Interference of deferasirox with assays for serum iron and serum unsaturated iron binding capacity during iron chelating therapy

Interference of deferasirox with assays for serum iron and serum unsaturated iron binding capacity during iron chelating therapy

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Running head: Interference of deferasirox with measurement of iron markers

Key words: Deferasirox, serum iron, unsaturated iron binding capacity (UIBC), iron chelation

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28 Nonstandard abbreviations: DFX, deferasirox; sFe, serum iron; UIBC, unsaturated iron binding capacity; DFX-Fe, DFX-iron complex; NTBI, non-transferrin-bound iron; Tf, transferrin; CPBA, competitive protein-binding analysis; Abs, absorbance
Introduction

Deferasirox (DFX) is a newly developed iron chelator that can be orally administered once a day, and is now used worldwide for the treatment of patients with iron overload, with high efficiency [1-6]. DFX is absorbed from the gastrointestinal tract with high bioavailability and then mainly binds to albumin in the serum. Although this mechanism has not yet been elucidated yet, DFX is considered to enter cells and to chelate iron within the cells in various organs with iron overload such as the heart and liver; this DFX-Fe complex then re-enters the systemic circulation where it is absorbed and finally taken up by hepatocytes in the liver. In the hepatocytes, the DFX-Fe complex is excreted into the bile; more than 80% of DFX is excreted in the feces.

Serum iron (sFe) and unsaturated iron binding capacity (UIBC) have been widely used as useful diagnostic markers in patients with various diseases in which iron homeostasis is dysregulated, and for monitoring the therapeutic progress of such patients. However, we have experienced an unexplainable increase in sFe or UIBC in some patients. We also encountered a patient who showed unexplainable changes in serum markers for iron. The patient was diagnosed with myelodysplastic syndrome and required frequent transfusions of concentrated red blood cells because of progressive anemia. At the time when the total transfusion exceeded 40 units, serum ferritin value reached 2969.6 ng/mL, and therefore, administration of DFX was started. As a result, serum ferritin, non-transferrin-bound iron (NTBI) and the density of the liver determined by computed tomography gradually decreased, indicating that iron
overload was improving. However, we found abnormally high sFe and UIBC values after starting administration of DFX (Supplemental Data Figure 1). Firstly, we speculated that iron chelation might induce transferrin (Tf) production in the liver; however, the direct measurement of Tf concentration was unexpectedly low. Besides, UIBC value determined by competitive protein-binding analysis (CPBA) was also low. The assay principles of the sFe and UIBC measuring systems used widely all over the world are based on photometrical measurement of chelating chromophores bound to iron. Therefore, we hypothesized that those assays might have been affected by administration of DFX or DFX-iron complex (DFX-Fe) present in serum, and investigated the effects of DFX and DFX-Fe on those assays.

1. Materials and methods

1.1. Measurement of sFe and UIBC

For measurement of sFe, Fe³⁺ was firstly released from diferric Tf in serum in an acidic milieu and then reduced to Fe²⁺ by ascorbic acid. Fe²⁺ produced was then chelated by a chromogen and colorimetrically measured as the sFe value.

For measurement of UIBC, iron with known content was added first to serum to fully saturate free apo-Tf in serum. Iron not bound to apo-Tf was chelated by a chromogen, and then the UIBC value was calculated by subtracting the unconsumed iron from the added iron.

Four commercially available assay systems each for sFe and UIBC were tested in the present study. (Table 1) [7-12] The following were used as iron chelators and
chromogens: 3-(2-pyridyl)-5,6-bis(4-phenylsulfonicacid)-1,2,4-triazine (FerroZine),
3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt (Ferene),
2-nitroso-5-(N-propyl-N-sulfopropylamino)-phenol (nitroso-PSAP), and
bathophenanthroline sulfonate (bathophenanthroline).  A general-purpose automated
analyzer, 7180 Clinical Analyzer (Hitachi High-Technologies Corporation, Tokyo,
Japan), was used for all assay kits, following the manufacturers’ instructions.

