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The Iron Content of Serum Ferritin: Physiological Importance and Diagnostic Value

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Summary: In this paper we present a method for determining the iron saturation of ferritin as a possible independent predictor of iron stores. Serum ferritin was purified by immunochemical precipitation, and could be completely recovered from serum without any contamination from transferrin. The iron content of the precipitated ferritin was determined by flameless atomic absorption spectrophotometry (FAAS) and the ferritin-iron saturation was calculated using the original serum ferritin concentration. The intra- and inter-assay variation coefficients were 4.2% and 13.4% respectively.

The first results with this assay indicate that serum ferritin contains a considerable amount of iron. Furthermore the results show that the iron saturation of ferritin in patients with acute phase response is significantly lower than the saturation found in healthy volunteers (19.3% and 24.3% respectively). These results suggest a possible role for the ferritin-iron saturation in the assessment of iron stores in patients suffering from acute phase response. In addition, the considerable amount of iron in ferritin suggests the need to revise the physiological role of this substance in relation to the serum iron homeostasis.

Introduction

For the determination of the iron status in anaemic patients several indicators can be used, such as serum iron, transferrin, transferrin iron binding capacity and ferritin. Ferritin is the method of choice to estimate the iron stores. It has been proposed that circulating ferritin may act as a transport protein for iron between the reticuloendothelial cells and the erythron, but the reported low iron contents of serum ferritin, even in iron overload states, makes this unlikely (1, 2). Several authors demonstrated the existence of ferritin receptors on the surface of erythrons and macrophages, however the function of these receptors is still unknown (3–4). With this in mind we were interested in the iron content of serum ferritin.

Ferritin acts as a positive acute phase reactant. Therefore, especially in chronic disease, the measurement of ferritin is not a reliable analyte for assessing the iron stores. Iron deficiency can be obscured by high ferritin concentrations due to the acute-phase response. The ferritin level in serum may also be increased due to liver cell damage or to malignancies. *Witte* et al. (6, 7) suggested that ferritin concentrations, if corrected for the acute phase response, could still be used to estimate the iron stores in bone marrow, even in patients with chronic disease. The applied nomograms describing the relation between ferritin concentration and C-reactive protein or erythrocyte sedimentation rate values, in order to diagnose or exclude iron deficiency anaemia in patients with chronic disease. This was done in an effort to minimize the numbers of bone marrow examination needed. In our institute the data of *Witte* et al. (6, 7), were not confirmed by *Coenen* et al. (8).

Recently, new efforts were undertaken to tackle this problem. A working hypothesis was formulated: In the case of inflammation and iron deficiency anaemia, the iron content of the increased level of ferritin is low.

To test this hypothesis a simple method had to be developed in order to determine the iron content of serum ferritin.

This paper deals with the description of this technique and gives the first results obtained with this method. During the European Iron Club meeting in Hamburg in September last year, part of these results were presented (9).

Materials and Methods

Flameless atomic absorption measurements were performed on a Perkin Elmer (Überlingen, Germany) model 3030 spectrophotometer with deuterium background correction, equipped with a PE HGA500 graphite furnace atomizer (programmable) and a PE AG40 autosampler. The iron hollow cathode lamp was from Instrumentation Laboratories (Palermo, Italy) no. 89225. Instrumental conditions are summarized in table 1.

Tab. 1 Instrumental conditions for the determination of iron by flameless atomic absorption spectrophotometry

Sample volume 20 µl
Fe hollow cathode lamp 30 mA
Slit width 0.2 nm, alternate
Wavelength 248.3 nm
Deuterium background detector on graphite furnace tube with *L'vov* platform, pyrolytic coated
Purge gas, argon: 330 ml/min
Calibration auto select
Iron standards 0, 25, 50, and 100 µg/l

Dry cycle	130 °C	10 s ramp	20 s hold
	250 °C	10 s ramp	5 s hold
Char cycle	850 °C	5 s ramp	5 s hold
	1400 °C	5 s ramp	5 s hold
	1400 °C	1 s ramp	5 s hold[1]
Atomize	2400 °C	0 s ramp	5 s hold[12]
Clean	2700 °C	1 s ramp	3 s hold
Cool	20 °C	1 s ramp	20 s hold

[1] Gas flow interrupt
[2] Signal processing, peak area, 5 s after start atomizer cycle
Calibration mixtures were prepared from a ferric nitrate standard solution 1 g/l, Spectrosol, BDH Chemicals) in 200 mmol/l nitric acid.

The ferritin assay was performed on the Stratus fluorometric analyzer using the Stratus ferritin fluorometric enzyme immunoassay-kit (B5700-56, Baxter Diagnostics Inc., Deerfield, IL, USA) according to the manufacturers protocol.

Beads coated with anti-ferritin antibodies were from Biorad (Quantimmune®-Ferritin-IRMA, Biorad, Clinical Division, Hercules, CA, USA). Before use the beads were washed in *Sørensen* buffer, in order to remove the radioactively labelled antibodies, and stored at 4 °C.

