Radioimmunoassay of serum ferritin

W. M. DEPPE, S. M. JOUBERT, AND P. NAIDOO

From the Department of Chemical Pathology, Faculty of Medicine, University of Natal, Durban, South Africa

SUMMARY Purified human spleen ferritin was labelled with ^{125}I . On Sepharose 6-B gel filtration four species of labelled products were separated: a component with a higher molecular weight than ferritin; a component which is eluted in the same volume as unlabelled ferritin; and two labelled compounds with molecular weights lower than ferritin. When these labelled materials were used in a double antibody radioimmunoassay, the high molecular weight fraction showed variable and high non-specific binding and was poorly displaced by unlabelled ferritin; the fraction behaving like true ferritin gave good standard curves and showed non-specific binding of less than 1%. The remaining two components showed poor binding to rabbit antiferritin. Using labelled material from the second fraction, a double antibody radioimmunoassay capable of measuring $2\mu\text{g}$ ferritin protein/litre of serum was developed. Inter- and intra-assay variation was between 3% and 8% over a concentration range of 0 to $250\mu\text{g}$ ferritin protein/litre. Good agreement between serum ferritin levels assayed by the present method and by an immunoradiometric method was obtained. Labelled ferritin was stable for at least six weeks. The simplicity of the methodology makes it possible to assay serum ferritin in large batches.

The introduction of an immunoradiometric assay for ferritin in serum by Addison *et al.* (1972) heralded the advent of intensive studies related to ferritin behaviour in serum in a wide variety of clinical conditions. Soon after the publication of this analytical procedure, Miles *et al.* (1974) published a two-site variation in the immunoradiometric procedure, and these two methods became the mainstay of serum ferritin assays.

Both procedures suffered the inherent disadvantages of immunoradiometric techniques, and most laboratories which introduced these techniques probably experienced some difficulties (Crosby, 1976). In our own laboratory the method of Addison *et al.* (1972) was satisfactorily established after some modification.

The first reference to a radioimmunoassay procedure appeared in 1975 shortly after we began work on a similar method (Niitsu *et al.*, 1975). Details of procedure given in this publication were, however, too fragmentary for reproduction. More detailed radioimmunoassay procedures were subsequently published (Marcus and Zinberg, 1975; Luxton *et al.*, 1977).

In the present communication the radioimmunoassay methodology developed in our own laboratory

for liver and spleen ferritin phenotypes is reported with particular reference to the occurrence of ferritin labelled products which cause high non-specific binding in the assay unless they are removed. Inasmuch as the radioimmunoassay of ferritins will possibly be extended to cover the main phenotypes in the foreseeable future (Hazard and Drysdale, 1977), the importance of reliable procedures is self-evident.

Material and methods

PREPARATION OF FERRITIN

Ferritin was isolated from human spleens by combining a number of methods. From the heated aqueous extract of homogenised spleen, crude ferritin was precipitated by half-saturated ammonium sulphate (Granick, 1946) and, after collection by centrifugation, dissolved in the smallest possible volume of isotonic saline (9.0 g/l) and dialysed exhaustively against distilled water to remove saline and ammonium sulphate. A precipitate which formed during dialysis was removed by centrifugation at 4000 g. Crude ferritin was recovered from the clear supernatant by centrifugation at 95 000 g (Penders *et al.*, 1968), and the pellet was redissolved in a minimal quantity of isotonic saline and submitted to gel filtration on Sepharose 6-B

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(Pharmacia, Uppsala, Sweden). During gel filtration three major protein components were separated; the fraction corresponding to the amber colour of ferritin was collected and the partially purified ferritin was isolated by centrifugation at 95 000 *g*. The precipitate was once again dissolved in a small volume of isotonic saline and resubmitted to gel filtration on Sepharose 6-B. This eluate contained only a single protein peak. Ferritin was recovered from the pooled fractions corresponding to the centre of the protein peak by centrifugation at 95 000 *g*.