1.2. Investigation of effects of DFX on assays
DFX was provided by Novartis Pharma AG (Basel, Switzerland), and resolved
in 50 mM borate (pH 9.0) to adjust the final concentration to 3.3 mM.  To examine the
effect of DFX on the assays of sFe and UIBC, samples were prepared by adding 0-300
µM DFX to pooled human serum or 15 randomized human serum samples.  Pooled
human serum and 15 randomized human serum samples were independently obtained
from different institutes.  The sFe value was determined as 11.6 µM in pooled human
serum, and as 2.5-33.7 µM in the randomized human serum samples; however, no other
clinical information (including the conditions of iron metabolism) was available.  The
concentrations of DFX were settled from the data previously reported; the
concentrations of free DFX were observed to be 30-90 µM /L within 24 hours of the oral
administration of 35 mg/kg DFX [13].  The UIBC value in the randomized human
serum samples ranged from 2.1 to 88.2 µM.
In addition, it is possible that DFX-Fe might have been present in the serum
during iron chelation therapy in iron-overload patients, and therefore, the effect of
DFX-Fe was also investigated. At first we mixed iron ammonium citrate solution and DFX solution at 37°C which resulted in the formation of a DFX-Fe complex. Using high performance liquid chromatography, we confirmed that the simple mixture of DFX and Fe led to their binding at a DFX:Fe ratio of 2:1. However, other complexes that showed a DFX:Fe ratio of 1:1 or 3:1 were also observed. We could however not determine if all the iron in the solution were bound to DFX; there is therefore the possibility of the presence of free iron other than the DFX-Fe complex if we simply add the DFX-Fe complex directly in the serum. This phenomenon may lead to misunderstanding of the effect of DFX-Fe on the measuring system, so that DFX itself was added to the iron solution at various DFX:Fe ratios. The DFX:Fe ratio was set at 1:1 to 3:1, and the changes in absorbance (Abs) were determined to confirm the effect of DFX-Fe.

1.3. Measurement of NTBI

Measurement of NTBI was performed by methods previously reported [14,15].

1.4. Statistical analysis

All experiments were performed at least twice, independently of each other; substantially the same results were obtained. Data obtained was analyzed using the Student’s t-test; a p value of 0.05 was considered to be statistically significant.

2. Results
2.1. Effect of iron-free DFX on measurement of sFe

After measurement, it was found that in all four measuring systems (sFe.A, sFe.B, sFe.C, sFe.D), addition of iron-free DFX at 60-300 μM to pooled human serum had no significant influence on sFe values (Figure 1A). The reaction time for color development of serum containing 300 μM DFX was not delayed compared to that of serum without DFX in all four measuring systems, indicating that there was no competition for iron between chromogen and iron-free DFX (data not shown). sFe measurement of randomized human serum samples with 0-60 μM DFX showed no difference at all (Supplemental Data Figure 2).

2.2. Effect of iron-free DFX on measurement of UIBC in pooled human serum

Addition of iron-free DFX gave positive errors in UIBC values when pooled human serum was used as a sample (Figure 2A). The increase in UIBC values seemed to be dependent on the concentrations of the added DFX. The degree of positive error observed differed among the measuring systems; positive errors were especially small when Ferene was used as a chromogen (UIBC.F). The measurement step of iron not bound to apo-Tf was not delayed, indicating that the reaction between the chromogen and Fe$^{2+}$ was not influenced by iron-free DFX (data not shown). Therefore, DFX was thought to be bound to the iron contained in the first reagent, as is apo-Tf, leading to positive errors in UIBC values. To determine the binding between DFX and Fe$^{3+}$ in the reagents, the reaction time was changed (3, 5 and 10 min); UIBC values increased as the reaction time was increased (Figure 2B). Addition of 60 μM DFX led to an
increase in UIBC values in all randomized human serum samples. The degree of increase was 0.6-28 μM in all samples regardless of the original UIBC value (Supplemental Data Figure 3). The change in UIBC values observed in the serum samples was also different among the measuring systems.

