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

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

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12 Japan

13

14 Running head: Interference of deferasirox with measurement of iron markers

15

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

18

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27

28 Nonstandard abbreviations: DFX, deferasirox; sFe, serum iron; UIBC, unsaturated iron

29 binding capacity; DFX-Fe, DFX-iron complex; NTBI, non-transferrin-bound iron; Tf,

30 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

53 overload was improving. However, we found abnormally high sFe and UIBC values
54 after starting administration of DFX (Supplemental Data Figure 1). Firstly, we
55 speculated that iron chelation might induce transferrin (Tf) production in the liver;
56 however, the direct measurement of Tf concentration was unexpectedly low. Besides,
57 UIBC value determined by competitive protein-binding analysis (CPBA) was also low.
58 The assay principles of the sFe and UIBC measuring systems used widely all over the
59 world are based on photometrical measurement of chelating chromophores bound to
60 iron. Therefore, we hypothesized that those assays might have been affected by
61 administration of DFX or DFX-iron complex (DFX-Fe) present in serum, and
62 investigated the effects of DFX and DFX-Fe on those assays.
63

64 **1. Materials and methods**

65 **1.1. Measurement of sFe and UIBC**

66 For measurement of sFe, Fe^{3+} was firstly released from diferric Tf in serum in
67 an acidic milieu and then reduced to Fe^{2+} by ascorbic acid. Fe^{2+} produced was then
68 chelated by a chromogen and colorimetrically measured as the sFe value.
69 For measurement of UIBC, iron with known content was added first to serum
70 to fully saturate free apo-Tf in serum. Iron not bound to apo-Tf was chelated by a
71 chromogen, and then the UIBC value was calculated by subtracting the unconsumed
72 iron from the added iron.
73 Four commercially available assay systems each for sFe and UIBC were tested
74 in the present study. (Table 1) [7-12] The following were used as iron chelators and

75 chromogens: 3-(2-pyridyl)-5,6-bis(4-phenylsulfonicacid)-1,2,4-triazine (FerroZine),

76 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt (Ferene),

77 2-nitroso-5-(N-propyl-N-sulfopropylamino)-phenol (nitroso-PSAP), and

78 bathophenanthroline sulfonate (bathophenanthroline). A general-purpose automated

79 analyzer, 7180 Clinical Analyzer (Hitachi High-Technologies Corporation, Tokyo,

80 Japan), was used for all assay kits, following the manufacturers' instructions.

81

82 **1.2. Investigation of effects of DFX on assays**

83 DFX was provided by Novartis Pharma AG (Basel, Switzerland), and resolved

84 in 50 mM borate (pH 9.0) to adjust the final concentration to 3.3 mM. To examine the

85 effect of DFX on the assays of sFe and UIBC, samples were prepared by adding 0-300

86 μM DFX to pooled human serum or 15 randomized human serum samples. Pooled

87 human serum and 15 randomized human serum samples were independently obtained

88 from different institutes. The sFe value was determined as 11.6 μM in pooled human

89 serum, and as 2.5-33.7 μM in the randomized human serum samples; however, no other

90 clinical information (including the conditions of iron metabolism) was available. The

91 concentrations of DFX were settled from the data previously reported; the

92 concentrations of free DFX were observed to be 30-90 μM /L within 24 hours of the oral

93 administration of 35 mg/kg DFX [13]. The UIBC value in the randomized human

94 serum samples ranged from 2.1 to 88.2 μM.

95 In addition, it is possible that DFX-Fe might have been present in the serum

96 during iron chelation therapy in iron-overload patients, and therefore, the effect of

97 DFX-Fe was also investigated. At first we mixed iron ammonium citrate solution and
98 DFX solution at 37°C which resulted in the formation of a DFX-Fe complex. Using
99 high performance liquid chromatography, we confirmed that the simple mixture of DFX
100 and Fe led to their binding at a DFX:Fe ratio of 2:1. However, other complexes that
101 showed a DFX:Fe ratio of 1:1 or 3:1 were also observed. We could however not
102 determine if all the iron in the solution were bound to DFX; there is therefore the
103 possibility of the presence of free iron other than the DFX-Fe complex if we simply add
104 the DFX-Fe complex directly in the serum. This phenomenon may lead to
105 misunderstanding of the effect of DFX-Fe on the measuring system, so that DFX itself
106 was added to the iron solution at various DFX:Fe ratios. The DFX:Fe ratio was set at
107 1:1 to 3:1, and the changes in absorbance (Abs) were determined to confirm the effect
108 of DFX-Fe.
109
110 **1.3. Measurement of NTBI**
111 Measurement of NTBI was performed by methods previously reported [14,15].
112
113 **1.4. Statistical analysis**
114 All experiments were performed at least twice, independently of each other;
115 substantially the same results were obtained. Data obtained was analyzed using the
116 Student's t-test; a p value of 0.05 was considered to be statistically significant.
117
118 **2. Results**

119 **2.1. Effect of iron-free DFX on measurement of sFe**

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

128

129 **2.2. Effect of iron-free DFX on measurement of UIBC in pooled human serum**

130 Addition of iron-free DFX gave positive errors in UIBC values when pooled
131 human serum was used as a sample (Figure 2A). The increase in UIBC values seemed
132 to be dependent on the concentrations of the added DFX. The degree of positive error
133 observed differed among the measuring systems; positive errors were especially small
134 when Ferene was used as a chromogen (UIBC.F). The measurement step of iron not
135 bound to apo-Tf was not delayed, indicating that the reaction between the chromogen
136 and Fe^{2+} was not influenced by iron-free DFX (data not shown). Therefore, DFX was
137 thought to be bound to the iron contained in the first reagent, as is apo-Tf, leading to
138 positive errors in UIBC values. To determine the binding between DFX and Fe^{3+} in
139 the reagents, the reaction time was changed (3, 5 and 10 min); UIBC values increased as
140 the reaction time was increased (Figure 2B). Addition of 60 μM DFX led to an

