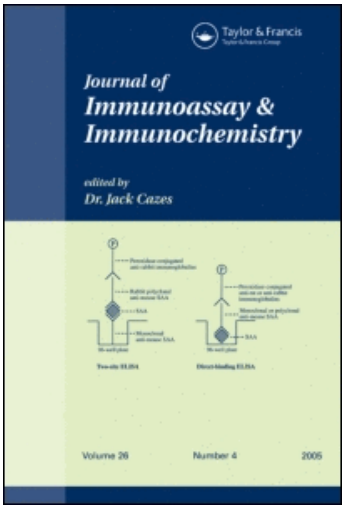

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MEASUREMENT OF FERRITIN LEVELS: COMPARISON OF A COMMERCIAL IRMA TO AN IN-HOUSE ELISA METHOD

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ABSTRACT

The purpose of this study was a comparison of a commercial IRMA to an in-house ELISA method. Because IRMA methods are considered to be very sensitive and results of these assays are widely accepted, the ferritin levels of 378 school children were determined in parallel with a commercial IRMA kit (Becton Dickinson Co., Orangeburg, NY) and the ELISA. For organizing reasons plasma was used for the ELISA, whereas serum was used for the IRMA. Regression analysis of the pairs of values, taking the IRMA reading as the independent variable, yielded a coefficient of variation $r = 0{,}90$ (Spearman, two-tailed, $p = 0{,}01$) and the straight line $y = 0{,}99x + 3{,}13$. Using this ELISA technique described by DAKO, ferritin levels in plasma were, on average, 13,2% higher than in serum taken at the same time. The ELISA

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technique described was found to have good accuracy and the speed and ease with which it may be carried out makes it suitable for a large number of samples.

INTRODUCTION

Iron deficiency is the most frequently encountered nutritional deficiency in man. It is estimated that 500 to 600 million people suffer from iron deficiency. Many more have depleted iron stores and are at risk for the development of anemia.(1)

This high prevalence, and the fact that some of the consequences of severe iron depletion are long-lasting and possibly irreversible, have led international organizations like WHO, as well as national agencies, to make reduction or elimination of iron deficiency a major goal.(2)

Laboratory measurement of serum ferritin has emerged during the past two decades as the single most important indicator of iron deficiency. It is the focal point of any iron assessment, whether performed to screen healthy individuals for latent iron deficiency, to diagnose iron deficiency in overtly anemic patients, or to assess iron stores in population surveys.(3)

The ferritin assay is widely used in hematology and in clinical chemistry laboratories and many kits (RIA, IRMA, or ELISA) are available commercially. The radioimmunoassay is based on a competitive reaction between the unlabelled and labelled antigen, whereas IRMA and ELISA are based on a non-competitive reaction between the sample and the labelled antibody.(4)

The purpose of the present study was to compare a Sandwich ELISA technique (DAKO) for the determination of ferritin to a commercial IRMA. The validity of the ELISA was checked by comparing the ferritin results obtained by this ELISA with those obtained by a ferritin ^{125}I IRMA (Becton Dickinson Co., Orangeburg, NY).

EXPERIMENTAL

Blood samples were obtained from 378 6–11-year-old primary school children from a subeconomic community, KwaZulu-Natal, a rural mountanous area, approximately 50 km northwest of Durban, South Africa. These school children are part of a food fortification study of the National Research Programme for Nutritional Intervention of the South African Medical Research Council.

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Ethical approval for the study was obtained from the Ethics Committee of the Medical Research Council. Written informed consent was obtained from the parents or guardians of all participants prior to the study and permission from the relevant Department of Education and the local community leaders.

Preparation of Specimen for Analysis

During the field study, plasma was collected using the Becton Dickinson Vacutainer System, EDTA. The blood was centrifuged at 3000 rpm with a Hettich EBA 8S centrifuge.

Storage

The plasma and serum samples of the school children were stored at $-80^{\circ}C$.

IRMA

Becton Dickinson Co., Orangeburg, NY.

ELISA

An in-house Sandwich ELISA technique, described by DAKO, was used (manufacturer's instructions: Sandwich ELISA, DAKO, Denmark).

