

Biophysical Comparability of the Same Protein from Different Manufacturers: A Case Study using Epoetin Alfa from Epogen[®] and Eprex[®]

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ABSTRACT: This study focuses on the development and application of biophysical methodology to characterize conformations of Epogen[®] and Eprex[®], the injectable formulations of recombinant human Epoetin alfa produced by different manufacturers and commonly used for the treatment of renal anemia. In these studies Eprex, from prefilled syringes, and Epogen bulk product formulated in a buffer similar to the Eprex formulation, were purified by anion-exchange chromatography. Analytical ultracentrifugation studies of the purified main peak from each sample demonstrated that Epogen contains a single component with an *s* value of 2.51 while Eprex contains a single component with the same molecular weight but with an *s* value of 2.44 suggesting a slight difference in hydrodynamic structure. The degree of α -helicity was compared by far-UV circular dichroism and shown to contain slight differences. Intrinsic tryptophan fluorescence and near-UV circular dichroism were assessed and demonstrated additional differences between the proteins. Finally, the global stability of the proteins was monitored using thermal unfolding monitored by far-UV circular dichroism. The Epoetin alfa of Epogen demonstrated complete reversibility while the Epoetin alfa purified from Eprex demonstrated only 80%–85% thermal reversibility when heated to 100°C. Together the data indicate that the proteins are not structurally identical.

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INTRODUCTION

Structural differences between proteins may occur because of oligomerization, *in vivo* modification or degradation of the protein primary sequence, differences in glycan composition or structure, or changes in the tertiary conformation. While comparability of protein oligomeric

states and chemical compositions can be analyzed routinely using chromatography and mass spectrometry, comparing protein conformational states presents a more challenging problem since protein conformational changes can be very subtle and sensitive to experimental conditions. Differences in protein structures between protein products have been a major concern in the biopharmaceutical industry and regulatory agencies worldwide and more recently with respect to follow-on biologics and biosimilars.¹ By contrast, research communities have not yet placed significant emphasis on biophysical comparability of

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the same protein expressed in different laboratories. The reasons for this lack of emphasis could be many fold. First, most proteins expressed for research are not for human or animal use, thus the safety risk is not as significant as with biopharmaceutical proteins. Second, many research proteins are small and perceived to have fewer degradation pathways and products. Finally, many of the studies involve protein mutants and native proteins produced in the same laboratory are used for comparison. This work outlines experiments designed to study aspects of conformational comparability using Epoetin alfa produced by different manufacturers and supplied in different formulations as an example.

Human erythropoietin is a 165-amino acid, glycosylated protein containing four sites for carbohydrate attachment, 3 N-linked and 1 O-linked.²⁻⁵ The protein stimulates erythropoiesis by simultaneously binding 2 cell-surface receptors on erythroid progenitor cells triggering maturation through a number of stages to red blood cells.⁶⁻⁸ The recombinantly produced human erythropoietins sold under the trade names Epogen[®]/Procrit[®] and Eprex[®] are manufactured by Amgen and Ortho Biotech Products, L.P., respectively. The protein formulations contain

additives to prevent adsorption and stabilize the protein. These include human serum albumin (HSA) for Epogen[®] and polysorbate 80 for Eprex[®]. While both proteins are manufactured in Chinese hamster ovary cells and have the same amino acid sequence, these products are manufactured separately and may have slightly different glycan structures, isoform distributions, impurities, and stabilities.

The objective of this experiment is to determine whether there are detectable differences in the conformation of Epoetin alfa from Eprex and Epogen. Because the spectral properties of a protein molecule depend upon the molecular environments and mobilities of its chromophores,^{9,10} several features of Epoetin alfa allow us to characterize its conformation and monitor for changes using classical biophysical techniques. The NMR solution structure of an *E. coli* produced rHuEPO with Asn to Lys mutations at positions 24, 38, and 83 and a Met-Lys added to the N-terminus (Fig. 1A)¹¹ indicates that Epoetin alfa adopts a four-helix bundle structure in solution. Consequently, changes in helicity of Epoetin alfa can be monitored by far-UV circular dichroism. Additionally, three tryptophan residues (Trp51, Trp64, and Trp88) can be used to discern changes in their local environments using Trp fluorescence

