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A sensitive time-resolved immunofluorometric assay of ferritin in serum with monoclonal antibodies

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Summary

We describe a new non-isotopic immunoassay for the quantitation of ferritin in serum. Ferritin is first immunoextracted from serum with a monoclonal antibody immobilized in white microtiter wells. A second biotinylated antibody is then added. The bound biotinylated antibody is quantified by a bridge reaction with streptavidin labeled with the europium chelate 4,7-*bis*(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) in the presence of excess Eu^{3+}. The fluorescence on the bottom of the dry microtiter well consisting of monoclonal antibody-ferritin-monoclonal antibody-biotin-streptavidin-BCPDA-Eu^{3+} is then measured with a time-resolved fluorometer. The assay is sensitive (0.5 μg/l), precise (CV's 3–9%) and accurate (average recovery 102%). Results compared well ($r = 0.99$) with those of a widely used RIA procedure.

Introduction

Ferritin is a molecule consisting of a protein shell (apoferritin), and an interior ferric oxyhydroxide core capable of binding up to 4000 iron atoms. The protein shell is composed of 24 nearly identical subunits. The overall molecular weight of ferritin is approximately 450000 [1–3].

Ferritin is one of the major intracellular iron-storage proteins, occurring in several isoforms in a variety of tissues, in particular in hepatocytes and reticuloen-

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dothelial cells of the liver, spleen and bone marrow. Isoferritins from various sources show some degree of structural and immunological differences [3–6]. This heterogeneity may, in part, be responsible for the observed variations in serum ferritin concentrations determined by a number of immunoassays [7–9].

Serum ferritin is considered one of the most useful indicators of iron status in the body [10–13]. Levels are decreased early in the development of iron-deficiency anemia and increased in iron overload states [11,14,15]. However, elevated ferritin levels inconsistent with the status of the iron stores may also be observed in certain pathological conditions such as malignancy, inflammation and liver disease [3,8,10].

Analytical procedures for quantitation of ferritin in serum most commonly include radioimmunoassays [8,9,16], and immunoradiometric procedures [8,9,11]. Recently, a number of alternative immunoassays involving various non-radioactive detection systems have been developed. Examples of such non-isotopic immunoassays for ferritin include polyclonal or monoclonal antibodies labeled with enzymes [17–19], luminescent [20] or fluorescent [21] probes.

We have developed a new non-isotopic immunoassay for the quantitation of ferritin in serum. In the assay, ferritin is reacted with a monoclonal anti-ferritin antibody immobilized in wells of microtiter strips. A second biotinylated anti-ferritin monoclonal antibody is then added to the reaction mixture and reacts with the already captured ferritin molecules. The degree of binding of the biotinylated antibody which is proportional to the ferritin concentration in the sample, is quantified by a bridge reaction with streptavidin labeled with multiple residues of the europium chelate 4,7-*bis*(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), in the presence of excess europium. The fluorescence of the final complex, consisting of antibody-ferritin-antibody-biotin-streptavidin-BCPDA-Eu^{3+}, is measured on the dried solid-phase with an automated time-resolved fluorometer. The method described is not affected by contamination with exogenous europium which constitutes the major disadvantage of time-resolved fluorescence immunoassays involving europium-labeled antibodies. This is due to the fact that in the proposed assay, BCPDA and not europium is used as the immunological label.

Materials and methods

Instrumentation

For solid-phase time-resolved fluorometric measurements we have used the CyberFluor 615 ™ Immunoanalyzer. The instrument has automatic data reduction capabilities [22,23]. Radioactivity counting was performed with the LKB Wallac 1275 Minigamma Counter.

Reagents

Chemicals

Human spleen ferritin (type V), and human heart ferritin (type VII) were purchased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (BSA),

and streptavidin were also from Sigma. Europium(III) chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI. Sulfosuccinimidyl 6-(biotinamide) hexanoate (NHS-LC-Biotin) was from Pierce Chemical Co., Rockford, IL. White opaque 12-well microtiter strips, Microfluor (TM), were products of Dynatech Laboratories Inc., Alexandria, VA.

