Low percentage of aluminoxamine and ferrioxamine in uremic serum after desferrioxamine administration

Pilar Menéndez-Fraga,1 Jose L. Fernández-Martín,1 Elisa Blanco-González,2 and Jorge B. Cannata-Andía1*

HPLC was used to study the effectiveness of two different desferrioxamine (DFO) administration strategies (15 mg/kg DFO, 1 h or 44 h before dialysis) on generation of aluminoxamine and ferrioxamine in five hemodialysis patients. The percentage of ultrafilterable aluminum and iron in these patients was also investigated by electrothermal atomic absorption spectrometry. The administration of DFO in both schemes increased the ultrafilterable serum aluminum concentrations from a mean of 17.1 ± 1.6% to a mean of 75.7 ± 14.1%. However, 1 h after DFO infusion, only 38.8 ± 7.7% of the total serum aluminum was bound to DFO; 44 h after DFO infusion, only 15.8 ± 8.0% was bound. Similar results were obtained for ferrioxamine. These results suggest that the ultrafilterable serum fraction contains aluminum and iron chelated by DFO and by DFO metabolites, which retain similar metal-chelating abilities.

Aluminum intoxication has represented a major cause of morbidity and mortality in dialyzed uremic patients during the last two decades and has been documented to cause neurological, skeletal, and hematopoietic toxicity (1). Improvement in mild cases of dialysis encephalopathy may occur with the use of appropriate water treatment for dialysis and after the cessation of oral aluminum ingestion (2). In some cases, however, aluminum removal must be increased by adequate techniques (3).

It is now well established that serum aluminum is largely bound (>80%) to high molecular weight proteins (4–9). Therefore, its removal by dialysis is limited unless a chelating agent such as desferrioxamine is used.

Desferrioxamine (DFO)3 (M, 560) is a naturally occurring trihydroxamic acid obtained by fermentation of the bacterium, Streptomyces pilosus. DFO effectively chelates trivalent ions such as iron and aluminum. The above chelated compounds are called ferrioxamine (FeDFO, $K_s = 10^{31}$) (10) and aluminoxamine (AlDFO, $K_s = 10^{22}$) (11), respectively. DFO has been widely used since 1963 to remove iron via the kidneys in patients with iron overload (12). In contrast, the chelator has been used in the diagnosis and therapy of aluminum accumulation/toxicity in dialysis patients only since 1979 (13).

Given the increasing number of reports dealing with side-effects resulting from DFO therapy and the variation in interindividual sensitivity to the drug, assessing the optimal therapeutic schedule of DFO treatment is imperative. Many protocols have varied the time of infusion, i.e., before (14), in the first (15) or last (16) hours, or after dialysis (17). Postdialysis administration has been advocated to avoid intradialysis loss of free DFO, whereas predialysis infusion has been proposed to exploit the intradialysis removal and reduce the risk of toxicity that is possibly linked to high peak values of aluminum. Nevertheless, thus far no definitive evidence has been provided to support a unique choice.

In view of the above information, this study was undertaken to investigate the effect of two periods of time (1 and 44 h) between DFO infusion and dialysis on the formation of AIDFO and FeDFO in serum from patients receiving a single dose of 15 mg/kg of DFO.

1 Bone and Mineral Research Unit, Hospital Central de Asturias, Instituto Reina Sofía de Investigación Nefrológica, Julián Clavería s/n, Oviedo 33006, Spain.
2 Department of Physical and Analytical Chemistry, Faculty of Chemistry, University of Oviedo, Oviedo 33006, Spain.
*Author for correspondence. Fax 34-8-5106142; e-mail cannata@hca.es.
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3 Nonstandard abbreviations: DFO, desferrioxamine; FeDFO, ferrioxamine; AIDFO, aluminoxamine; ETAAS, electrothermal atomic absorption spectrometry; and HD, hemodialysis.
Materials and Methods

REAGENTS
Desferrioxamine mesylate (Desferal®) was obtained from Ciba-Geigy. Ferric chloride and aluminum chloride atomic absorption spectroscopy standard solutions (Titrisol®) were purchased from Merck. HPLC grade methanol and acetonitrile were purchased from Teknokroma (Barcelona, Spain). All other chemicals were of the highest analytical grade (Merck). Ultrapure water (specific resistivity, 18 megohm/cm) was obtained by purifying distilled water in a NANOpure system (Barnstead, Boston, MA).