141 increase in UIBC values in all randomized human serum samples. The degree of
142 increase was 0.6-28 μM in all samples regardless of the original UIBC value
143 (Supplemental Data Figure 3). The change in UIBC values observed in the serum
144 samples was also different among the measuring systems.
145
146 **2.3. Effect of DFX-Fe on measurement of sFe**
147 Firstly, sFe levels were determined in 36 μM Fe^{3+} solution with added 33, 65,
148 and 98 μM DFX, and found not to differ from those in Fe^{3+} solution without DFX
149 (Figure 3). Usually, DFX binds to Fe^{3+} in a mixed solution and forms a complex, so
150 that the fraction that should be measured as sFe is reduced in the solution. However,
151 even in that situation, the measuring systems could not show the decreased levels of sFe,
152 indicating that DFX-Fe may also possibly be measured as sFe in those measurement
153 systems. Therefore, it is likely that DFX-Fe may also be measured as sFe,
154 undistinguishable from Tf-bound iron, in serum samples.
155
156 **2.4. Effect of DFX-Fe on measurement of UIBC**
157 Similarly to the result obtained on the effect of iron-free DFX on UIBC
158 measurement, the existence of DFX-Fe led to positive errors (Figure 4A). The degree
159 of the positive errors observed in this experiment differed among the measuring kits, the
160 same as for the influence of iron-free DFX. To determine the possibility of
161 interference by Abs of DFX-Fe, we selected nitroso-PSAP (Shino-Test Corp., Tokyo,
162 Japan) as a model of a chromogen. Abs for nitroso-PSAP-Fe^{2+} was calculated by

163 subtracting the subsidiary Abs at 600 nm from the main Abs at 750 nm. On the other
164 hand, DFX-Fe showed the main Abs at 500 nm. Abs at 500 nm of DFX-Fe should be
165 offset by the 2-point-end method if Abs does not change during the whole measuring
166 procedure, but Abs at 500 nm of DFX-Fe increased when the second reagent for UIBC
167 measurement was added. This influenced and increased Abs of nitroso-PSAP-Fe^{2+} at
168 600 nm, leading to a decrease in the final Abs of nitroso-PSAP-Fe (Figure 4B). This
169 might have caused the positive errors in the UIBC values.
170
171 **3. Discussion**
172 In the present study, the interference of an iron chelator, DFX, in serum
173 samples on assays of sFe and UIBC, both of which have been widely used as clinical
174 markers for iron metabolism, was measured using a general-purpose automated analyzer.
175 The effects of DFX itself and DFX-Fe complex on those assays were determined
176 (Figure 5).
177 In this study, iron-free DFX was simply added to serum samples to determine
178 the effect of DFX itself on the sFe measurement systems. There was a possibility that
179 added DFX might remove iron from Tf and form DFX-Fe complex immediately, but we
180 believed that the main portion of DFX should be iron-free because the added DFX
181 amount was much higher than the pooled human serum's sFe concentration of 11.6 μM.
182 Therefore, the effect of iron-free DFX was thoroughly investigated in this study.
183 Initially, we presumed that the competition for iron between chromogens and
184 iron-free DFX decreased the production of chromogen-Fe, finally leading to decreased

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

192 On the other hand, DFX-Fe measurement of sFe was certainly influenced by
193 DFX-Fe; DFX-Fe seemed to have been measured as sFe undistinguishable from
194 Tf-bound iron. Our results suggested that DFX-Fe readily released iron in an acidic
195 milieu, like Tf, because the pH in the first step might have been lowered by addition of
196 the first reagent. There was a possibility that a hydroxyl group in the DFX molecule
197 dissociated in an acidic milieu, resulting in separation of DFX from iron. In iron
198 chelation therapy for patients with iron overload, the existence of DFX-Fe in serum
199 would be plausible, and DFX-Fe might have increased the sFe value. Lebitasy et al.
200 [16] reported that there was interference of DFX-Fe but no effect of iron-free DFX on
201 the assay of sFe with a slide cartridge-type measuring kit utilizing
202 N-{4-[2,4-bis(1,1-dimethylpropyl)phenoxy]butyl}-5-methoxy-6-[(2,3,6,7-tetrahydro-8-
203 1H,5H-benzoquinolizine-9-yl)azo]-3-pyridinesulfonamide as a chromogen. In our
204 present measurement, the results furthermore proved that this phenomenon was
205 generally observed in various commercially available colorimetric measuring systems
206 of sFe, utilizing a general-purpose automated analyzer. This information should be of

207 great significance for clinical laboratories and clinicians involved in the treatment of
208 patients with iron overload.

209 Concerning the measuring system for UIBC, the first step was performed at the
210 appropriate condition for binding of apo-Tf to Fe^{3+} contained in the reagent. Therefore,
211 if DFX is added in this situation, DFX can easily bind to Fe^{3+}, as does apo-Tf. The
212 remaining Fe^{3+} that does not bind to apo-Tf or DFX will be reduced to Fe^{2+} by ascorbic
213 acid at the next step. At this step, DFX-Fe will not release iron, so that the amount of
214 iron bound to the chromogen might decrease, leading to increased UIBC values. The
215 reason for the difference in UIBC values among the measuring kits might be the
216 difference in pH of the reagents or the difference in affinity of the chromogens used in
217 each kit. The final pH values, after adding the second reagents of the UIBC.E,
218 UIBC.G, and UIBC.H kits were approximately 8.5, but that of the UIBC.F kit was 7.8
219 (data not shown). A slightly acidic milieu might have led to easy removal of iron from
220 DFX-Fe, so that the UIBC.F kit might have been less influenced than other kits.

221 Measurement of serum UIBC values was also influenced by DFX-Fe. Our
222 results using nitroso-PSAP showed one possible explanation, that this came from the
223 Abs of DFX-Fe. Therefore, UIBC assay systems would have been affected both by
224 iron-free DFX and DFX-Fe. This study proved the interference of DFX itself and
225 DFX-Fe on the UIBC assays.

226 During iron chelation therapy for iron overload, DFX-Fe comes from organs
227 with iron deposition, such as the liver and heart. In other words, DFX-Fe may increase
228 when iron chelation therapy is successful, and unexpected high sFe values are observed

229 during iron chelation therapy. However, even though DFX-Fe increased in serum,
230 DFX-Fe will finally be excreted mainly in the stool after being taken up from the serum
231 by hepatocytes again. Besides, NTBI level gradually decreased unless sFe value
232 increased during iron chelation therapy with DFX in our patients, suggesting that the
233 binding of DFX and iron was so tight that DFX-Fe was not detected as NTBI; thus,
234 DFX-Fe might not be harmful to organs compared to NTBI.

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

248 In conclusion, the commonly used laboratory assessment method available to
249 support the clinical therapy of iron overload states could be interfered with by the therapy

250 itself and so careful attention should be paid during therapy in order to understand the

251 laboratory data. Alternative methods should also be considered for precise evaluation.