Buffers

The coating buffer was a 0.05 mol/l carbonate solution, pH 9.6. The blocking buffer was 0.1 mol/l carbonate solution (pH 8.3) containing 10 g BSA and 0.5 g sodium azide/l. The assay buffer was 0.01 mol/l Tris solution (pH 8.5) containing 29.8 g KCl, 10 g BSA and 0.5 g sodium azide/l. The streptavidin-europium buffer was a 0.05 mol/l Tris solution (pH 7.8) containing 9 g NaCl, 10 g BSA and 0.5 g sodium azide/l. The wash solution was a 9 g/l NaCl solution containing 0.5 ml polyoxyethylene-sorbitan monolaurate (Tween 20)/l.

Ferritin standards

A reference preparation of the first international standard of human liver ferritin for immunoassay (80/602) was obtained from the National Institute of Biological Standards and Control (NBSC, Holly Hill, London). The preparation was reconstituted as directed and diluted in normal equine serum (Gibco Laboratories, Grand Island, NY) to give the desired standard concentrations.

Monoclonal antibodies

Two mouse monoclonal antibodies raised against human spleen ferritin were used. The antibodies were immunoglobulin fractions purified from the ascites fluid by ion-exchange chromatography and were obtained from CyberFluor Inc., Toronto, Canada. The coating antibody has an affinity constant of 0.9×10^{11} $(mol/l)^{-1}$. The detection antibody has an affinity constant of 1.5×10^{11} $(mol/l)^{-1}$.

Specimens and comparative method

Human serum samples stored at $-20°C$ for no longer than 1 month were provided by Dr. M. D'Costa (Mount Sinai Hospital, Toronto, Ontario). Lyphocheck immunoassay control sera (human) levels I, II, and III were from Bio-Rad Clinical Division, Richmond, CA.

A commercially available radioimmunoassay (Becton-Dickinson Immunodiagnostics, Orangeburg, NY) was used as a comparative method. It is a competitive RIA technique based on a double antibody separation.

Biotinylation of monoclonal antibody

The biotinylation procedure used has been previously described [23,27]. After dialysis, the biotinylated anti-ferritin antibody was tested at various dilutions to determine the optimal concentration for the assay. Routinely, the stock preparation stored at 4°C, is diluted in the assay buffer just before use to give a working biotinylated antibody solution of 10 μg/ml.

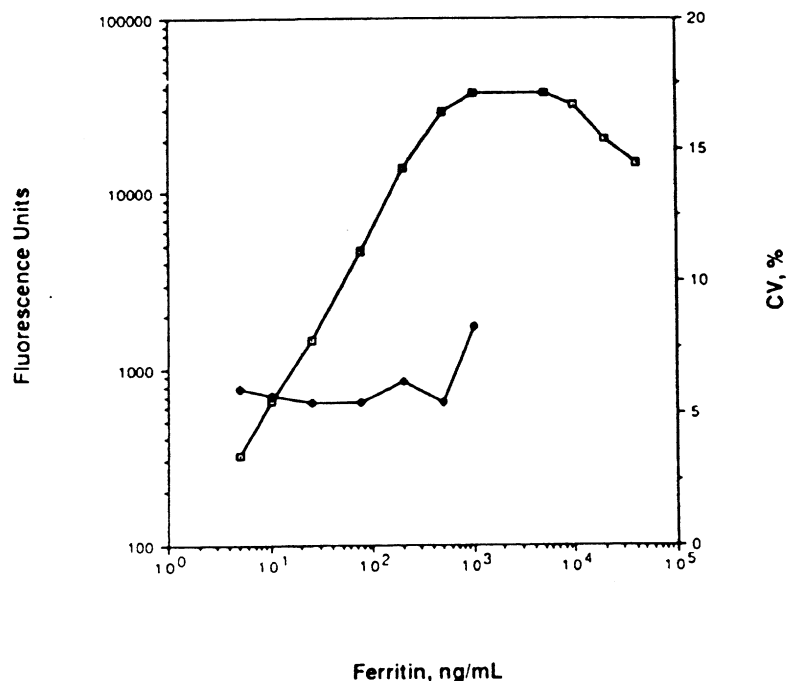

Fig. 1. Calibration curve and precision profile for the proposed ferritin assay. The fluorescence of the zero standard has been subtracted from all other fluorescence readings.