APPARATUS
The chromatographic determination of DFO, AlDFO, and FeDFO in the serum ultrafiltration fraction was carried out with a partially inert HPLC system equipped with the following LKB components: two high pressure pumps (Model 2150) with titanium pump heads, an injection valve (Model 2154–002) with a 200-μL loop of titanium, a variable-wavelength spectrophotometric detector (Model 2151) with a 10-μL flow cell, and a recording integrator (Model 2221). All connections used titanium tubing (LKB, Model 2135–602). The chromatographic separation was performed on a Tracer Spherisorb ODS-2 column [10-μm beads, 250 × 4.0 (i.d.) mm, Teknokroma] preceded by a guard column (ODS-TRC-160; Teknokroma).

Determination of aluminum and iron concentrations in the serum samples and the ultrafiltration fractions was performed by Electrothermal Atomic Absorption Spectrometry (ETAAS), using the following Perkin–Elmer equipment: a graphite furnace (Model HGA-600) coupled to an atomic absorption spectrophotometer (Model Z-3030) possessing Zeeman background correction, an automatic sampler (Model AS-60), and a PR-100 printer. All the instrumentation was housed in a cleanroom equipped with a filtered laminar air supply to avoid sample contamination from aluminum in dust.

Ultramicrofiltration experiments were carried out using an Amicon Micropartition System (MPS-1) fitted with Amicon YM5 membranes (nominal cutoff, M, 5000).

Statistical comparisons were performed using Student’s t-test for paired data incorporated into the Systat, Ver. 5.2, statistical package for the Macintosh.

PATIENT PROTOCOL
Five patients on regular hemodialysis (HD) were studied during a period of 2 weeks, following the protocol in Fig. 1. Our procedures complied with the Helsinki Declaration of 1975 for human subjects, as revised in 1983. DFO was administered at a dose of 15 mg/kg body weight. The DFO was diluted in physiological solution and then infused for 30 min by endovenous route either at the end of the dialysis (Fig. 1, dialysis A) or 1 h before the dialysis (Fig. 1, dialysis C). The dialyses were performed using cuprophane membranes for ~4 hours.

Blood samples (3–4 mL) were collected from the arterial line at the beginning (Pre-HD), middle (Half-HD), and at the end (Post-HD) of the three dialysis sessions under study (Fig. 1, A-C). In the case of dialysis C, an additional sample was taken at the beginning of the next dialysis (Fig. 1, dialysis D).

SAMPLE STORAGE AND PREPARATION
Blood was allowed to clot and was centrifuged for 20 min at 800g. Serum was transferred into polystyrene tubes and stored at −20 °C until time of analysis.

Before HPLC analysis, serum samples (700 μL) were placed in the ultramicrofiltration cell and centrifuged at room temperature at 2600g for 40 min. This protein-free ultrafiltrate was injected (200 μL) into the HPLC system. The recovery of DFO, AlDFO, and FeDFO from serum samples with this ultramicrofiltration procedure was ~90% (18).

HPLC PROCEDURE
AlDFO, FeDFO, and DFO concentrations in serum ultrafiltrates were determined by reversed-phase HPLC, using our previously reported method (18). Briefly, the three solutes investigated were separated with a mobile phase of 130 mL/L acetonitrile in phosphate buffer (5 mmol/L, pH 3.5) at a flow rate of 1.5 mL/min on a C18 column and detected by ultraviolet absorption at 210 nm (DFO) and 220 nm (AlDFO and FeDFO).

