Concerns over iron toxicity related to this “oversaturation” of transferrin have recently been applied to circumstances in which iron is given intravenously as either iron dextran (used in the US) or iron gluconate to patients on hemodialysis receiving erythropoietin therapy (3, 4). A recent study describing oversaturation of transferrin, as calculated by the measurement of high serum iron concentrations shortly after intravenous iron gluconate treatment, suggested that with this product, transferrin saturation is commonly >100% (serum iron/total iron binding capacity for transferrin binding sites × 100 = % saturation) (3). When transferrin is fully saturated, serum may contain “free” iron or iron loosely bound to other plasma proteins, a situation that can cause acute toxicity, including tissue damage, cardiac arrhythmias, and/or hypotension (1, 2). It has been suggested that this oversaturation soon after intravenous iron infusion in hemodialysis patients may account for these types of complications (3). However, parenteral iron is a necessary treatment in this patient population, and similar complications, particularly hypotension, are common during hemodialysis sessions even when iron is not given (4).

Because iron bound to iron dextran or iron gluconate does not cause acute iron toxicity (5), we hypothesized that serum assays for iron measure the iron present in these complexes after intravenous infusion, which produces misleadingly high results for the oversaturation of transferrin.

Pharmacokinetic studies have indicated that after infusion, serum concentrations of dextran-bound iron are distributed almost entirely in the plasma, with removal limited to the reticulo-endothelial system at a constant rate (3, 5–7). Previous studies in iron-deficient patients have suggested that dextran has a variable, but in some patients a relatively long, half-life of >20 h in the circulation (5–7). In a recent study, a mean half-life of 58.9 h was measured in patients with iron deficiency and chronic disease, including renal failure patients on hemodialysis receiving Dexferrum (8). Various modifications of methodology have given conflicting results as to whether iron as iron dextran is measured as “serum iron” (5, 7). When one methodology was used (5), <3% of the iron present as iron dextran was measured (5), and it was suggested this small percentage was bioavailable iron already dissociated from the iron dextran complex (5). However, another study, in which a different methodology was used, indicated that serum iron measurements up to 2 weeks after iron dextran injection could overestimate the amount of serum iron bound to transferrin (bioavailable iron); this overestimation was attributed to dissociation of the iron from iron dextran in the assay used (6, 7).

The potential for incorrect diagnosis of oversaturation of transferrin may increase with the introduction of an iron gluconate preparation that recently was approved for use in the US (9). To address this oversaturation question and to assess routine clinical monitoring guidelines (4), we measured serum iron in vitro in the presence of added iron gluconate as well as iron dextran.
Two methods for serum iron determination were used. One method uses an acetate buffer (pH 4.5) with 15 g/L hydroxylamine hydrochloride (Sigma Diagnostics) to release the iron from transferrin (10). The initial absorbance is read at 560 nm on a Hewlett Packard 8452A diode array spectrophotometer. The released iron reacts with 8.5 g/L Ferrozine reagent during a 15-min incubation at 37 °C to produce a magenta-colored complex, which is read a second time. Because the assay is linear, a single calibrator with an iron concentration of 84.7 μmol/L (500 μg/dL) is used. The second method, often used in European countries (11) and now used in the US in certain automated systems, uses an acetate buffer (0.1 mol/L, pH 4.8) containing ascorbic acid (56.8 mmol/L) and guanidine (6 mol/L) to release transferrin-bound iron. The absorbance is measured at 595 nm (3, 11) before and after a 5-min incubation at 37 °C with 36 mmol/L Ferrene S. Again the assay is linear, and a single calibrator is used [30–84.7 μmol/L (177–500 μg/dL)]. Neither of these methods measures the iron present in heme. We used the form of iron dextran currently used in Europe and approved for use in the US, sodium ferric gluconate complex in sucrose (Ferrlecit), from R & D Laboratories, and iron dextran (InFed) from Schein Pharmaceuticals.

The results obtained for each of the two methods after various incubation times are shown in Fig. 1. Serum iron was measured in samples obtained after blood was drawn into red-stoppered tubes, allowed to clot, and centrifuged to separate the serum. Iron as iron gluconate or iron dextran was added at a concentration of 204 μmol/L (1206 μg/dL). This is the amount of added iron, based on what is known about iron dextran distribution and kinetics (see above) (3, 5–7), that would be expected to be present in a patient’s serum after approximately one half-life of disappearance of the iron preparations if 100 mg was given intravenously; this same amount would be present at least several half-lives (i.e., 1 week or more) after “total dose” iron dextran infusion (1000 mg) (12). The total iron present was verified by the addition of sodium dithionite, an agent known to release iron from gluconate or dextran complexes (5). The method that uses the acetate/hydroxylamine hydrochloride buffer system measured 2.2% (± 1.2%) of the iron in iron dextran after the recommended 15-min incubation, whereas the ascorbic acid/guanidine method measured 7.3% of the iron after the recommended 5-min incubation period. Both assays measured significantly more iron when iron gluconate was added: 7.0% (± 0.4%) in the acetate/hydroxylamine hydrochloride method vs 33% (± 11.0%) in the ascorbic acid/guanidine method. When both assays and five different concentrations of iron gluconate from 51 to 408 μmol/L (301 to 2412 μg/dL) were used, the percentage of iron measured from the added iron gluconate remained similar (± 3%) over the entire range of concentrations. In other experiments, both iron compounds were added to the serum within 1 h prior to the assay (as shown in Fig. 1) or 6 and 24 h prior to the assay and kept at room temperature with no significant change in the amount of iron released (difference <10%).