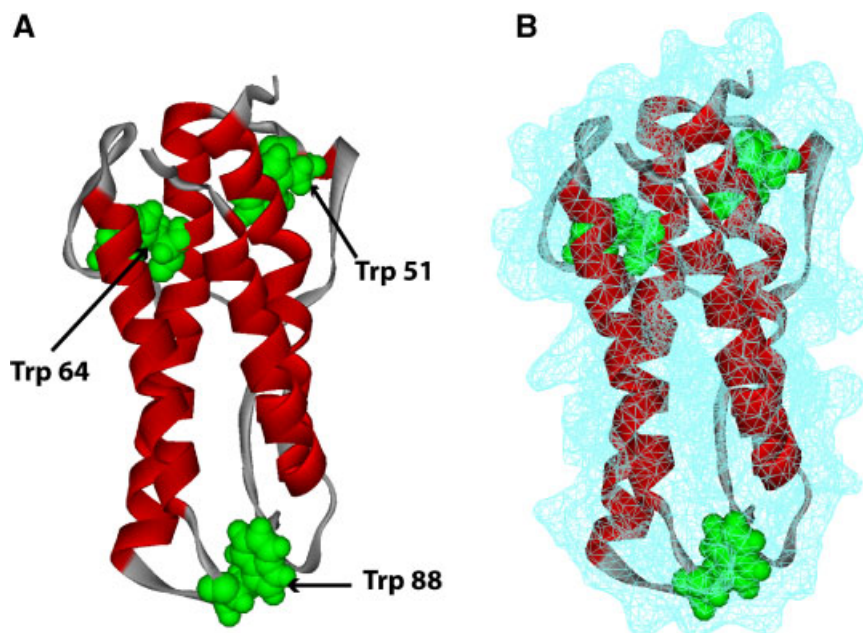


Figure 1. NMR structure of *E. coli* produced Epoetin alfa (PDB: 1BUY) (A) showing the four-helix bundle tertiary structure and three tryptophan residues and (B) showing hydration surface (1.4 Å water radius).

emission spectroscopy and near-UV circular dichroism. Trp88 is within vanDer Waals contact of the Cys29–Cys33 disulfide so that its fluorescence emission is quenched.¹² The observed fluorescence is from Trp51 and Trp64 near the high and low affinity receptor binding sites. Changes in the structure of the hydration surface, the structure presented to the immune system (Fig. 1B), may lead to immunogenicity¹³ and can be characterized by analytical ultracentrifugation analysis of the proteins hydrodynamic properties.^{14,15} Finally, the global stability and potential structural differences may be magnified by thermally stressing the protein and monitoring unfolding profiles and reversibility.

EXPERIMENTAL SECTION

Materials

Epogen bulk was obtained from Amgen, Inc. (Thousand Oaks, CA) at 3.5 mg/mL in 20 mM sodium citrate and 100 mM sodium chloride at pH 6.9. The protein was stored at 4°C in the dark and used prior to its expiry date. Eprex 10000 (1 mL at 10000 IU/mL or 83 µg/mL) and Eprex 1000 (0.5 mL at 2000 IU/mL or 17 µg/mL) in prefilled syringes with coated rubber stoppers were purchased off the market from Europe. Eprex samples were purified at least 8 months prior to their expiry date (16 months before the first purification and 8 months before the second purification). The biophysical experiments were conducted within 2 weeks after purification. Polysorbate 80 was obtained from Croda (Edison, NJ). Inorganic salts were purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparations

Fifty thousand units (417 µg) of protein was diluted to 30 mL with water, and loaded on a 1 mL HiTrap Q HP column (Pharmacia Biotech, GE Healthcare Bio-Sciences Inc., Piscataway, NJ) equilibrated in 20 mM Tris at pH 8.4 (A buffer). After protein loading, the column was eluted with a linear gradient from 0% to 100% B buffer (200 mM NaCl and 20 mM Tris at pH 8.4) in 50 mL. The main peak of Eprex eluted at approximately 80–140 mM NaCl. A shouldering peak eluted at approximately 140–200 mM NaCl. Both fractions were collected separately and the overlapping fractions discarded. Epogen eluted at approximately 80–150 mM NaCl. A shoulder was

not evident. The collected peaks were concentrated and buffer exchanged to 10 mM sodium phosphate and 140 mM sodium chloride at pH 6.9 using Centricon YM-10 concentration devices (Millipore, Bedford, MA), then filtered through a 0.22 µm filter (Spin-X, Costar). The final stock concentration for the collected main peaks were approximately 581 µg/mL. Yields were greater than 95%. All steps were performed between 4 and 8°C. Unless otherwise noted, stock solutions were diluted to 0.1 mg/mL with 10 mM sodium phosphate and 140 mM sodium chloride at pH 6.9 prior to analysis. The purified samples are referred to as purified Epogen and purified Eprex.

An additional set of experiments was conducted starting with different lots of Epogen bulk and commercial Eprex 10000. Epogen bulk was incubated in the reported Eprex polysorbate formulation¹⁶ (20 mM sodium phosphate (mono and dibasic), 99 mM NaCl, 0.03% polysorbate 80, and 0.5% glycine) for 1 week at 4°C then purified as previously indicated. During purification of the second lot of Eprex a distinct shoulder on the lagging edge of the major Eprex peak was again observed. This peak was purified and referred to as the purified Eprex post peak. The similar peak observed during purification of the first lot was not purified at that time. Analysis of purified samples using evaporative light scattering analysis showed that the amount of polysorbate 80 left in the purified fractions was less than 0.0001% (w/v).

Analytical Ultracentrifugation

The solution molecular weights of the proteins were evaluated by sedimentation equilibrium measurements carried out with a temperature-controlled Beckman XL-I analytical ultracentrifuge equipped with an An-60 Ti rotor and a photoelectric scanner (Beckman Instruments, Palo Alto, CA). Protein samples were loaded in a double sector cell equipped with a 12 mm Epon centerpiece and a sapphire optical window. The reference compartment was loaded with the matching buffer. The samples were monitored at 280 nm at a rotor speed of 45000 rpm at 20°C for sedimentation velocity and at a rotor speed of 15000, 22000, and 27000 for sedimentation equilibrium.

Analysis of the raw data was carried out using SEDFIT v. 8.9 (a freeware developed by Dr. Peter Schuck of NIH) to obtain the concentration distribution ($c(s)$) as a function of sedimentation coefficient (s , svedberg).¹⁷ Both frictional ratio and

meniscus were fitted for each sample. The range of s -values for fitting was 0.5–20 S with a resolution of 200. Weight average s -values were obtained using a confidence level of 0.7. Sedimentation equilibrium data were analyzed by a nonlinear least squares approach using kDalton (Amgen Proprietary Software). The data were fit to the Lamm equation for a single species model

$$A_r = A_0 \exp \left\{ \frac{\omega^2}{2RT \times M(1 - (\bar{v})\rho)} \right\} (r^2 - r_0^2) \quad (1)$$

where A_r is radial absorbance, A_0 is the baseline absorbance, ω is the rotor speed (/s), R is the gas constant (J/mol·K), T is the Temperature (K), \bar{v} is the partial specific volume (mL/g), ρ is the density of solvent (g/mL), r is the variable radius, and r_0 is the meniscus radius. The \bar{v} value used for these experiments is 0.68 mL/g, which was calculated from amino acid and glycan composition using Sednterp software (John S. Philo, Amgen, Inc.). The discrepancy in \bar{v} between purified Eprex and purified Eprex was <0.5% since the MALDI-TOF-MS of both proteins were similar (data not shown).

