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A high-performance liquid immunoaffinity chromatography method for determining transferrin-bound iron in serum

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ABSTRACT

Background: The analyzed values in the ICSH reference method for serum iron analysis are affected by non-transferrin(Tf)-bound iron such as ferritin. Also, non-Tf-bound plasma iron (iron citrate) is present in iron-overloaded specimens from patients with hemochromatosis, which was measured as serum iron in previous methods. We developed a specific determination method for serum transferrin-bound iron (serum *t* Fe) by high-performance liquid immunoaffinity chromatography (HPLAC), and compared it with the ICSH method and a fully automated (FA) method.
Methods: Tf and *t* Fe were isolated from interferents in serum by HPLC using an immunoaffinity column. The concentration of *t* Fe isolated was determined by a colorimetric reaction using a highly sensitive chromogen.
Results: Interferents, except iron saccharate (detected at 5%), do not affect *t* Fe determination. Within-run and between-run imprecisions were in the ranges of 0.2–0.4% and 0.4–1.0% CV. The results of the HPLAC method correlated well with those of the ICSH method ($r = 0.9993$) and FA method ($r = 0.9984$).
Conclusions: In contrast to the ICSH and FA methods for determining serum iron, the HPLAC method is simple, highly precise and specific for serum *t* Fe, which can contribute to the measurement of iron status.

1. Introduction

Various laboratory tests have been developed for the diagnoses of iron deficiency and iron overload [1]. In all these tests, serum iron remains a useful marker, particularly in combination with serum transferrin (serum Tf) [2]. In serum iron analysis, there is a major problem concerning the accuracy of the reference method proposed by the International Committee for Standardization in Hematology (ICSH, 1978 [3] and 1990 [4]) [2,4–8]. False positive results occur when using ICSH methods owing to the release of iron from non-transferrin (non-Tf)-bound iron, such as ferritin [4,8–10], iron dextran for intravenous iron therapy [11], and iron desferrioxamine (ferrioxamine) in chelating agent therapy, except hemoglobin (Hb) in hemolyzed fresh serum, because of the low pH of the reaction mixture and the harsh deproteinization treatment using HCl, thioglycollic acid and trichloroacetic acid (TCA). Moreover, non-Tf-bound plasma iron (iron citrate) is present in iron-overloaded specimens from patients with hemochromatosis, which has been measured as serum iron in previous methods including the 1990 ICSH [4]. Interferences by monoclonal immunoglobulins (false positive or negative results) [12–16] and fibrinogen (false positive results, when using heparinized blood samples) [17,18], with potential interfering substances that are present in patient specimens such as hemolysis (Hb), icterus (bilirubin) and lipemia (expressed as triglycerides), are also generated in a direct colorimetric method using a fully automated analyzer (fully automated (FA) method), which is a routine analytical method. Therefore, the development of a new method is expected to overcome these problems encountered when using the 1990 ICSH and FA methods [2,7].

High-performance liquid immunoaffinity chromatography (HPLAC), which is based on high-performance liquid chromatography (HPLC) using an immunoaffinity column has been developed [19]. HPLAC for serum protein concentration determination involves the use of silica-bound antibodies (particularly Tf analysis) [20] or synthetic hydrophilic resin-bound antibodies [21]. This technique appears to be an attractive possibility for the complete isolation of Tf from interfering substances in serum.

Thus, we have designed a new method (HPLAC method) for the determination of only *t* Fe concentration. This study was conducted with the aims of (1) establishing a method of isolating Tf and its bound iron (*t* Fe) from interfering iron compounds particularly in serum by HPLAC and (2) establishing an accurate method of determining the concentration of *t* Fe isolated by colorimetric detection.

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2. Materials and methods

2.1. Serum samples

Serum samples from patients were collected and stored at 4 °C until use. Serum samples were filtered using a membrane filter (W-25-5, Tosoh, Japan) before HPLC. The study protocol was approved by the Kanagawa University of Human Service Research Ethics Committee, and written informed consent was obtained from all the patients.

2.2. Metal-free HPLAC system

Pyrex glassware and plastic ware were cleaned by the method of Brown et al. [22]. All chemicals were of the highest analytical grade and obtained from Wako Pure Chemical Ltd. (Japan), unless otherwise stated. The following aqueous solutions were prepared using metal-free (redistilled demineralized, <0.1 nmol/l iron contaminant) water: a regeneration buffer solution containing 0.02% Brij 35 and 0.5 mol/l NaCl in 20 mmol/l *N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer of pHs 7.0–7.5, an elution buffer solution containing 0.02% Brij 35 and 0.5 mol/l NaCl in 20 mmol/l citric acid buffer of pHs 1.1–1.6, and a color reagent solution containing 0.02% Brij 35 and 1 mmol/l 2-nitroso-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]phenol (nitroso-PSAP®, Wako) in 0.2 mol/l 2-amino-2-hydroxymethyl-1,3-propanediol (Tris). Nitroso-PSAP solution was prepared by adding 10 mmol/l sodium ascorbate to the color reagent solution. This solution was freshly prepared for each use. These solutions except the nitroso-PSAP solution remain stable for at least 6 months in a Teflon bottle at 4 °C. An immunoaffinity column (anti-Tf column) was prepared by coupling an anti-human Tf antibody (rabbit polyclonal, Dako Cytomation, Denmark) to a tresyl-activated support TSK® gel (support: synthetic hydrophilic resin; particle size: 10 μm; pore size: 100 nm; pH stability: 2.0–12.0, Tresyl-5PW, Tosoh) according to the method of Nakamura et al. [21]. The resulting affinity adsorbent was packed into a column (50 mm × 4.6 mm i.d. PEEK). The iron standard solution, approximately 35.8 μmol/l iron (2 g/l holo-Tf), was prepared by dissolving holo-Tf (purity ≤98%, #T4132, Sigma, St. Louis, MO) in the regeneration buffer solution. This standard solution must be prepared for each use, and iron concentration was analyzed by the 1990 ICSH method [4] using a 35.8 μmol/l iron (as working standard) prepared from the iron standard solution (1 mg Fe/ml in 0.2 mol/l HNO_3, #20247, Kanto Chemical Co., Inc., Japan).