Recovery was determined by assaying pooled serum samples which had been spiked with exogenous ferritin. The recovery of the added ferritin was found to be between 97 and 109% with an average value of 102% (Table III).

Cross-reactivity

To evaluate cross-reactivity, we determined the response of the assay to increasing concentrations of the human heart (75–500 μg/l) and spleen (75–500 μg/l) isoferritins. The apparent concentrations of the spleen and heart ferritin measured against the liver standards were about 103 and 15%, respectively. No cross-reactivity exists with the horse-serum ferritin.

TABLE I

Precision of the ferritin assay

Within-run (n = 12) ferritin (μg/l)			Between-run (n = 10) ferritin (μg/l)			Day-to-day (n = 16) ferritin (μg/l)		
Mean	SD	% CV	Mean	SD	% CV	Mean	SD	% CV
39.7	2.7	6.8	43.2	3.4	7.8	42.0	3.8	9.0
138.6	9.8	7.0	144.7	11.6	8.0	147.0	13.4	9.1
242.9	7.2	3.0	257.3	21.0	8.1	260.0	23.0	8.8

TABLE II

Dilution linearity of the ferritin assay

Samples [a]	Undiluted	2x	4x	8x	16x	32x
[1] Expected	–	196.6	98.3	49.1	24.6	12.3
Observed	393.2	207.6	102.7	55.6	30.6	16.8
[2] Expected	–	160.0	80.0	40.0	20.0	10.0
Observed	320.0	151.9	66.7	32.1	17.4	10.2
[3] Expected	–	153.9	76.9	38.5	19.2	9.6
Observed	307.8	159.6	85.0	44.7	23.9	13.0
[4] Expected	–	155.6	77.8	38.9	19.5	9.7
Observed	311.2	150.7	78.0	41.7	21.7	11.5

[a] Regression analysis of the expected (x) vs. measured (y) values (μg/l).
Sample 1, slope = 1.029, intcpt = 4.28, r = 0.9998.
Sample 2, slope = 0.9489, intcpt = −3.17, r = 0.9973.
Sample 3, slope = 1.014, intcpt = 4.81, r = 0.9997.
Sample 4, slope = 0.9500, intcpt = 3.44, r = 0.9998.

TABLE III

Recovery of ferritin from serum samples

Ferritin (μg/l)			
Added	Measured	Recovered	% Recovery
Sample 1			
0.0	34.5	–	–
50	87.5	53.0	106
100	136.7	102.2	102
Sample 2			
0.0	40.9	–	–
50	93.6	52.7	105
100	150.0	109.1	109
Sample 3			
0.0	48.6	–	–
50	99.4	50.8	102
100	150.8	102.2	102
Sample 4			
0.0	45.3	–	–
50	96.2	50.9	102
100	141.5	96.2	96
Sample 5			
0.0	10.8	–	–
50	60.2	49.4	99
100	108.2	97.7	97