As shown in Fig. 1, the change in iron released with increasing incubation times changed significantly when the ascorbic acid/guanidine buffer was used. Many clinical laboratories in the US now use automated systems with a similar ascorbic acid/guanidine buffer (Boehringer Mannheim) for serum iron determinations. This methodology might, based on minor differences in incubation time or other details of the assay, give results different from those shown in Fig. 1. Three different serum samples from three healthy subjects with different starting iron concentrations and the same samples containing 204 μmol/L iron as iron gluconate or iron dextran were sent to three clinical laboratories, one (Veterans Administration Hospital, Denver, CO) that uses a hydroxylamine method (Beckman) and two (University Hospital, Denver, CO, and Presbyterian-St. Luke’s Medical Center, Denver, CO) that use variations of an ascorbic acid method with different automated equipment (Hitachi 917 and Johnson & Johnson Vitros system, respectively). The results obtained with the hydroxylamine method (Table 1) for both iron dextran and iron gluconate were similar to our manual results shown in Fig. 1. The results obtained from the two variations of the ascorbic acid method are somewhat different from each other and the results in Fig. 1, but still show significantly increased release of iron from...
both complexes compared with the hydroxylamine method. When pooled serum samples (from three other subjects) containing two different concentrations of iron dextran or iron gluconate (204 and 33.9 μmol/L) were sent in duplicate to each laboratory, almost identical results were obtained for from each laboratory, as shown in Table 1.

Limited studies were performed using both iron gluconate and iron dextran diluted in pH 7.4 buffer, with the assay performed in the same neutral buffer (UIBC; Sigma Chemical Co.). In this assay, the iron present in iron dextran was either not measurable or barely measurable (<1%); the iron measurable in added iron gluconate was significantly higher (mean, 2.8%); than in duplicate samples assayed with pH 4.1 buffer (mean, 9.1%; P <0.05, paired t-test). Iron measured in pH 7.4 buffer clearly represents free iron present in the compound, although this may underestimate other loosely bound iron that may become bioavailable.

Taken together, these data suggest that both acidic buffers not only release iron from the transferrin-bound iron complex and measure free iron (see above), but also release some iron still bound to the gluconate and dextran complexes. The amount of iron released by the hydroxylamine method is relatively low and consistent for various assay conditions. The higher and more variable amounts of iron released in the ascorbic acid/guanidine method could lead to gross overestimations of serum iron if either of these iron complexes is present in the serum. Thus, serum obtained after infusion of 100 mg of iron as iron dextran or iron gluconate could, for example, add an additional 169 μmol/L (1206 μg%) of iron (the equivalent of one half-life), and iron measurements could falsely indicate oversaturation of transferrin (i.e., if 10–25% of this iron were released, an additional 16.9–42.4 μmol/L (16.9–302.5 μg%) would be measured in the serum iron assay).

Because of concerns about iron deficiency as well as iron toxicity, measurement of serum iron and calculation of the percentage of saturation are performed frequently on all hemodialysis patients receiving intravenous iron preparations (4). Our results indicate that the measurement of serum iron within 24 h after infusion, even if a patient shows signs indicative of iron toxicity, would yield misleading results for transferrin saturation with any of the present methodologies. In addition, measurement of serum iron to assess the response to intravenous iron as reflected by bioavailable iron (increased percentage of saturation) may still reflect the in vitro release of iron from these intravenous iron preparations, which may lead to a change in therapy based on guidelines that recommend using serum iron determinations to monitor iron dosing. For future pharmacokinetic studies of iron gluconate, extensive in vitro studies that include measurement of total iron and free iron (5) are necessary to determine any overestimation of “bioavailable iron” that may be related to the methodology used.

In summary, methodologies that measure serum iron, particularly those using an ascorbic acid/guanidine buffer method, will cause in vitro dissociation of iron bound to gluconate or dextran complexes. To ensure that only bioavailable iron is measured in patients treated with intravenous iron, as suggested by one guideline (4), it is best to wait at least 2 weeks after the last dose of intravenous iron. In addition, careful attention must be paid to any changes in the methodologies used by the laboratories that receive samples from dialysis centers.