The iron content of ferritin is determined as follows: 300 µl serum (with known ferritin concentration, not more than 1000 µg/l) are combined with an overestimate of 300 µl beads in an iron-free glass tube, and incubated for 30 minutes. The beads are separated from the supernatant by centrifugation (10 minutes at 3000 min^{-1}). Subsequently the beads are washed three times with 300 µl 9 g/l saline at 4 °C. Bidistilled water (300 µl) is added to the beads followed by an incubation of 60 minutes at 90 °C in a water-bath. After a 10 minute centrifugation (3000 min^{-1}) the iron content of the supernatant is determined with flameless AAS.

An experiment was performed to establish the washing procedure and the results are shown in table 2. From these results it can be concluded that after three washings most of the serum iron is removed and that the losses of ferritin and iron are acceptable.

Calculation of the ferritin iron saturation

The relative molecular mass of ferritin is 450 000, while the total number of iron molecules per molecule ferritin is reported to be around 4500 (1). The maximal amount of iron is taken as 100%. Thus, a ferritin concentration of x µg/l and an iron concentration in the ferritin of y µg/l, gives a saturation percentage (z%) of:

$$[(y / 55.8) / \{(x / 450\,000) \times 4500\}] \times 100\% = z\%$$

Results

Ferritin isolation from serum

In figure 1 the results are shown from an experiment in which a serum sample was incubated with beads coated with anti-ferritin antibodies. Ferritin removal from the serum is 100%, while the transferrin concentration is unaffected. The isolated ferritin is removed from the beads by heating at 90 °C 60 minutes; to estimate the iron content of the ferritin the iron content of the supernatant is determined by FAAS. In order to estimate whether all iron was removed from the beads, an experiment was performed in which the denaturation procedure with the ferritin coated beads with diluted (0.24 mol/l) HNO_3 was compared to the aqua dest. denaturation. Denaturation of the beads with HNO_3 did result in iron concentrations of the supernatant comparable to the denaturation with aqua dest., indicating that the removal of the iron is complete (data not shown). The ferritin content of the beads equals the content of the original serum sample because of the 100% removal of the ferritin from the serum (fig. 1). The intra- and inter-assay coefficients of variation of the new technique, described in this paper, were found to be satisfactory (4.2% and 13.4% respectively; tab. 3).

Tab. 2 Representative experiment of iron measurement in ferritin, in which the ferritin and iron losses during the several washing procedures are measured.

Washings	Ferritin concentration in washing solutions	Iron concentration in washing solutions		Iron concentration in solution after denaturation
	(µg/l)	(µg/l)	(%)	(µg/l)
0*	0.9	550	100	75
1	3.3	182	33	59
2	7.5	61	11	52
3	3.3	17	3	51
4	2.8	9	2	56
5	2.3	5	1	48

* Serum

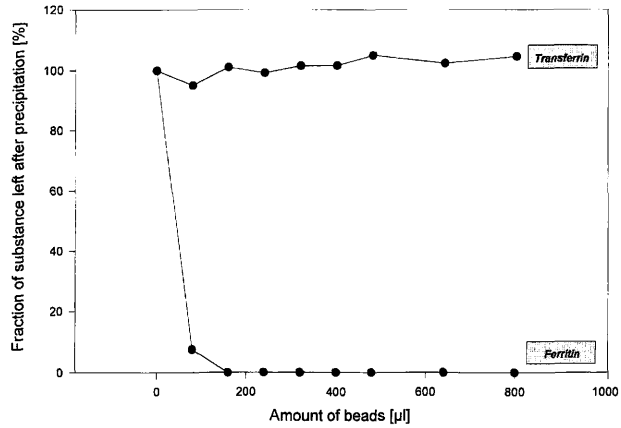

Fig. 1 Transferrin and ferritin concentration as a percentage of the initial value in a serum sample incubated with increasing amounts of anti-ferritin antibody coated beads.

Tab. 3 Intra- and inter-assay coefficients of variation of the iron content in ferritin.

Intra-assay CV[1]	Inter-assay CV[2]
4.2%	13.4%
Mean iron value: 108 µg/l	Mean iron value: 101 µg/l
SD: 4.6 µg/l	SD: 12.0 µg/l

[1] In one series of measurements the same sample was determined 10 times.
[2] On 10 consecutive days the same sample was measured once a day.

Linearity of the assay

The linearity of the assay was estimated by stepwise dilution (with saline) of a sample from a known haemochromatosis patient. The results are shown in figure 2. The linearity was acceptable until the Fe concentration dropped below 10 µg/l. So at a saturation of 20%, the detection limit of this ferritin assay was found to be 100 µg/l. In order to exclude matrix effects a serum sample with a very low ferritin concentration was used as a diluent, the results were comparable (data not shown).

Preliminary patient data

The ferritin iron saturation in the serum of 10 healthy male volunteers was measured. The iron saturation of ferritin was also determined in a group of 47 patients selected on the basis of a high erythrocyte sedimentation rate. Table 4 shows the comparison between healthy volunteers and patients with an acute phase reaction. From the range it can be seen that the iron ferritin saturation percentages vary from 5 to 50%, so the amount of iron in ferritin is considerable. Furthermore these data suggest that in patients with an acute phase reaction the iron saturation of ferritin is statistically significantly ($p < 0.05$) lower than in healthy controls.