The following equipment was used for the assay:

Well Plates: Nunc MaxiSorp, Code Nr. 439454
Ferritin Antibodies: Rabbit/Anti-Human; Ferritin; DAKO A/S, Denmark
Rabbit/Anti-Human; Ferritin; HRP; DAKO A/S, Denmark
Standard: Ferr/MYO T Control (Roche): $123\,\mu g/L \pm 12\,\mu g/L$
Controls: Ligand 1,2,3 (Chiron Diagnostics Corporation, East Walpole, MA, USA)
Automatic Washer: Denley Wellwash 4
ELISA Reader: Organon Technika, Microwell system, Reader 530 TC thermocontrol, first filter: 492 nm, reference filter: 690 nm
Computer Program: MIMS V3.20 SIL/MI94044
Graph type of calculation: straight line
Axis type: log/log

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Assay Procedure

In the following protocol, the standard level points and patient samples must be run in duplicate and simultaneously. Control sera should be run concurrently with patient samples.

1. Incubate wells overnight in the fridge with 100 µL 1 : 1000 diluted ferritin antibodies. Well plates should be covered with parafilm.
2. Wash well plates three times with wash buffer using an automatic plate washing machine.
3. Incubate wells with blocking buffer for 1 hour at 37°C.
4. Wash well plates three times with wash buffer using an automatic plate washing machine.
5. Incubate wells with 100 µL of 1 : 2 diluted plasma for 2 hours at room temperature.
6. Wash well plates three times with wash buffer using an automatic plate washing machine.
7. Incubate wells with 100 µL of 1 : 1000 diluted ferritin peroxidase antibody for 1 hour at room temperature.
8. Wash well plates three times with wash buffer using an automatic plate-washing machine.
9. Add 100 µL of chromogenic substrate to each well. Allow colour to develop in the dark at room temperature.
10. Stop the enzymic reaction after a sufficient colour development by adding 150 µL sulfuric acid to each well.
11. Measure the intensity of colour developed in each well in a colorimeter at 492 nm (reference filter 690 nm).

Pooled plasma was obtained by pooling the plasma of three adult males. The ferritin level of this pooled plasma was determined using Ferr/MYO T Control (Roche): 123 µg/L ± 12 µg/L as a standard. The ferritin level of the pooled plasma determined by ELISA was 183.84 µg/L. The pooled plasma was stored at −80°C. For the determination of the plasma ferritin levels of the school children, this pooled plasma was used as a standard. The pooled plasma was diluted to get the standard concentrations given in Table 1.

Linearity

The assay showed a linear range of 0–100 µg/L and plasma and serum samples were therefore diluted 1 : 2, 1 : 4 or 1 : 10 in coating buffer.

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Table 1. Standards of the ELISA

Number	Ferritin [μg/L]
1	45,960
2	27,567
3	18,384
4	12,868
5	9,192
6	1,838

Intra-assay and Inter-assay

The within-run precision (intra-assay) of the ELISA was found to have coefficients of variation of 2.2–5,3%. The between-run precision (inter-assay) of the ELISA exhibited a CV range of 7–13.5%, respectively.

RESULTS

Calculation of Ferritin Levels

To test how the standard curve looks, the ferritin standard Ferr/MYO T Control (Roche): 123 μg/L ± 12 μg/L was used. This ferritin standard was diluted in coating buffer to get a ferritin concentration of 100 μg/L and was then used in different dilutions to get different ferritin concentrations. (see Figure 1)

This type of curve can be used for reading off results, but it is inconvenient for direct computation. However, by transformation into log-log form, a straight line can be fitted to data, making complete automation in recording and calculation of results possible (Figure 2).

Certification of a Pooled Plasma

For the determination of the plasma ferritin levels of the school children, a pooled plasma with known content of ferritin was used as a standard. This pooled plasma was obtained by pooling the plasma of three healthy male adults who were supposed to have high ferritin levels. To certify the ferritin content of the pooled plasma, Ferr/MYO T Control (Roche): 123 μg/L ± 12 μg/L was used as a ferritin standard.