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Fig. 1. Design of the study.
A, dialysis prior to DFO infusion; B, dialysis 44 h after DFO infusion; C, dialysis 1 h after DFO infusion; and D, dialysis 49 h after DFO infusion. The arrows indicate when DFO was infused.
The calibration plots were linear over the range tested (1–18 mg/L for DFO, 0.5–3.3 mg/L for AlDFO, and 0.3–1.6 mg/L for FeDFO). The relative SD observed oscillated between 3.1% to 4.1% with a detection limit for supplemented ultrafiltrate serum (3 $\sigma_b$ criterion) of 0.14 mg/L for DFO, 0.10 mg/L for AlDFO, and 0.08 mg/L for FeDFO (18).

**Results and Discussion**

When serum samples were analyzed according to the HPLC procedure described above, DFO was detected only in the samples collected 1 h after DFO infusion, at the beginning of the dialysis C (Fig. 1). The concentration of DFO determined in the ultrafilterable fraction of these samples ranged from 0.6 to 11.8 mg/L (mean 3.35 ± 4.79 mg/L).

The total and ultrafilterable aluminum concentrations found in the samples analyzed are given in Table 1. As can be seen, 44 h after DFO infusion (dialysis B) the total aluminum in the serum increased about threefold, whereas the ultrafiltered aluminum increased by more than 10-fold because of the mobilization of this element from deposits in the body by chelation with DFO. As a result, the percentage of ultrafilterable aluminum increased from 17.1 ± 1.6% before DFO infusion to 78.6 ± 19.3% 44 h after DFO infusion (Table 1). Results obtained for the samples collected 1 h after DFO infusion (dialysis C) showed that, in this case, the total serum aluminum content did not increase, but again, ~72.9 ± 7.4% of aluminum was ultrafilterable (Table 1); there were no substantial differences in the ultrafilterable aluminum percentage between the two strategies of chelator administration. It appears that after 1 h, DFO is able to displace aluminum from serum proteins (transferrin) but not from other body deposits (e.g., bone). However, before dialysis D (48 h later), the concentrations of total and ultrafilterable serum aluminum increased (Table 1), again indicating the release of aluminum from deposits within the

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**Table 1. Aluminum distribution in serum from HD patients before and after DFO administration.**

<table>
<thead>
<tr>
<th>Time of dialysis</th>
<th>$A_{\text{TOTAL}}$ mean ± SD, µg/L</th>
<th>$A_{\text{UF}}$ mean ± SD</th>
<th>$A_{\text{AlDFO}}$ mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td>%</td>
<td>µg/L</td>
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<tr>
<td>(A) Prior to DFO infusion</td>
<td></td>
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<tr>
<td>Pre-HD$^c$</td>
<td>96.9 ± 35.3</td>
<td>16.7 ± 6.4</td>
<td>17.1 ± 1.6</td>
</tr>
<tr>
<td>Half-HD</td>
<td>93.8 ± 40.8</td>
<td>16.7 ± 9.8</td>
<td>17.9 ± 5.2</td>
</tr>
<tr>
<td>Post-HD</td>
<td>100.5 ± 44.7</td>
<td>12.6 ± 3.7</td>
<td>15.9 ± 11.7</td>
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<tr>
<td>(B) 44 h after DFO infusion</td>
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<tr>
<td>Pre-HD</td>
<td>297.2 ± 91.5</td>
<td>234.6 ± 101.4</td>
<td>78.6 ± 19.3</td>
</tr>
<tr>
<td>Half-HD</td>
<td>186.9 ± 62.1</td>
<td>148.8 ± 70.1</td>
<td>78.3 ± 21.9</td>
</tr>
<tr>
<td>Post-HD</td>
<td>132.8 ± 37.5</td>
<td>109.3 ± 39.2</td>
<td>82.5 ± 20.7</td>
</tr>
<tr>
<td>(C) 1 h after DFO infusion</td>
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</tr>
<tr>
<td>Pre-HD</td>
<td>114.6 ± 33.4</td>
<td>84.8 ± 31.1</td>
<td>72.9 ± 7.4</td>
</tr>
<tr>
<td>Half-HD</td>
<td>103.7 ± 32.9</td>
<td>80.1 ± 23.4</td>
<td>80.4 ± 22.0</td>
</tr>
<tr>
<td>Post-HD</td>
<td>102.5 ± 33.0</td>
<td>69.7 ± 23.1</td>
<td>70.1 ± 18.2</td>
</tr>
<tr>
<td>(D) 49 h after DFO infusion</td>
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<tr>
<td>Pre-HD</td>
<td>177.8 ± 63.5</td>
<td>124.9 ± 42.2</td>
<td>71.0 ± 11.7</td>
</tr>
</tbody>
</table>