Gradient (125 ml 0.025 M barbital buffer, pH 6.8, and 125 ml 0.025 M barbital buffer, pH 6.8, in 1.0 M NaCl) ion exchange chromatography on a 0.9 cm by 10 cm column of Whatman DE-52 ion exchange cellulose revealed that the ferritin was homogeneous after two gel filtrations. No further purification was undertaken and ferritin isolated in this manner was used for standards, as material for raising antibodies in rabbits, and in the labelling of ferritin. Ferritin was not crystallised because only the most basic isoferritins tend to crystallise (Drysdales *et al.*, 1977).

PREPARATION OF STANDARDS

The protein concentration of purified spleen ferritin was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard and exercising care that estimations were carried out in the linear range of the reaction. Stock standards of ferritin at a concentration of 10 mg/l were prepared in 0.05 M barbital buffer (pH 8.0) containing 18 g/l NaCl, 40 g/l bovine serum albumin, and 0.2 g/l sodium azide; suitable portions were frozen at -20°C until needed. On the day of assay a portion was removed and, after thawing, a series of working standards was prepared by dilution in the same buffer to cover the range 0.500 µg/l.

PREPARATION OF ANTIBODY TO FERRITIN

Five rabbits (mixed strains) were injected subcutaneously with 50 µg protein equivalent ferritin, which was prepared by homogenising a solution of the ferritin with an equal volume of complete Freund's adjuvant. At three-weekly intervals injection of ferritin in the same dosage was repeated for a total of three months. Antibody titre was assessed by measuring the final dilution necessary to bind 50% of labelled ferritin used in the assay.

LABELLING OF FERRITIN WITH ¹²⁵I

The method of labelling ferritin with ¹²⁵I was essentially based on the method of Hunter and Greenwood (1962): 50 µl of phosphate buffer

(0.5 M, pH 7.4) and 10 µl chloramine-T (1.5 g/l) were pipetted into a test tube. Into the capillary limb of a Pasteur pipette was drawn 10 µl Na¹²⁵I (1.0 mCi) solution in phosphate buffer, an air bubble, and, finally, 30 µl ferritin solution containing 10 µg ferritin. At zero time the contents of the Pasteur pipette was delivered into the test tube containing chloramine-T. The mixture was allowed to react for 30 seconds and then stopped by the addition of 50 µl sodium metabisulphite (2 g/l) followed by 5 µl of 1.0 M potassium iodide solution.

Labelled ferritin was separated from unreacted iodide in the initial stages of the study on 1 × 50 cm columns of Sephadex G-75 previously equilibrated with a solution of 0.02 M Tris-HCl buffer (pH 7.6) containing 0.01 M EDTA, 0.1 M NaCl, and bovine serum albumin at a concentration of 10 g/l. In subsequent labelling experiments, separation was achieved using 2 × 60 cm Sepharose 6-B columns pre-equilibrated with the same buffer.

Fractions of 1.0 ml of the eluate were collected from the Sepharose 6-B columns and counted. When storage was required sodium azide was added to a concentration of 0.5 g/l and the solutions were stored at 4°C.

DILUTION OF REAGENTS FOR ASSAY

Labelled ferritin, first and second antibody, and normal rabbit serum were diluted in 0.05 M barbital buffer (pH 8.0) containing 18 g/l NaCl, 5 g/l bovine serum albumin, and 0.2 g/l sodium azide.

ASSAY PROCEDURE

Assays were carried out in polystyrene tubes (LKB, Stockholm, Sweden) and were based on the principle of reacting ferritin with limited rabbit antibody and separating free and bound ferritin by an antirabbit antibody.

