Citation: Clin Transl Sci (2017) 10, 194–200; doi:10.1111/cts.12443

ARTICLE

Performance of Redox Active and Chelatable Iron Assays to Determine Labile Iron Release From Intravenous Iron Formulations

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Emerging data from global markets outside the United States, where many generic iron sucrose formulations are available, have revealed that non-US generic intravenous (i.v.) iron formulations may have iron release profiles that differ from the reference listed drug (RLD). The first generic i.v. iron approved in the United States was sodium ferric gluconate complex in 2011. We evaluated chelatable and redox labile iron assay methods to measure the amount of labile iron released from i.v. iron formulations in biorelevant matrices *in vitro*. The majority of published labile iron assays evaluated were not suitable for use *in vitro* due to overwhelming interference by the presence of the i.v. iron products. However, an optimized high-performance liquid chromatography (HPLC)-based method performed well for use *in vitro* labile iron detection in a biorelevant matrix. Application of this method may enhance bioequivalence evaluation of generic i.v. iron formulations in the future.

Clin Transl Sci (2017) **10**, 194–200; doi:10.1111/cts.12443; published online on 3 February 2017.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Studies have identified safety issues with labile iron release from iron formulations but translation to bioequivalence evaluation has not been studied.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ Currently, there have been no published studies systematically evaluating the various labile iron assays for potential *in vitro* application to enhance current bioequivalence regulatory guidance.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
✔ This study evaluated four assays, two based on chelation methodology and two based on redox methodology, for use *in vitro*. We found that of the assays studied only an HPLC assay based on chelation with desferroximine was viable for *in vitro* use.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
✔ Further study of this assay method *in vivo* may inform an *in vitro in vivo* correlation model to augment bioequivalence requirements for generic intravenous iron formulations.

Intravenous (i.v.) iron products are widely used to treat anemia of various etiologies, including chronic kidney disease (CKD), chronic inflammatory disease, heavy uterine bleeding, and malignancy-related anemia.[1] Current commercially available intravenous iron formulations consist of an iron oxyhydroxide core surrounded by a carbohydrate shell of various sizes and polysaccharide branch characteristics. These products are formulated as colloidal suspensions of nanoparticles.[2–4] The manufacture of these iron-carbohydrate formulations is sensitive to pH, temperature, and other conditions in the manufacturing process, presenting challenges to reproducible manufacturing of i.v. iron formulations to be considered for generic approval.[4] However, it has been shown that complexes of similar molecular weight can be synthesized using multiple different manufacturing procedures, suggesting that the iron complex may be thermodynamically stable.[5]

The available branded i.v. iron formulations differ with regard to stability profile and pharmacokinetic disposition, which directly impacts the rate and extent of labile (i.e., free or non-transferrin bound) iron release from the iron–carbohydrate complex.[6,7] Emerging data from Europe, South America, and Asia, where many non-US generic iron sucrose formulations are available and in widespread clinical use, have shown that non-US generic i.v. iron formulations may not be therapeutically equivalent and may have increased oxidative stress induction.[8–10] It has been hypothesized that these observations arise due to differences in the stability profile and labile iron release from the non-US generic i.v. iron formulations compared with the reference listed drug (RLD).[8]

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Received 8 November 2016; accepted 19 December 2016; published online on 3 February 2017. doi:10.1111/cts.12443

In March 2011, the first generic i.v. iron, sodium ferric gluconate complex (SFGC), was approved in the United States. SFGC was rated AB bioequivalent to Ferrlecit.[11,12] The prescribing information (PI) states "Direct movement of iron from sodium ferric gluconate complex in sucrose to transferrin was not observed"; however, the methods that support this statement were not described.[12] The current draft guidance for sodium ferric gluconate recommends comprehensive physicochemical characterization of the test and reference products, and suggests the possible use of multiple labile iron assessment approaches.[13] Identification of a candidate assay that is suitable for measurement of labile iron both *in vitro* and *in vivo* would facilitate the development of an *in vitro* to *in vivo* correlation (IVIVC) model to enable prediction of serum labile iron *in vivo.* Such a model could be used to augment physicochemical characterization and improve equivalence testing for candidate generic i.v. iron formulations. The objective of this study was to evaluate redox active and chelatable iron assays for their suitability to measure labile iron release from intravenous iron formulations *in vitro.*

