthy young man.

assay variation calculated as the coefficient of variation from nine repeated assays of control samples on three levels of ferritin concentration was: 8% for 19 arb. U/l, 6.5% for 73 arb. U/l and 4.4% for 180 arb. U/l. The average detection limit ($p=0.05$) of the nine assays was 4.6 arb. U/l of serum.

The dose–response curve for serial dilutions of the reference standard, diluted in 25% serum, paralleled those for dilutions of sera with high ferritin content (Fig. 1). A fresh serum sample from a healthy young male (80 arb. U of ferritin per litre) was chromatographed on a Sephadex G 200 column and all fractions were assayed for ferritin content. The sensitivity was 1.5 arb. U/l. Two immunoreactive peaks eluted soon after the void volume were detected (Fig. 2). This elution pattern for immunoreactive ferritin was almost identical with that obtained for the radioactivity when ^{125}I-labelled ferritin added to normal serum was gel chromatographed on the same Sephadex G-200 column.

The levels of ferritin in serum in normal males and females and male blood donors is shown in Fig. 3. The geometric mean for males was given the value of 100 arb. U/l (range expressed as 95% limits: 40.3–246), which was significantly ($p<0.001$) higher than that for women, 66 arb. U/l (range 26–173). Male blood donors had significantly ($p<0.002$) lower levels than other normal men; geometric mean 68.5 arb. U/l.

The 4 patients with liver cirrhosis had values of 261, 292, 983 and 2496 arb. U/l. The highest value recorded, 3640 arb. U/l, was in a patient with chronic renal failure and iron overload due to repeated transfusions and oral iron therapy. Her bone marrow was stained for hemosiderin and showed a strongly positive reaction. One patient with pure red cell aplasia, who had had several blood transfusions, rose from 433 arb. U/l to 1404 arb. U/l during a 3 month period of repeated transfusions.

The ferritin levels in serum during induction of anaemia and after iron treatment of 3 healthy young men is shown in Fig. 4. Ferritin fell to a subnormal mean value when iron stores were depleted (no marrow hemosiderin could be detected). When iron

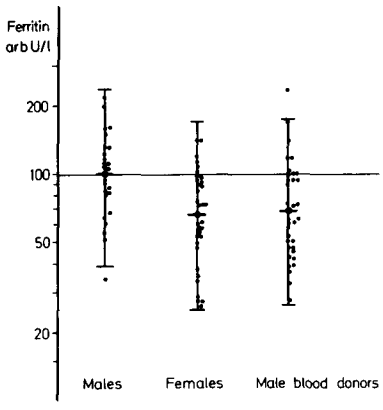

Fig. 3. Serum ferritin levels in normal males, females and male blood donors.

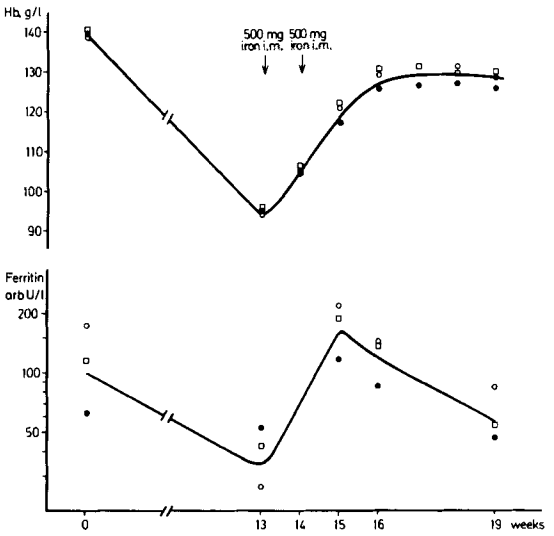

Fig. 4. Mean Hb concentrations and serum ferritin levels in 3 healthy young men during phlebotomy and iron treatment.

was injected, ferritin levels rose, in one case almost 10-fold. The iron dose was chosen so that iron stores would again be depleted before the initial haemoglobin level was reached, as also occurred: there was no stainable iron in the bone marrow and serum ferritin levels fell again to low levels.

DISCUSSION

One prerequisite for achieving high sensitivity in a competitive inhibition radioimmunoassay technique for ferritin using labelled ferritin is the labelling of this protein to a sufficiently high specific activity. Using the conjugation technique with ^{125}I-labelled ester, a specific activity of about 10 Ci/g was obtained in the present study, which is about 1 000 times higher than that obtained by the chloramine-T technique (12). The labelled ferritin had to be further purified by gel chromatography, as less than 30% of the ^{125}I-labelled protein was immunoreactive. It seems likely that this was due mainly to heterogeneity of the ferritin preparation used for labelling, and, to a lesser degree, a result of the labelling procedure, since this method is expected to damage the protein very little (3). Two variants were used for coupling of antibodies. The observation of a higher value for the slope and a lower ferritin concentration at 50% inhibition using the indirect vs. the direct coupling method is in agreement with results from assay of gonadotropins (19).

Support for a high specificity of the ferritin assay was obtained from several experiments with the method. On gel chromatography of serum, the immunoreactivity was eluted in the same position as labelled ferritin. There was no difference in relative ferritin activity in serum when the three different antisera were compared in the assay. The relation between mean ferritin concentration in serum from healthy men and women was similar to that reported by other investigators (1, 4, 8, 9, 16). Male blood donors had significantly lower mean ferritin concentrations than other healthy males of the same age. The results of the clinical material and the variation in immunoreactive ferritin concentration in serum for the three men participating in the phlebotomy study with iron therapy give further evidence for the high specificity of the ferritin assay. Several types of ferritin have been isolated from various tissues in man (6, 10) and some of them have been shown to cross-react immunologically (2, 13). However, it is not known to what degree they cross-react in our radioimmunoassay system. The values for precision and sensitivity of the method were acceptable for the application of the method for the assay of ferritin in serum clinical studies.

The method, which is technically simple to perform in a clinical chemical laboratory, was robust and suffered from only a small intra- and inter-assay variation. There was no detectable change in the activity of control samples when different batches of labelled ferritin were compared. The labelled ferritin could be used for at least four months. All three antisera could be used for a large number of tests and with such high "titres" it is unlikely that this solid phase technique will be found wasteful of antibodies when compared with other radioimmunoassay systems (18).

For several reasons, we have chosen to express the activity in relation to the mean activity obtained in healthy men, no blood donors, 20–30 years of age. There is no international standard preparation of ferritin. We had no evidence for the homogeneity of the ferritin preparation which was available. Furthermore, Stauffer & Greenham (15) have recently shown that variations in iron content of the ferritin molecule constitute a serious source of error in estimations of ferritin protein by the method of Lowry et al.

Expressing results in relation to a normal population simplifies calibration of new reference standard

solutions in our own laboratory and facilitates comparisons of our results with those from other laboratories.

In conclusion: a method is described for the assay of ferritin in serum, which is based on the use of labelled ferritin in a solid phase system and which has been found to be a robust method suitable for clinical investigations.

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