Protein Isolated from Biopharmaceutical Formulations Cannot be Used for Comparative Studies: Follow-up to "A Case Study using Epoetin Alfa from Epogen and EPREX"

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ABSTRACT: In the biotechnology area, the issue of comparability with an innovator product is complex. Ideally, a side-by-side comparison of physical properties would be part of the demonstration of comparability. However, biogeneric companies do not have access to the bulk drug substance from the innovator company for biophysical comparison, and isolation of protein from marketed product cannot be guaranteed to produce material that is identical to the bulk drug substance from which it was prepared. In a recently published study, protein was isolated from marketed product and comparative studies performed. In a follow-up investigation of the published work, we demonstrate here that even a simple isolation procedure can significantly compromise the protein, which raises serious questions about the interpretation of that study, and in a broader context the value of any studies done with such "out-of-process" protein.
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INTRODUCTION

The expansion of the market for protein therapeutics shows no signs of abating. Over the past three and a half years, North American and European Regulatory Agencies have approved at least 32 biopharmaceuticals for human use.[1] As this market matures, it creates the potential for the introduction of biosimilars, a term referring to an off-patent biological medicinal product produced by a manufacturer other than the originator which is similar, but not identical, to the originator product.[1] This raises the issue of how to determine the degree of comparability of a biosimilar with the innovator protein.

The FDA approved Omnitrope® as a "follow-on protein product" of the non-glycosylated growth hormone Genotropin®. It was defined as a "follow-on protein product" rather than a generic substitute for Genotropin[2] since a generic is defined as being bioequivalent to the originator product and two medicines are bioequivalent only when they contain the same amount of an identical active moiety.

It should be stressed that it is not possible for another manufacturer to duplicate the original production process of the innovator,[2] thus the terms 'generic biosimilars' or 'generic biopharmaceuticals' are inappropriate. This point has been emphasized in the 'Guidelines on Similar

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Biological Medicinal Products' that has been recently released by the European Medicines Agency, in which they state, 'by definition, similar biological medicinal products are not generic medicinal products.'[3]

It is accepted that new manufacturers will need to ensure that their biosimilar product has a similar efficacy and safety profile to the innovator product through more extensive clinical trials than the limited testing required for generic versions of low molecular weight chemical drugs. In developing the characterization profile of the biosimilar bulk drug substance, it is generally acknowledged that the biosimilar manufacturer would not have access to the innovator bulk drug substance, their validated test methods and other necessary information that would allow an exhaustive comparison with the innovator product.[3]

A source of the originator bulk drug substance would be of great value to a biosimilar company. Deechongkit *et al*. recently published a paper, Biophysical Comparability of the Same Protein from Different Manufacturers: A Case Study using Epoetin Alfa from Epogen® and Eprex®,[4] in which they attempted to recreate EPREX bulk drug substance by isolating it from EPREX formulated product, obtained on the open market. The procedure used for the isolation of the protein from the formulated product as well as Epogen bulk drug substance in an EPREX-like buffer used a commercially available ion exchange column, eluted with an apparently innocuous buffer (a sodium chloride gradient in 20 mM Tris buffer at pH 8.4). The subsequent comparative biophysical characterization studies (AUC, CD, UV and fluorescence) suggested conformational differences between the proteins from these two manufacturers. The work presented here shows that some of their chromatographic results are artifactual. Further, the isolation process itself irrevocably compromised the isolated proteins, rendering them different from the bulk drug substances and thereby making any subsequent comparative studies of questionable value. We also specifically discuss how the problems created by the extraction procedure may have significantly altered their data and its interpretation.

We have reproduced part of their work, the analysis of protein purified from EPREX formulated product. Subsequent biophysical characterization studies of EPREX bulk drug substance put through an analogous isolation method have shown that the resultant protein displays significant differences relative to EPREX bulk drug substance when compared to an appropriate control sample. In this paper, we present a portion of this work that demonstrates that the seemingly innocuous isolation procedure has altered the protein and generated artifactual results. We have purposely not included Epogen or any other erythropoietin from another company in our studies because of the inability to run proper controls due to the lack of access of the corresponding bulk drug substances. Also it is our position that it is inappropriate to isolate protein from commercial product from the market for comparative studies.

EXPERIMENTAL

Materials

Four samples (with multiple replicates of each) were analyzed in this study. They include EPREX bulk drug substance, EPREX formulated product, and protein isolated from both EPREX bulk drug substance and EPREX formulated product using HiTrap columns. EPREX bulk was obtained from Ortho Biologics, Inc., Manatí, Puerto Rico. EPREX prefilled syringes were obtained from Janssen-Cilag, Schaffhausen, Switzerland. HiTrap columns were purchased from GE Healthcare. Reagents were obtained from J.T. Baker and were the highest quality available.

Protein Isolation

A HiTrap Q HP (1 mL) column was prepared by passage of 10 mL of 10 mM Tris, pH 6.4 (buffer A) through the column, followed by 10 mL of 10 mM Tris, 1.0 M NaCl, pH 6.4, and the column was then re-equilibrated in buffer A. Samples were diluted to ~8 μg/mL with buffer A and loaded onto the HiTrap Q HP column at a flow rate of 1 mL/min. The column was washed with 10 mL of buffer A, followed by a single step elution with 10 mM Tris, 250 mM NaCl, pH 6.9. Fractions of 1 mL were collected and each fraction scanned by UV from 240 to 320 nm to isolate the individual fractions containing all eluted EPO molecules. Fractions containing EPO were pooled and dialyzed three times against 100 equivalent volumes of 20 mM citrate, 100 mM NaCl, pH 6.9 (CBS) at 2–8°C. The final dialysis buffer was retained as a blank for all subsequent sample analysis.