METHODS

Matrices and timepoints

To compare labile iron release *in vitro*, available i.v. iron formulations, Venofer, Ferrlecit, generic sodium ferric gluconate complex (NDC 00591-0149-87, Watson Laboratories, Parsippany, NJ), InFeD, Feraheme, and a preclinical investigational formulation GE121333 were incubated in 150 mM saline and in a biorelevant matrix (rat serum).[14] Rat serum was selected as the biorelevant matrix for *in vitro* labile iron release profiling to optimize comparison of *in vitro* release with *in vivo* plasma concentration time profiles in this well studied preclinical model.[8] Concentrations of 0.95 mg/mL were used to simulate the predicted maximal plasma concentration (C_{max}) after an intravenous injection of 40 mg/kg of elemental iron in rats from each of the six products. The 40 mg/kg dose was selected to limit the need for dilution of the agents, which could impact formulation stability and labile iron release profiles.[8] To expand the chemical classes of agents evaluated, we additionally tested GEH121333, which is a research-stage iron oxide nanoparticle formulation with a PEG-based coating.[14] These samples were assayed at frequent prespecified timepoints (0, 5, 15, 30, 60, 90, 120, and 150 min) using both redox active and chelatable iron assay methodologies. Characterization of the rate and extent of labile iron release for each formulation as a cumulative consequence of direct, spontaneous release from the iron formulation was then evaluated between assays and products. Assays were compared by limits of detection (LOD), practical limitations, and limitations for *in vitro* performance.

Labile iron measurement

Further details on assay methods are provided in the **Supplementary Information**.

Redox active assays

Rhodamine conversion assay

In this assay, sodium ascorbate causes labile iron to undergo redox cycling. The resulting radicals are detected using dihydrorhodamine (DHR) 123 (nonfluorescent in the absence of reactive oxygen species (ROS); catalyzed in the presence of ROS to cationic DHR 123, which exhibits a green fluorescence). The addition of an iron-selective chelator (deferiprone) in a second reagent solution (solution B) is utilized to assess the specific involvement of labile iron, which is detected by a quenching of the fluorescent signal. The rate (slope) of DHR fluorescence in the presence or absence of the iron chelator is calculated. The procedure was adapted from the methods published by Esposito *et al.*[15]

Bleomycin detectable iron (BDI) assay

Capitalizing on the ability of the chemotherapeutic agent bleomycin to induce oxidative damage to deoxyribonucleic acid (DNA) in the presence of ferrous iron (Fe2+), this assay is used to indicate the presence of labile iron in an oxidative state capable of catalyzing the generation of hydroxyl radicals. In the originally published methodology for the BDI assay, DNA damage is measured by the formation of malondialdehyde (MDA) from the 2' deoxyribose moiety of DNA by the thiobarbituric acid test, which measures the resultant TBA-MDA chromophore.[16] Limitations of conventional methods include exposure of the sample to harsh conditions (heat) that may induce secondary oxidative stress reactions and detection of byproducts of lipid peroxidation other than MDA. These limitations may be avoided through use of a modified procedure where DNA damage in the presence of bleomycin, ascorbic acid, and iron is determined by the fluorescence of the interchelating compound ethidium bromide.[16]

Chelatable iron assays

Desferoxamine chelatable iron (DCI) assay

Using methodology previously published, this assay detects labile iron utilizing fluoresceinated desferoximine (FL-DFO).[17] The fluorescence signal of this reagent is stoiciometrically quenched in the presence of labile iron. Two reagents (A and B) are prepared to perform the assay. In samples treated with Reagent A the labile iron binds to the Fl-DFO present. The action of iron binding to Fl-DFO results in a quenching of the total fluorescence of the compound. In samples treated with Reagent B containing nonfluorescent DFO, labile iron binds the nonfluorescent DFO with a higher affinity than the Fl-DFO. The Reagent B sample is used to correct for non-iron factors present in the serum that may affect the fluorescence measurement (e.g., turbidity, absorbance). Therefore, the ratio of fluorescence of Reagent A/B is calculated to normalize the samples. The ratio of the fluorescence of Reagent A/B yields a measure of the labile iron present in the sample (e.g., ratio ≥ 1 indicates little to no detectable iron in the sample, while a ratio of <1 indicates the presence of iron). The ratio of Reagent A/B is inversely proportional to the concentration of chelatable labile iron present in the serum sample.

