

An automated immunoradiometric assay for ferritin

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SYNOPSIS A semi-automated method for the immunoradiometric assay of ferritin has been developed. Results obtained with serum samples show excellent correlation between this method and the previously described assay. A further useful reduction in the assay time may be achieved by incubating for 3 hours at 37°C instead of 24 hours at 4°C.

The assay of serum ferritin is a valuable method for assessing the iron status of normal subjects and patients with iron deficiency or iron overload (Jacobs, Miller, Worwood, Beamish, and Wardrop, 1972; Lipschitz, Cook, and Finch, 1974; Jacobs and Worwood, 1975). The immunoradiometric assay of Addison, Beamish, Hales, Hodgkins, Jacobs, and Llewellyn (1972) is sensitive and economical of reagents but is not appropriate for handling large numbers of samples. This paper describes a semi-automated immunoradiometric assay using 'Anal-matic' equipment (Searle Instruments Ltd, Harlow, Essex) which is particularly suitable for large batches of sera.

The Analmatic is a discrete automated analysis system. A wide range of reaction tube sizes may be handled, and racks of up to 100 tubes at a time may be taken through a number of processes such as sampling, diluting, reagent addition, and centrifugation. Syringe pumps carry out sampling and reagent addition, and plates, holding up to six syringes, may be rapidly interchanged.

Materials and Methods

PREPARATION OF REAGENTS

The horse spleen ferritin immuno-adsorbent was prepared as previously described (Addison *et al.*, 1972) and was washed six times with 0.05 M veronal buffer before use. This buffer contained 10.3 g/l barbitone sodium: 6 g/l sodium chloride: 0.2 g/l sodium azide: 5 g/l bovine serum albumin (Cohn Fraction V; Sigma London Chemical Company Ltd) adjusted to pH 8.0 with 5 M hydrochloric acid. All buffers were made up in distilled water. Human spleen ferritin was prepared by the method of Aherne and Worwood (1974) and antibodies to this

protein were extracted from a rabbit antiserum and iodinated (Addison *et al.*, 1972). The labelled antibodies were coupled to horse spleen ferritin immuno-adsorbent (Addison *et al.*, 1972) and stored at -20°C in aliquots containing approximately 2 µCi. A control serum was prepared from blood taken from 10 male laboratory staff and 0.5 ml aliquots were stored at -20°C.

PROTOCOL FOR THE AUTOMATED ASSAY

A thawed aliquot of ¹²⁵I-antibody coupled to immuno-adsorbent was washed on filter paper (No 41, Whatman Biochemicals Ltd, Maidstone, Kent) with 40 ml of veronal buffer followed by 5 ml pH 3.0 hydrochloric acid. A fraction of the ¹²⁵I-antibody was then eluted with 3 ml pH 2.5 hydrochloric acid into veronal buffer (25 ml per rack of 100 tubes). Standard ferritin solutions were prepared by diluting an aliquot of the stock standard (stored at a concentration of 10 mg/l at -20°C) to concentrations of 1-100 µg/l with veronal buffer. Serum samples were diluted as required with the same buffer. Both standards and sera were diluted with a Hook and Tucker Mark II dilutor (Hook and Tucker Ltd, Croydon, Surrey) giving dilutions of 10, 25, 50, and 100 fold. Standard solutions, controls, and test sera, in a volume of approximately 1 ml, were placed in 50 cups in the sample rack of the Analmatic preparation unit, and duplicate 50 µl samples were taken and diluted with 240 µl of labelled antibody in 75 mm × 11 mm polystyrene tubes (L P 3, Luckham and Co Ltd, Burgess Hill, Sussex). These tubes were held in a reaction rack fitted with plastic tube holders for 100 tubes. The syringes and probes for this stage were fitted to the first syringe plate. The racks of tubes were incubated for 20-24 hours at 4°C. Washed immuno-adsorbent was diluted approximately 200 times with veronal buffer and gently stirred with a magnetic stirring bar. Using the second

syringe plate and duplicate syringes, 500 μ l of this immunoadsorbent suspension was added to each tube. After 30 minutes at room temperature the rack of 100 tubes was placed in the Analmatic 100 centrifuge and centrifuged at 3000 rev/min for 12 minutes. The rack was placed in the preparation unit in the sample rack position and a 350 μ l aliquot of supernatant was taken from each tube and washed into an L P 3 tube with 1 ml of distilled water. This stage was carried out with a single probe on the second syringe plate. The sampling rate is 20 minutes per 100 tubes but it may be carried out in duplicate (10 minutes per 100 tubes). Up to three racks of 100 tubes may be handled in any one batch. The assay tubes were each counted for 10 minutes in an automatic gamma counter.