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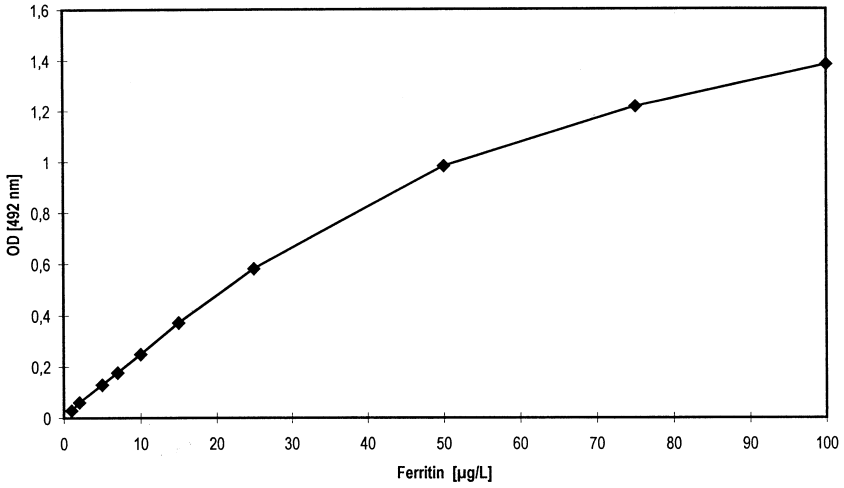

Figure 1. Composite standard curve. Ferr/MYO T Control (Roche): 123 µg/L ± 12 µg/L was used as a ferritin standard. The ODs were measured at 492 nm.

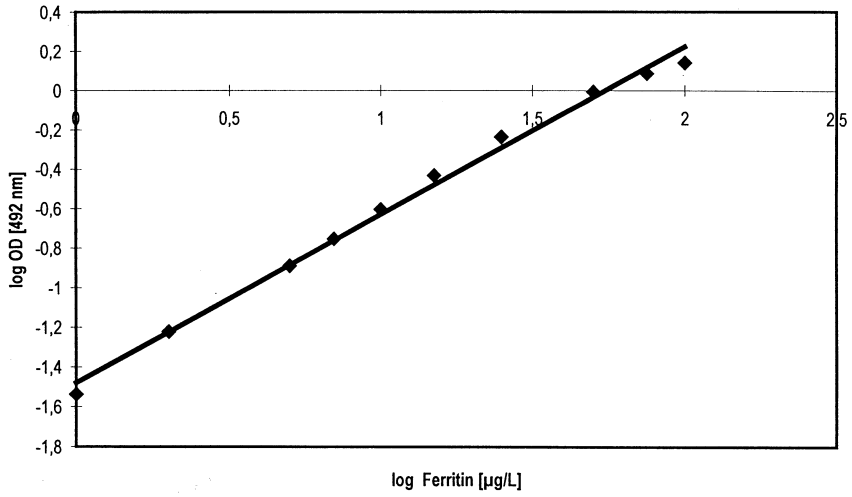

Figure 2. Plot of log OD against log dose of ferritin. Data the same as in Fig. 1.

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This ferritin standard was diluted in coating buffer to get a ferritin concentration of 100 μg/L. Pooled plasma and standard were then diluted the following way: 1 : 4; 1 : 6.67; 1 : 10; 1 : 14.29; 1 : 20; 1 : 50; 1 : 100 (Figure 3).

The pooled plasma was reading higher than the ferritin standard. The results of the differently diluted standard yielded the following straight line: $y = 0.95x - 1.57$. Regression analysis (Spearman, two-tailed) of the pairs of values yielded $r = 1$ ($p = 0.01$). According to this calibration curve the ferritin content of the pooled plasma was 183.84 μg/L. The ferritin content of the pooled plasma determined by IRMA was 186.86 μg/L.

Comparison of Ferritin Values Obtained by IRMA and ELISA

The ferritin levels of 378 school children were determined in parallel with a commercial IRMA kit and the ELISA. For organizing reasons serum was used for the IRMA and plasma for the ELISA. The plasma samples for the ELISA were diluted 1 : 2. One plasma sample was not in the linear range of the ELISA (0–100 μg/L). Therefore, the plasma sample was diluted 1 : 4 and determined again (Figure 4).