Fluorescein-conjugated desferoxamine (Fl-DFO) was synthesized following the protocol detailed by Su *et al.*[18]

High-performance liquid chromatography (HPLC) detection of chelatable iron (HPLC-DFO)

Using methodology adapted from Tesoro *et al.,*[19] chelatable iron was detected following chelation with 20 mM desferrioxamine (BioVision, Milpitas, CA) and quantified by integration of the colored ferioxamine peak following HPLC separation.

Table 1 Summary of labile iron assays evaluated *in vitro*

Labile iron assay	Assay method	Approximate LOD[a]	Practical limitations	*In vitro* limitations
Rhodamine fluorescence Conversion	Redox active iron	30 μM Fe	Reaction product is very sensitive in ambient conditions and degrades rapidly.	Abolished signal in the presence of agent complex.
Bleomycin detectable iron (BDI)	Redox active iron	10 μM Fe	Multiple reagents and pipetting steps required may reduce accuracy. Narrow assay dynamic range (10-100μM).	Strong interference in the presence of agent complex.
Directly chelatable iron (DCI): FL-DFO	Chelatable iron	2 μM Fe	Narrow assay dynamic range (~2-~60μM).	Abolished fluorescence in the presence of agent complex.
HPLC-DFO	Chelatable iron	50 μM Fe[b]	Duration to complete analysis.	Apparent kinetic increase of labile iron upon incubation with DFO when agents are present (correctable using kinetic analysis to back-calculate labile iron at t = 0).

[a]The assay limit of detection (LOD) as employed was estimated in y as the intercept plus 3 times the standard error of the fit.
[b]Routinely achievable, sufficient for scope of work.

RESULTS

Of the four assays evaluated, only an HPLC-based chelatable iron assay that utilizes desferroximine as a chelator (HPLC-DFO) was considered viable for *in vitro* application. (**Table 1**). Although the other three assays demonstrated good performance with ferric chloride standard solutions, they all exhibited complete and prohibitive interference, when executed in the presence of the i.v. iron formulations themselves.

For the rhodamine conversion assay the reaction product (cationic rhodamine 123) is highly sensitive to ambient conditions and began to degrade rapidly at the completion of the study. In addition, incubation of the iron agent in the presence of 0.95 mg/mL of Ferrlecit produced a near complete attenuation of signal (**Supplementary Figure S1**). Thus, the presence of the i.v. iron formulation itself produces an interference causing a complete loss of detectable signal in the rhodamine conversion assay. Due to this interference, the rhodamine conversion assay is not viable for assessing labile iron in the presence of concentrations intended to simulate a predicted C_{max} of a 40 mg Fe/kg dose selected for potential application to IVIVC modeling.

When incubated with i.v. iron formulations, the BDI assay reported similar apparent mM levels of labile iron among the i.v. iron formulations studied. However, these findings were also viewed as suspect because of the minimal variation between the agents (**Figure 1**) and because the response was inconsistent with the assessed labile iron levels for the agents observed by the other assays in this study, in particular the HPLC-DFO assay. To test whether the i.v. iron formulations interfered with the assay, an experiment was conducted using single-stranded DNA consisting of two test groups: 5–500 μM Iron (III) Chloride and 5–500 μM Iron (III) Chloride in the presence of 0.95 mg/mL of Ferrlecit. As seen in **Figure 2**, incubation of iron in the presence of a 40 mg/kg equivalent of Ferrlecit produces a significant attenuation of the percent fluorescence. Furthermore, this effect was seen in the reaction tube and not the 0% and 100% fluorescence control samples, showing that the attenuating effect of the i.v. iron formulations in this assay is a result of a direct effect of the formulations on the bleomycin reaction responsible for DNA degradation causing effects on fluorescence output of the