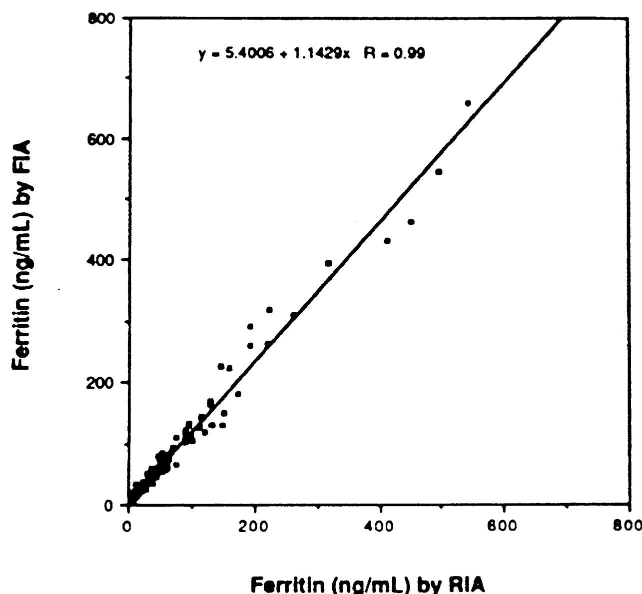

Fig. 2. Comparison of results between the proposed method and a widely used radioimmunoassay procedure for 104 serum samples.

Correlation with radioimmunoassay

Ferritin in 104 clinical samples was assayed by the present method (y) and a commercial RIA kit (x) (Fig. 2). There was a good agreement between the results obtained by the two procedures. The regression equation was: $y = 5.40 + 1.14x$, $r = 0.99$.

Discussion

Measurements of ferritin have been traditionally performed by radioimmunoassays [8,9,16] and immunoradiometric [1,8,9] methods, both of which involve the use and disposal of radioisotopes. The fluorescent europium complexes are being increasingly used for development of alternative non-isotopic immunoassays [22,24,25]. Time-resolved fluorescence immunoassays achieve sensitivities equivalent to or better than those obtained by using radiolabeled tracers [26–30] because of the effective background signal rejection during measurements. However, when europium-labeled antibodies are employed, the assays are sensitive to interference from environmental europium present in dust and water, as well as on skin surfaces. The present methodology avoids the problem by using BCPDA as label and performing the assay in the presence of a saturating concentration of europium (10^{-5} mol/l). The use of a biotin-streptavidin bridge system in the design of the assay has a number of additional advantages which have been discussed elsewhere [22,27].

The performance characteristics of the assay are comparable to those reported for radioimmunoassays [8,9,16], immunoradiometric assays [8,9,11,31,32], and enzyme

immunoassays [17–19]. The detection limit is about 0.5 μg/l, with an almost linear dose-response curve covering a 1 000-fold dynamic range. Several factors, including the amplification achieved by the use of multiple streptavidin labeling, the rejection of background fluorescence by time-resolved fluorometry and the use of high concentrations of monospecific antibodies, contribute to the high sensitivity of the assay.

A number of reports deal with the 'high-dose hook effect' associated with non-competitive immunoassays for ferritin [32,33]. Such assays may give a serum ferritin value in the normal range when the true value is highly elevated. This is particularly misleading when evaluating patients with iron-overload and iron-storage disease with ferritin concentrations reaching or exceeding 10 000 μg/l. In the present assay, fluorescence intensity from samples with ferritin concentration from 500 to 10 000 μg/l remain above that of the highest standard point (500 μg/l). A reduction in the assay response is seen only at ferritin concentrations above 15 000 μg/l.

It has been suggested that the immunological heterogeneity of ferritin may be a cause of variability in serum ferritin determinations [7–9]. This is because anti-ferritin antibodies used in the development of the assays have been raised against different tissue sources of ferritin [9]. Evaluating the response of the proposed assay to the major isoferritins, we found 100% cross-reaction with spleen and liver ferritin but only about 15% of the heart ferritin was detected. This is expected because heart ferritin is known to be acidic and immunologically different from liver or spleen ferritin [4,5,7,8]. This specificity is similar to that reported for the available immunoassays which recognize 100% and 12–33% of the spleen and the heart isoferritins, respectively [9].

In conclusion, we describe a new non-isotopic immunoassay for the measurement of ferritin in serum. The assay is based on the 'sandwich' principle and uses a detection system that has been described recently. The assay is highly sensitive and specific with good performance characteristics and a protocol suitable for use in routine clinical laboratories as well as in population screening programs.

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