HPLC Analysis

Analysis of samples by HPLC was performed according to the method of Deechongkit[4] using a Dionex AS500 metal-free HPLC system equipped with an AD20 UV detector set at 280 nm. Additional analysis was done with the same gradient profile but using 10 mM Tris, pH 6.9 (buffer A) and 10 mM Tris, 200 mM NaCl, pH 6.9 (buffer B).

Analytical Ultracentrifugation

Two replicate aliquots were taken from each sample. Samples at a concentration of 0.5 mg/ml were loaded into cells with two-channel charcoal-epon centerpieces with 12 mm optical path length. The dialysate was loaded in the reference channel. Those loaded cells were then placed into an AN-60Ti analytical rotor, loaded into a Beckman Optima XL-A analytical ultracentrifuge, and brought to 20°C. The rotor was then brought to 3000 rpm and the samples were scanned at 279 nm to confirm proper cell loading. The rotor was then brought to the final run speed of 60000 rpm. Scans were recorded at this rotor speed approximately every 4 min for ~5.5 h (80 total scans for each sample).

The data were analyzed using the $c(s)$ method developed by Peter Schuck at the NIH and implemented in his analysis program SEDFIT (Version 8.9).[5] In this approach many raw data scans are directly fitted (~33000 data points for each sample in this case) to derive the distribution of sedimentation coefficients, while modeling the influence of diffusion on the data in order to enhance the resolution. The method works by assigning a diffusion coefficient to each value of sedimentation coefficient based on the assumption that all species have the same overall hydrodynamic shape (with the shape defined by the frictional coefficient ratio relative to that for a sphere, f/f_0). A maximum entropy regularization probability of 0.683 (1σ) was used; removal of time-invariant noise was not employed.

A solvent density of 1.00555 g/ml and viscosity of 1.0316 cp at 20°C were calculated using the program SEDNTERP by John Philo, David Hayes, and Tom Laue.[6] Together with the published partial specific volume for EPO[7] of 0.698 ml/g the ratio of standardized ($s_{20,w}$) to raw sedimentation coefficients was then calculated as 1.0472.

Ultraviolet Spectroscopy

Two liters of 10 mM Tris, pH 6.9 and 2 L of 20 mM Tris buffer, pH 8.4 were prepared. Two EPREX bulk drug substance solutions were prepared by diluting (1:15) EPREX bulk drug substance with each of the Tris buffers to a final volume of 6.0 ml at an approximate concentration of 0.2 mg/ml. Weighed aliquots (ca. 4 ml) of the samples were loaded into 10000 MWCO Slide-a-lyzer cassettes (Pierce) and dialyzed against 500 mL of the corresponding Tris buffers. Three buffer exchanges were performed over 48 h, and the final dialysis buffers were retained for use as blanks for both samples and protein concentrations determined. Final dialysis volumes were obtained by weighing the recovered material and calculating volume based on the density of water corrected for the temperature of the laboratory. Data was obtained on a Beckman DU 7500 spectrophotometer using a 10 mm path length cell from 230 to 360 nm to encompass the aromatic and light scattering regions of the protein spectrum. UV/Vis concentration determinations were performed at 280 nm using the extinction coefficient $\varepsilon_{0.1\%} = 0.743$[7,8] in citrate buffered saline at pH 6.9. Data was converted to ASCII format for off-instrument plotting. The curve of the pH 8.4 sample was adjusted to have identical 280 nm absorbance to the pH 6.9 sample for comparative purposes.

RESULTS

The protein from EPREX bulk drug substance and material expressed from EPREX prefilled syringes was isolated using HiTrap Q HP columns. The concentration of the isolated protein was determined using the absorbance at 280 nm.

The high-resolution sedimentation coefficient distributions for the samples are shown in Figures 1–3. These graphs are much like chromatograms, with the vertical axis giving the concentration and the horizontal axis showing the separation based on the sedimentation coefficient. Each distribution has been normalized to account for the minor concentration differences among the samples, with the total area under the curve set to 1.0 so that the area for each peak gives the fraction of that species. All of these graphs have the same scale to facilitate comparison. The inset in each graph shows the same data at a 100-fold expanded vertical scale so that the minor peaks can be seen.