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253 *content of this paper and have met the following 3 requirements: (a) significant*

254 *contribution to the conception and design, acquisition of data, or analysis and*

255 *interpretation of data; (b) drafting or revising the article for intellectual content; and*

256 *(c) final approval of the published article.*

257

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259 *all authors completed the Disclosure of Potential Conflict of Interest form. Potential*

260 *conflicts of interest:*

261

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271 **References**

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

323

Abbreviation	sFe Kit (Manuacturer)	Chromogen
sFe.A	Fe (Roche Diagnostis GmbH, Mannheim, Germany)	Ferrozine
sFe.B	IRON-SL ASSAY (Sekisui Diagnostics Ltd., Kent, UK)	Ferene
sFe.C	QuickAuto Neo Fe (Shino-Test Corp., Tokyo, Japan)	Nitroso-PSAP
sFe.D	LtypeWAKO Fe･N (Wako Pure Chemical Industries Ltd., Osaka, Japan)	Bathophenanthroline

324

	UIBC Kit (Manufacturer)	
UIBC.E	UIBC (Roche Diagnostics GmbH, Mannheim, Germany)	Ferrozine
UIBC.F	UIBC ASSAY (Sekisui Diagnostics Ltd., Kent, UK)	Ferene
UIBC.G	QuickAuto Neo UIBC (Shino-Test Corp., Tokyo, Japan)	Nitroso-PSAP
UIBC.H	LtypeWAKO UIBC (Wako Pure Chemical Industries Ltd., Osaka, Japan)	Bathophenanthroline

325

326 **Figure legends**

327

328 **Fig. 1.**

329 Effect of iron-free DFX on measurement of sFe. In all measuring systems (sFe.A,

330 sFe.B, sFe.C, sFe.D), there was no significant influence on sFe value.

331

332 **Fig. 2.**

333 (A) Effect of iron-free DFX on measurement of UIBC in pooled human serum using

334 four measuring systems (UIBC.E, UIBC.F, UIBC.G, UIBC. H). Iron-free DFX gave

335 positive errors in UIBC values. (B) UIBC values increased as the reaction time

336 increased.

337

338 **Fig. 3.**

339 Effect of DFX-Fe on measurement of sFe. sFe levels were determined in 36 μM Fe^{3+}

340 solution with added 33, 65, and 98 μM DFX. Even in this simple mixture, DFX was

341 expected to chelate Fe^{3+} and form a complex, so that sFe levels were expected to

342 decrease substantially; however, sFe values were found not have changed at all after

343 measurement, indicating that neither of the measuring systems could distinguish free

344 Fe^{3+} from iron bound to DFX.

345

346 **Fig. 4.**

347 (A) Effect of DFX-Fe on measurement of UIBC. Existence of DFX-Fe led to positive

348 errors. (B) Changes in absorbance (Abs) of DFX-Fe by adding the first reagent (R1)

349 and the second reagent (R2) in the measuring system. Abs of DFX-Fe increased when

350 R2 for UIBC measurement was added.

351

352 **Fig. 5.**

353 (A) Influence of DFX on sFe measurement. DFX-Fe was measured as sFe

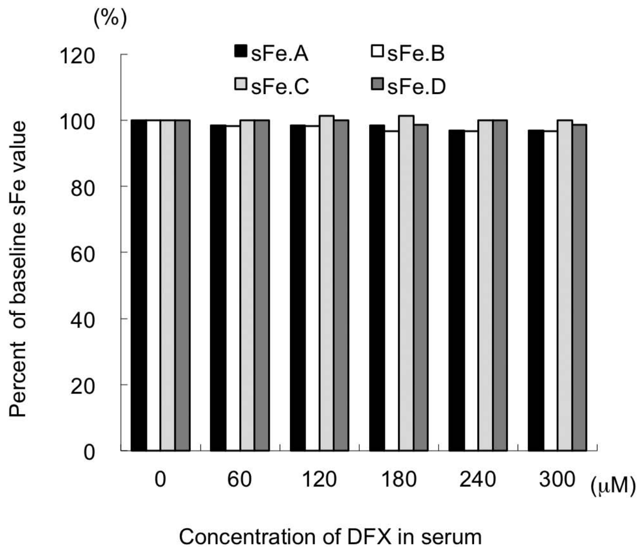
354 undistinguishable from Tf-bound iron. (B) Influence of DFX on UIBC measurement.

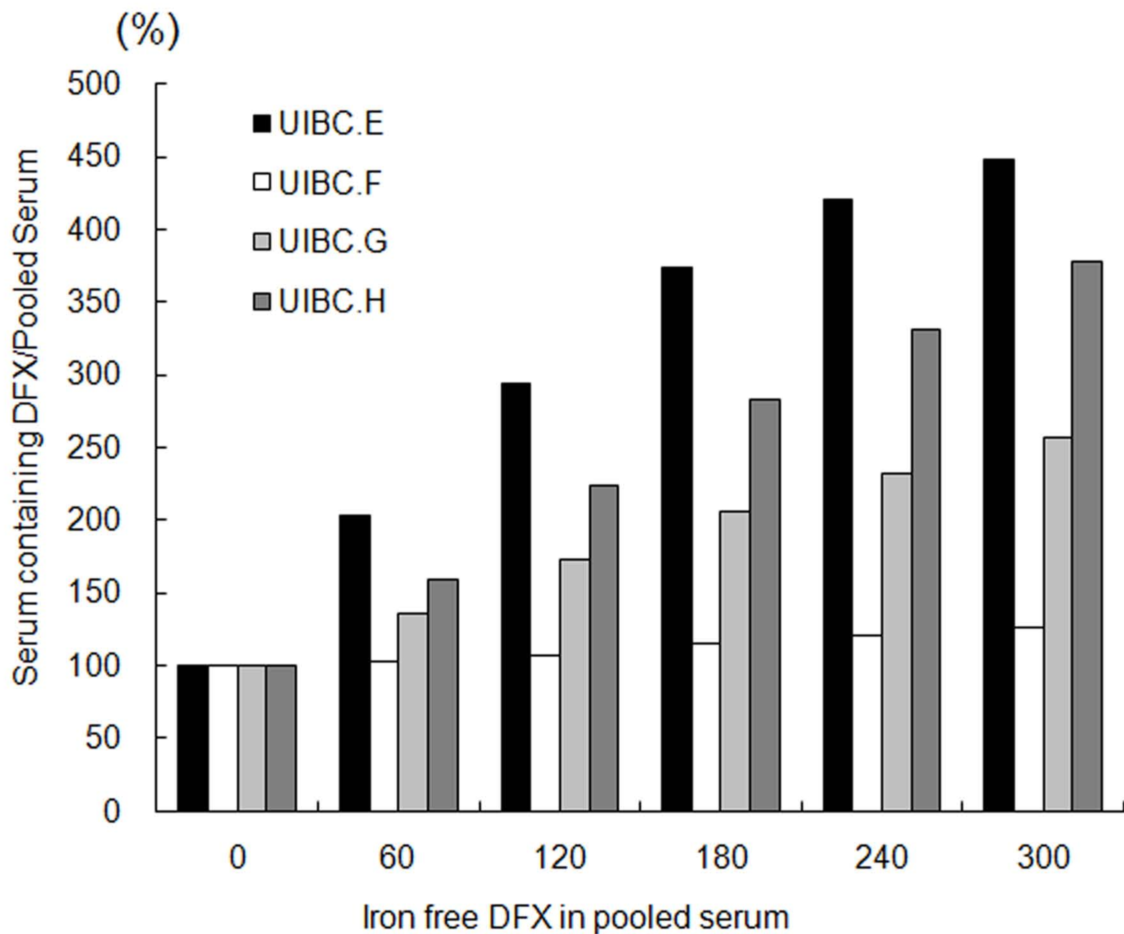
355 Iron-free DFX should be bound to iron in the reagent. The absorbance (Abs) of