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References

Table 1. Concentration of added iron in serum. a

<table>
<thead>
<tr>
<th>Analyzer (buffer)</th>
<th>Serum Plus</th>
<th>Change, μmol/L</th>
<th>Change, %</th>
<th>Serum Plus</th>
<th>Change, μmol/L</th>
<th>Change, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iron dextran,</td>
<td></td>
<td></td>
<td>Iron gluconate,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hitachi 917 (ascorbic acid)</td>
<td>13.2</td>
<td>22.0</td>
<td>8.8</td>
<td>4.3</td>
<td>46.9</td>
<td>33.7</td>
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<tr>
<td>Johnson &amp; Johnson (ascorbic acid)</td>
<td>15.9</td>
<td>31.5</td>
<td>15.6</td>
<td>7.6</td>
<td>70.3</td>
<td>54.4</td>
</tr>
<tr>
<td>Beckman (hydroxylamine)</td>
<td>14.4</td>
<td>17.8</td>
<td>3.4</td>
<td>1.7</td>
<td>26.3</td>
<td>11.9</td>
</tr>
</tbody>
</table>

a Iron (204 μmol/L) added as iron dextran or iron gluconate.
Simultaneous Measurement of Allantoin and Urate in Plasma: Analytical Validation and Potential Clinical Application in Oxidant:Antioxidant Balance Studies, Iris F.F. Benzie,1,2 Wai-yuen Chung,1 and Brian Tomlinson1

In humans, allantoin is formed by nonenzymatic oxidation of urate; it may, therefore, be useful in assessing oxidative stress (1, 2). Most published methods involve separate analysis of urate and allantoin and require extraction, hydrolysis, and derivatization procedures (1–6). The primary aim of this study was to evaluate a slightly modified version of an HPLC assay described by Lux et al. (7) for the simultaneous measurement of urate and allantoin. A secondary aim was to explore the clinical utility of allantoin as a biomarker of oxidative stress, the hypothesis being that in disease associated with increased oxidative stress, allantoin increases because of an increased “oxidative turnover” of urate. The final aim of the study was to investigate the effect of age on urate and allantoin concentrations.

Allantoin and uric acid were from Sigma; 1-heptanesulfonic acid, sodium salt monohydrate was from Sigma-Aldrich; potassium dihydrogen phosphate was from Merck; sodium hydroxide was from Riedel-de Haen; orthophosphoric acid was from BDH, and Moni-Trol Level 1 Chemistry Control Serum was from Dade International. MilliQ water (Millipore ultra-pure water system; Millipore) was used for preparation of all solutions. Aqueous stock solutions of allantoin (1000 μmol/L) and urate (2000 μmol/L) were prepared and stored at 4 °C.

Because urate reportedly is less stable at alkaline pH (2), the stability of the stock urate calibrator (pH 9.4) was assessed by repeated measurements of Moni-Trol control at pH 9.4 for 10 min to remove protein and other molecules of mobile phase. Ultrafiltrates (20 μL) was injected into the HPLC system, which comprised an isocratic pump (ISCO model 2350 pump with a 20-μL looped Valco manual injector; ISCO), a variable wavelength absorbance detector (ISCO model V4 detector with 5-mm flow cell path), a guard column (Spherisorb C18, 5 mm, 10 × 4.6 mm i.d.), and a reversed-phase analytical column (ISCO C18, 5 μm, 250 × 4.6 mm i.d.). The mobile phase was aqueous 5 mmol/L potassium dihydrogen phosphate containing 5 mmol/L 1-heptanesulfonic acid (ion-pairing reagent) and adjusted to pH 3.1 using orthophosphoric acid. The flow rate was 1.0 mL/min, and detection was at 210 nm.

The peak heights of allantoin and urate were measured manually, using chromatograms recorded by a chart recorder fitted within the detector. By plotting peak height against calibrator concentration, we constructed calibration curves for allantoin and urate. We calculated the concentration (μmol/L) of allantoin or urate in each sample, using peak height over the slope of the calibration curve. The purities of the compounds of interest were not assessed, but were assumed on the basis of previously published data (7).

Because urate reportedly is less stable at alkaline pH (2), the stability of the stock urate calibrator (pH 9.4) was assessed. To check whether membrane filtration caused loss of analyte, fresh fasting, heparinized plasma was analyzed with and without filtration. Linearity was assessed by repeated measurements of Moni-Trol control at various concentrations. Recovery was assessed by the addition of allantoin (25 μmol/L) and urate (250 μmol/L) to pooled plasma. A signal-to-noise ratio of 3:1 was used to determine detection limits.

This study was approved by the Ethics Subcommittee of the Hong Kong Polytechnic University, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 1996.

Fasting heparinized plasma samples were obtained from 40 apparently healthy volunteers [23 men, ages 20–55 years; mean (SD), 30.3 (11.9) years; and 17 age-matched women] and 64 subjects with non-insulin-dependent diabetes mellitus [NIDDM; 27 men, ages 32–86 years, mean (SD), 63.4 (14.1) years; and 37 age-matched women]. The NIDDM subjects had been assessed clini-