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Quantitative Determination of Non-Haem Iron and Ferritin Iron in Bone Marrow Using Flameless Atomic Absorption Spectrophotometry

A Comparative Study on the Cytological and Chemical Determination of the Bone Marrow Iron Content

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Summary: A method for non-haem iron analysis in bone marrow aspirates using graphite furnace atomic absorption spectrophotometry has been developed. Bone marrow aspirates were obtained from patients with various disorders. A good correlation is observed between chemical and cytological assessment of total non-haem iron in bone marrow. An intra-assay coefficient of variation of 9.0% was observed.

The ferritin-iron concentration was also determined and a CV_{duplo} of 11% was found. The ferritin iron concentration increased with an increasing total iron content until saturation of ferritin appeared to be reached at about 3 g ferritin per kg protein. It was concluded that the quantitative determination of bone marrow iron can be of value in the diagnosis and investigation of both hypo- and hyper-ferraemic disorders.

Introduction

Estimation of bone marrow iron by cytological staining is often considered as the "golden standard" for diagnosis. However, the degree of iron staining in bone marrow does not necessarily reflect the amount of total iron as detected by elementary analysis, but is also related to the chemical nature of iron compounds such as transferrin, ferritin, haemosiderin and the occurrence of molecular aggregates. Moreover, cytochemical estimation is a qualitative method and provides no quantitative data of total iron stored in bone marrow.

For these reasons we developed a method for iron analysis in bone marrow aspirates using graphite furnace atomic absorption spectrophotometry. The procedure corresponds, with modifications, to a method for the determination of iron in liver tissue (1), which was earlier developed in our laboratory.

An interesting feature is the comparison of data obtained for total non-haem iron, haemosiderin iron and ferritin iron concentrations in bone marrow, with results of visual evaluation of cytological iron staining (Prussian blue).

Patients

Iliac crest bone marrow aspirates were obtained from 62 patients with various disorders (Departments of Internal Medicine and Rheumatology).

Methods

Sample preparation

An aliquot of the bone marrow aspirate was used for routine cytological examination. The remaining aspirate was washed in saline and after centrifugation bone marrow remained afloat. The washing procedure to remove blood was repeated twice.

The isolated bone marrow was homogenized (Potter homogenizer) in 2 ml 9 g/l NaCl solution. For acid digestion, 100 µl bone marrow homogenate was added to a mixture of 300 µl 98% H_2SO_4 (BDH Chemicals) and 150 µl 30% H_2O_2. Acid digestion was performed at 120 °C until samples were colourless. After cooling, 1 ml H_2O was carefully added and mixed. Blanks (100 µl) and standards (100 µl) were treated similarly before measurement.

Atomic absorption spectrophotometry

Flameless atomic absorption measurements were performed on a Perkin Elmer (Überlingen, D) model 3030 spectrophotometer with deuterium background correction, equipped with a PE HGA 500 graphite furnace atomizer (programmable) and PE AG 40 autosampler. The iron hollow cathode lamp was from Instrumentation Laboratory No. 89225 (Paderno, Italy).

Instrumental conditions are summarized in table 1.

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

Sample volume 20 µl
Fe hollow cathode lamp 30 mA
Slit with 0.2 nm, alternate
Wavelength 248.8 nm
Deuterium background corrector on
Graphite furnace tube with *L'vov* plattform, pyrolytic coated
Purge gas, argon: 300 ml/min
Calibration auto-select
Standards 0, 600, 1200, 1800 µg/l iron

Dry cycle	130 °C	15 s ramp	10 s hold
Char cycle	500 °C	5 s ramp	5 s hold
	1000 °C	2 s ramp	2 s hold
	1000 °C	1 s ramp	5 s hold[1])
Atomize	2400 °C	0 s ramp	6 s hold[1]), [2])
Clean	2700 °C	1 s ramp	4 s hold
Cool	20 °C	1 s ramp	10 s hold

[1]) Gas flow interrupt
[2]) Signal processing, peak area, 3 s after start atomize cycle

Calibration mixtures (0, 600, 1200 and 1800 µg/l) were prepared from a ferric nitrate standard solution (1 g/l, Spectrosol, BDH Chemicals) in 200 mmol/l nitric acid.

Determination of iron compounds

Haem iron concentration is estimated colorimetrically as haemocyanide according to the method of *Zuyderhoudt* (2).