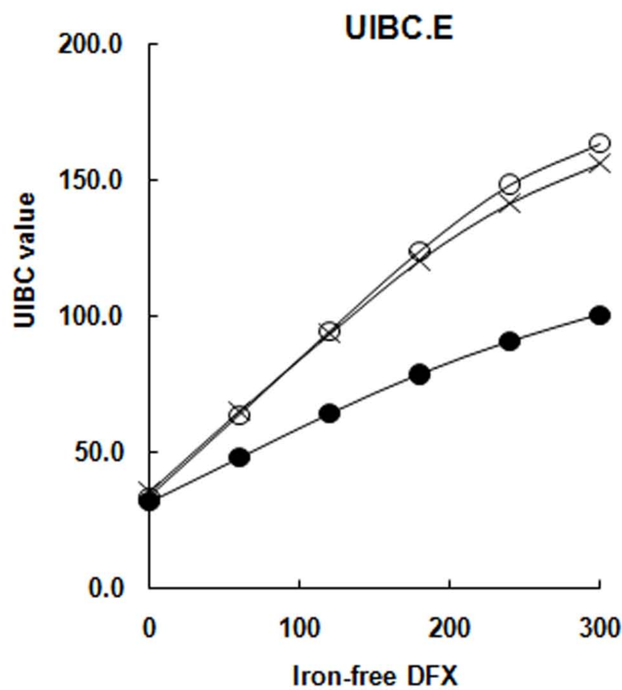
356 DFX-Fe might have influenced Abs of chromogen-iron, leading to positive errors for

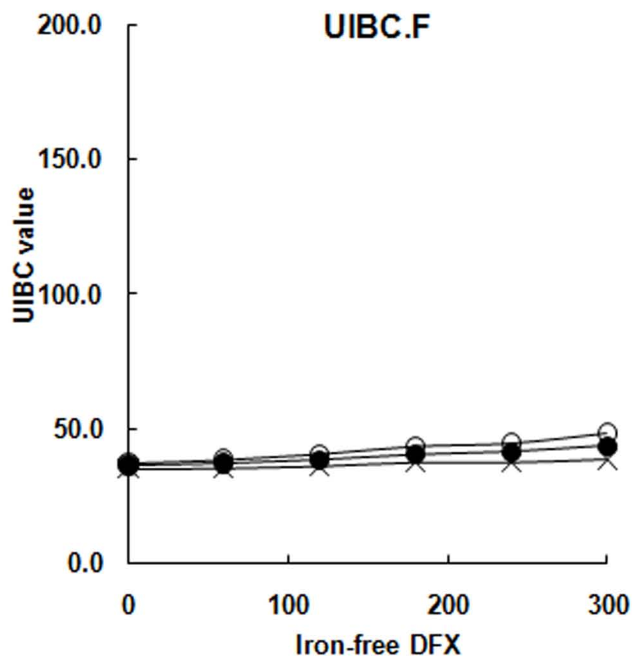
357 UIBC values. DFX: deferasirox, UIBC: unsaturated iron binding capacity, Abs:

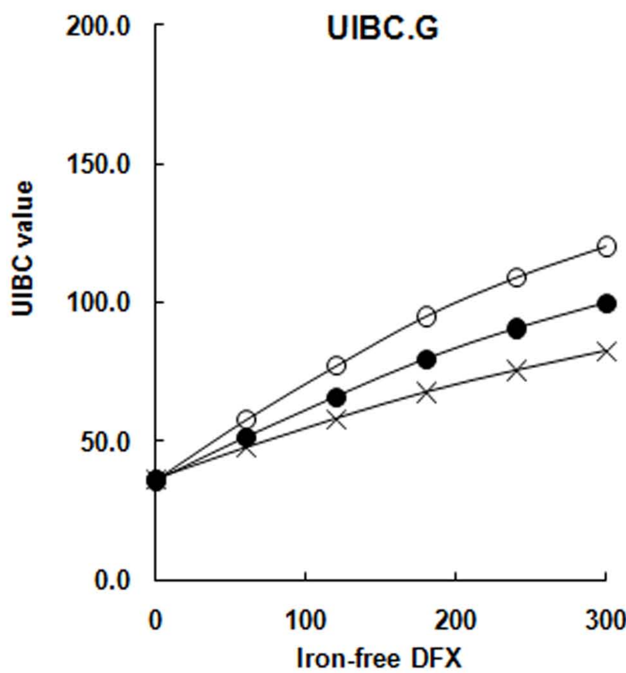
358 absorbance, Tf: transferrin

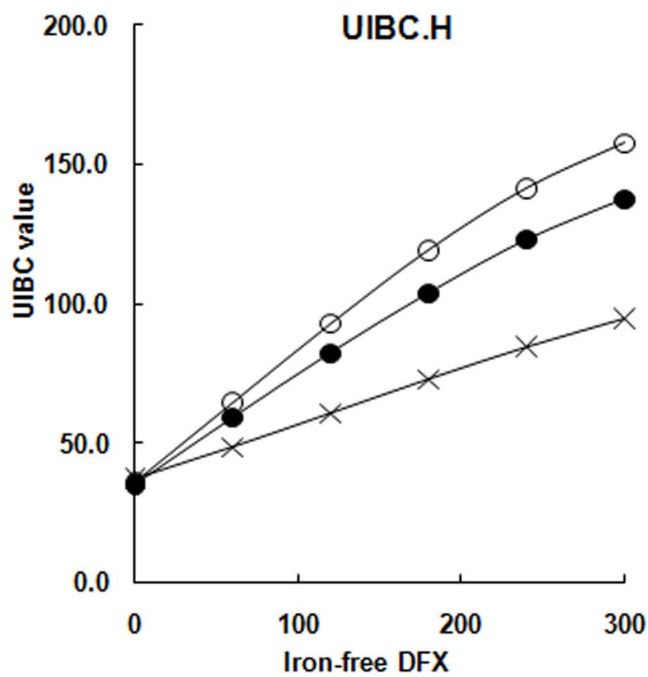

(%)
120
100
80
60
40
20
0
sFe.A
sFe.B
sFe.C
sFe.D
Percent of baseline sFe value
0
60
120
180
240
300
(μM)
Concentration of DFX in serum

