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Non-transferrin-bound iron assay system utilizing a conventional automated analyzer

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ABSTRACT

Background: Iron is an essential metal in the body, but its excessive accumulation causes damage in various organs through free radical production. Iron homeostasis is therefore tightly regulated. However, when iron balance collapses, such as in prolonged transfusion, transferrin (Tf) is fully saturated and non-Tf-bound iron (NTBI) appears in the serum. Monitoring serum NTBI levels is therefore crucial in the assessment of the clinical status of patients with iron overload, since NTBI is associated with cellular and organ damage. Several methods for NTBI determination have been reported, but these are extremely complicated and very few laboratories can quantify NTBI at present.

Methods: We established a novel assay system utilizing automated analyzers that are widely used in clinical laboratories for diagnostic testing. In this assay, NTBI is chelated by nitrilotriacetic acid (NTA), after which the iron is reduced and transferred to nitroso-PSAP, a chromogen.

Results: The assay shows excellent linearity, reproducibility, and compatibility with HPLC, one of the most reliable conventional methods for NTBI quantification.

Conclusions: Our novel method for NTBI measurement is high-throughput and may be a useful and powerful tool in the study of the physiological and clinical importance of NTBI.

1. Introduction

Iron is an essential metal in the body, but iron overload causes organ damage through free radical production. Iron homeostasis is therefore tightly regulated under normal conditions by orchestration of proteins related to iron metabolism [1–4]. Iron circulates in serum by binding to its specific carrier protein, transferrin (Tf). In healthy individuals, only 20–30% of Tf is usually saturated with iron, so that during a period of iron burden (such as in red blood cell transfusion or iron administration) iron is immediately trapped by iron-free apo-Tf. However, in situations where the balance of iron metabolism collapses, Tf is fully saturated and non-Tf-bound iron (NTBI) appears in serum.

NTBI refers to all forms of iron in plasma that is not bound to Tf and was first measured by Hershko et al. [5] in 1978. Iron binds to various ligands other than Tf, including albumin and citrate, implying that NTBI is present in heterogeneous forms [6–8]. However, these binding affinities are extremely weak compared to the iron–Tf complex and so NTBI can also be considered as "free iron" in serum. Tf-bound iron is taken up via a specific receptor, transferrin receptor 1 (TfR1), which is expressed in almost all cells and permits strict regulation of the cellular delivery of Tf-bound iron. In contrast, there is no known regulatory mechanism for NTBI uptake, and thus NTBI can easily enter cells and produce free radicals through the Fenton reaction, resulting in cell and organ damage [9,10]. The fraction of NTBI that is both redox-active and susceptible to chelation is sometimes referred to as labile plasma iron (LPI). LPI is considered to be a major component of NTBI, and is responsible for cytotoxic activity [11].

Abbreviations: Tf, transferrin; NTBI, non-transferrin-bound iron; TfR1, transferrin receptor 1; NTA, nitrilotriacetic acid; Nitroso-PSAP, 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)-amino] phenol; Bathophenanthroline, 4,7-diphenyl-1,10-phenanthroline; Ferene, 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt; Ferrozine, 3-(2-pyridyl)-5,6-bis(4-sulfophenyl)-1,2,4-triazine; sFe, serum iron.

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Clinically, NTBI is increased during iron overload in various disease states [12]. However, NTBI is decreased below the detection limit by long-term therapy with iron chelators such as deferoxamine and deferasirox in thalassemic patients and by venesection in hemochromatotic patients [13,14]. Therefore, measurement of NTBI levels is important for evaluating and monitoring the risk of iron toxicity [15]. Several methods for NTBI determination have been established [16–20], but it is still difficult to measure NTBI in blood due to the complex nature of the procedures involved and difficulty in eliminating iron contamination in reagents and equipment. Because of these problems, only a limited number of institutions can quantify NTBI at present.

We first focused on an NTBI assay using high-performance liquid chromatography (HPLC). The principle of this method is based on Gosriwatana et al. [21]: NTBI in serum is initially chelated by nitrilotriacetic acid (NTA) and then transferred to another chelator, CP22, which produces a chromogenic effect when bound to iron. The level of NTBI is quantified by measuring the peak absorbance at 450 nm using a non-metal HPLC. However, the sensitivity of previously reported HPLC-based methods are considered to be unsatisfactory, as these methods cannot be used to measure serum samples containing low concentrations of NTBI, especially samples from healthy individuals. We previously reported that iron contamination during HPLC-based assay procedures can cause elevated blank values, which leads to a decrease in its analytical sensitivity. By extensive reduction of contaminated iron from the reagents and HPLC devices, we achieved very low reagent blanks and successfully improved the sensitivity of our HPLC method, as a result of which we were able to detect serum NTBI in normal individuals [22]. However, our improved non-metal HPLC method for NTBI quantification still needs further modification and may not be suitable for routine laboratory testing because of the following reasons: (i) expensive materials such as CP22, columns and non-metal HPLC devices are required for analysis, (ii) a multi-step sample pretreatment is required, and the whole procedure takes 2 h to complete, and (iii) the assay rate is therefore quite limited. These problems prevent handling of a large number of clinical samples and make it difficult to apply NTBI analysis in clinical practice.