Results

The results described in this section were obtained with assays consisting of batches of 200 tubes. Four replicate estimations were performed for each point of the standard curve, two at the beginning of the first rack and two at the end of the second rack. A typical standard curve is shown (fig 1). For a series of seven assays the coefficient of variation ranged from 3% at the zero point to 1% for the highest point on the standard curve. There was no significant difference between the results for the standards at

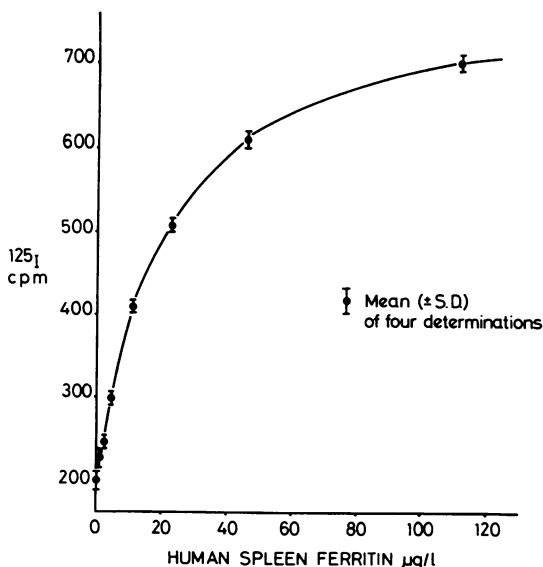


Fig 1 Standard curve for the automated assay of human spleen ferritin. Total radioactivity 775 ct/min: 75% of the counts were bound by the immunoadsorbent in the absence of ferritin.

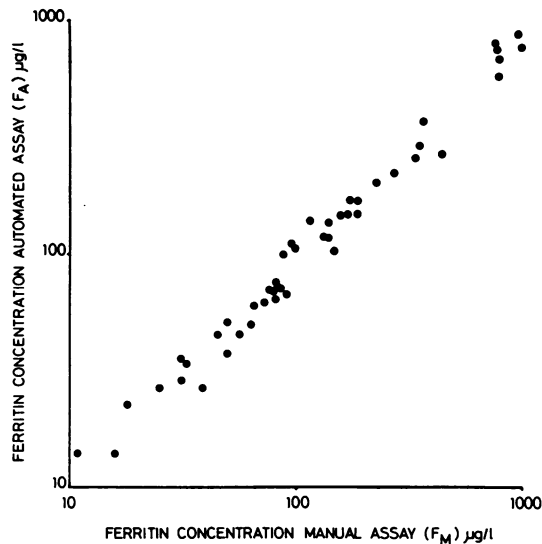


Fig 2 Comparison between the automated and manual assays. The correlation coefficient $r = 0.98$. The linear regression equation is $F_A = 0.87F_M + 4.0$.

the beginning of the assay and those at the end. The variation within an assay was assessed by carrying out eight determinations in duplicate on each of three sera. For serum A a mean value of 59.3 μ g/l was obtained with a standard deviation of 2.8 μ g/l; for serum B, 477 \pm 28 μ g/l; for serum C, 787 \pm 44 μ g/l. The coefficients of variation for these were 4.7, 5.9, and 5.6% respectively. The variation between assays was determined on a series of seven assays carried out over a period of six weeks. In each assay four determinations of a control serum were made. The mean value of this control serum for the seven assays was 73.6 \pm 4.0 μ g/l, coefficient of variation 5.4%. In this series, 432 sera (random samples from the population of a Welsh town) were assayed in duplicate. In 87% of the sera each duplicate was within 10% of the mean of the two values and in 76% within 5% of the mean. Carry-over was estimated in a series of six assays in which three samples containing no ferritin (veronal buffer) followed a standard solution of 100 or 200 μ g/l. The mean value of ferritin concentration in the first 'zero' was 0.47% of the previous standard although in one case a value of 1% was found. Nine sera with ferritin concentrations ranging from 48 to 7000 μ g/l were assayed at three dilutions. There was no consistent change in the ferritin concentration of the sera with increasing dilution.