$^a$ Al, aluminum; UF, ultrafilterable.

$^c$ Corresponding dialysis session in Fig. 1.

$^b$ Pre-HD sample taken at beginning of dialysis session; half-HD sample taken midway through dialysis session; post-HD sample taken at end of dialysis session.
body. The results reported above confirm our group’s previous results (22).

The concentration of aluminum bound to DFO in the serum samples was also determined. The percentage of serum aluminum bound to DFO was significantly higher when DFO was infused 1 h before dialysis (38.8 ± 7.7% compared with the percentage bound when the drug was administered 44 h before dialysis (15.8 ± 8.0%) (P < 0.02; Table 1). Results showed that only a small amount of ultrafilterable aluminum was bound to DFO. Considering the low concentrations of free DFO in these samples, probably because DFO is metabolized rapidly (23) and because the binding region in nearly all the metabolites of DFO remain unaffected and thus retain similar metal-chelating abilities (24, 25), the most reasonable explanation these results is that the aluminum present in the ultrafiltrate is bound to both DFO and DFO metabolites. Thus, as shown in Table 1, 44 h after DFO infusion, the concentration of aluminum bound to DFO is similar to that obtained 1 h after drug administration. However, the total ultrafilterable aluminum concentration is higher. This suggests that DFO and DFO metabolites are able to remove aluminum from stores in the body.

Fig. 2 shows the changes in total, ultrafilterable, and DFO-bound serum aluminum concentrations vs time after DFO infusion. As expected, in both strategies of DFO administration, the concentration of aluminum bound to DFO decreased continuously during the intradialytic period (HD-B and -C) because of the removal of the low molecular weight (M_r 584) AlDFO complex by dialysis. The amount of aluminum bound to DFO also decreased during the interdialytic period (Fig. 2, HD-C and -D), in this case, probably because DFO was metabolized.

In contrast, the total aluminum concentration in serum and the ultrafilterable fraction decreased intradialytically (elimination by dialysis of aluminum bound to DFO and DFO metabolites) but not interdialytically (Fig. 2) because DFO and DFO metabolites removed aluminum from stores in the body and from serum transferrin during the interdialytic period (between dialyses C and D). As a result, the percentage of ultrafilterable aluminum observed after DFO administration remained virtually unchanged during all periods of time investigated, in agreement with our previous results (22), whereas the percentage of aluminum bound to DFO decreased with time during (dialyses B and C) and between (dialyses C and D; Table 1) dialysis sessions.

The data in Table 1 also show that, after DFO infusion, ~76% of total serum aluminum is present in the ultrafilterable fraction and that this percentage does not seem to be influenced by the time (1 and 44 h) between DFO infusion and dialysis (22). However, the percentage of aluminum bound to DFO decreases from 38.8% 1 h after DFO infusion to 15.8% 44 h after DFO infusion.