2.3. Effect of DFX-Fe on measurement of sFe

Firstly, sFe levels were determined in 36 μM Fe³⁺ solution with added 33, 65, and 98 μM DFX, and found not to differ from those in Fe³⁺ solution without DFX (Figure 3). Usually, DFX binds to Fe³⁺ in a mixed solution and forms a complex, so that the fraction that should be measured as sFe is reduced in the solution. However, even in that situation, the measuring systems could not show the decreased levels of sFe, indicating that DFX-Fe may also possibly be measured as sFe in those measurement systems. Therefore, it is likely that DFX-Fe may also be measured as sFe, undistinguishable from Tf-bound iron, in serum samples.

2.4. Effect of DFX-Fe on measurement of UIBC

Similarly to the result obtained on the effect of iron-free DFX on UIBC measurement, the existence of DFX-Fe led to positive errors (Figure 4A). The degree of the positive errors observed in this experiment differed among the measuring kits, the same as for the influence of iron-free DFX. To determine the possibility of interference by Abs of DFX-Fe, we selected nitroso-PSAP (Shino-Test Corp., Tokyo, Japan) as a model of a chromogen. Abs for nitroso-PSAP-Fe²⁺ was calculated by
subtracting the subsidiary Abs at 600 nm from the main Abs at 750 nm. On the other hand, DFX-Fe showed the main Abs at 500 nm. Abs at 500 nm of DFX-Fe should be offset by the 2-point-end method if Abs does not change during the whole measuring procedure, but Abs at 500 nm of DFX-Fe increased when the second reagent for UIBC measurement was added. This influenced and increased Abs of nitroso-PSAP-Fe$^{2+}$ at 600 nm, leading to a decrease in the final Abs of nitroso-PSAP-Fe (Figure 4B). This might have caused the positive errors in the UIBC values.

3. Discussion

In the present study, the interference of an iron chelator, DFX, in serum samples on assays of sFe and UIBC, both of which have been widely used as clinical markers for iron metabolism, was measured using a general-purpose automated analyzer. The effects of DFX itself and DFX-Fe complex on those assays were determined (Figure 5).

In this study, iron-free DFX was simply added to serum samples to determine the effect of DFX itself on the sFe measurement systems. There was a possibility that added DFX might remove iron from Tf and form DFX-Fe complex immediately, but we believed that the main portion of DFX should be iron-free because the added DFX amount was much higher than the pooled human serum’s sFe concentration of 11.6 μM. Therefore, the effect of iron-free DFX was thoroughly investigated in this study.

Initially, we presumed that the competition for iron between chromogens and iron-free DFX decreased the production of chromogen-Fe, finally leading to decreased
sFe values; however, after measurement it was found that addition of DFX did not
influence the sFe values. The reaction time curve also showed that there was no delay
despite the presence of DFX. In all measurement systems we tested, the first step of
the reaction was carried out at an acidic pH with ascorbic acid as a reductant. In such
a condition, the iron removed from Tf must have changed to Fe$^{2+}$ immediately;
therefore, no competition for iron between the chromogen and DFX should have
occurred, because the affinity between Fe$^{2+}$ and DFX was extremely low.

On the other hand, DFX-Fe measurement of sFe was certainly influenced by
DFX-Fe; DFX-Fe seemed to have been measured as sFe undistinguishable from
Tf-bound iron. Our results suggested that DFX-Fe readily released iron in an acidic
milieu, like Tf, because the pH in the first step might have been lowered by addition of
the first reagent. There was a possibility that a hydroxyl group in the DFX molecule
dissociated in an acidic milieu, resulting in separation of DFX from iron. In iron
chelation therapy for patients with iron overload, the existence of DFX-Fe in serum
would be plausible, and DFX-Fe might have increased the sFe value. Lebitasy et al.
[16] reported that there was interference of DFX-Fe but no effect of iron-free DFX on
the assay of sFe with a slide cartridge-type measuring kit utilizing
N-\{4-[2,4-bis(1,1-dimethylpropyl)phenoxy]butyl\}-5-methoxy-6-\[(2,3,6,7-tetrahydro-8-
1H,5H-benzoquinolizine-9-yl)azo\]-3-pyridinesulfonamide as a chromogen. In our
present measurement, the results furthermore proved that this phenomenon was
generally observed in various commercially available colorimetric measuring systems
of sFe, utilizing a general-purpose automated analyzer. This information should be of
great significance for clinical laboratories and clinicians involved in the treatment of patients with iron overload.