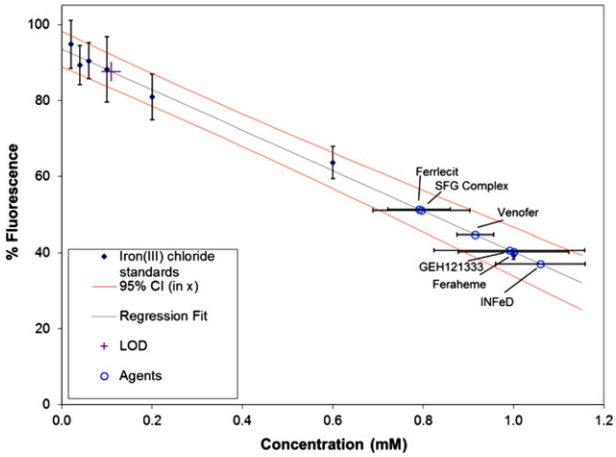

Figure 1 BDI assay calibration and agents. A representative calibration curve generated using 2 mg/mL DNA in PBS at 37°C is shown (blue diamonds) for iron(III) chloride standards ranging in concentration from 20 μM to 1 mM. Example readings of the six agents are also shown for i.v. iron formulations as labeled at concentrations of 0.95 mg Fe/mL.

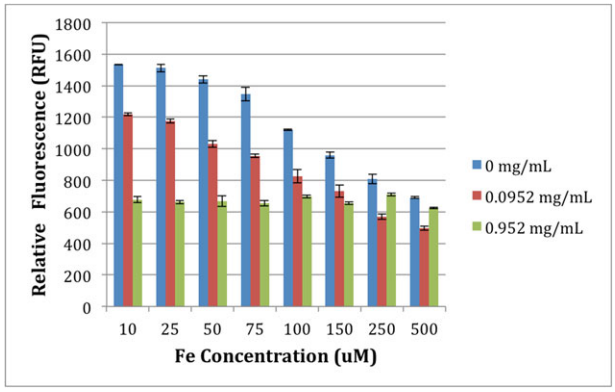

Figure 2 BDI Assay in the presence and absence of Ferrlecit. Incubation of iron (III) chloride in the absence of Ferrlecit (blue) as compared with incubation in the presence of 0.095 mg/mL and 0.95 mg/mL concentrations of Ferrlecit. An attenuation of fluorescence signal attributable to labile iron is observed with increasing Ferrlecit agent concentration, suggesting assay interference.

assay. While the mechanistic nature of this interfering reaction was not studied, the BDI assay is therefore shown to not be suitable for measurements of samples in which unknown concentrations of i.v. iron formulations may be present.

Calibration of the DCI assay was performed with Iron (III) Chloride Hexahydrate. No significant change in fluorescence of samples treated with Reagent A was seen in concentrations greater than 64.6 μM or less than 1.5 μM. Furthermore, samples treated with Reagent B showed no decrease in fluorescence intensity at concentrations $<$500 μM (**Supplementary Figure S2**). However, a significant reduction in Reagent B fluorescence occurred following incubation of i.v. iron formulations in serum and in phosphate-buffered saline (PBS) samples. While these findings suggest a significant interference, an interaction of the i.v. iron formulation with a serum component is unlikely, as a similar effect was seen in both PBS and fresh rat serum. A possible explanation is the presence of large amounts of labile iron in i.v. iron formulation incubated samples, which quickly saturates the nonfluorescent DFO present in Reagent B. To test this, we increased the nonfluorescent DFO 5-fold in Reagent B; however, no effect was observed on the apparent inhibition of fluorescence, suggesting that this is not due to labile iron in excess of the unlabeled DFO pool in Reagent B. Therefore, these experiments strongly suggest an interaction between the Fl-DFO and the i.v. iron formulation is capable of quenching Fl-DFO fluorescence, making impossible the measurement of labile iron in the presence of the i.v. iron formulation. Optical absorbance by the i.v. iron formulation at the measured wavelengths (485 nm) may be an additional factor in the interference. These findings demonstrate that the DCI assay is not suitable for *in vitro* samples containing i.v. iron formulations, and provided the stimulus for application of an HPLC separation method to avoid readout interferences caused by the presence of the iron formulations.