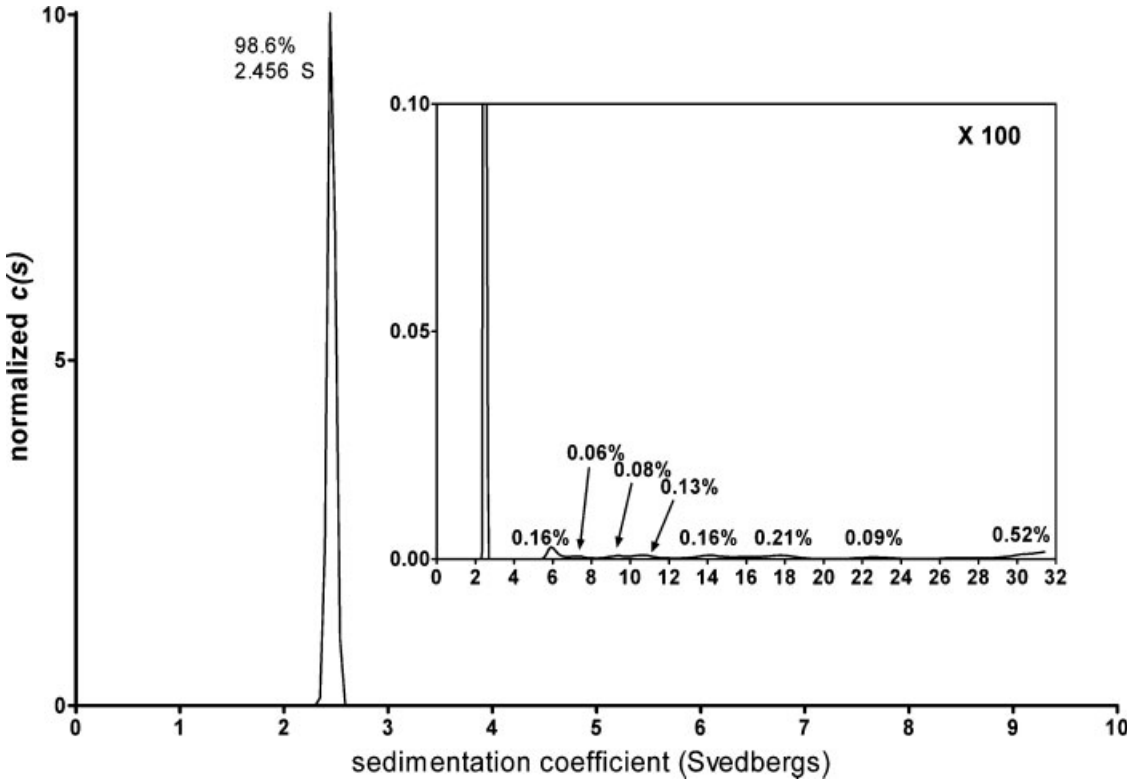

Figure 1. Normalized sedimentation coefficient distribution obtained from the first aliquot of EPREX bulk drug substance showing a main peak at 2.456 S corresponding to 98.6% of the total area. The insert is a 100-fold expansion to show the levels of aggregates.

Distribution graphs are shown for one of the two aliquots of each sample.

Figure 1 shows the distribution of EPREX bulk drug substance. It contains a total of 1.4% aggregates with the main peak at 2.456 S. The sedimentation coefficient distribution for the protein resulting from the HiTrap processing of EPREX bulk drug substance (Fig. 2) has its main peak at an equivalent position (2.458 S). However, the percent of aggregates has increased from 1.4% to 3.6%, with the dimer being the predominant species at 1.1%. Figure 3 is the distribution graph of the protein resulting from the HiTrap processing of EPREX manufactured product. The main peak is at 2.454 S and the total percent of aggregates is 3.5% with the predominant species being the dimer at 1.2%. These numbers are in close agreement with those obtained for the protein processed from EPREX bulk drug substance (Fig. 2). Two aliquots were taken from each sample. Figures 1–3 represent the results from one set of aliquots. The results for the individual sedimentation coefficients and aggregate contents for both sets of aliquots are given in Table 1. For all samples, there is good agreement between the two aliquots.

Knowing that the HiTrap processing method resulted in a higher level of aggregates in the isolated protein, we examined the differences between the HiTrap procedure presented here and the Deechongkit *et al*. procedure. Their method uses a pH 8.4 buffer while we used a pH 6.9 buffer. Aliquots of EPREX bulk drug substance, which was not available to Deechongkit *et al*., were dialyzed into either Tris buffer at pH 6.9 or Tris buffer at pH 8.4. The recovery of the two samples was determined to be 95% at pH 6.9 (835 μg from 875 μg) and 94% at pH 8.4 (792 μg from 845 μg) at the end of the dialysis procedure. UV spectra were collected on both samples and the pH 8.4 spectrum was normalized to the absorbance of the pH 6.9 sample at 280 nm for comparative analysis. Figure 4 shows that the different pH buffers result in non-identical spectra, particularly in the region above 300 nm. The pH 8.4 sample has a higher absorbance above 300 nm than the pH 6.9 sample. This increase above 300 nm in the pH 8.4 buffer is indicative of light scattering,