Size Exclusion Chromatography

Size exclusion chromatography was performed on an Agilent 1100 HPLC (Palo Alto, CA) using two 7.8 mm × 300 mm TosoHaas TSKGel G3000_{SWXL} (Montgomeryville, PA) columns connected in tandem and equilibrated with 20 mM sodium phosphate and 140 mM sodium chloride, pH 6.9. Protein load was 20 μg and eluted in the same buffer. The flow rate was 0.5 mL/min and the column temperature was 25°C.

Absorbance Measurements

Absorbance spectra were collected on an Agilent 8853 UV/Vis spectrophotometer, using a photodiode array detector. Spectra were collected from 200 to 1100 nm. To eliminate any scattering, spectra were subtracted using the corresponding buffer blank for purified Epogen and purified Eprex samples. The buffer blank was not subtracted from the commercial Eprex or formulated Epogen samples. The molar extinction coefficient used for the concentration calculations is 0.74 (ϵ^{280} , /((mg/mL)·cm). The value was determined empirically based on the absorbance and mass of the protein including the carbohydrate moiety.

Fluorescence Emission Measurements

Fluorescence measurements were carried out on an AVIV model ATF-105 automated titrating differential/ratio spectrofluorometer. Emission spectra were recorded from 300 to 450 nm in 1 nm steps with excitation at 280 nm using a 1 × 0.4 cm cuvette.

Circular Dichroism Measurements

Measurements were carried out on Jasco J-810 spectropolarimeter and a Jasco J-720 spectropolarimeter. Comparability of the measurements on both spectrophotometers was confirmed using Epogen bulk standard. All the wavelength scan measurements were conducted at 25°C using 1.0 × 0.2 cm rectangular quartz suprasil cuvettes for far-UV CD and 0.6 × 1.0 cm rectangular quartz suprasil cuvettes for near-UV CD (Hellma Cells, Inc., Plainview, NY) in peltier temperature controlled cell holders. Far-UV circular dichroism spectra were taken from 200 to 260 nm at 20 nm/min with 8 s response time using 0.1 mg/mL solutions of samples. Near-UV circular dichroism spectra were taken from 320 to 260 nm at 10 nm/min with a 16 s response time using a 0.4 mg/mL solution. Mean residue ellipticities (MRE, deg cm²/dmole) were calculated using Equation (2),

$$\text{MRE} = \frac{M_0 \times \theta}{10 \times l \times c} \quad (2)$$

where M_0 is mean residue weight (112.12 Da/residue), θ is observed ellipticity (mdeg), l is light path (cm), and c is concentration (mg/mL).¹⁸

Thermal unfolding experiments were monitored with far-UV CD at 219 nm. The temperature range utilized was from 0 to 100°C with a 0.2°C step size at 1°C/min heating rate and 16 s response time unless otherwise stated. After thermal unfolding, the samples were cooled down to 25°C for final wavelength scan measurement. The fraction unfolded was calculated by normalizing the thermal unfolding profiles.^{19,20}

Glycan Measurements by Capillary Electrophoresis

Glycan measurements were carried out on a Beckman MDQ capillary electrophoresis utilizing a dynamic coating capillary zone electrophoresis (CZE) method. The separation of rHuEPO isoforms is accomplished using 8 M urea in 300 mM phosphate buffer at pH 4.2 and detected at UV 200 nm. Approximately 100 μL of the sample was

transferred into each PCR vial and placed in microvial holders (P/N 970657, Beckman Coulter). The microvials were subsequently placed in the sample rack of the instrument. A Beckman CE PA800 capillary electrophoresis instrument equipped with a PDA detector, fixed wavelength monitoring at 200 nm, and a 50 $\mu\text{m} \times 60$ cm (50 cm effective length) uncoated fused silica capillary was used for the analysis. The procedure involved rinsing the capillary in forward wash mode with 0.1 N HCl for 2 min, water for 2 min at 20 psi then 0.1 N NaOH for 2 min at 20 psi followed with water for 2 min. After the treatment, the capillary was filled (forward mode) with eCAP amine regenerator solution (Beckman, P/N 477433) for 3 min at 20 psi followed by phosphate buffer wash for 3 min at 20 psi. At this point the sample was injected by pressure at 0.5 psi for 15 s followed by buffer injection at 0.2 psi for 15 s. Samples were separated at 15 kV (reverse polarity) for 60 min. The experiment was carried out with the temperature of the capillary maintained at 25°C.