Representative HPLC-DFO response curves for $FeCl_3$ in saline and in rat serum are shown in **Figure 3**. This assay as implemented also demonstrated the greatest assay dynamic range ($\sim$50 μM to at least 2 mM) of the assays tested in this study. The assay was not optimized in this work to minimize the LOD given that the labile iron of the agent samples in this study were in the hundreds of μM range; indeed a sub-μM LOD has been reported for a similarly implemented assay showing further improvement in LOD may be possible.[20] However, the LOD was routinely achievable and more than sufficient for the scope of work described. Accuracy of the HPLC-DFO assay was assessed in several ways. First, the calibration standards were run in triplicate to allow determination of the coefficient of variance (CV, as the ratio of SD to mean) as a function of iron concentration after collection of each calibration curve. Typically, in both saline and serum the CV was less than $\sim$2% for iron standard concentrations greater than 500 μM. Between 100 and 500 μM, the CVs were typically $<$10%. At and below the LOD ($\sim$50 μM), the CVs increased to $\sim$50–100%. As a second assessment of accuracy and repeatability, a one-time experiment tested repeated measures of a 500-μM spike sample in rat serum. For 12 repeated measures over 2 days, the average measured was 491 $\pm$ 33 μM ($\mu \pm$ SD) for a recovery of 98.3% and a CV of 6.7%. There was no apparent trend in these repeated measures over the course of the experiment, suggesting that kinetic effects following incubation of free iron with DFO are negligible.

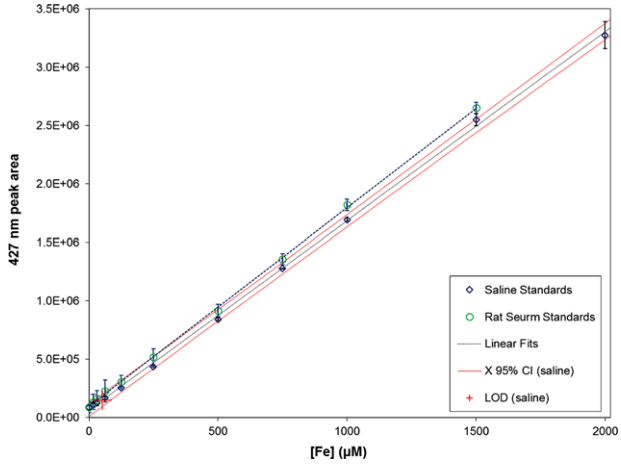

Figure 3 Representative Fe-DFO HPLC response curves for $FeCl_3$ in saline and in rat serum. Plots of the Fe-DFO peak area at 427 nm following triplicate HPLC analysis vs. input iron concentration were linear with $R^2 > 0.999$ and were comparable for 150 mM saline and for rat serum. Red lines represent 95% confidence intervals (CIs) of iron concentration for given HPLC response measurement, and the red plus represents the LOD for the calibration (both shown only for saline for clarity; similar CIs and LOD were determined for serum). The linear regression equations for saline was 427 nm peak area $=$ 1620([Fe] (μM)) $+$ 65595 with an $R^2 = 0.9994$, and for rat serum was 427 nm peak area $=$ 1699([Fe] (μM)) $+$ 99610 with an $R^2 = 0.9996$. The subtle difference in the calibration line slope is likely attributable to HPLC performance differences over a period of months between collection of these examples; standard calibration curves were generated concurrently for use with every run of sample batches.

When the i.v. iron formulations were tested, each showed an increase in Fe-DFO peak area as a function of incubation time in the presence of DFO. A similar, time-dependent increase in Fe-DFO signal was not observed after a 3-h incubation at ambient temperature when $FeCl_3$ was used as the iron source. Thus, it was hypothesized that the continuing increase in Fe-DFO peak area as a function of incubation time was due to a kinetic release of labile iron from the i.v. iron formulations.

To test this hypothesis, i.v. iron formulations were diluted as described above into either saline or serum and incubated at ambient temperature for either 15 or 180 min in the absence of DFO (**Figure 4a,b**). Once this initial preincubation was complete, DFO was then added and the resulting solutions were repeatedly analyzed by HPLC at longitudinal timepoints. The resulting natural logarithm transformed labile iron concentrations vs. time data were fit by linear regression (**Figure 4a,b**). This regression permitted estimation of the concentration of labile Fe at t $=$ 0 (y-intercept) because it is not technically feasible to instantaneously measure the labile iron for each i.v. iron formulation present upon addition of DFO (t $=$ 0) (**Table 2**). Incubation of the i.v. iron formulations in saline or serum for 15 or 180 min allowed for identification of kinetic release of iron in the absence of DFO in either medium. Our results show that there is negligible difference between