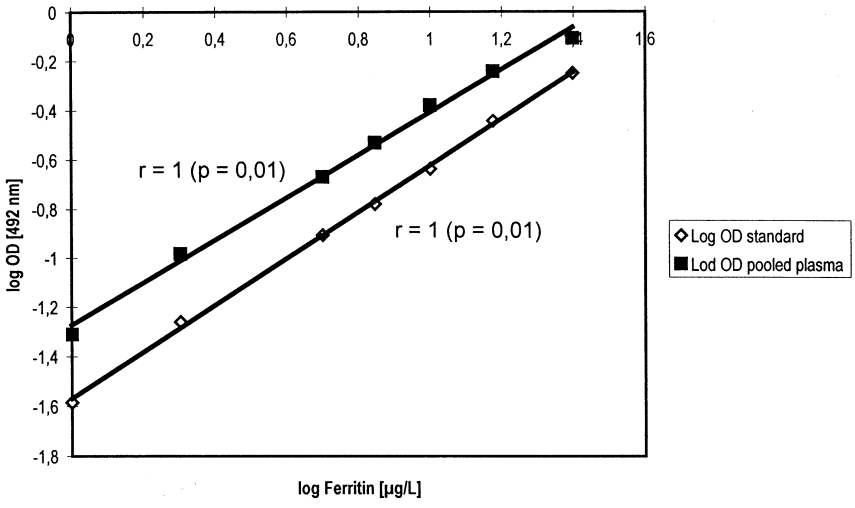

Figure 3. Comparison of ferritin standard and pooled plasma used in the same dilutions. ODs were measured at 492 nm. Pooled plasma and standard were diluted the following way: 1 : 4; 1 : 6.67; 1 : 10; 1 : 14.29; 1 : 20; 1 : 50; 1 : 100.

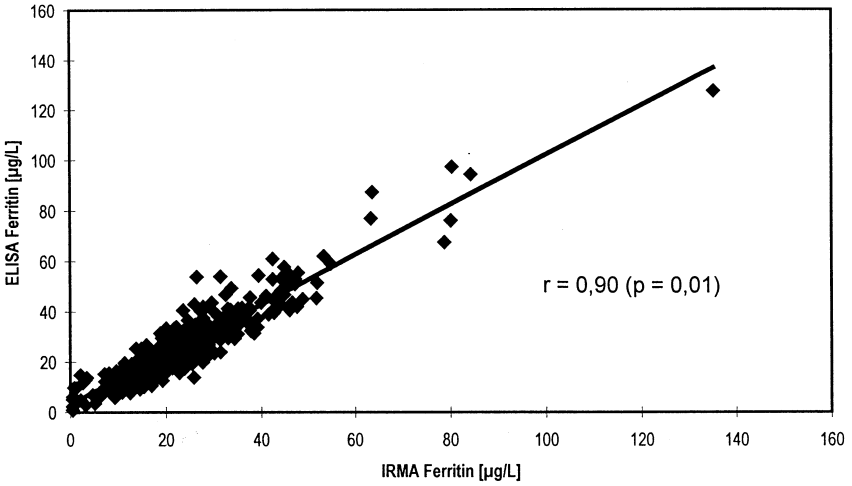

Figure 4. Comparison of results for ferritin (µg/L] obtained by IRMA and ELISA. Blood samples of 378 school children of were used for this comparison. For organizing reasons, serum was used for the IRMA and plasma for the ELISA.

Regression analysis (Spearman, two-tailed) of the pairs of values yielded $r = 0.90$ ($p = 0.01$) and the straight line $y = 0.99x + 3.13$.

A simple plot of the results of one method against those of the other is a useful start, but it will be difficult to assess between-method differences. A plot of the difference between the methods against their mean may be more informative (Figure 5).(5)

The mean difference in ferritin value (IRMA − ELISA) and the 95% predication interval for the difference in ferritin values were −2.87 µg/L and −13.80 to 8.06 µg/L for samples with ferritin concentrations ranging from 0 to 140 µg/L. Thus, on average, the ELISA gave ferritin values that were 2.87 µg/L higher than those obtained by IRMA.

The geometric means and ranges for ferritin ($n = 378$) measured by IRMA and ELISA were 23.52 (range: 0.87–127.62) and 26.39 (range: 0.5–135.15) µg/L. On average, ferritin levels analysed with the ELISA were 12.2% higher than those analysed with the IRMA (Figure 6).

