proANP as a marker for heart failure could be missed with a nonfasting sample. If the clinical presentation is unclear, such an occurrence could have dire consequences for the patient with values that are otherwise in the diagnostically “gray zone.” For now, the recommendation is that proANP measurements be made on fasting patients. Future studies must also examine the effects of meal intake on the active hormones (ANP and BNP), for which their profiles with respect to food intake are completely unknown. Finally, investigations for mechanistic insights into this phenomenon should address the possible relationship between insulin and natriuretic peptide secretion to explain the current finding and perhaps the reportedly low plasma proANP and proBNP concentrations in obese persons.

In conclusion, plasma proANP concentrations, but not proBNP concentrations, are affected by meal intake, with a decrease of 50% after 2 h. This observation needs to be confirmed in clinical studies of proANP. If the results are confirmed, the findings may provide new biological insight into insulin-resistant persons or patients with diabetes.

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Jens P. Goetze*
Department of Clinical Biochemistry
Rigshospitalet
University of Copenhagen
Copenhagen, Denmark

*Address correspondence to the author at:
Department of Clinical Biochemistry
Section 3014, Rigshospitalet
Blegdamsvej 9, DK-2100
Copenhagen, Denmark
Fax +45-3545-2880
E-mail JPG@dadlnet.dk

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Comparison of Bleomycin-Detectable Iron and Labile Plasma Iron Assays

To the Editor:

Non–transferrin-bound iron (NTBI)1 is detected in the plasma of patients with diseases in which the transferrin-binding capacity for iron is exceeded by a massive iron overload, such as in certain forms of thalassemia and hemochromatosis, repeated blood transfusions, or bone marrow failure. NTBI may occur in plasma as insoluble polynuclear ferrhydrate species, as Fe(III) citrate or acetate complexes, or bound to certain flavonoids, amino acids, albumin, or modified albumin. Excess iron is harmful because it causes oxidative stress, accumulates in and damages tissues, and enhances the growth of pathogens. Therefore, the measurement of NTBI is of clinical importance, because NTBI may be used as an indicator of systemic iron overload and iron toxicity (1, 2).

We compared 2 NTBI assays: a labile plasma iron (LPI) assay that uses desferrioxamine as the iron chelator (3) and a microwell modification of a bleomycin-detectable iron (BDI) assay (2). The BDI assay is an indirect method based on the formation redox-active complexes between NTBI in the sample and the bleomycin reagent. The Fe(II)–bleomycin complex then degrades DNA in the sample mixture via a free-radical reaction, which can be measured photometrically. The detection limit of our BDI assay was 0.05 μmol/L, and the interassay CV was 18% and 9.8% at 0.2 μmol/L and 1.5 μmol/L, respectively (2). The LPI assay is based on the measurement of the redox-active and readily chelatable fraction of serum NTBI. The assay measures iron-catalyzed radical generation in the presence of a low ascorbate concentration. Radical generation is measured with the fluorogenic redox-sensitive probe dihydrorhodamine 123 (Sigma-Aldrich), and iron-catalyzed radical generation is calculated by subtracting the radical generation in the presence of 100

1 Nonstandard abbreviations: NTBI, non–transferrin-bound iron; LPI, labile plasma iron (assay); BDI, bleomycin-detectable iron (assay); TS, transferrin saturation.
concentration was 0.12 μmol/L, which is calculated as 3 SDs above the mean of a zero sample (n = 8). The interassay CV was 11% and 12% at 0.3 μmol/L (n = 8) and 2.1 μmol/L (n = 8), respectively. The transferrin concentration was measured, and the percentage of transferrin saturation (TS) was calculated as previously described (2).

We measured NTBI and TS in 78 serum samples from 5 leukemia patients undergoing allogeneic hematopoietic stem cell transplantation, as described in a previous report (4). The median serum NTBI concentration was 0.12 μg/L (95% CI, 0.06–0.21 μg/L) and 0.10 μg/L (95% CI, 0.05–0.16 μg/L) as measured with the BDI and LPI assays, respectively. The results were correlated according to the equation: y = 1.33x – 0.06 μg/L, where x and y are the NTBI concentrations obtained with the BDI and LPI assays, respectively (r = 0.329, Deming regression). There was no statistically significant difference between the NTBI results obtained by the 2 methods (P = 0.329, paired t-test). The median TS in the samples was 89.9% (95% CI, 83.1%–92.4%). The NTBI concentration measured by both methods was correlated with TS (P ≤ 0.0001). The NTBI concentrations in samples with TS <87% were low: For the BDI assay, all NTBI results except one (NTBI, 0.21 μg/L; TS, 13.6%) were <0.1 μmol/L. For the LPI assay, all NTBI results were <0.17 μmol/L (Fig. 1).

The exact biochemical nature of NTBI is unknown, and analytical approaches to measure NTBI vary with respect to testing principle and practical application. In 2005, an international round robin for the quantification of serum NTBI compared 8 methods in 6 different laboratories (5). Seven of the methods were chelating assays based on 5 different chelators, and one was our BDI assay (2). The methods differed considerably in their NTBI results, and the BDI assay measured the lowest NTBI concentrations (5). The mobilization of NTBI by chelators from complexes in which NTBI is not available for the bleomycin reagent may explain this discrepancy. The LPI and BDI methods produced concordant results in the present study, suggesting that both methods measure the same form of NTBI in plasma (r = 0.87).