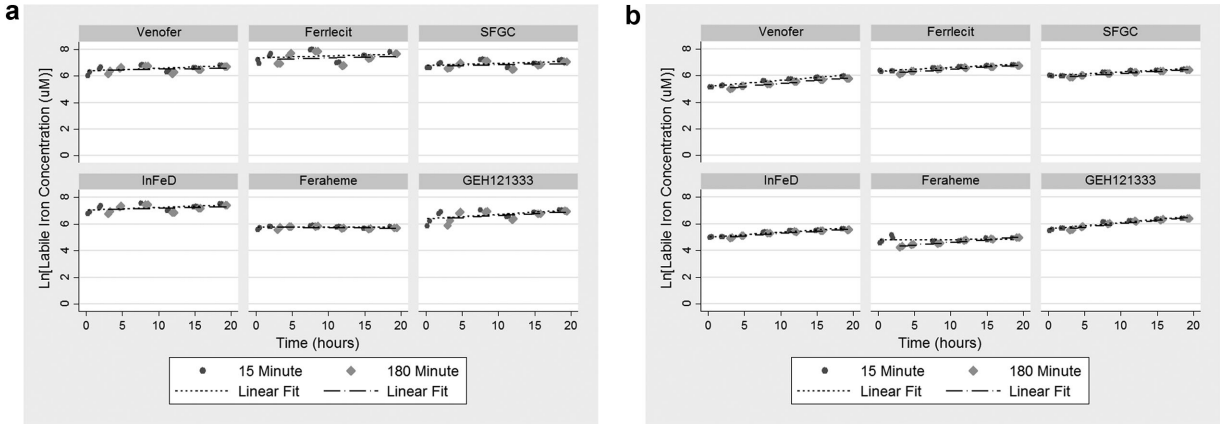

Figure 4 Natural logarithm of the Fe-DFO peak area as a function of time following addition of DFO and linear regression analyses for i.v. iron formulations (0.952 mg/mL) incubated in 150 mM saline (**a**) or rat serum (**b**) for 15 or 180 min prior to the addition of DFO.

Table 2 Chelatable iron concentrations following preincubation in either 150 mM saline or rat serum

		150 mM saline		Rat serum	
IV iron formulation	**Preincubation time[a]**	**[chelatable Fe] (μM)**	**± 95% CI**	**[chelatable Fe] (μM)**	**± 95% CI**
Ferrlecit	15 min	959	120	595	23
	180 min	756	42	514	27
SFG Complex	15 min	616	17	411	19
	180 min	549	15	378	15
INFeD	15 min	801	46	155	11
	180 min	835	29	151	8
Venofer	15 min	392	33	138	23
	180 min	397	18	80	4
Feraheme	15 min	220	13	278	24
	180 min	236	18	268	21
GEH121333	15 min	347	82	174	8
	180 min	531	43	148	7

[a]Iron concentrations were determined using the calculated Fe-DFO peak area at t = 0 from linear regression of the Fe-DFO peak area as a function of time following addition of DFO.

the fits for the solutions incubated for either 15 or 180 min prior to the addition of DFO for all agents tested (**Figure 4a,b**). The absence of differences at t = 0 suggests that additional iron is not released by any of the commercial i.v. iron formulations in the absence of DFO chelator for the conditions tested here (i.e., 0.95 mg/mL agent concentration, tested over a 3-h time frame). While the data for GEH121333 in saline suggest there may be an increase in the chelatable Fe concentration, the difference in the linear fits as a function of incubation time prior to DFO addition is likely due to the nonlinearity of the labile iron concentration at <3-h incubation times with DFO (**Figure 4a**). While the reason for the observed nonlinearity is not known for GEH121333, exclusion of the data for the <3-h timepoints as recommended yields little if any difference as a function of incubation time and suggests that additional iron is not released following dilution into 150 mM saline on the time scale studied. A similar analysis was also conducted following dilution of the i.v. iron formulations in rat serum. Similar to the results described above, little to no difference was observed at t = 0 as a function of incubation time without DFO, suggesting the absence of a kinetic release upon incubation in serum for up to 3 h (**Figure 4b**) Thus, the HPLC-DFO assay does not exhibit any apparent issues with interference from i.v. iron formulations *in vitro*. A representative HPLC chromatogram, collected with 1 mM FeCl3·6H2O, is shown in **Figure 5**.