The *non-haem* bone marrow *iron* concentration is the total iron concentration of native homogenates as determined by elementary analysis, minus the haem iron concentration.

Ferritin for iron determination was isolated from the bone marrow homogenate by heat treatment (70–75 °C, 5 minutes) and centrifugation as described by *Zuyderhoudt* (2). Haem-iron and haemosiderin iron precipitate at this temperature. After centrifugation, ferritin iron was measured in the supernatant using flameless atomic absorption spectrophotometry.

The *haemosiderin* concentration was calculated by subtraction of the ferritin concentration from the total non-haem bone marrow iron concentration.

All iron determinations were expressed in g/kg protein in the native homogenate. The protein concentration was determined according to *Lowry* (3).

Cytological bone marrow examination

Cytological bone marrow smears, after staining with *May Grünwald Giemsa* and *Perl*'s iron stain, were used for visual estimation of iron storage. The grade of iron staining was classified by microscopic evaluation of macrophage staining (negative, trace, positive, strongly positive) and sideroblast staining (> 20% positive/negative) and presence of ringsideroblasts. Iron deficiency was defined as present when macrophages were judged negative or trace-positive.

Reagents

Unless specified otherwise, all reagents were purchased from Merck (Darmstadt, D).

Results

Precision of the elementary iron analysis

The total assay variation was obtained by digesting two different bone marrow homogenates, ten times each. Total iron concentration was determined in each sample using flameless AAS. Overall coefficients of variation were 7.6% (mean concentration 639 µg/l) and 7.5% (mean concentration 1593 µg/l).

Figure 1 presents the results obtained for total bone marrow iron concentrations determined by flameless atomic absorption spectrophotometry, compared with microscopic evaluation of cytological bone marrow in staining.

Figure 2 presents the correlation between the total bone marrow iron concentration and the bone marrow ferritin iron concentration, determined by isolation and chemical analysis.

Figure 3 presents the relation between the total bone marrow iron concentration and the bone marrow haemosiderin concentration.

Discussion

The detection and quantitation of iron compounds in tissue has long been a problem (4). Until recently, histological methods were considered as golden standards. The visualisation of iron was generally based on the formation of Prussian blue or similar compounds. Microscopic evaluation in order to quantitate the amount of iron present is, however, hampered by different reactivities of iron compounds to form Prussian blue complexes, and also by observer bias. Sensitive flameless atomic absorption spectrophotometry combined with mineralisation of the tissue samples

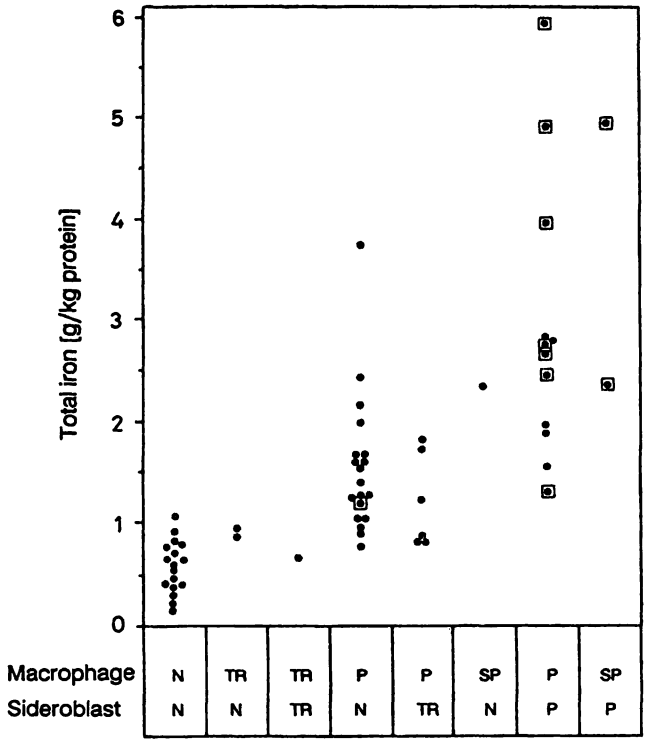

Fig. 1. Comparison of results obtained for iron determined by flameless atomic absorption spectrophotometry and cytological evaluation of iron staining in bone marrow; ⊡ indicates presence of ringsideroblasts.
N = negative
TR = trace
P = positive
SP = strongly positive

