Comparison of Bathophenanthroline Sulfonate and Ferrene as Chromogens in Colorimetric Measurement of Low Hepatic Iron Concentration, Laurence Pieroni,1 Lina Khalil,1 Frederic Charlotte,2 Thierry Poynard,3 Annie Piton,1 Bernard Hainque,1 and Françoise Imbert-Bismut 1*

Sensitive and accurate measurement of hepatic iron concentration (HIC) is required to investigate liver fibrogenesis (1) and its influence on the outcome of interferon therapy for chronic viral hepatitis C (2, 3). Hepatic iron content can be measured by a quantitative chemical method and/or evaluated by semiquantitative histologic scoring. Quantitative chemical methods assess all liver iron forms, whereas histologic scoring evaluates only the hemosiderin form. A colorimetric method using bathophenanthroline sulfonate as chromogen was recommended in 1978 by the International Committee for Standardization in Hematology (ICSH) for determination of serum iron (4, 5). It was adapted by Barry and Sherlock (6) to the determination of HIC, and we recently evaluated it for measurement of low HIC (7). In 1990, the ICHS replaced bathophenanthroline sulfonate with ferrene, a more sensitive chromogen, in the determination of serum iron (8). The aim of the present study was to evaluate the replacement of bathophenanthroline sulfonate with ferrene to improve the sensitivity of the colorimetric determination of low HIC.

We used samples of a frozen Wistar rat liver for quality control and determination of reliability criteria of both assays. We compared the results obtained with the two chromogens on 66 liver biopsies from patients with chronic liver diseases hospitalized in the Department of Hepatogastroenterology of the Pitié-Salpêtrière Hospital. The clinical diagnoses of these patients are summarized in Table 1. We determined the CV for HIC measurements on two separate samples from the same liver specimen for each chromogen on 38 human liver biopsies. Histologic iron scoring was according to the method of Deugnier et al. (9); among the 66 biopsies, 20 had no stainable iron (score of 0), and the 46 others exhibited iron overload (score ≥6). Biopsies were fixed in 40 g/L formaldehyde and embedded in paraffin as part of routine histologic processing for better preservation and transport.

Paraffin-embedded tissues were heated at 60°C to remove the paraffin and washed in three successive baths of xylene followed by three baths of ethanol. Liver samples were dried at 120°C for 24 h and weighed on a microbalance with 0.01 mg precision. The mean dry weight (dw) of the liver samples was 1 mg (SD, 0.4 mg; range, 0.5–2.5 mg). Liver samples were digested with an equivolume mixture of sulfuric acid and nitric acid for 5–8 min over a low flame. The working solutions were prepared by adding 10 mL of H2O (10). Samples of thawed rat liver were dried, weighed, and treated as described above.

HIC was measured according to the colorimetric method described by Barry and Sherlock (6). The iron in 3 mL of each working solution was reduced with 50 µL of thioglycolic acid (Sigma Aldrich) and 2.5 mL of an acetic acid–acetate buffer (Merck Eurolab) at pH 4.5, which is optimal for the reduction of ferric to ferrous iron and for absorbance measurements of iron complexed with bathophenanthroline sulfonate (4,7-diphenyl-1,10-phenanthroline disulfonic acid), disodium salt or ferene [3-(2-pyridyl)-5,6-bis[2-(5-furyl sulfonic acid)]-1,2,4-triazine] (11).
Samples were then incubated with 0.4 mL of bathophenanthroline sulfonate or ferene (3 μmol/L; Sigma Aldrich) for 1 h at 37 °C. The absorbances were read against a blank on a Beckman DU 640 spectrophotometer at 535 nm for bathophenanthroline or at 592 nm for ferene as the chromogen.

All chemical reagents were iron-free grade. The absorbance of the blank had to be <0.006 to verify the absence of contaminating iron. A calibration curve was constructed, using serial dilutions of a solution of 180 μmol/L ferrous ammonium sulfate (Merck Eurolab). Results were expressed in μmol/g dw of liver tissue. When we used bathophenanthroline sulfonate as chromogen, HIC was =20 μmol/g dw for a histologic iron score of 0.

We performed regression analysis using the method of Passing and Bablok (12). The differences between the chromogen-related iron measurements in relation to the mean of the two paired values were estimated by Bland–Altman plots (13).

The within-day reproducibility of the method was evaluated on the same rat liver tissue. Ten liver samples were treated as described above. The iron content was assayed twice with each chromogen on the different working solutions. The CVs were 11% (mean of the two paired values were estimated by Bland–Altman plots (13).

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For 11 patients with a histologic score of 0 (iron concentration, 4.3–15.0 μmol/g dw), HIC was determined with each chromogen in two different samples from the same liver biopsy (1.32 ± 0.45 mg dw). The SD for a single iron determination gave CVs of 9.8% and 11% for the bathophenanthroline and ferene methods, respectively. For 27 patients with a histologic iron score ≥6 (iron concentration, 14.0–89.0 μmol/g dw) tested in duplicate (dry weight of liver samples, 0.92 ± 0.32 mg) the SD for iron determination gave CVs of 15% for both the bathophenanthroline and ferene methods.

A linear calibration was verified for the two chromogens for iron concentrations of 0.3–30.0 μmol/L. The detection limit of the assay measured on 10 blank replicates was 0.30 μmol/L for bathophenanthroline and 0.16 μmol/L for ferene. The sensitivity of the assay, defined as the slope of the calibration curve, was 0.215 with bathophenanthroline and 0.333 with ferene. The intercept (absorbance) was 0.007 for bathophenanthroline and 0.008 for ferene.