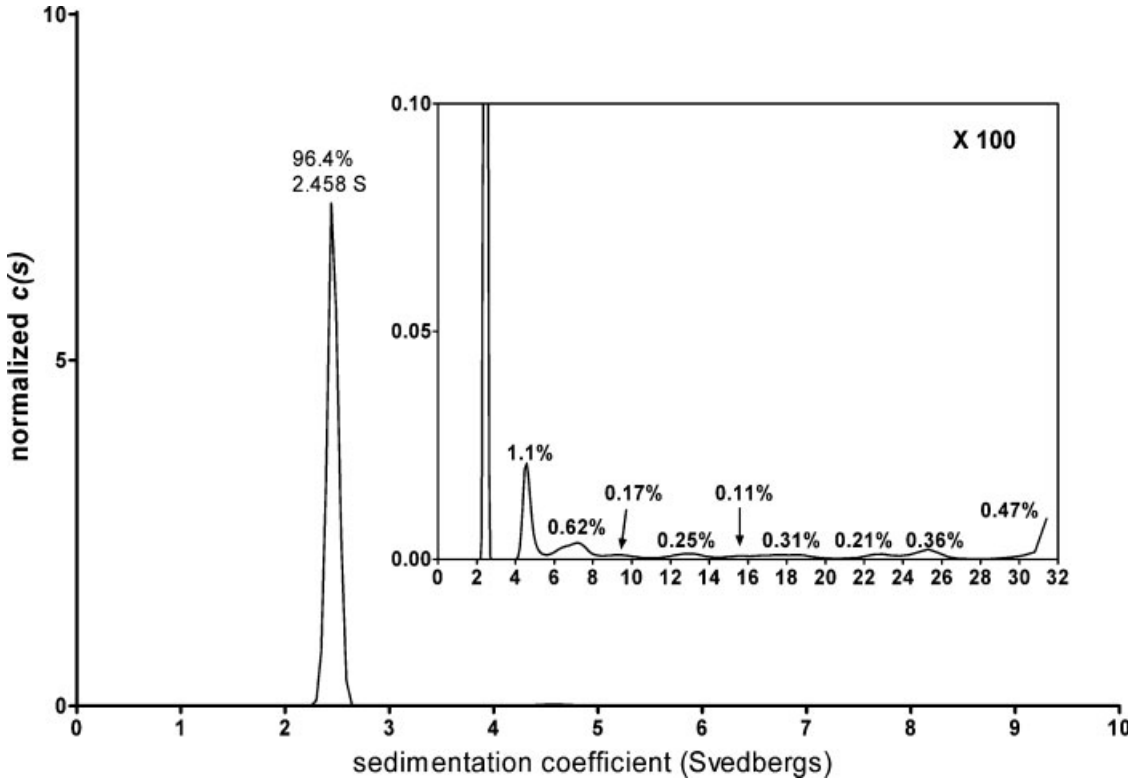

Figure 2. Normalized sedimentation coefficient distribution obtained from the first aliquot of the protein isolated from EPREX bulk drug substance showing a main peak at 2.458 S corresponding to 96.4% of the total area. The insert is a 100-fold expansion to show the levels of aggregates. The aggregate level has increased from 1.6% before isolation to 3.6% after isolation.

presumably from the formation of aggregates. Additionally, the absorbance maxima for the sample at pH 8.4 was blue-shifted to 282 nm in contrast to the sample at pH 6.9 with an absorbance maxima at 280 nm, indicative of changes in the environment of aromatic residues, perhaps associated with the observed aggregation at pH 8.4.

Replication of the chromatography conditions defined in the Deechongkit paper (20 mM Tris, pH 8.4) successfully reproduced the trailing peak (referred to as the "purified EPREX post peak") in the 140–200 mM NaCl concentration range of the gradient after the elution of erythropoietin in the 100–140 mM NaCl concentration range (Fig. 5, *red* chromatographic trace). When identical chromatographic conditions were used with EPREX bulk drug substance, this peak was not observed (Fig. 5, *blue* chromatographic trace). This trailing peak was also present when the EPREX formulation buffer was injected (Fig. 5, *black* chromatographic trace). This trailing peak is not observed if EPREX final product undergoes HiTrap processing at pH 6.9 rather than 8.4 (data not shown) or if EDTA is included in the analysis. Thus the extraction procedure at pH 8.4 appears to have produced a new peak, apparently resulting from some component in the EPREX formulation buffer and cations in the chromatographic system.

DISCUSSION

The approval of a human therapeutic is the culmination of years of work on the evaluation of its safety and efficacy, including exhaustive documentation on its physical/biophysical and chemical characterization and that its manufacturing process is reproducible. Numerous authors have pointed out the differences between small molecule drugs and biologics.[8,9,11] Small molecule drugs are low molecular weight compounds synthesized from standard reagents and chemi-

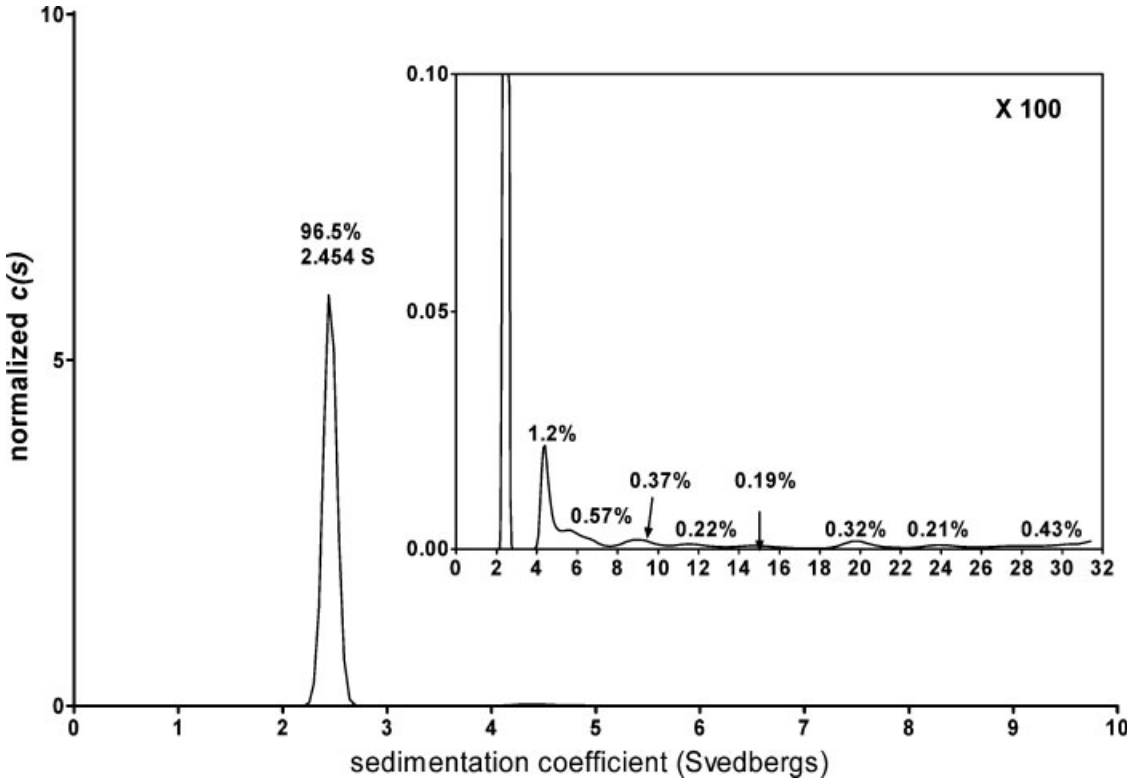

Figure 3. Normalized sedimentation coefficient distribution obtained from the first aliquot of the protein isolated from EPREX formulated product showing a main peak at 2.454 S corresponding to 96.5% of the total area. The insert is a 100-fold expansion to show the levels of aggregates. The aggregate level is 3.5% after isolation.