We compared NTBI assays with serum samples from patients undergoing stem cell transplantation for hematologic malignancies (2). Therapies related to iron chelation are of special interest for this patient group. Avoiding NTBI formation is also an important therapeutic goal in other diseases in which NTBI has been observed, i.e., hemochromatosis, hepcidin or ferroportin deficiency, β-thalassemia, liver diseases, end-stage renal disease, diabetes, and myelodysplastic syndrome (1). The LPI assay detects the redox active NTBI, i.e., the potentially toxic species of plasma NTBI, and may therefore be more relevant for clinical use than the BDI assay.

**Fig. 1.** Serum NTBI (S-NTBI) concentration as measured by the BDI (■) and LPI (♦) assays and compared with the TS percentage.

An increased NTBI concentration was detected in samples when the TS value was >87%.

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**References**

To the Editor:

25-Hydroxyvitamin D (25-OH-D) is the most appropriate marker for monitoring vitamin D storage status. The 2 major forms of 25-OH-D [25-OH-D₂ (D₂) and 25-OH-D₃ (D₃)], differ slightly in molecular structures but have essentially the same physiological activities. Therefore, both D₂ and D₃ should be measured to assess vitamin D status.

Recognizing the ability of an assay to quantify both the D₃ and D₂ forms equally is therefore crucial.

The analytical performance of several total 25-OH-D assays approved by the US Food and Drug Administration is well documented (1); however, information is limited on how D₂ affects assay accuracies. Le Goff et al. (2) demonstrated the inconsistency between several assays in their cross-reactivities toward D₂. The size of their study was small, however, and the range of D₂ percentages (D₂%) was unknown. We used a larger sample size than previous studies and a D₂% interval of 1.3%–91.2% [3.4–95.1 ng/mL (8.5–238 nmol/L)] and evaluated the influence of D₂% on the accuracies of the following assays: DiaSorin Liaison, Abbott Architect, Roche Cobas, and Siemens Centaur. We developed and validated a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay as the reference method.

Samples were prepared for the LC-MS/MS assay by mixing with internal standard (D₁-d₆/D₂-d₆), ZnSO₄ solution, and methanol; liquid–liquid extraction (hexane); drying; and reconstitution. The liquid chromatography method was established with a Phenomenex 2.6-μm Kinetex C18 column (2.1 × 50 mm) at 35 ºC and a flow rate of 0.35 mL/min. Mobile phases A and B were deionized water and LC-MS-grade methanol, respectively, both containing 1 mL/L formic acid and 2 mmol/L ammonium acetate. The linear gradient of the mobile phase was 60% to 100% B in 3.5 min, and the total length of the liquid chromatography run was 8 min. A Waters Micromass Quattro Micro mass spectrometer, operated with positive electrospray ionization, was used to acquire chromatograms with the following ion transitions: D₃, m/z 401.0 → 159.1; D₂, m/z 413.0 → 355.2; D₃-d₆, m/z 407.0 → 159.1; and D₂-d₆, m/z 419.0 → 355.2.

The between-day CVs were between 5.1% and 12% at D₂, D₃, and total D concentrations of 12.9–143 ng/mL (32.2–358 nmol/L). The matrix effect was negligible. The linearity intervals for D₃, D₂, and total D were 3.0–200 ng/mL (7.5–500 nmol/L), 1.0–150 ng/mL (2.5–375 nmol/L), and 3.0–350 ng/mL (7.5–875 nmol/L), respectively. The recoveries were 83.8%–109.2% (mean, 95.9%). The lower limits of quantification for D₃ and D₂ were 3.0 ng/mL (7.5 nmol/L) and 1.0 ng/mL (2.5 nmol/L), respectively. We tested the 2011 College of American Pathologists proficiency testing set ABVD-01–05 with the assay, and all results were within ±25% of target values (mean bias, −1.2%). Interference by 3-epi-25-OH-D₂ was negligible, because our study included only samples from adults (21–97 years) (3).

We randomly chose samples from 149 adult patients submitted for 25-OH-D testing by the Abbott Architect assay. All samples were tested by the LC-MS/MS assay (63 contained D₂), and subsets of these samples were aliquoted for the following assays: 75 samples (31 with D₂) by the Liaison assay, 64 samples (20 with D₂) by the Centaur assay, and 55 samples (25 with D₂) by the Cobas assay. Total 25-OH-D values obtained with each commercial assay were compared with those obtained by LC-MS/MS. Deming regression analysis and the determination of percent bias were performed under 2 scenarios: (a) samples with D₃ only vs. (b) all samples. The regression equations and the coefficients of determination (r²) obtained for each scenario are shown in Fig. 1A.

For the Liaison assay, the 2 scenarios yielded similar coefficients of determination; however, these values were different for all of the other assays. The regression slopes and r² values of the Architect and Cobas assays both de-