Distribution of Ferritin Levels Determined by ELISA and IRMA

Figure 6 shows that the distribution curve of the IRMA is more symmetrical than the distribution curve of the ELISA.

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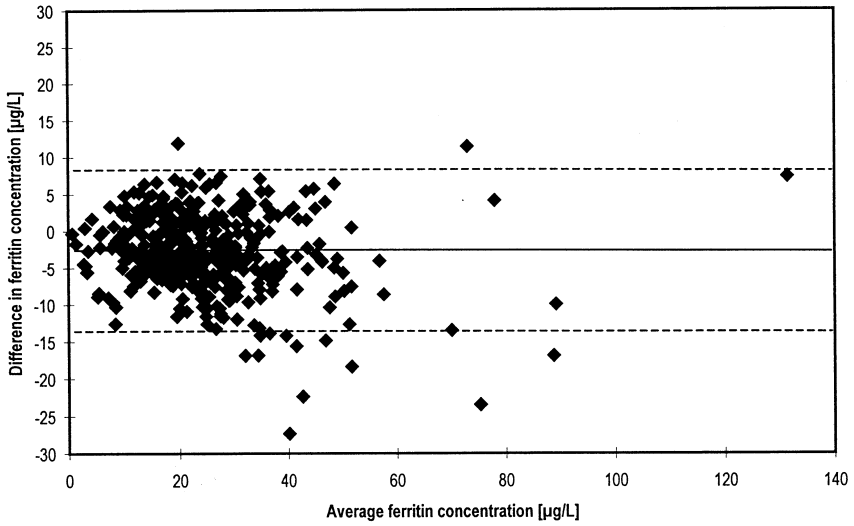

Figure 5. Relationship between the difference in ferritin values determined by IRMA and ELISA against the mean ferritin concentration by the two methods for samples with concentrations in the 0–140 µg/L range. The solid and dashed lines represent the mean and mean ± 2 SD, respectively, of the difference in ferritin values by the two methods.

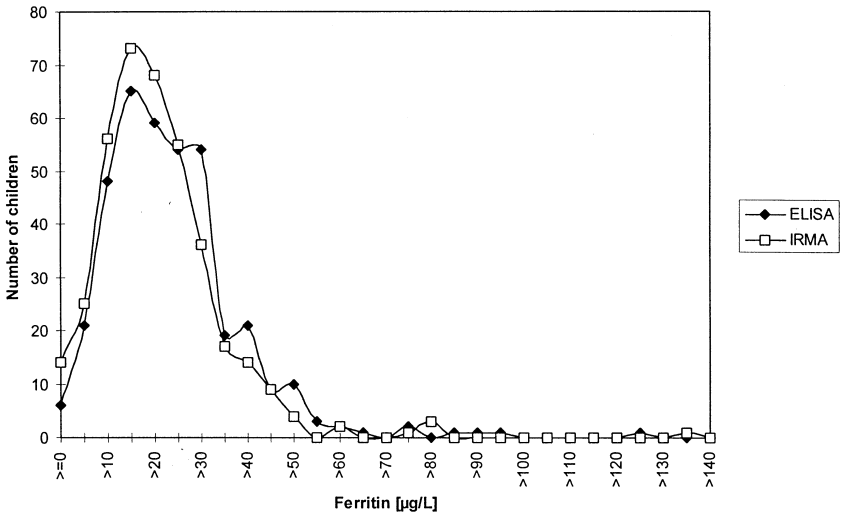

Figure 6. Plot of number of children against ferritin values obtained by ELISA and IRMA.

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Difference Between Plasma and Serum Ferritin Levels

To test if there is a difference between plasma and serum ferritin levels, blood was drawn from volunteers ($n = 11$). Blood was processed and stored at -80°C. Plasma and serum samples were diluted 1 : 4 (Table 2).

Regression analysis (Spearman, two-tailed) of the pairs of values yielded $r = 0.982$ ($p = 0.01$) and the straight line $y = 0.89x - 1.47$ (Figure 7).