To confirm the ideas discussed above, the aluminum distribution in serum ultrafiltrate was investigated. A serum sample from a patient collected at the beginning of the dialysis (dialysis B, 44 h after DFO infusion) with a total aluminum content of 372 μg/L was analyzed for AlDFO according to the HPLC procedure with UV detection described in Materials and Methods. In addition, column fractions were collected manually (750 μL) in aluminum-free automatic sampler cups and immediately analyzed off-line for aluminum by ETAAS, using the operating conditions described in Materials and Methods. The results are shown in Fig. 3. The peak (detected at 220 nm) that eluted at 9.15 min corresponds to the quantitative elution of the AlDFO complex that we reported previously (18). The aluminum concentration correspond-
to this peak (aluminum bound to DFO) was determined by HPLC with UV detection to be 89.6 µg/L, only 25% of the total aluminum (355 µg/L) present in the sample injected. The UV elution profile in Fig. 3 also shows the presence of important peaks at retention times up to 5 min, which correspond to different unidentified UV-absorbing species present in ultrafilterable serum (18). The ETAAS elution profile of total aluminum (bar graph in Fig. 3) indicates that aluminum is present in fractions (9–10 min) corresponding to elution of the AIDFO complex detected at 220 nm (Fig. 3). These fractions contain ~23% of the total aluminum injected, in agreement with the results reported above for AIDFO, using UV detection. In addition, it can be observed in Fig. 3 that aluminum is also present in the fractions at 1–5 min; the amount of aluminum found in these fractions represents 65% of the total aluminum injected (total aluminum recovery ~90%).

According with Sing et al. (24) and Lehmann and Heinrich (25), the main metabolites of DFO are produced by bioconversion of the terminal amino group of the DFO molecule into a carboxyl group, leading to a shortening of the hydrocarbon chain length of DFO, in which methylene groups are removed but the hydroxamic chelation sites remain intact. Therefore, the complexes of aluminum with these DFO metabolites are less hydrophobic (fewer -CH2- groups) than the AlDFO complex. Consequently, they are expected to elute faster than AlDFO in reversed-phase liquid chromatography, where solution retention occurs mainly by hydrophobic interaction with the hydrocarbon stationary phase. In fact, this chromatographic behavior has been already described for iron bound to DFO and DFO metabolites (23–25). Therefore, the aluminum peaks detected by ETAAS (bar graph in Fig. 3) at retention times <5 min could correspond to aluminum bound to DFO metabolites. Unfortunately, these peaks cannot be detected by UV absorption at 220 nm because in our chromatographic system they are overlapped by the peaks produced by other UV-absorbing serum components (Fig. 3).

Other authors, however, have proposed the formation of unknown species (aluminum-binding proteins or a ternary complex of aluminum-DFO-protein) after the infusion of DFO. The molecular weight of those species has been reported to be 8000 (26, 27) or in the range between 20 000 and 60 000 (28). It has been suggested that the presence of this unknown species is induced by high serum aluminum concentrations, even without the administration of DFO (26). The molecular weight of the unknown species of aluminum found in our study must be <5000 because this is the molecular weight cutoff used to deproteinate the serum samples before the chromatographic process. Thus, the aluminum-induced Mr 8000 protein described before (26) cannot explain our findings.

Our findings also suggest that a great part of the aluminum removed with the use of DFO is not in the form of aluminum-DFO complex (Fig. 3). Additional speciation studies after DFO administration are necessary to know the serum carriers of aluminum and the mechanism of action of DFO for the treatment of aluminum overload.

Table 2 shows the results obtained for the determination of total and ultrafilterable iron concentrations in the serum samples included in this study. As expected, a small amount of the total serum iron concentration is found in the ultrafilterable fraction after DFO infusion; therefore, only 6.8 ± 6.6% and 11.3 ± 5.4% of total iron was ultrafiltered 44 h and 1 h after DFO infusion, respectively (Table 2). These results agree with previous reports.