cals, whereas biologics are higher molecular weight materials produced by complex processes. The processes used for the preparation and purification of a protein determine its integrity; it is "process-defined." The same protein, subjected to different processes, will in general not be identical. While a protein therapeutic has a defined amino acid sequence, differences in its production can result in the same protein having subtle changes in glycosylation, impurities or aggregate levels, even though they demonstrate similar clinical efficacy. Such production differences can include factors such as changes in raw materials suppliers or specifications, media composition, cell culture conditions, pH, oxygen, temperature, time, scale of fermentation, changes in fermentation site or facility, column or resin changes, size of column, cleaning and storage conditions, purification protocols, addition, substitution or elimination of a specific process step and changes in the scale of the downstream processing.[12] Because of the multitude of

Table 1. Monomer Sedimentation Coefficient and Total Percent of Aggregates as Determined by Sedimentation Velocity

	Aliquot 1		Aliquot 2		Mean	
	$s_{20,w}$ (S)	% Aggregate	$s_{20,w}$ (S)	% Aggregate	$s_{20,w}$ (S)	% Aggregate
EPREX bulk drug substance	2.456	1.4	2.463	0.8	2.459	1.1
Protein extracted from EPREX bulk drug substance	2.458	3.6	2.459	2.6	2.459	3.1
Protein extracted from EPREX formulated product	2.454	3.5	2.463	3.6	2.458	3.6

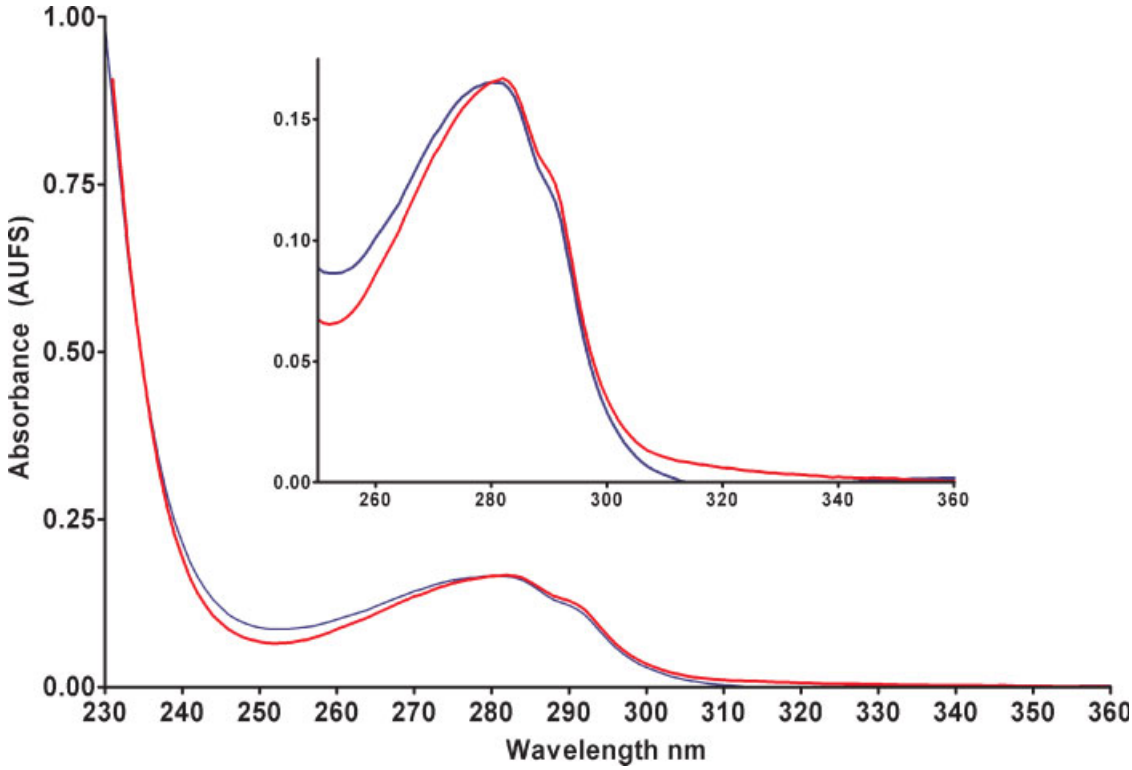

Figure 4. Ultraviolet spectra of EPREX bulk drug substance dialyzed into Tris buffers at pH 8.4 (*red*) and 6.9 (*blue*). Concentrations were determined independently and the pH 8.4 spectrum was normalized to match the absorbance maximum of the pH 6.9 sample at 280 nm. The inset shows the increase in absorbance in the 300–340 nm region of the pH 8.4 sample when compared to the pH 6.9 sample. This increase is attributed to light scattering that may be caused by aggregate formation.

variables to control, it is virtually impossible for two different companies, producing the same protein, to generate structures of absolute identity. Thus a major problem with the Deechongkit paper is the misconception that they are comparing "the same protein."

An ion exchange chromatography procedure, a HiTrap Q HP column, was used to isolate the protein from EPREX formulated product obtained from the market and this protein was compared to that isolated from Epogen bulk drug substance in an EPREX-like buffer using the same procedure.[4] The proteins isolated by this procedure can be termed "out-of-process" material since there is no *a priori* reason that they should be identical to "process-defined" protein. Even if the original purification process for EPREX bulk drug substance were used to isolate protein from EPREX formulated product, the resulting protein would not necessarily be identical to EPREX bulk drug substance since that process would not have been validated for removal of formulation excipients. Although erythropoietin is a robust molecule with good stability, as we have demonstrated here its treatment under "non-process" conditions can compromise its integrity.