The metal-free HPLAC system used consisted of the following: an anti-Tf column; a Rheodyne® 9725i sample valve fitted with a 2-ml PEEK sample loop (IDEX Corporation, Lake Forest, IL); a 0.1-ml GASTIGHT® sample injection syringe (Pt-Rh needle, #81008, Hamilton, Reno NV); an LC-10i HPLC pump (2 sets, Shimadzu, Japan); an SPD-20A UV detector for Tf analysis (set in the 0–2 absorbance range and at 290 nm) and an SPD-20AV UV–VIS detector for iron analysis (set in the 0–0.5 absorbance range and at 756 nm) fitted with a PEEK flow cell (Shimadzu); and an AC-5700 microfraction collector (Atto, Japan). The system was also equipped with a ChromatoDAQ® integrator (input 1000 mV, Ulvac, Japan).

A sample was passed through the anti-Tf column and UV detector, and joined the flow from the HPLC pump for the nitroso-PSAP solution. Then, the flow was passed through a reaction coil (PEEK tube, 3 m × 0.5 mm i.d.) and the UV–VIS detector. In all the experiments, HPLC was carried out at a flow rate of 1 ml/min at room temperature (25 ± 1 °C). All the buffers were degassed before use. Prior to the first use of a column, 20 mmol/l EDTA solution (pH 8.3) was pumped through the column for 30 min to remove iron contaminants in the new column. The column was first washed with the regeneration buffer solution for 10 min and twice (20 min) with the measuring reagent blank, in each experiment. Before every injection, the needle of the sample syringe was wiped with a piece of metal-free paper ("Techno Wipe C100-S", Nippon Paper Crecia Co., Ltd., Japan). To prevent cross contamination, the sample valve was washed with 3 ml of water after injection of samples and the elution buffer solution. Firstly, a 50-μl sample was injected into the column. After 2 min, 2 ml of the elution buffer solution was injected using a 5-ml plastic syringe. Eight minutes after the injection of the elution buffer and re-equilibration with a fresh regeneration buffer solution, the column is ready for re-use. That is, the analysis time per sample is 10 min. The reagent blank was prepared similarly, using the regeneration buffer instead of a serum sample. The peak height (mV) was measured at 290 (Tf) and 756 nm (iron) against metal-free water (baseline, zero). Tf peak height (mV) was measured at 290 nm because the sensitivity was too high at 280 nm against the baseline zero. The *t* Fe concentration in serum was calculated by comparing the peak height (mV) of the serum sample against the reagent blank with that of the standard. The column filled with the regeneration buffer must be stored at 4 °C until the next experiment. Finally, the system except the column was sequentially washed for 15 min each with 0.2% Brij 35 and water.

2.3. Method comparisons

Two analytical methods for serum iron were compared: the 1978 ICSH method with modifications using 1-ml serum samples and a highly sensitive chromogen, Ferene-S® (the 1990 ICSH method [4]), and an FA method using a highly sensitive chromogen, Nitroso-PSAP (a commercial kit "Quick-Auto-Neo-Fe", involving a specific calibrator (#326041701), Shino-Test Corporation, Japan), with a Cobas Integra® 800 analyzer.

Statistical analysis was carried out using Analyse-it® ver. 2.22 (Analyse-it Software, Ltd., UK). Method comparisons included using a paired *t*-test, regression lines calculated according to the procedures of Passing–Bablok and agreement assessed in accordance with the Bland–Altman method.

3. Results

3.1. Isolation of Tf and its bound iron

The elution profiles of Tf (290 nm) and *t* Fe (756 nm), which were isolated from a pooled patient serum (approximately 21.5 μmol/l iron) and standard (2 g/l holo-Tf) as described above, are shown in Fig. 1. By decreasing the column pH from 7 to 1, *t* Fe eluted faster than Tf. Also, this fact was confirmed by determining Tf and *t* Fe in the column effluents (0.2 ml) using latex agglutination immunoassay (LAIA) [23] and graphite furnace atomic absorption spectrometry [24]. The *t* Fe elution times of the pooled patient serum and standard

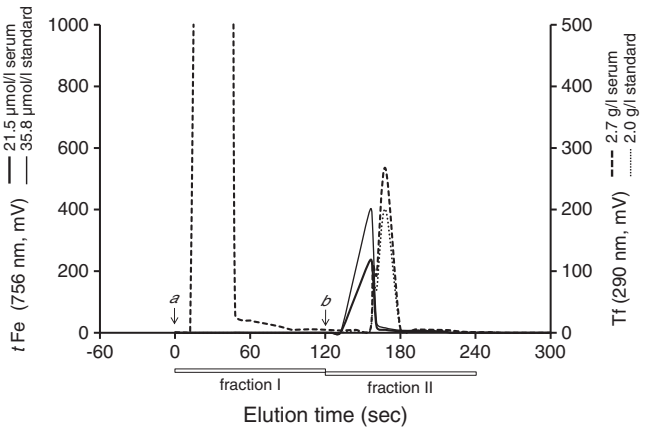

Fig. 1. Elution profiles of Tf and *t* Fe. (*a*) Injected 50-μl sample; (*b*) injected 2-ml elution buffer (20 mmol/l citrate, pH 1.3) with 0.5 mol/l NaCl. Flow rate: 1 ml/min. Temperature: 25 ± 1 °C.