To make NTBI quantification more convenient, an automated system that can be used in clinical laboratories for diagnostic testing is desired. Some advantages of the automated NTBI quantification method are: i) simple sample preparation without complicated sample pretreatment steps, ii) easy usability without special techniques and devices, iii) applicability for widely-used automated biochemical analyzers, and iv) a high-throughput assay at a low cost.

2. Materials and methods

2.1. Chromogen screening

The chromogens used were 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)-amino] phenol (nitroso-PSAP) (Dojindo); 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) (Dojindo); 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (ferene) (Sigma); and 3-(2-pyridyl)-5,6-bis(4-sulfophenyl)-1,2,4-triazine (ferrozine) (Dojindo).

Standard metal solutions (iron, copper, cobalt and nickel) (Wako) were adjusted to 2 mmol/l, and mixed with chromogens to make a final metal concentration of 20 μmol/l. The spectrum of these chromogen–metal mixtures was scanned.

2.2. Reagents for automated analysis

Reagent-1 (R1) was prepared by mixing Tris buffer and NTA (iron-mobilizing ligand). Reagent-2 (R2) contained Tris buffer, ascorbic acid and nitroso-PSAP (iron-chelating chromogen). R1 and R2 were titrated to pH 8.0 and loaded onto automated analyzers. Final concentrations of Tris buffer, NTA, ascorbic acid and nitroso-PSAP were set at 0.1 mol/l, 0.07 mmol/l, 1.32 mmol/l and 0.09 mmol/l, respectively. Reagents were kept stable for at least 6 months by storing at 10 °C.

2.3. Settings for the automated analyzer and assay method

NTBI was measured using a HITACHI 7700 clinical analyzer with the following settings: sampling volume: sample/R1/R2 = 15 μl/150 μl/50 μl; assay wavelength: main/sub = 750 nm/600 nm; assay method: 2-point end; and reading point: first/last = 16/34. In the assay method, samples and R1 (NTA) were first dispensed into reaction cells on the automated analyzer. After mixing and 5-min of incubation, R2 (nitroso-PSAP with ascorbic acid) was added to the cells and left for 5 more min. The increase in absorbance at 750 nm was used to calculate the amount of NTBI present in serum samples. Total assay time was only 10 min for each sample. 2-point linear regression calibration function was used for the analysis. The calibration stability was confirmed to be stable at least for 6 months. In the experiments using blood samples from humans, serum was selected as an acceptable sample type; plasma samples obtained with heparin showed substantially equal values compared with serum (data not shown), but plasma samples obtained with citrate or ethylenediaminetetraacetic acid were considered to be inappropriate because these anticoagulants chelate iron.

2.4. Measurement of NTBI and Tf-bound iron by the automated system

The iron contents of iron citrate (Shino-Test), 5% bovine serum albumin (BSA) (Nacalai Tesque) and human holo-Tf (Sigma) were determined using a serum Fe (sFe) assay kit, which utilizes nitroso-PSAP as a chromogen (QuickAuto Neo Fe, Shino-Test). NTBI concentration in these samples was also measured by the automated system and the percentage of NTBI to the iron contents was calculated as the recovery rate of NTBI.

2.5. Dialysis of holo-Tf solution

Human holo-Tf solutions (15 or 70 μmol/l iron was bound) were dialyzed with 50 mmol/l Tris buffer (pH 8.0) using dialysis tubing (EIDIA Co., Ltd.) 3 times to remove contaminated iron. Iron and NTBI levels in the dialyzed holo-Tf were quantified by a sFe kit and the automated system, respectively. The percentage of NTBI to the iron contents was calculated as the recovery rate of NTBI.

2.6. Intra- and inter-assay CVs

The intra-assay CV was established using serum samples with low (0.35 μmol/l), medium (0.55 μmol/l) and high (0.95 μmol/l) NTBI levels. Each sample was measured in 20 replicates in the same run. The inter-assay CV was established using the same serum samples mentioned above. Samples were measured in 5 replicates for 20 days.

2.7. Limit of blank (LOB), limit of detection (LOD) and limit of quantification (LOQ)

LOB and LOD were defined as $LOB = Mean_{blank} + 1.645 \times (S.D._{blank})$ and $LOD = LOB + 1.645 \times (S.D._{low\ concentration\ sample})$, respectively, where $Mean_{blank}$ and $S.D._{blank}$ are the mean and S.D. of 6 blank samples assayed in 5 replicates for 5 days, and $S.D._{low\ concentration\ sample}$ is the S.D. of 10 low concentration samples assayed in 5 replicates for 5 days. LOQ was determined as the lowest concentration that results in a CV of 20%. Samples for LOQ determination were assayed in 5 replicates for 5 days.

2.8. Linearity of the assay

Linearity was assessed by serial dilution of 20 μmol/l iron citrate. Each dilution point was assayed in 5 replicates and S.D. was calculated.