To 100 µl volumes of working standards (0.500 µg/l protein equivalent ferritin) and 100 µl volumes of serum to be assayed were added: 100 µl of ¹²⁵I-labelled ferritin (35 000 cpm); 100 µl rabbit anti-ferritin serum (diluted in assay buffer to give 65% binding to the labelled ferritin); 100 µl normal rabbit serum diluted in assay buffer to a dilution of 1 in 100. In addition, control tubes containing buffer instead of rabbit antiferritin serum were set up for measuring non-specific binding. The assays were carried out in duplicate. After mixing, the tubes were incubated for 24 h at 4°C when 100 µl donkey anti-rabbit serum (1 in 20 dilution in assay buffer; RD17 Wellcome Reagents Ltd, Beckenham, Kent) were added. Following a further 20 h incubation at 4°C, samples, standards, and controls were diluted with 1.0 ml isotonic saline (9 g/l) containing Tween 20 (1 g/l) and centrifuged in the cold for 30 minutes at

2400 g. Supernatant fluid was discarded and the precipitate was washed with 1.0 ml of the saline-Tween solution, centrifuged again, and, after drainage of the tubes, counted in a Packard auto-gamma scintillation spectrometer.

IMMUNORADIOMETRIC ASSAY OF FERRITIN

The method followed was essentially that of Addison *et al.* (1972). However, in our hands, this method gave reliable and reproducible results only when ferritin was purified as described above and the labelled antibody isolated by affinity chromatography was further purified by Biogel P-2 filtration (Miles *et al.*, 1974). Forty-six serum specimens were assayed for ferritin content using this assay and compared with the results obtained by radioimmunoassay.

Results

LABELLING OF FERRITIN WITH ^{125}I

Separation of labelled ferritin from unreacted ^{125}I on Sephadex G-75 or Sephadex G-25 as used by Marcus and Zinberg (1975) and Luxton *et al.* (1977) results in the appearance of labelled ferritin in the eluate corresponding to the void volume of the column as a homogeneous peak, and initially this material was used in assays. However, the control tubes indicated variable recovery of radioactivity (non-specific binding) which was unacceptably high (7-20%). On ageing, non-specific binding tended to increase in all batch preparations of labelled ferritin isolated by Sephadex G-75 gel filtration. Gradient ion exchange chromatography on Whatman DE-52 cellulose showed heterogeneity of the labelled material, and separation of the iodination products was hence attempted by Sepharose 6-B gel filtration.

Figure 1a demonstrates a typical example of the radioactive elution peaks observed during Sepharose 6-B gel filtration. The first peak appears in the region corresponding to the void volume of the column, and therefore these labelled products have a much greater molecular weight than ferritin; the second peak corresponds to the elution volume in which ferritin is expected; the third and fourth peaks contained labelled products smaller than ferritin; and the fifth peak is unbound ^{125}I .

In Fig. 1b the non-specific binding, expressed as a percentage of total counts, is plotted for the elution peaks demonstrated in Figure 1a. From these plots it is clear that the high molecular weight components are associated with high non-specific binding. The percentage of non-specific binding was not constant but varied with each iodination, and in some experiments it was as high as 40%. The remaining elution peaks all showed very low non-specific binding.

Fig. 1 (a) Elution profile of iodination products of ferritin from Sepharose 6-B. (b) Non-specific binding of ^{125}I -iodinated products, expressed as a percentage of total counts, for elution peaks shown in (a).

Material from the first elution peak is unsuitable for use as labelled antigen. Quite apart from the high and variable non-specific binding, it is poorly displaced by unlabelled ferritin and hence lacks sensitivity in measuring ferritin.

Labelled material from the second elution peak generated very satisfactory standard curves (Fig. 2), and non-specific binding was always less than 1%; radioactive yield in this fraction was generally about 15% of the radioactive dose used in labelling; in the presence of excess antiferritin antibody 93-95% of the radioactivity of this fraction was bound after incubation at 4°C for 24 hours; 98% of the radioactivity was precipitable by trichloroacetic acid. Material from this peak was therefore regarded as true labelled ferritin and was used in all subsequent studies as labelled ferritin.

The material from the third and fourth elution peaks showed very poor binding to antiferritin antibody and was not used.