Concerning the measuring system for UIBC, the first step was performed at the appropriate condition for binding of apo-Tf to Fe\(^{3+}\) contained in the reagent. Therefore, if DFX is added in this situation, DFX can easily bind to Fe\(^{3+}\), as does apo-Tf. The remaining Fe\(^{3+}\) that does not bind to apo-Tf or DFX will be reduced to Fe\(^{2+}\) by ascorbic acid at the next step. At this step, DFX-Fe will not release iron, so that the amount of iron bound to the chromogen might decrease, leading to increased UIBC values. The reason for the difference in UIBC values among the measuring kits might be the difference in pH of the reagents or the difference in affinity of the chromogens used in each kit. The final pH values, after adding the second reagents of the UIBC.E, UIBC.G, and UIBC.H kits were approximately 8.5, but that of the UIBC.F kit was 7.8 (data not shown). A slightly acidic milieu might have led to easy removal of iron from DFX-Fe, so that the UIBC.F kit might have been less influenced than other kits. Measurement of serum UIBC values was also influenced by DFX-Fe. Our results using nitroso-PSAP showed one possible explanation, that this came from the Abs of DFX-Fe. Therefore, UIBC assay systems would have been affected both by iron-free DFX and DFX-Fe. This study proved the interference of DFX itself and DFX-Fe on the UIBC assays.

During iron chelation therapy for iron overload, DFX-Fe comes from organs with iron deposition, such as the liver and heart. In other words, DFX-Fe may increase when iron chelation therapy is successful, and unexpected high sFe values are observed.
during iron chelation therapy. However, even though DFX-Fe increased in serum, DFX-Fe will finally be excreted mainly in the stool after being taken up from the serum by hepatocytes again. Besides, NTBI level gradually decreased unless sFe value increased during iron chelation therapy with DFX in our patients, suggesting that the binding of DFX and iron was so tight that DFX-Fe was not detected as NTBI; thus, DFX-Fe might not be harmful to organs compared to NTBI.

Deferoxamine (DFO) was widely used as an iron chelator until the introduction of DFX as the novel iron chelator. Moreover, clinical concerns had been reported; for example, the measurements of sFe and total iron binding capacity (TIBC) were considered not to be useful in acute iron overload and during iron chelation therapy with DFO [17, 18]. Although there should be differences in the biological behavior of DFO and DFX, our present results indicated that careful attention should be paid when these markers are observed during iron chelation therapy with DFX. We therefore recommend careful observation when high serum sFe values or unexplainable UIBC values are observed prior to the cessation of iron chelation therapy. We also recommend the direct measurement of Tf by nephelometry or radio assay in the measurement of UIBC values [19], although there is no other useful method to measure sFe values, once unexplainable values of sFe or UIBC are observed during iron chelation therapy using DFX.

In conclusion, the commonly used laboratory assessment method available to support the clinical therapy of iron overload states could be interfered with by the therapy
itself and so careful attention should be paid during therapy in order to understand the
laboratory data. Alternative methods should also be considered for precise evaluation.
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References


Table 1. Assay systems for measuring serum iron and unsaturated iron binding capacity evaluated in the present study.