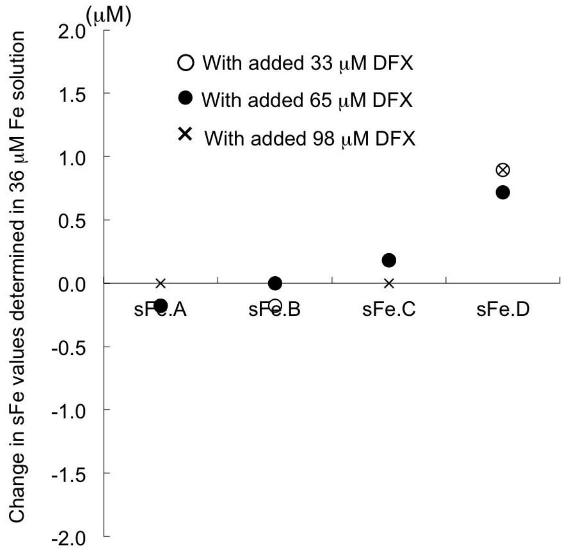

(%)
Serum containing DFX/Pooled Serum
500
450
400
350
300
250
200
150
100
50
0
UIBC.E
UIBC.F
UIBC.G
UIBC.H
0
60
120
180
240
300
Iron free DFX in pooled serum

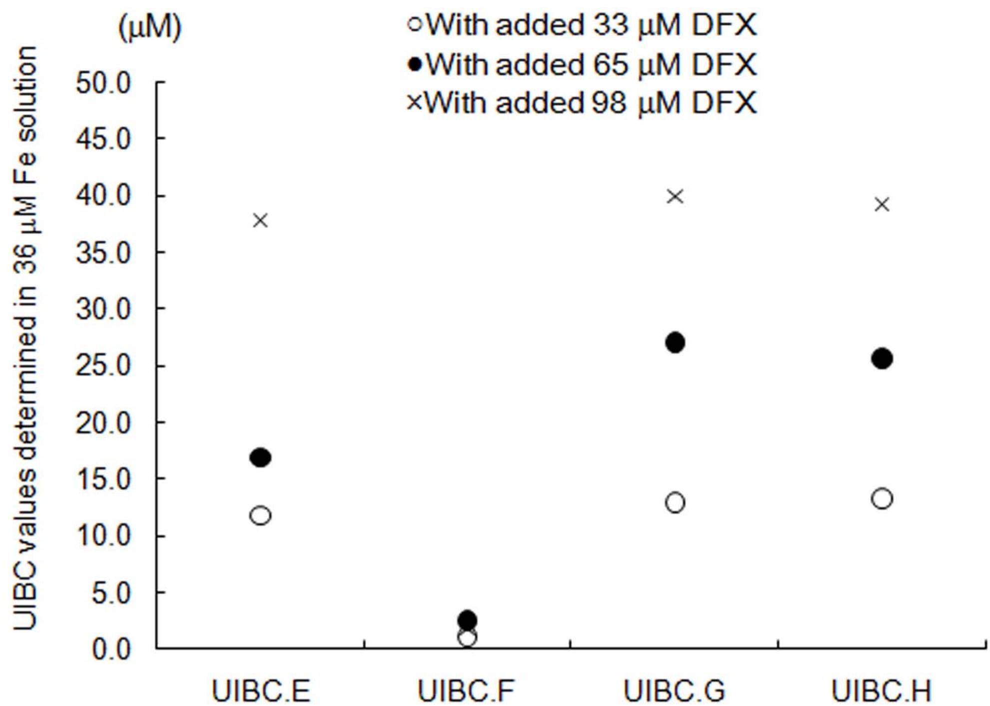

UIBC.E
200.0
150.0
100.0
50.0
0.0
UIBC value
0
100
200
300
Iron-free DFX


UIBC.F
200.0
150.0
100.0
50.0
0.0
UIBC value
0
100
200
300
Iron-free DFX

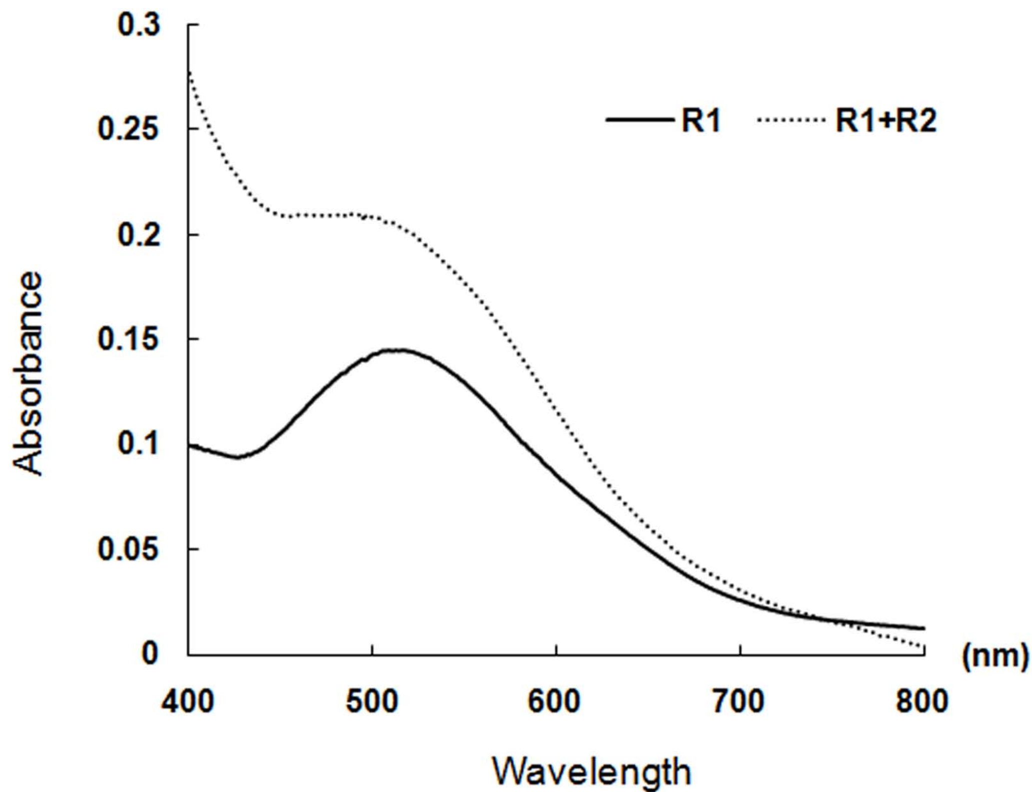

UIBC.G
200.0
150.0
100.0
50.0
0.0
UIBC value
0
100
200
300
Iron-free DFX


UIBC.H
200.0
150.0
100.0
50.0
0.0
UIBC value
0
100
200
300
Iron-free DFX


(μM)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
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
sFe.A
sFe.B
sFe.C
sFe.D