FERRITIN ANTIBODY TITRE

The five rabbits used in generating antiferritin antibodies all produced good responses, and the titres varied between 1 in 7.5×10^5 and 1 in 4.0×10^6 (final dilutions).

Titre in this context was defined as the final dilution necessary to bind 50% of the radioactive ferritin used in the assay.

Fig. 2 Composite standard curves (± 1 SD) generated in five consecutive assays using labelled ferritin recovered from the second elution peak in Fig. 1a.

STANDARD CURVES

Figure 2 represents a composite standard curve generated from five consecutive assays (mean ± 1 SD). The ratio of bound radioactivity (B) to bound radioactivity in the zero standard (B_0) is plotted against the logarithm of the standard concentrations. The B/ B_0 ratio obtained for the standard concentration of 2 $\mu\text{g/l}$ was significantly different from zero ($P < 0.005$).

The values obtained on serial dilution of four sera with different ferritin concentrations are graphically presented in Figure 3. It will be noted that the ferritin concentrations, as determined, vary linearly with the dilution, and on extrapolation all four plots passed through the origin.

PRECISION OF ASSAY

As a measure of intra-assay variation 130 serum samples were assayed in duplicate, and the coefficient of variation between duplicates was calculated. The results are summarised in Table 1 and indicate an intra-assay coefficient of variation of 3.6% to 8.1%.

Inter-assay variation was assessed by assaying five different serum samples in five consecutive assays. The results are summarised in Table 2, and the coefficient of variation between assays was much the same as determined for the intra-assay study.

Fig. 3 Plot of ferritin values obtained by assay when four sera with different ferritin concentrations were serially diluted.

Table 1 Intra-assay coefficient of variation of serum ferritin assays*

	Range (μl)				
	0-50	51-100	101-150	151-200	201-250
CV %	3.6	4.0	5.3	8.1	7.4
Mean $\mu\text{g/l}$	24	72	126	174	232
n	68	33	17	7	5

*Coefficient of variation calculated from duplicates of a single assay as:

$$\text{CV \%} = \sqrt{\frac{\sum(d^2)}{2n \times m}} \times \frac{100}{1}$$

where d = difference between duplicates
n = number of duplicate pairs
m = mean

EQUILIBRATION TIME AND TEMPERATURE

Reaction times for the first reaction were studied at 4°C and 37°C. At 4°C the reaction equilibrium is established at 20 h; at 37°C reaction equilibrium is reached at 6 h.

For second antibody maximal radioactive yield is achieved in the precipitate after 5 h at 4°C; at 37°C the reaction is rapid, and when dextran is added to the reaction mixture (Martin and Landon, 1975) maximum radioactive recovery is achieved after 30 minutes in the precipitate.

Table 2 Inter-assay coefficient of variation of five consecutive serum ferritin assays

Sample no.	Assay					Mean	SD	CV%
	1	2	3	4	5			
1	36	31	37	38	37	35.8	2.8	7.8
2	68	67	65	70	68	67.6	1.8	2.7
3	137	126	129	135	129	131.2	4.6	3.5
4	143	143	134	159	138	143.4	9.5	6.6
5	283	253	279	244	260	263.8	16.8	6.4

COMPARISON OF RADIOIMMUNOASSAY WITH IMMUNORADIOMETRIC TECHNIQUE

Figure 4 illustrates a scatter diagram in which 46 serum samples were assayed by the present method (RIA) and compared with values obtained by the immunoradiometric technique (IRA) as modified. The same standard was used in both procedures. Good agreement between the two methods was obtained; the IRA method gave slightly higher values ($r = 0.98$; linear regression: $y = 1.02x + 3.37$ where x is the value obtained by RIA).

A further 29 serum specimens were assayed by the present method and compared with a commercially available kit based on the two-site assay of Miles *et al.* (1974) (Hoechst, Behring Institute, West Germany). In the assays using the present method our own standard was used; in kit assays the standard supplied was used. The kit method gave substantially higher results ($r = 0.98$; linear regression $y = 0.92x - 7.7$ where x is the value obtained by kit).