Carbohydrate Analysis

Oligosaccharide mapping was carried out on Dionex Bio LC unit equipped with a pulsed amperometric detector (PAD), after released carbohydrate from the sample by PNGase F(QA-Bio, E-PNG01). Prior to LC separation the carbohydrate moieties were released from the protein according to the following method. First, all samples were diluted to 0.1 mg/mL in water. Commercial Eprex (10000 IU) was used without dilution. To 200 μL of sample was added 40 μL of 100 mM sodium phosphate, pH 8.5 and 4 μL of PNGase F(QA-Bio, catalog #E-PNG01) followed by incubation at 37°C for 48 h. The released glycan structures were separated on a Dionex CarboPac PA-100, 4 \times 250 mm (p/n 43055) column with a guard column (p/n 43054) in front. The flow rate was 0.5 mL/min. The column was equilibrated under initial conditions (50 mM Sodium acetate/50 mM sodium hydroxide) for a minimum of 20 min prior to injection of the sample. Following injection of the sample the column was washed for another 15 min using the initial conditions. The glycan was eluted with a gradient of 50–150 mM sodium acetate in 50 mM sodium hydroxide over 70 min followed by a gradient of 150–450 mM sodium acetate in 50 mM sodium hydroxide in 24 min. Finally the column was washed for 10 min with 450 mM

sodium acetate/450 mM sodium hydroxide, and re-equilibrated to 50 mM sodium acetate/50 mM sodium hydroxide over 15 min.

RESULTS

Global Folds of Epoetin Alfa: Hydrodynamic Property Comparisons

Sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation (AUC-SV and AUC-SE, respectively) were used to characterize the hydrodynamic properties of the Epoetin alfa. Sedimentation velocity profiles for purified Epogen and purified Eprex were analyzed using size distribution as a function of sedimentation coefficient (Fig. 2A and B).¹⁵ Each peak in the $c(s)$ plot represents a macromolecular component in the solution and is described by its sedimentation coefficient in the table. Purified Epogen contains a single component with an s value of 2.51 s while purified Eprex contains a single component with an s value of 2.44 s (Tab. 1). To ensure that the difference in the $c(s)$ profiles did not arise from the purification process, sedimentation velocity experiments were conducted on samples prior to purification of the Epogen bulk and commercial Eprex. Epogen bulk has a profile equivalent to that of the purified Epogen while the commercial Eprex profile is similar to that of the purified Eprex. Based on sedimentation equilibrium analysis, the average molecular weight for all samples is the same and equal to the monomeric molecular weight of intact Epoetin alfa (30.4 kD). Since the molecular weight for all samples is the same, the calculated hydrodynamic radii for the 2.51 s and 2.44 s components are 3.16 and 3.24 nm, respectively (Tab. 1). Nevertheless, the average s value for Eprex and Epogen remains different regardless of the regularization limit. Because the observed differences in hydrodynamic radii could arise from the data fitting parameters size-exclusion chromatography was also used to characterize hydrodynamic properties. The difference in size was also observed (Fig. 2C and D), where the retention time of Eprex is lower than that of Epogen.

Absorbance Spectra of Commercial and Purified Eprex With Epogen

To eliminate possible protein concentration effects on the biophysical measurements, the concentrations of the purified Epogen and purified