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<th>Abbreviation</th>
<th>sFe Kit (Manufacturer)</th>
<th>Chromogen</th>
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<tr>
<td>sFe.A</td>
<td>Fe (Roche Diagnostics GmbH, Mannheim, Germany)</td>
<td>Ferrozine</td>
</tr>
<tr>
<td>sFe.B</td>
<td>IRON-SL ASSAY (Sekisui Diagnostics Ltd., Kent, UK)</td>
<td>Ferene</td>
</tr>
<tr>
<td>sFe.C</td>
<td>QuickAuto Neo Fe (Shino-Test Corp., Tokyo, Japan)</td>
<td>Nitroso-PSAP</td>
</tr>
<tr>
<td>sFe.D</td>
<td>LtypeWAKO Fe·N (Wako Pure Chemical Industries Ltd., Osaka, Japan)</td>
<td>Bathophenanthroline</td>
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<tr>
<th>Abbreviation</th>
<th>UIBC Kit (Manufacturer)</th>
<th>Chromogen</th>
</tr>
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<tbody>
<tr>
<td>UIBC.E</td>
<td>UIBC (Roche Diagnostics GmbH, Mannheim, Germany)</td>
<td>Ferrozine</td>
</tr>
<tr>
<td>UIBC.F</td>
<td>UIBC ASSAY (Sekisui Diagnostics Ltd., Kent, UK)</td>
<td>Ferene</td>
</tr>
<tr>
<td>UIBC.G</td>
<td>QuickAuto Neo UIBC (Shino-Test Corp., Tokyo, Japan)</td>
<td>Nitroso-PSAP</td>
</tr>
<tr>
<td>UIBC.H</td>
<td>LtypeWAKO UIBC (Wako Pure Chemical Industries Ltd., Osaka, Japan)</td>
<td>Bathophenanthroline</td>
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Figure legends

Fig. 1.
Effect of iron-free DFX on measurement of sFe. In all measuring systems (sFe.A, sFe.B, sFe.C, sFe.D), there was no significant influence on sFe value.

Fig. 2.
(A) Effect of iron-free DFX on measurement of UIBC in pooled human serum using four measuring systems (UIBC.E, UIBC.F, UIBC.G, UIBC.H). Iron-free DFX gave positive errors in UIBC values. (B) UIBC values increased as the reaction time increased.

Fig. 3.
Effect of DFX-Fe on measurement of sFe. sFe levels were determined in 36 μM Fe³⁺ solution with added 33, 65, and 98 μM DFX. Even in this simple mixture, DFX was expected to chelate Fe³⁺ and form a complex, so that sFe levels were expected to decrease substantially; however, sFe values were found not have changed at all after measurement, indicating that neither of the measuring systems could distinguish free Fe³⁺ from iron bound to DFX.

Fig. 4.
(A) Effect of DFX-Fe on measurement of UIBC. Existence of DFX-Fe led to positive errors. (B) Changes in absorbance (Abs) of DFX-Fe by adding the first reagent (R1) and the second reagent (R2) in the measuring system. Abs of DFX-Fe increased when R2 for UIBC measurement was added.

Fig. 5.

(A) Influence of DFX on sFe measurement. DFX-Fe was measured as sFe undistinguishable from Tf-bound iron. (B) Influence of DFX on UIBC measurement. Iron-free DFX should be bound to iron in the reagent. The absorbance (Abs) of DFX-Fe might have influenced Abs of chromogen-iron, leading to positive errors for UIBC values. DFX: deferasirox, UIBC: unsaturated iron binding capacity, Abs: absorbance, Tf: transferrin
Change in sFe values determined in 36 μM Fe solution

- ○ With added 33 μM DFX
- • With added 65 μM DFX
- × With added 98 μM DFX
(A) Sample → R1: Acidic milieu → Protein denaturing → Fe$^{3+}$ → Fe$^{2+}$ → R2: Coloring after binding of Fe$^{2+}$ and chromogen → Measure Abs

- [Iron-free DFX (-), DFX-Fe (-)]
  - diferric-Tf
  - apo-Tf

- [Iron-free DFX (+)]
  - No influence on sFe value

- [DFX-Fe (+)]
  - Positive error in sFe value

(B) Sample → R1: pH 8.5, Fe$^{3+}$ (Tf binding to iron) → R2: Coloring after binding of iron not bound to Tf and chromogen → Measure Abs

- [Iron-free DFX (-), DFX-Fe (-)]
  - holo-Tf
  - apo-Tf

- [Iron-free DFX (+)]
  - Decrease of remaining iron

- [DFX-Fe (+)]
  - Abs of DFX-Fe$^{3+}$ influenced Abs of chromogen-iron
  - Positive error in UIBC values

Legend:
- Fe$^{3+}$
- Fe$^{2+}$
- Fe$^{3+}$ in reagent
- Chromogen
- DFX
- Chromogen-Fe$^{2+}$
- DFX-Fe$^{3+}$