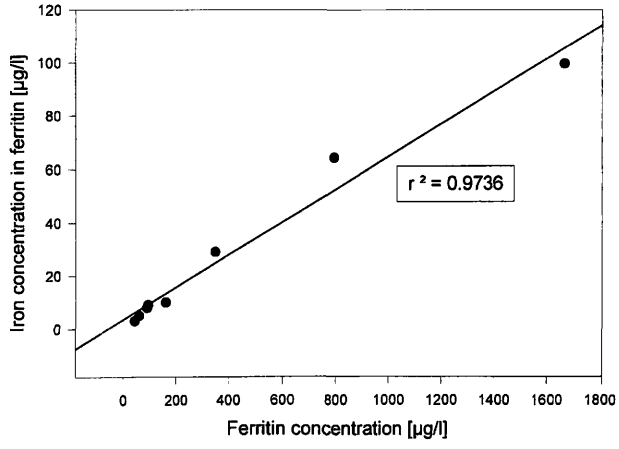

Fig. 2 Linearity of the assay. Each point is measured in triplicate.

Discussion

The iron saturation of ferritin was found to be rather high, up to 50%. A ferritin concentration of 200 µg/l with an iron content of 40% contributes around 6% to the total serum iron concentration. The biological role of this considerable amount of circulating iron store still has to be elucidated. However, a dynamic role for serum ferritin with respect to iron transport is not unlikely since the presence of ferritin receptors on lymphoid and erythroid cells has been known for many years (3–5, 10, 11). In addition *Meyron-Holtz* et al. (4) recently reported that ferritin bound to its receptor is internalized and may be important as a donor of iron in erythroid cells.

Earlier investigations describe lower saturation percentages of ferritin, 2.5–11% molar percentages (12–15). The method presented in this paper is much more direct than the methods which were applied by earlier investigators. They may have lost iron from the ferritin during laborious and time consuming-chromatographic isolation procedures, using elution buffers without iron. *Herbert* et al. (16) recently published an abstract in which they reported iron saturation percentages of 11–50%, which is in line with our results.

With the technique presented here it will be possible to study the ferritin iron saturation in various pathological conditions, such as patients with anaemia and chronic disease. The preliminary data obtained with patients having acute phase response are in line with our working hypothesis that the iron saturation of ferritin is lower in these patients. The results described in this paper are supported by the work of *Herbert* et al. (16), who stated in their abstract that inflammation is characterized by an increased number of ferritin molecules each containing a "subnormal" quantity of iron.

Ferritin molecules are composed of 24 subunits. A common structure of most ferritin protein molecules is the composition of different ratios of the primary ferritin subunit chains: the L (light) subunit M_r 19 500 and the H (heavy) subunit M_r 21 000 (17). It is known that ferritin synthesis under influence of acute phase cytokines such

Tab. 4 Iron saturation of ferritin in 47 patients having an acute phase reaction, compared to 10 healthy volunteers.

	Healthy volunteers (n = 10)	Patients with acute phase (n = 47)
Ferritin concentration (µg/l) mean (SD)	111.8 (81.6)	445.2 (56.6)
Iron saturation of ferritin (%) mean (SD)	24.3 (8.2)*	19.3 (7.4)*
(range)	(11–37)	(5–42)

* significant difference $p < 0.05$ (*Students* t test)

as the interleukins (IL) IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) is more in favour of the H subunit, while under influence of iron both subunits are up-regulated (18). If such a shift is considered during the acute response in our group of patients, theoretically a maximum shift would result in M_r of 504 000 (24 H subunits). This means an increase in M_r of 8%, which results in an increase of the calculated iron saturation of ferritin of 8%. This may compensate for the observed decrease of iron-saturation of ferritin of 5% (tab. 4) in this group of patients, although such a dramatic shift in isoforms is unlikely. Glycosylation could have been another disturbing factor, since it is known that serum ferritin is also composed of a glycosylated subunit (M_r 23 000). However we do not have to take this into account because the serum ferritin and liver- and spleen ferritin, in the applied assay, react in the same way (manufacturers' information). This indicates that the epitopes used in this assay are unaffected by glycosylation.

The group of patients which were investigated was very heterogenous with respect to the cause of the acute phase response. Further investigation on patients with well-defined conditions causing an acute phase response, for instance rheumatoid arthritis, are necessary to prove the value of this method in clinical practice.

Instead of a bone marrow biopsy, the iron saturation of the ferritin may also be helpful in assessing the iron stores of patients with anaemia and chronic disease. At the moment this work is in progress in our institute. Furthermore the ferritin iron saturation is of interest when dealing with haemochromatosis patients with respect to the monitoring of therapy. Further insight into the kinetics of iron transport and storage processes may help in the diagnosis and follow-up of iron deficiency, while the role of ferritin as a transporter of iron has to be reconsidered.

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