DISCUSSION

Clinical use of i.v. iron colloidal suspension formulations began in the late 1950s, which preceded the nanomedicine exploration frontier.[4] Considering the rising use of i.v. iron formulations for a number of chronic diseases in the context of a cost-constrained healthcare environment, it is reasonable to speculate that use of generic i.v. iron formulations will increase as they become available.[1] The complexity of i.v. iron formulations differentiates them from traditional small molecules and as such they have been described as "nonbiologic complex drugs" by non-US regulatory groups.[21] Based on experience from generic formulations outside the US, creating an exact copy of the RLD is challenging.[4] Thus, it is important to improve and sophisticate analyses to evaluate abbreviated new drug applications (ANDAs) for these products.

The relevance of potential reduced stability profiles of generic i.v. iron formulations is related to formulation-based

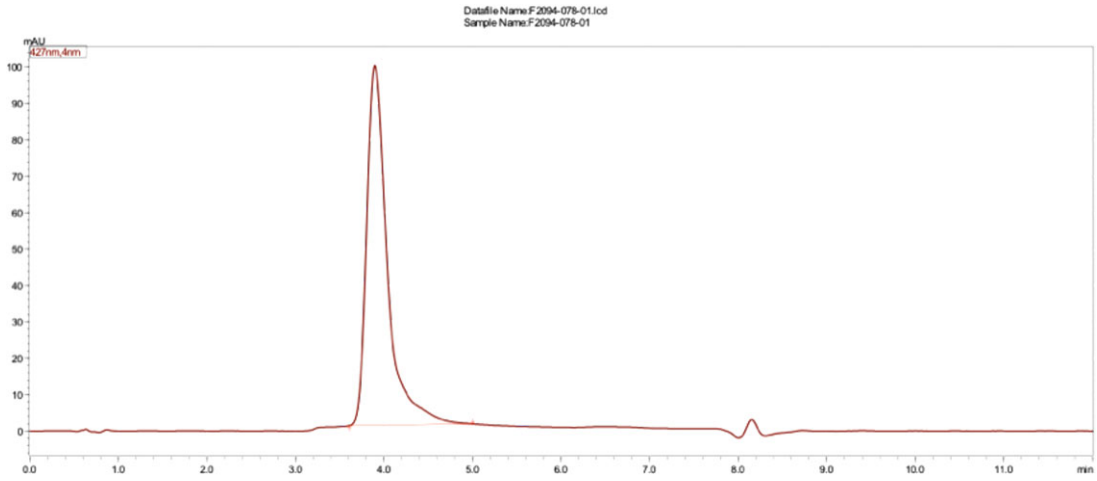

Figure 5 Representative HPLC chromatogram at 427 nm. The peak at 3.9 min is the Fe-DFO chelate.

labile iron release after administration. Among available i.v. iron formulations, products with smaller particle sizes are more labile and more likely to release labile iron directly into the plasma (i.e., before metabolism by RES).[6] We observed that the smallest molecular weight formulations (Ferrlecit and SFGC) had higher labile iron release profiles *in vitro*. The hypothesis for the pathogenesis of acute oxidative stress induced by intravenous iron formulations is the direct release of iron from the iron–carbohydrate structure resulting in transient concentrations of labile plasma iron. Labile iron can participate in Fenton chemistry and the Haber–Weiss reaction promoting formation of highly reactive free radicals such as the hydroxyl radical.[22] The proposed biologic targets of labile-iron-induced oxidative stress may include systemic cellular components including endothelial cells, myocardium, liver, as well as low-density lipoprotein and other plasma proteins. An additional concern regarding appearance of labile plasma iron is the potential for easily accessible iron to augment bacterial growth and increase the risk of infection.[23]

As we have investigated and confirmed in our *in vitro* analyses in a biorelevant matrix (rat serum), labile iron release profiles differ among available i.v. iron formulations. We have shown that the measured labile iron concentrations for most formulations were notably lower in the rat serum matrix vs. saline, with the lone exception being Feraheme. Spike recovery data presented show that recovery of free iron in serum is complete for our assay conditions, suggesting that these observed reductions in measured labile iron in serum may be a result of stabilization of the colloidal dispersions by the presence of serum proteins. It is important to acknowledge that although some non-US generic i.v. iron formulations may not be differentiable based on certain physicochemical characteristics and may have met pharmacopeia criteria, they may potentially nonetheless still appear to exhibit differential toxicity profiles *in vivo*.[8,9] Thus, it is important to establish comprehensive physicochemical characterization including labile iron release and the existing data in the literature considered in tandem with an IVIVC model would be useful to further inform bioequivalence of i.v. iron formulations filing ANDAs.