Rationale for our Purification Protocol

While the Deechongkit group used a 20 mM Tris buffer at pH 8.4 and a linear gradient of sodium chloride for isolation of the protein from the HiTrap columns, we chose to use a 10 mM Tris buffer, a slightly lower pH (6.9) and a step gradient of sodium chloride. The choice of a pH of 6.9 was based on data showing that erythropoietin is more stable at 6.9 than at higher pH,[8] consistent with our in-house observations. A lower buffer concentration (10 mM Tris) was used to load the HiTrap column to favor retention of all potential binding species in the sample. The choice of a stepwise over linear sodium chloride

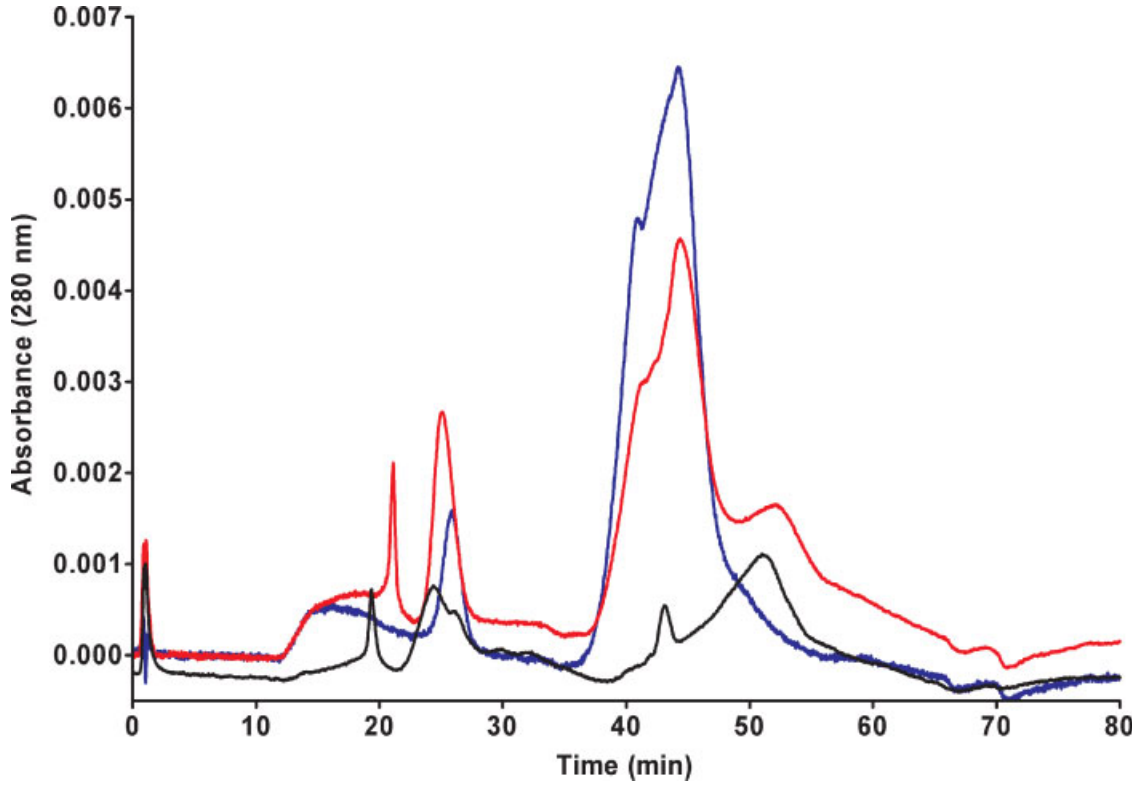

Figure 5. Hi-Trap elution profiles at 280 nm of EPREX bulk drug substance (*blue*), EPREX formulated product (*red*) and EPREX formulation buffer (*black*). The elution buffer is the Tris pH 8.4 buffer used by Deechongkit *et al*. The non-protein, post-erythropoietin eluting peak observed by Deechongkit *et al*. at ca. 50 min is present in EPREX formulated product and EPREX formulation buffer but not in EPREX bulk drug substance, indicating that its source is the formulation buffer.

gradient was to ensure that all materials were eluted and recovered from the column and to guarantee that no selective fractionation occurred which might enhance the appearance of aggregates or degradants that may form as a result of the isolation procedure. The elution step used 250 mM sodium chloride, a higher salt concentration than that used in the Deechongkit study, to maximize recovery of column-bound materials.

New Impurities are Created during EPREX Isolation at pH 8.4

An unidentified peak eluting after erythropoietin was detected in the 140–200 mM portion of the NaCl gradient by Deechongkit *et al*., which they termed the "purified EPREX post peak." This peak was determined to be non-proteinaceous.[4] Running the EPREX formulation buffer under their chromatographic conditions readily identified this peak as associated with the formulation and not the protein. Analysis of the formulation components revealed that this peak is an artifact of the Deechongkit method (Fig. 5). The fact that this peak was not observed in Ref. 4 for the Epogen that was extracted from an EPREX-like formulation proves that the components of that formulation were not equivalent to those in the commercial EPREX product samples.