The results of a comparison with the manual assay are shown (fig 2). Sera were assayed in four

batches by both automated and manual techniques, and each sample was assayed in duplicate at one dilution. The results were accepted if each duplicate was within 10% of the mean of the two values. If the ferritin concentration in any diluted serum was less than 5% of that of the previous specimen the result was rejected because of the possibility of carry-over producing an incorrect value. There is excellent correlation between the results produced by the two methods but the automated assay gives values which are approximately 10% lower than those given by the manual assay.

It is of interest to note that the assay may be carried out with a much shorter incubation period. Identical sets of tubes were incubated at 37°C for periods of 2, 3, and 4 hours. Binding of labelled antibody by ferritin appeared to be complete after 3 hours. Comparative assays were carried out, as described above, and incubated at either 37°C for 3 hours or 4°C for 24 hours. There was no significant difference in the results obtained for serum samples with a wide range of ferritin concentrations (5 µg/l to 6000 µg/l).

Discussion

The method described demonstrates that an immunoradiometric assay for ferritin may be partially automated using commercially available equipment. In order to achieve this a number of changes have had to be made in the method. For example, the volumes used are greater than those in the manual assay, and a relatively small volume of sample is diluted with a large volume of antibody (the reverse applies to the manual assay). As the majority of sera are diluted at least 50 × with buffer the use of buffer containing 40 g/l albumin was discontinued. It was expected that dispensing the immuno-adsorbent would present problems but few difficulties were experienced. It was, however, particularly important that the syringes used at this stage were adequately primed. We recommend at least 20 consecutive fillings of the syringes. With the Analmatic system the addition of immuno-adsorbent to 100 tubes takes 10 minutes using two syringes. The incubation time of 30 minutes is measured from the time the immuno-adsorbent is added to the first tube. As all tubes are then centrifuged at the same time this means that there is a time range for the incubation of 20 to 30 minutes. Results show that this time variation does not cause a significant difference in the binding of free antibody by the immuno-adsorbent at the beginning or end of a batch.

The automated assay is quicker and less tedious than the manual version. The time taken to load and prime the apparatus and to add samples, standards

and antibody to 200 tubes is 30 minutes. The corresponding stages for the manual assay require 80 minutes. On the second day of the assay the time required to add immuno-adsorbent, centrifuge, and remove an aliquot of supernatant for counting is reduced from 130 to 80 minutes. For both procedures a similar time is required to dilute samples and standards, elute antibody, and wash immuno-adsorbent. The total time, excluding counting, for the automated assay is 3 hours 50 minutes as against 5 hours 35 minutes for the manual assay. For much of the time the Analmatic equipment is unattended, permitting the operator to carry out other related procedures. If the actual labour times are compared, there is a considerable difference between the time of 2 hours 10 minutes for the automated technique and the 5 hours 15 minutes required for the manual method. The automated assay uses twice as much labelled antibody and immuno-adsorbent but these are easily prepared in larger batches. The small extra cost of these reagents is compensated by a saving in the cost of the reaction tubes used in the automated assay. If more than 150 samples are assayed each week, then even allowing for the cost of the equipment it is more economical to use the automated assay. With increasing workload greater savings may be achieved. The assay gives results which are slightly but consistently lower than those obtained with the manual method. A number of factors may be responsible as no single variation in the method has been found to correct this difference.

Crosby (1974) has suggested that the assay of serum ferritin will provide 'a most practical way to establish the presence of iron depletion in nutritional surveys'. The automated assay described in this paper and the use of shorter incubation times will make easier the handling of large numbers of serum samples necessary for such surveys.

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