In our analyses, the HPLC-DFO chelatable labile iron assay performed better than the other assays when tested at higher concentrations of i.v. iron formulations, and provided the widest dynamic range of the assays tested. Addition of the HPLC-based separation step in this new assay format eliminates potential confounding of response by the presence of the nanoparticulate iron formulations and/or media components that are otherwise present in previously reported incubate-and-read DFO chelation assay formats. Labile iron concentrations were detected by the bleomycin detectable iron assay *in vitro*; however, interference in the presence of the agents is problematic. Other studies have used the bleomycin detectable iron assay to determine labile iron concentrations in *ex vivo* spiked rat serum samples.[7] However, the doses utilized in those experiments were much lower and necessitated a several-fold dilution that is far greater than what is recommended by the prescribing information. This can compromise the stability of the i.v. iron formulation.[24] Although typical clinical i.v. push doses for the various i.v. iron formulations range from 2–15 mg/kg, this would require substantial dilution for *in vitro* analysis and would not be representative of the formulations stability profile when administered to patients undiluted by i.v. push.[25,26] Our approach to the doses used in this study minimized the need for dilution and would be sufficient to determine a release profile *in vivo* over time. The rhodamine fluorescence conversion and fluorescence-based directly chelatable iron assays were determined not to be viable for *in vitro* analysis due to reduced or no signal in the presence of high concentrations of the i.v. iron formulations. Among four assays evaluated to detect labile iron *in vitro*, the HPLC-based DFO chelatable iron assay was considered most viable for potential use to evaluate comparative labile iron release from i.v. iron formulations.

There are several limitations of the current study. Our evaluation of available *in vitro* labile iron assays may not have been exhaustive, and in particular we considered evaluating an additional reported chelatable iron assay utilizing the metalosensor calcein[27] to detect labile iron. However, this assay could not be tested as part of this study because the

key reagent, a calcein–iron complex, is no longer available commercially. An additional limitation is that we only examined single lot of each product studied. There have been data suggesting lot-to-lot variations in the physicochemical characteristics and emergence of clinical adverse events.[8,23] The only US Food and Drug Administration approved generic product (SFGC) was studied in this series of experiments. It would be advantageous to test other non-US generic i.v. iron formulations available in the global market to further evaluate *in vitro* labile iron release and to inform further IVIVC development.

In summary, published assay methodologies to detect labile iron have limitations with regard to equivalence evaluation of RLD and generic products. This necessitates additional evaluation of these formulations in biorelevant matrices *in vitro* and *in vivo*. This is the first study to evaluate all of the commercially available i.v. iron formulations including the only Food and Drug Administration approved generic product to quantitate labile iron release *in vitro*. We determined that an HPLC-DFO chelatable labile iron assay performed optimally *in vitro* with relevant concentrations of i.v. iron formulations diluted in rat serum designed to simulate maximal plasma concentrations *in vivo*. This assay also provided the widest dynamic range of the assays tested. Thus, future IVIVC modeling efforts will benefit from using this new assay approach to compare labile iron release from i.v. iron formulations *in vitro*.

Acknowledgments. Disclosure Funding for this presentation was made possible, in part, by the Food and Drug Administration through grant 1U01FD004889-01.

Author Contributions. A.B.P., D.E.M., B.A.B., V.E.C., M.P.P., W.J., and N.Z. wrote the article; A.B.P., D.E.M., designed the research; B.A.B., V.E.C., performed the research; A.B.P., D.E.M., B.A.B., V.E.C., M.P.P., W.J., and N.Z. analyzed the data.

Conflict of Interest. Views expressed in written materials or publications and by speakers and moderators do not necessarily reflect the official policies of the Department of Health and Human Services, nor does any mention of trade names, commercial practices, or organization imply endorsement by the United States Government.

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Supplementary information accompanies this paper on the *Clinical and Translational Science* website. (http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1752-8062)