With use of the approach of Bland and Altman (Figure 8), the mean difference in ferritin value (plasma minus serum) and the 95% predication interval for the difference in ferritin values were 7.89 µg/L and −8.16 to 23.94 µg/L for samples with ferritin concentrations ranging from 0 to

Table 2. Number of Subjects Classified to Have Low Ferritin Levels and of Subjects Classified Iron-Deficient by IRMA Compared with ELISA

Method	Low Ferritin Levels (< 20 µg/L)	Severe Iron Depletion (< 10 µg/L)
IRMA	170	39
ELISA	141	27

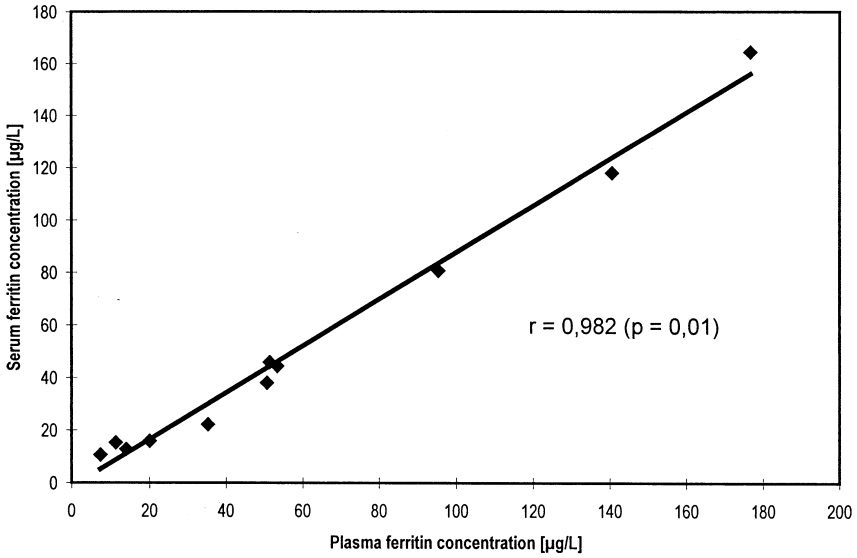

Figure 7. Plot of serum ferritin concentrations against plasma ferritin concentrations.

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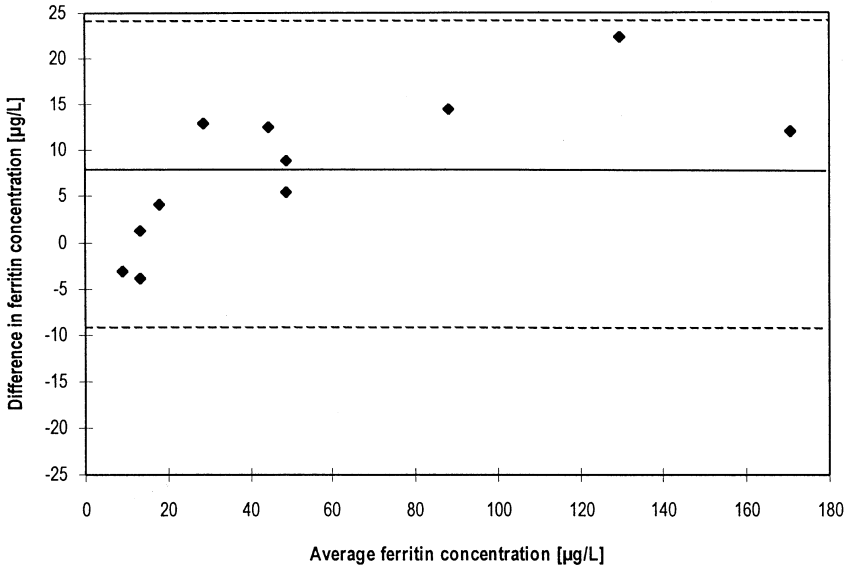

Figure 8. Relationship between the difference in ferritin values determined in plasma and serum against the mean ferritin concentration by the two methods for samples with concentrations in the 0–180 µg/L range. The solid and dashed lines represent the mean and mean ± 2 SD, respectively, of the difference in ferritin values by the two methods.

180 µg/L. Thus, on average, ferritin values obtained from plasma were 7.89 µg/L higher than those obtained from serum.

The geometric means and ranges for ferritin ($n = 11$) measured in plasma and serum were 59.60 (range: 7.41–176.56) and 51.71 (range: 10.58–164.48) µg/L. On average, plasma ferritin levels were 13.2% higher than serum ferritin levels.