The HIC of 66 patients with chronic liver disease was determined using the two chromogens. The correlation of the two methods was assessed by Passing–Bablok analysis. Comparison of the methods gave a slope of 1.00, an intercept of −1.00, and a correlation coefficient of 0.99. A Bland–Altman difference plot for the paired values is shown in Fig. 1. The differences between the paired values are shown on the y-axis, and the means are shown on the x-axis. The mean difference and the SD of the differences are depicted parallel to the x-axis. Fig. 1 shows that eight of the paired values were outside the 95% confidence interval. Four values were close to the lower limit of agreement (−2.6), whereas the other four were close to the upper limit of agreement (5.0 and 6.0 vs 4.2).

Colorimetric methods for determination of hepatic iron content have been shown to be accurate and sensitive. The flameless atomic absorption spectrophotometry method has been described as sensitive, but it requires costly equipment. Recently, magnetic resonance imaging was shown to distinguish mild from severe iron overload in the range of 45–134 μmol/g dw (14). However, at lower concentrations, variability and reproducibility of the method were not investigated. We previously reported the evaluation and validation of a colorimetric method described by Barry and Sherlock (6) with the aim of measuring low HIC (7). To improve the sensitivity of this colorimetric assay, we evaluated the performance of ferene as a chromogen. Indeed, ferene has a higher absorptivity than bathophenanthroline sulfonate (33 850 vs 22 369 L·mol⁻¹·cm⁻¹) and has been shown to be more sensitive in the determination of serum iron (11).

The CV for iron concentrations in duplicate specimens from liver samples from patients with a histologic score of 0 was slightly higher with ferene (11% vs 9.8%). Barry and Sherlock (6) reported a CV of 7.0%, but the dry weights of their liver samples were heavier. In specimens from patients with an histologic iron score ≥6, the CVs for determination of HIC were comparable for ferene and bathophenanthroline.

The within-day reproducibility of the method, using rat liver samples, was better when ferene was used as the chromogen (9.4% vs 11%) and was comparable to other colorimetric assays (15). The day-to-day CV was better when ferene was used. Comparison of the two methods
by Passing–Bablok analysis showed that results obtained with the two chromogens were correlated. The Bland–Altman difference plot (Fig. 1) showed that eight of the paired values were outside of the 95% confidence interval (−2.6 and 4.2). These values fell nearly within the limits of agreement, and medical acceptance remained possible.

The determination of low HIC with ferene gave better sensitivity (0.333 vs 0.215) and a lower detection limit (0.15 vs 0.30 μmol/L) than with bathophenanthroline. Moreover, this chromatogen is less expensive than bathophenanthroline.

In conclusion, ferene, which is less expensive than bathophenanthroline sulfonate, slightly improves the sensitivity of the colorimetric measurement of low HIC. Ferene can beneficially replace bathophenanthroline sulfonate in HIC determinations as recommended by the ICSH for serum iron (8).

References


Osteoprotegerin in Serum as a Novel Marker of Bone Metastatic Spread in Prostate Cancer, Klaus Jung,† Michael Lein,† Katharina von Hösslin,† Brigitte Brux,‡ Dietmar Schnorr,† Stefan A. Loening,† and Pranav Sinha‡ (Departments of †Urology and ‡Laboratory Medicine, University Hospital Charité, Humboldt University Berlin, D-10098 Berlin, Germany; †address correspondence to this author at: Department of Urology, Research Division, University Hospital Charité, Humboldt University, Schumannstrasse 20/21, D-10098 Berlin, Germany; fax 4930-450-515904, e-mail klaus.jung@charite.de)

Prostate cancer (PCa) is the most frequent carcinoma in men and is often complicated by skeletal metastasis (1). Because bone scintigraphy, the standard method of monitoring metastatic bone involvement, is expensive, lacks specificity, and is not particularly suitable for the follow-up of patients, various metabolic bone markers have been studied as indicators for bone metastasis in PCa patients (2, 3). Markers that reflect osteoblast proliferation, e.g., skeletal alkaline phosphatase (sALP), are reportedly useful, which is consistent with the osteoblastic reactions seen in the skeletal metastases (4).

The balance between osteoblastic and osteoclastic activity in bone is essentially influenced by osteoclastogenesis. The latter is regulated by three proteins: receptor activator of nuclear factor-κB (RANK), which is expressed on osteoclast precursor cells; its ligand (RANKL), which is expressed on the surface of preosteoblastic and stromal cells; and osteoprotegerin (5, 6). The interaction of RANKL with RANK stimulates the differentiation of osteoclasts, whereas osteoprotegerin blocks this process by functioning as a decoy receptor for RANKL. This novel cytokine system appears to play an important role in the establishment of bone metastases in PCa patients (7). Osteoprotegerin has recently been found to be overexpressed in bone metastases of these patients (8). This overexpression could indirectly favor osteoblastic reactions by inhibiting osteoclastogenesis and might explain the bone lesions typically seen PCa patients. Because tissue overexpression of proteins often is reflected in blood, osteoprotegerin could be a potential serum marker for diagnosis of bone metastasis.

Using a recently introduced novel osteoprotegerin assay, we performed the present study to evaluate the diagnostic validity of osteoprotegerin as a potential bone metastasis marker in PCa in comparison with sALP and cross-linked N-telopeptides of type I collagen (NTx), which represent osteoblastic and osteoclastic markers, respectively.

The study included 36 male controls (mean age, 51.4 years) with no history of prostate diseases and normal digital rectal examinations, 35 patients with benign prostatic hyperplasia (BPH; mean age, 67.1 year), and 93 patients with carcinoma of the prostate (mean age, 66.2 years). Seventeen of the carcinoma patients had bone metastases, whereas 76 were without bone metastases. All of the men were investigated in the Department of Urology of the Humboldt University or the affiliated outpatient department. We used