(μM)
○With added 33 μM DFX
●With added 65 μM DFX
×With added 98 μM DFX
UIBC values determined in 36 μM Fe solution
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
UIBC.E
UIBC.F
UIBC.G
UIBC.H


R1
R1+R2
Absorbance
0.3
0.25
0.2
0.15
0.1
0.05
0
400
500
600
700
800
(nm)
Wavelength

(A)

Sample

R1

Acidic milieu

Protein denaturing

$Fe^{3+} \longrightarrow Fe^{2+}$

R2

Coloring after binding of Fe^{2+} and chromogen

Measure Abs

0 min

5 min

10 min

[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

0 min

5 min

10 min

[Iron-free DFX (-), DFX-Fe (-)]

holo-Tf

apo-Tf

[Iron-free DFX (+)]

Decrease of remaining iron

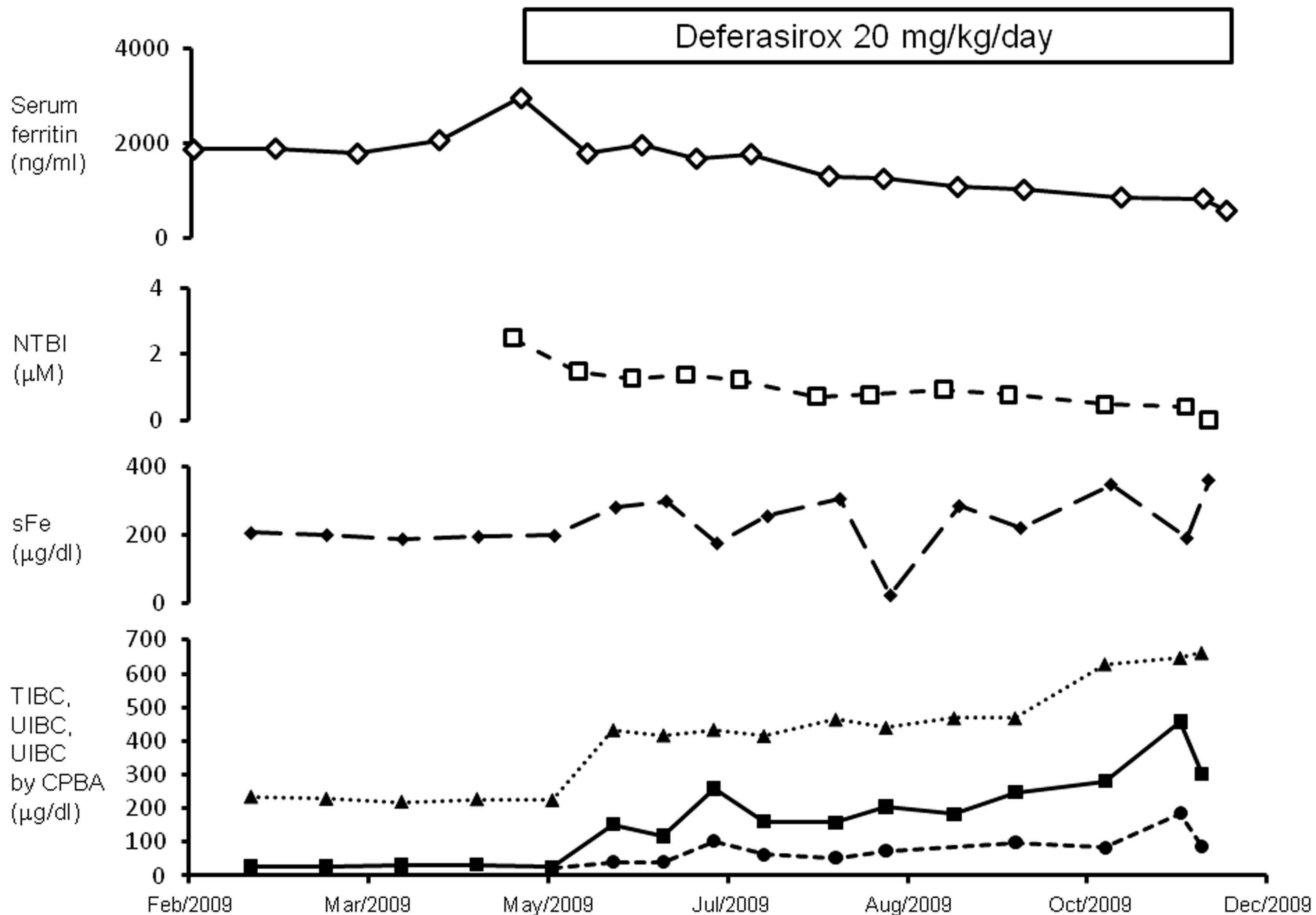
[DFX-Fe (+)]