DISCUSSION

The advent of enzyme-linked immunosorbent assays has made possible the measurement of minute concentrations of clinically valuable components with the same ease and accuracy as that obtainable before only by RIA or IRMA methods.(6)

The validity of the ELISA was checked by comparing the ferritin results obtained by this ELISA with those obtained by a ferritin ^{125}I

IRMA (Becton Dickinson Co., Orangeburg, NY). For organizing reasons, plasma was used for the ELISA and serum for the IRMA. Different standards were used for the two assays. For 378 children, the coefficient of correlation between the two methods was 0.90 ($p = 0.01$), with the equation for the regression line being $y = 0.99x + 3.13$.

The mean difference in ferritin value (IRMA − ELISA) was −2.87 μg/L for samples with ferritin concentrations ranging from 0 to 140 μg/L. Thus, on average, the ELISA gave ferritin values that were 2.87 μg/L higher than those obtained by IRMA. On average, ferritin levels analysed with the ELISA were 12.20% higher than those analysed with the IRMA. Though linear regression gives a good correlation, the 95% predication interval for the difference in ferritin values (−13.80 to 8.06 μg/L) is quite high. These findings can be explained by the fact that plasma was used for the ELISA and serum for the IRMA, because of concentration differences between serum and plasma.

The effect of the difference on the classification of subjects as iron deficient could be of greater importance. With use of ferritin < 10 μg/L as the definition of severe iron depletion, the classification of subjects as iron deficient by ELISA was about 31% less than by IRMA, whereas, with use of ferritin < 20 μg/L, the classification of subjects with low ferritin values by ELISA was about 17% less than by IRMA.

As mentioned before, for organizing reasons, plasma was used for the ELISA and serum for the IRMA. Several authors describe that there are concentration differences concerning serum and plasma. Conradie and Mbhele reported, in 1980, that ferritin levels in plasma had been, on average, 12% higher than in serum taken at the same time using their ELISA technique for the determination of ferritin. Using the ELISA technique described by DAKO, ferritin levels in plasma were, on average, 13.2% higher than in serum taken at the same time. This finding agrees well with the observation of Conradie and Mbhele.

According to these facts, one can suggest that this might be the reason for the higher results obtained by ELISA. One can also suggest that this may, in part, be attributable to different standards used in each method or differences in anti-ferritin antibody specificity. Differences between assay results could also relate to the method of assay.

The within-run precision (intra-assay) of the ELISA was found to have coefficients of variation of 2.2–5.3%, whereas the within-run precision of the IRMA yielded a coefficient of variation of 6.0% (Marais, personal communication). The between-run precision (inter-assay) of the ELISA for the concentration range of exhibited a CV range of 7.0–13.5%, respectively. The between-run precision of the IRMA yielded a coefficient of variation of 12.0% (Marais, personal communication). That means the CVs of the

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within-run precision and of the between-run precision of ELISA were lower than those obtained by IRMA.

The main advantages of ELISA over IRMA are that the reagents are much more stable and considerably less costly, and that the equipment used is simpler, cheaper and usually already available in most laboratories.(6) This assay has a prolonged shelf life and utilizes a colorimetric substrate that requires short incubation. The CVs of the within-run precision and of the between-run precision of the ELISA were lower than those obtained by IRMA. Another important advantage of ELISA over IRMA is that the ELISA technique is not bound up with radioactivity.

The speed and ease with which it may be carried out makes it suitable for large numbers of samples. It was found that a trained person could comfortably complete the assay in 5 hours with 100 samples per day being the upper limit.

Although several IRMA and RIA kits are available for the quantitation of serum ferritin the costs of these assays are prohibitive because of factors such as the need for properly equipped and licensed laboratories, automated gamma counters and facilities for the disposal of the radioactive waste.(6) Examination of ELISA showed that it was potentially a much cheaper technique, yet one which could be as sensitive, accurate and easy to perform as the IRMA method. The costs for a single determination of ferritin using the IRMA are 7.29 Rand whereas the costs for a ferritin determination (duplicate) are about 3 Rand. The relative cheapness of the enzyme label is perhaps a far more important consideration, especially in developing countries.(6)

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