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**Table 2. Iron distribution in serum from HD patients before and after DFO administration.**

<table>
<thead>
<tr>
<th>Time of dialysis</th>
<th>FeTOTAL mean ± SD, µg/L</th>
<th>FeFeDFO* mean ± SD, µg/L</th>
<th>FeUF mean ± SD, µg/L</th>
<th>FeUF/DFO mean ± SD, µg/L %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) 5 h prior to DFO infusion</td>
<td></td>
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<tr>
<td>Pre-HD</td>
<td>1079.5 ± 567</td>
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<td>&lt;LOD</td>
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<tr>
<td>Half-HD</td>
<td>904.7 ± 314</td>
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<td>&lt;LOD</td>
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<tr>
<td>Post-HD</td>
<td>946.7 ± 488.2</td>
<td></td>
<td>&lt;LOD</td>
<td></td>
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<tr>
<td>(B) 44 h after DFO infusion</td>
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</tr>
<tr>
<td>Pre-HD</td>
<td>1685.6 ± 1348.1</td>
<td>73.7 ± 38.9</td>
<td>6.8 ± 6.6</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Half-HD</td>
<td>1217.5 ± 331.3</td>
<td>31.52 ± 13.3</td>
<td>3.0 ± 2.5</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Post-HD</td>
<td>1195.9 ± 334.1</td>
<td>27.2 ± 12.2</td>
<td>2.5 ± 1.3</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>(C) 1 h after DFO infusion</td>
<td></td>
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</tr>
<tr>
<td>Pre-HD</td>
<td>1011.9 ± 549.1</td>
<td>95.4 ± 18.5</td>
<td>11.3 ± 5.4</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Half-HD</td>
<td>1164.4 ± 506.6</td>
<td>60.4 ± 23.3</td>
<td>5.9 ± 2.9</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Post-HD</td>
<td>1227 ± 93.2</td>
<td>62.5 ± 19.7</td>
<td>6.3 ± 3.6</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>(D) 49 h after DFO infusion</td>
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<tr>
<td>Pre-HD</td>
<td>1331.4 ± 413.6</td>
<td>30.6 ± 13.7</td>
<td>2.5 ± 1.4</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

*Fe, iron; UF, ultrafilterable; LOD, limit of detection.

* Corresponding dialysis session in Fig. 1.

* Pre-HD sample taken at beginning of dialysis session; half-HD sample taken midway through dialysis session; post-HD sample taken at end of dialysis session.
that DFO cannot displace in vivo iron from the serum protein carrier transferrin (29), because replacement occurs slowly in vitro (30).

Again, as in the case of aluminum, not all of the iron present in the ultrafiltrate was bound to DFO. In fact, the concentration of iron as FeDFO found in the samples investigated was always below the detection limit (7 μg/L) of the HPLC procedure used (18). These results confirm our idea that DFO and DFO metabolites are implicated in the mobilization of iron and aluminum in the body. In fact, Sing et al. (24) have described the presence of three iron chelates, ferrioxamine, and two iron-binding DFO metabolites in the serum ultrafiltrate and urine of two patients treated with DFO.

Finally, regarding the patient benefit of using our new protocol of DFO administration (i.e., predialysis infusion), we want to stress that the most important goal we want to achieve with DFO is the removal of the greatest amount of aluminum with the least risk of toxicity for the patient. Previous studies have shown great efficacy in the removal of aluminum, with lower interdialytic peaks of serum aluminum, when DFO is administered 1 h before HD (14). The present study confirms previous findings (14), demonstrating that using DFO before dialysis allows us to obtain lower serum peaks of total and ultrafilterable aluminum at the beginning and at the end of dialysis, with a lower chance of aluminum redistribution in tissues during the interdialytic period (Table 1). This fact may decrease the possibility of reentrance of aluminum into some organs, e.g., the brain, limiting the toxic effect of the use of DFO (14). The delayed effect of a single infusion of DFO observed in the second and third dialyses (Fig. 2), also suggests that a lower dose of DFO could be effective, as has been recently described in studies using 5 mg/kg (31) and very low doses of DFO, such as 0.5 mg/kg (32). The combination of all of these new approaches—new schedules of DFO administration and lower doses—may contribute to the reduction of the toxicity of DFO without a loss of efficacy.

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References