Abs of DFX-Fe^{3+} influenced Abs of chromogen-iron

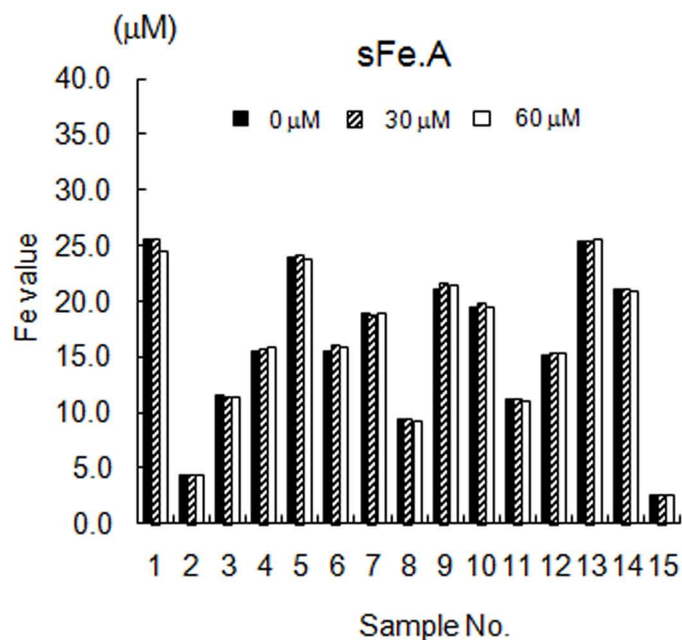
Positive error in UIBC values

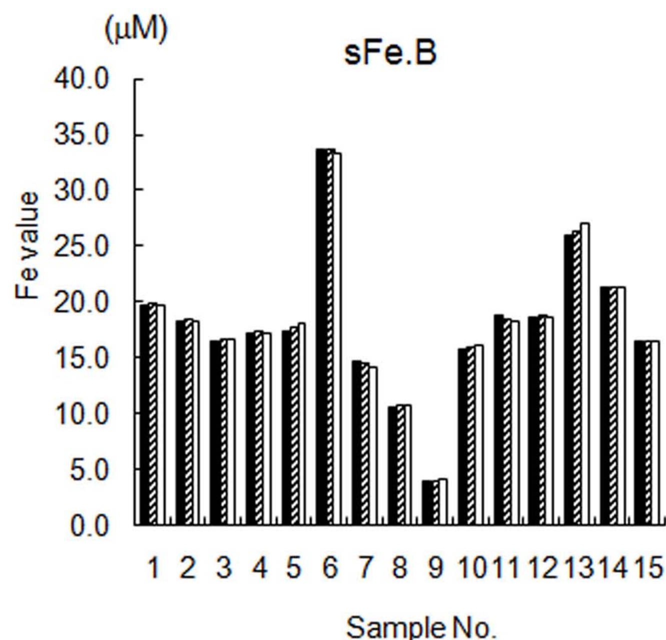
Fe^{3+} Fe^{2+} Fe^{3+} in reagent Chromogen DFX

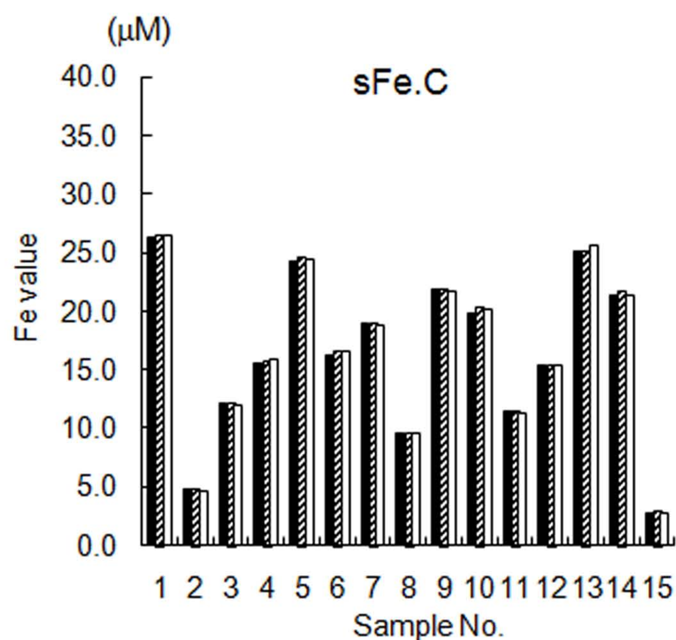
Chromogen-Fe^{2+} DFX-Fe^{3+}

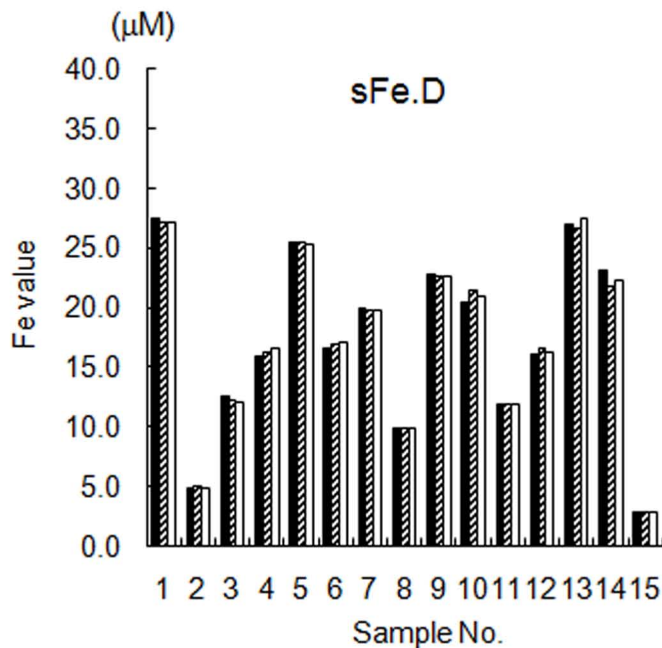


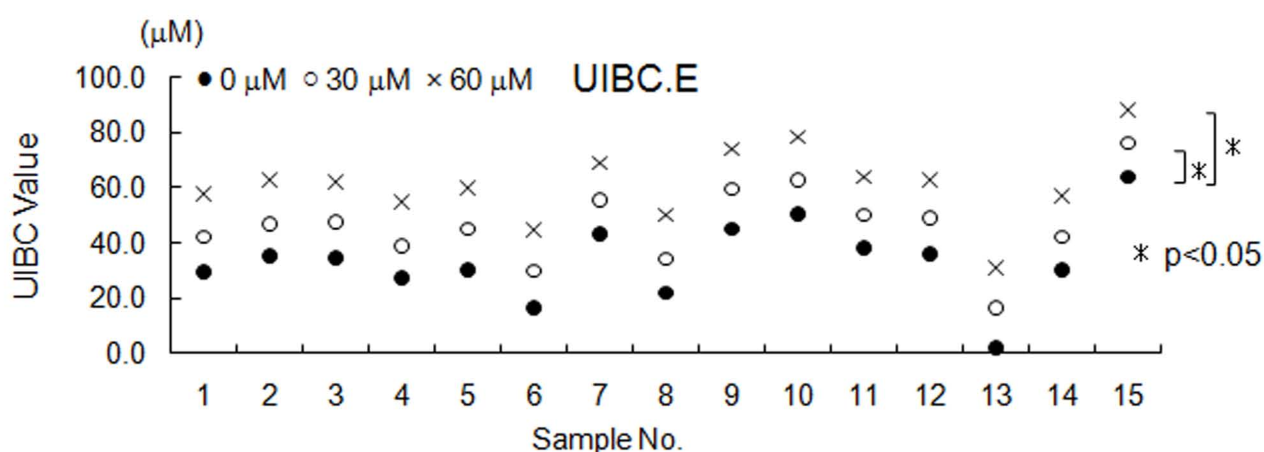
Deferasirox 20 mg/kg/day
Serum ferritin (ng/ml)
4000
2000
0
NTBI (μM)
4
2
0
sFe (μg/dl)
400
200
0
TIBC, UIBC, UIBC by CPBA (μg/dl)
700
600
500
400
300
200
100
0
Feb/2009
Mar/2009
May/2009
Jul/2009
Aug/2009
Oct/2009
Dec/2009