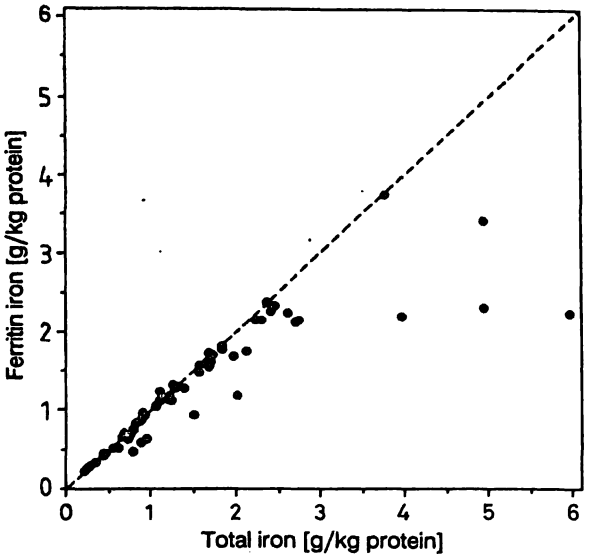

Fig. 2. Correlation between total bone marrow iron and bone marrow ferritin iron. The dashed line represents the hypothetical outcome, if all the liver iron is stored as ferritin iron.

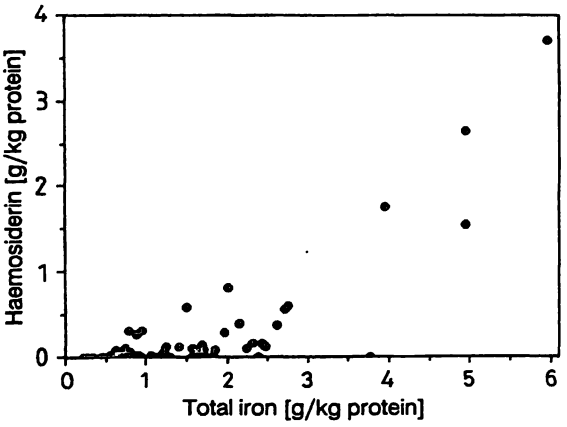

Fig. 3. Relation between the total bone marrow iron concentration and bone marrow haemosiderin concentration.

provides the possibility of accurate and precise determination of elementary iron, regardless of the original compound from which it originates. Such a procedure was developed for the determination of iron in liver tissue (1), and with modification it appears to be applicable to the quantitative determination of iron in bone marrow.

Regarding the qualitative aspects of the histological/cytological assessment of storage iron in bone marrow aspirates, an acceptable correlation with the chemical determination method has been observed. There is much overlap of the histological categories. This is not surprising because classification is the result of microscopic evaluation, which has its drawbacks as discussed earlier. In our opinion, total iron determination by the described quantitative accurate chemical analysis should replace "iron content evaluation" by qualitative visual estimation.

The amount of bone marrow iron per kg protein is in accordance with earlier results (5) and those obtained with a spectrophotometric method of *Kerr* (6).

An interesting feature of course is the relative distribution of iron in several compounds in bone marrow. We found a correlation between total iron and ferritin iron (fig. 2). Up to a total iron concentration of 3 g/kg protein, a more or less linear correlation was observed. The ferritin iron appeared to reach a level of saturation at elevated iron concentrations, whereas the concentration of the insoluble iron compound haemosiderin still increased.

Increase in ferritin iron up to a certain level, after which iron accumulates in the form of haemosiderin, was also found in liver tissue. An upper level of ferritin iron per kg protein is also seen in investigated liver tissue (1). We found a similar value of 3 g/kg protein.

A less clear-cut relationship is observed between the total iron and haemosiderin (fig. 3), although there is a positive correlation, especially at elevated values, after ferritin saturation. The increase in haemosiderin iron seems to be prognostic for iron accumulation/overloading (7). The clinical implication of the measurement of the ferritin iron versus haemosiderin iron ratio for individual patients, however, still needs to be evaluated.

The precise and accurate measurement of iron deficiency or iron accumulation in bone marrow is important. Critical evaluation of ferriprive anaemia is still based on iron deficiency in the bone marrow. A reliable method for determination of iron content is now available using mineralisation and subsequent flameless atomic absorption spectrophotometry. In our opinion this procedure provides a true standard and should replace the visual "iron estimation" by the histological/cytological procedure; the latter provides, however, additional information, such as e. g. the presence or absence of ring sideroblasts.

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