Protein Quality is Compromised by the Isolation Procedure

While in our view it is inappropriate to isolate protein from commercial product for purposes of comparison, we repeated the isolation procedure using HiTrap Q HP columns to purify protein from several lots of EPREX product using a pH that is more appropriate for erythropoietin. We have confirmed that the HiTrap process does indeed

compromise the material when compared to EPREX bulk drug substance. UV scans of EPREX bulk drug substance dialyzed into either their 20 mM Tris buffer, pH 8.4 or our 10 mM Tris buffer, pH 6.9 (Fig. 4) showed an increased level of absorbance above 300 nm at pH 8.4, indicative of light scattering in the sample,[10,11] presumably due to aggregate formation. This indicates that EPO has a greater tendency to aggregate at pH 8.4, which may reflect the p$I = 8.4$ of the protein moiety, although the glycosylated EPO has a pI of 3–4. Narhi *et al*. also observed that the stability of EPO follows the pI of the protein moiety, since the stability increases as the pH approaches the pI of the protein moiety, not the whole protein.[12]

The fact that the isolation procedure induces aggregates that were not present in the bulk drug substance can also clearly be seen from our sedimentation velocity studies. This conclusion can only be made when the bulk drug substance from which the formulated product was produced is available for comparison. Figure 2 shows the sedimentation coefficient distribution for the protein that resulted from the HiTrap isolation processing of EPREX bulk drug substance at pH 6.9. The monomer sedimentation coefficient is unchanged from that seen for EPREX bulk drug substance (mean of 2.459 S for both samples) but the aggregate level was significantly increased from 1.4% before processing to 3.6% after processing. When the protein isolated from EPREX formulated product was examined using AUC, it showed a monomer peak at 2.454 S (mean of 2.458 S for both samples) with a total aggregate content of 3.5%. The lack of significant aggregate levels in EPREX bulk drug substance, coupled with the similarity in the aggregate profile and total aggregate levels shown in Figures 2 and 3 for the HiTrap processed samples strongly suggest that the isolation procedure using the HiTrap column is responsible for aggregate formation. The Deechongkit *et al*. AUC studies also show the formation of aggregates as a result of the isolation procedure (Figs. 2A and B from Ref. 4), although there is no quantitative data given on aggregate levels and no scale is given for the portion of the graphs where the aggregates are visible.

The net result of the HiTrap isolation procedure is material that is representative of neither EPREX bulk drug substance nor the protein in EPREX formulated product, rendering the results of any subsequent comparative biophysical characterization studies of questionable value, as we will discuss in detail next.

The use of Extracted Proteins may have Significantly Affected the Results and Interpretation in the Deechongkit Study

CD and Fluorescence Spectra

Our UV spectroscopy studies (Fig. 4) showed that the protein isolation procedure produces a significant increase in turbidity due to light scattering. The presence of significant light scattering can lead to an incorrect determination of protein concentration using the absorbance at 280 nm, which would affect the interpretation of both the CD and fluorescence studies. The lower CD and fluorescence intensity for the purified EPREX, but with an identical spectral shape, that was reported by Deechongkit *et al*. might be explained by an overestimated protein concentration for the purified EPREX. It is not possible to determine if this light scattering was seen in the published study and the appropriate correction was made[13] since their UV spectra (run in PBS at pH 6.9 after isolation in Tris at pH 8.4) terminated at 300 nm (Fig. 3, Ref. 4). Although it cannot be known for certain whether the protein concentration was overestimated due to light scattering, an error in protein concentration appears to be the most likely explanation for their observed lower CD intensity over the entire far-UV region. If the monomer has a different polypeptide structure, as they postulate, it would not cause a uniform decrease in intensity, but instead would alter the spectral shape because even a fully disordered structure has strong far-UV CD signals.

Effects on AUC Data and Interpretation

The Deechongkit study reports different sedimentation coefficients for EPREX- and Epogen-erythropoietin (2.44 S and 2.51 S, respectively), although it is unclear whether these are raw sedimentation coefficients or standardized $s_{20,w}$ values, without attributing the difference to any specific factor(s). They also report identical weight-average molecular weights for the various processed and unprocessed materials as determined by sedimentation equilibrium. However given the presence of different levels of aggregates in the samples (most notably the differences between Epogen bulk drug substance and HiTrap-processed Epogen), the molecular weights as determined by sedimentation equilibrium analysis should not be identical, and in particular the value for HiTrap-processed Epogen should be

higher than the reported monomer value of 30.4 kDa.[7] Even if all four samples studied in the Deechongkit paper were completely homogeneous (aggregate free), one would expect to see random variations in the molecular weight values, which are not evident (Table 1 in Ref. 4). No error bars were given for these sedimentation equilibrium results, and it is unclear how many samples of each protein stock were examined. The original exhaustive sedimentation equilibrium study of EPO by Yphantis and co-workers[7] was only able to measure its molecular weight with an uncertainty of ±400 (±1.3%). A study of stem cell factor using three protein samples and three rotor speeds, and the same data analysis software used by Deechongkit *et al*., gave the molecular mass with an uncertainty of ±1.8%.[14] Thus, it is unlikely that the precision of their sedimentation equilibrium data is better than ±1.5%, and it could easily be a factor of two to three worse. Therefore it is unclear whether the reported difference of 2.9% between the sedimentation coefficients for the monomer from extracted Epogen and extracted EPREX is really indicative of differences in conformation or differences in monomer molecular mass (due to differences in glycosylation).

Deechongkit *et al*. used their AUC data to derive hydrodynamic radius values for these samples with a stated precision of ±0.01 nm (±0.3%). However that stated precision apparently assumes zero uncertainty in the molecular mass (hydrodynamic radius values derived from sedimentation coefficients cannot be more accurate than the uncertainty in buoyant molecular mass). Since they used the measured monomer sedimentation coefficient to calculate the hydrodynamic radius, it is the monomer buoyant mass that must be used in calculating the hydrodynamic radius. Therefore unless their sedimentation equilibrium data have a precision better than 2.5%, and unless those data can clearly be demonstrated as measuring the monomer mass, their conclusion that the hydrodynamic radius of EPREX is 2.5% larger than that for Epogen is not actually supported by their AUC data. Further, these hydrodynamic radius values appear to have either been calculated incorrectly or to have assumed some different (unstated) value for the monomer mass or partial specific volume. For example, a $s_{20,w}$ value of 2.44 S for extracted EPREX monomer, molecular mass of 30.4 kDa, and partial specific volume of 0.68 mL/g gives a hydrodynamic radius of 3.52 nm (calculated by SEDNTERP[6]) rather than 3.24 nm as they report (and the other values in their Table 1 appear to be underestimated by a similar factor).