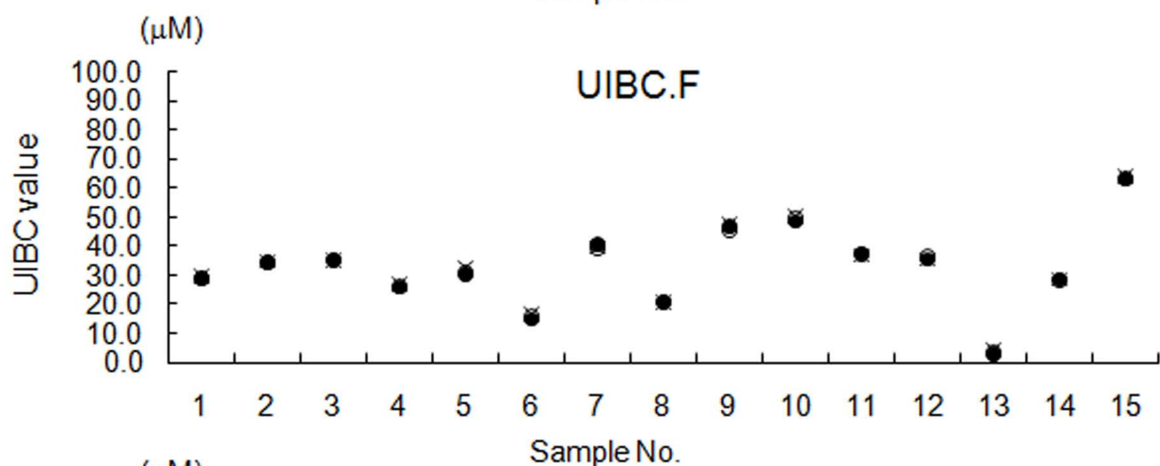

(μM)
sFe.A
0 μM
30 μM
60 μM
Fe value
Sample No.

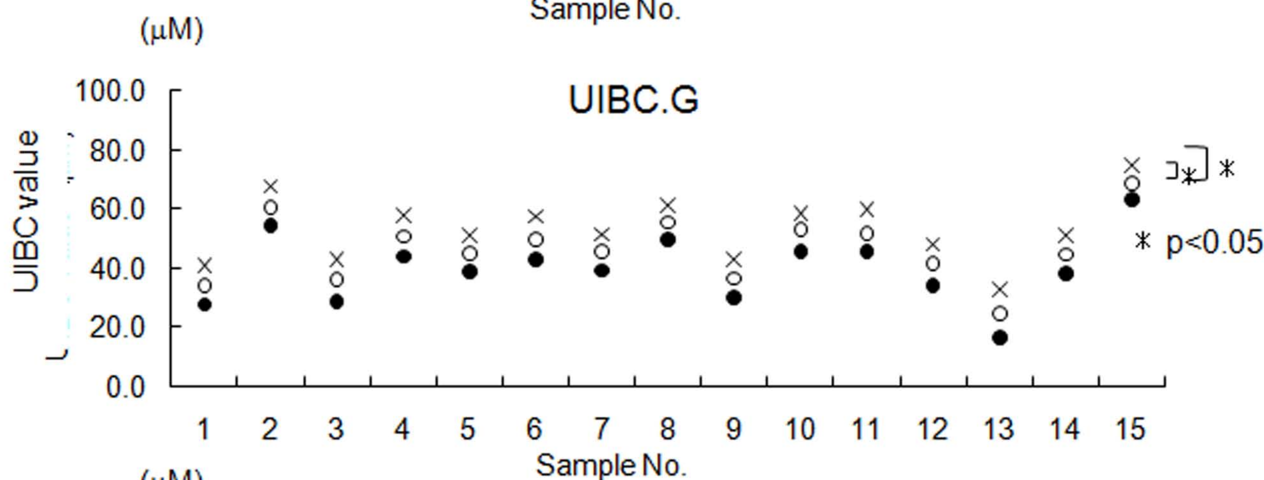

(μM)
sFe.B
Fe value
Sample No.

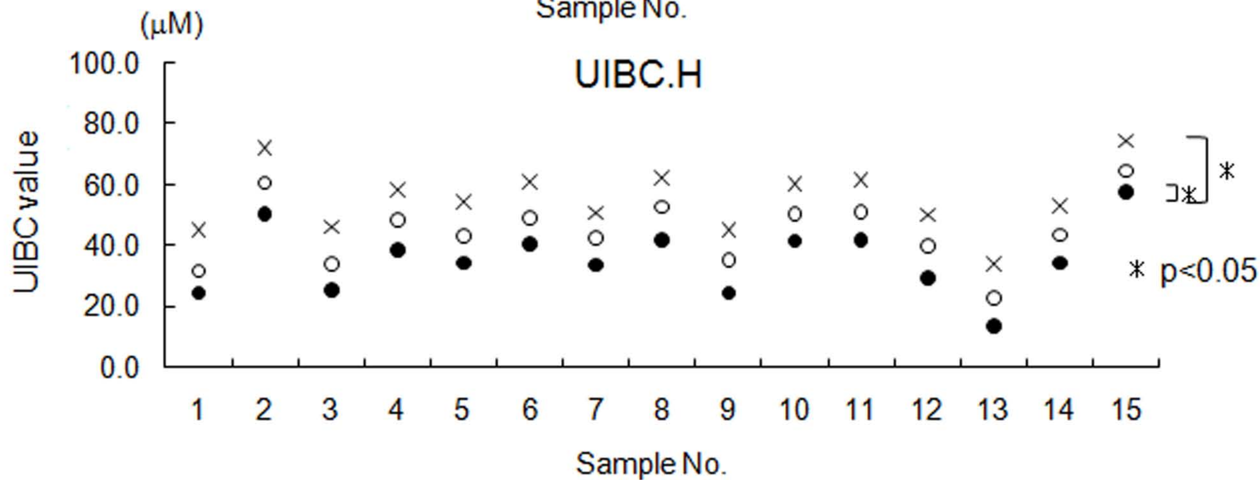

(μM)
sFe.C
Fe value
Sample No.


(μM)
sFe.D
Fe value
Sample No.


(μM)
● 0 μM ○ 30 μM × 60 μM
UIBC.E
UIBC Value
100.0
80.0
60.0
40.0
20.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sample No.
*
*
* p<0.05


(μM)
UIBC.F
UIBC value
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sample No.


(μM)
UIBC.G
UIBC value
100.0
80.0
60.0
40.0
20.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sample No.
*
*
* p<0.05


(μM)
UIBC.H
UIBC value
100.0
80.0
60.0
40.0
20.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Sample No.
*
*
* p<0.05