Fig. 4 Scatter diagram of values obtained when 46 sera were assayed by radioimmunoassay (RIA) described in text compared with values obtained by an immunoradiometric technique. Values are expressed as μ g ferritin protein/l.

Discussion

In the present study no attempt was made to 'optimise' the assay described for the reason that laboratories differ in terms of both requirements and circumstances. However, it is clear that very large batches of assays may be undertaken, and the assay system can conveniently be adapted to provide results within 36 hours or less within an optimised system.

A number of aspects are critical to establishing successful assays; the labelled ferritins should be readily displaceable from antibody by unlabelled ferritins to permit equilibration and to provide sensitivity in assay; non-specific binding should be as low as possible and should not vary during ageing of the labelled ferritins; the quality of the labelled material should be predictable and should be easy to prepare on a reproducible basis.

Results presented indicate that unless high molecular labelled products are removed from the labelled products, the requirements outlined would not obtain. In our hands, the labelled product which results after Sephadex gel separation showed not only variable non-specific binding but a variable sensitivity and, more disturbing, substantial variation in the rate of ageing for different batch preparations of labelled ferritin. Luxton *et al.* (1977) made similar observations, finding a decrease in immunoreactivity and loss of sensitivity with ageing. However, isolation of the labelled ferritin by the methods outlined resulted in a product which was stable for at least six weeks in its immunoreactive properties and sensitivity. The exact nature of damage to the ferritins during iodination is not known, and milder methods of iodination may well be rewarding in terms of product yield and half-life time stability.

Recently it has been demonstrated that the multiple isoferritins found in most human tissues appear to be hybrid molecules composed of two subunit types combined in different proportions (Adelman *et al.*, 1975). The different isoferritin populations can be distinguished immunologically (Arosio *et al.*, 1976; Hazard, *et al.*, 1977), and their distribution among the various tissues differs; in liver

and spleen one type predominates; in heart and tumours the more acidic type of iso-ferritins is found. It may therefore be expected that there will be immunological cross-reactivity between the iso-ferritin phenotypes. Inasmuch as the acidic 'carcino-fetal' iso-ferritins have gained prominence as markers in various forms of cancer (Marcus and Zinberg, 1975; Hazard and Drysdale, 1977) it is quite obvious that a problem arises in interpreting the results of ferritin assays in serum when using an antibody raised to iso-ferritins in which particular types predominate. Hazard and Drysdale (1977) have demonstrated the marked differences in serum values obtained in a variety of cancers when using two different antibodies raised to iso-ferritins isolated from liver and Hela cells, respectively. In the present study, no attempt was made to quantitate the cross-reactivity between the antibody raised to human spleen ferritins and the more acidic types of iso-ferritin. This will be dealt with in a subsequent publication. It should, however, be borne in mind that the antibodies used in the present study would be expected to bind preferentially to the liver-type of iso-ferritins and hence would tend to underestimate iso-ferritins originating from malignant tissues.

At first sight the heterogeneity of both tissue and serum iso-ferritins (Drysdale *et al.*, 1977) creates apparent dilemmas from the point of view of assay design and in the preparation of standards. However, if it is correct that iso-ferritins are hybrid molecules of only two, or possibly three, subunit types and if the immunological identity of these phenotypes is sufficiently distinct to permit reasonable discrimination, it should be possible to run one or more assays in parallel to discriminate between native ferritin and those originating from tumours. Hazard and Drysdale (1977) applied this approach to the problem of tumour-associated ferritins, and their results clearly indicate that there is promise in attempting to assay the main phenotypes separately. It is equally clear that this approach will place a premium on standardised methodology, which, in turn, requires common standards and appropriate antibodies for inter-laboratory comparison.

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Requests for reprints to: Dr W. M. Deppe, Department of Chemical Pathology, Faculty of Medicine, University of Natal, Box 17039, Congella, 4013, Natal, South Africa.