A further important point is that for any comparison of hydrodynamic or other biophysical properties of heavily glycosylated proteins such as EPO it is essential to make a distinction between differences in polypeptide structure versus carbohydrate structure. From Figure 6A (capillary electrophoresis of erythropoietin samples) and Figure 6B (ion exchange chromatography of carbohydrate analysis of erythropoietin samples) in the Deechongkit paper it is clear that there are carbohydrate differences between Epogen and the protein isolated from EPREX manufactured product. Although they refer to the carbohydrate profiles as "similar", clearly similar does not equate with identical. Variations in carbohydrate content and structure would be expected to result in different frictional coefficients, and hence different sedimentation coefficients, since the frictional coefficient is quite sensitive to disordered, flexible regions as well as overall shape. Differences in carbohydrate structure can alter AUC results[15] without affecting polypeptide structure.[16] Even if the total carbohydrate content of EPREX and Epogen is identical, any difference in the average glycosylation at individual amino acid residues will likely lead to a difference in frictional coefficient. Therefore the variations in sedimentation coefficients in the Deechongkit paper must be evaluated by considering both carbohydrate as well as polypeptide structural differences. In fact, their CD thermal unfolding data points to a high similarity of polypeptide structure. The thermal unfolding profiles are nearly identical between these proteins, an indication of identical melting temperature and enthalpy of thermal unfolding. This in turn indicates that they have identical intramolecular packing and further supports the notion that the observed differences in the spectral properties between the purified Epogen and EPREX are due to errors in the protein concentration estimate.

Polysorbate 80 Effects

The Deechongkit study used a different source of polysorbate 80 than our qualified raw material to prepare an "EPREX-like" formulation using Epogen bulk drug substance and then used HiTrap columns to isolate the protein. The composition and quality of the material vary significantly, not

only between suppliers but also from batch to batch. The effect of different compositions and impurity profiles from different sources of polysorbate 80 may alter the resulting aggregate profile.

The potential effect of residual polysorbate 80, its impurities and degradants must also be considered when assessing differences in the biophysical characterization presented in Ref. 4. They report the use of evaporative light scattering to validate the removal of polysorbate 80 to "less than 0.0001% (w/v)." In our hands the level of detection of polysorbate 80 by evaporative light scattering, as determined using a 200 μL injection volume, is 0.003% (10% of the concentration of polysorbate 80 in EPREX).

HPLC experiments with polysorbate 80 in our labs[17] as well as in those of outside investigators (H. Schellekens, personal communication) have shown the tenacity with which polysorbate 80 adheres to columns and will continue to leach into the eluate of subsequent injections. Further, measurements of residual polysorbate 80 do not necessarily imply equivalent removal of its impurities or degradants (e.g. PEG-oleate). The presence of low levels of polysorbate 80 and/or its impurities and degradation products may affect sedimentation coefficient values, fluorescence intensity and CD thermal melting results.

Deechongkit *et al*. observed the loss of reversibility of thermal melting and a different CD spectral shape at 100°C for the purified EPREX. EPO is characterized by a relatively low melting temperature but high reversibility of melting,[18] but does aggregate upon excessive heating. It has previously been shown that heating EPO to only 79°C in phosphate buffers results in aggregate formation and heating even at 60°C in phosphate buffers, pH 7.4, generates dimers after 2 h and larger aggregates after a prolonged incubation to 24 h.[18] The observed differences in CD melting profile for the purified EPREX can simply be explained by this excessive heating to 100°C (which even the authors describe as "extreme"). These extracted samples started off with raised levels of aggregates, as shown by their sedimentation velocity data, and those aggregates will affect the spectrum and especially the reversibility of unfolding. Further, any differences in the composition of the solvent, such as residual polysorbate 80 or its impurities, between purified EPREX and Epogen can alter the aggregation tendency (or rates of chemical degradation) at such high temperatures.

Are Biophysical Studies of Extracted Proteins Meaningful?

It can be argued that even though the extraction procedure alters the protein, the comparison between the different manufacturers is valid because Epogen bulk was also subjected to this extraction procedure. But does this really provide a proper control? First, if actual drug products are going to be compared, then fairness would require starting with drug product, purchased on the open market, for both products. Second, given that in this case the extraction clearly damages the protein, then is any comparison of damaged samples really valid or significant, even if the damage is actually equivalent for both? Third, because the starting materials inevitably contain different buffer components and different impurities, the extracted samples will inevitably not be equivalent. Indeed, the fact that the "purified EPREX post peak" was not seen when Deechongkit *et al*. extracted Epogen bulk from their simulated EPREX formulation proves that these starting materials were non-equivalent in a significant way. As we discussed above, different polysorbate 80 samples have quite different impurities and degradation products, so this is one important source of non-equivalence after extraction.

The statement by Deechongkit *et al*. that their paper is "... the first study to systematically characterize the structural conformation of the same protein produced by different manufacturers" is unsupported by a critical examination of their methods and results. Their paper should rather be cited as the first example of why a comparative study of this type may give misleading results. Their inability to isolate protein from finished product that is representative of "process defined" bulk drug substance highlights one of the difficulties for companies working on the introduction of biosimilars to the market. The degree of similarity will be important in defining the level of clinical studies required for approval of biosimilars. The question is whether the protein is sufficiently comparable that its safety, efficacy and level of immunogenicity would be similar to the innovator product, such that only limited clinical studies would be required.

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