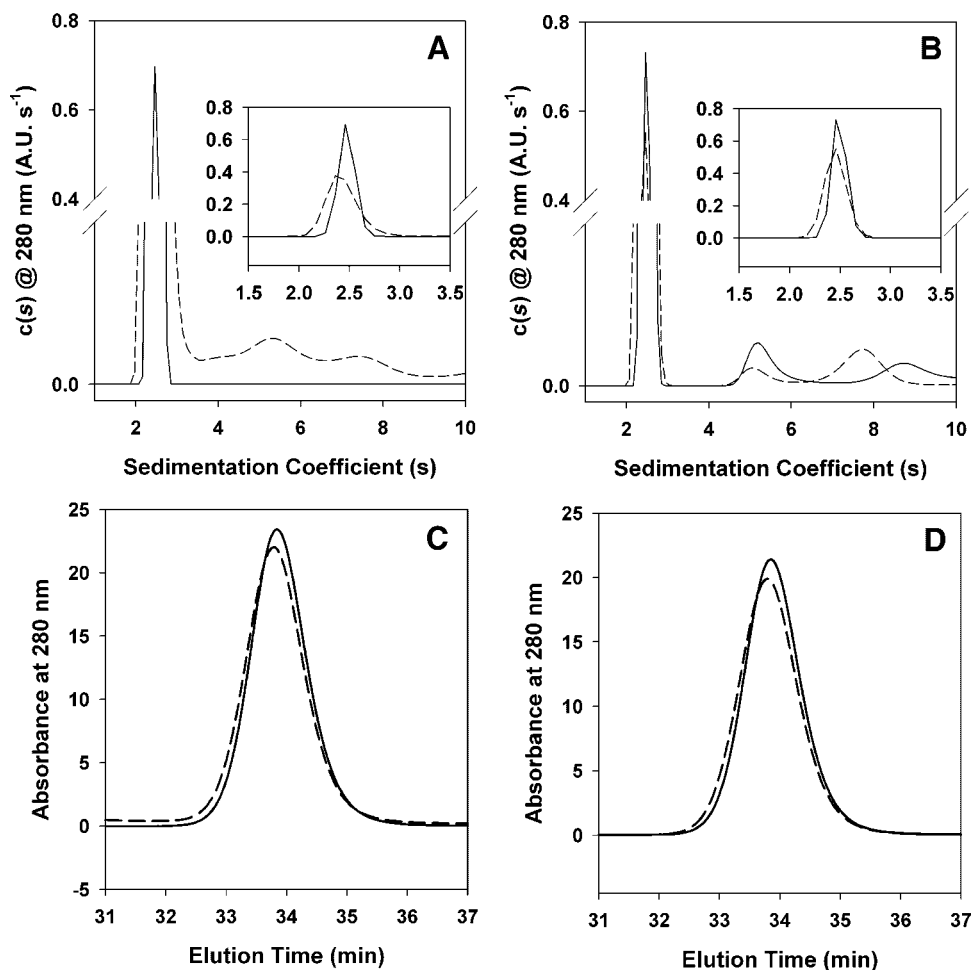


Figure 2. Sedimentation velocity analysis of (A) commercial Eprex 10000, (---) and Epogen bulk (—), and (B) purified Eprex (---) and purified Epogen (—). Zoom views are shown in the insets. Distribution plots are similarly reproducible and only the plots from the first set are shown. Size exclusion chromatograms of (C) Commercial Eprex 10000, (---) and Epogen bulk (—), and (D) purified Eprex (---) and purified Epogen (—).

Table 1. Sedimentation Coefficients, Distributions, Hydrodynamic Radii, (R_h) and Molecular Weight Determined by Sedimentation Equilibrium Analysis for Samples Shown in Figure 2

Protein	Average s Value	MW (kD)	R_h (nm)
Purified Epogen	2.51 ± 0.01 (2.52 ± 0.01)	30.4	3.16 ± 0.01 (3.15 ± 0.01)
Epogen Bulk	2.51 ± 0.01 (2.52 ± 0.01)	30.4	3.16 ± 0.01 (3.15 ± 0.01)
Purified Eprex	2.44 ± 0.01 (2.46 ± 0.01)	30.4	3.24 ± 0.01 (3.22 ± 0.01)
Eprex 10000	2.44 ± 0.01 (2.46 ± 0.01)	30.4	3.24 ± 0.01 (3.22 ± 0.01)

The numbers in parentheses are from the second set of samples. Data are the average of three independent measurements with standard error.

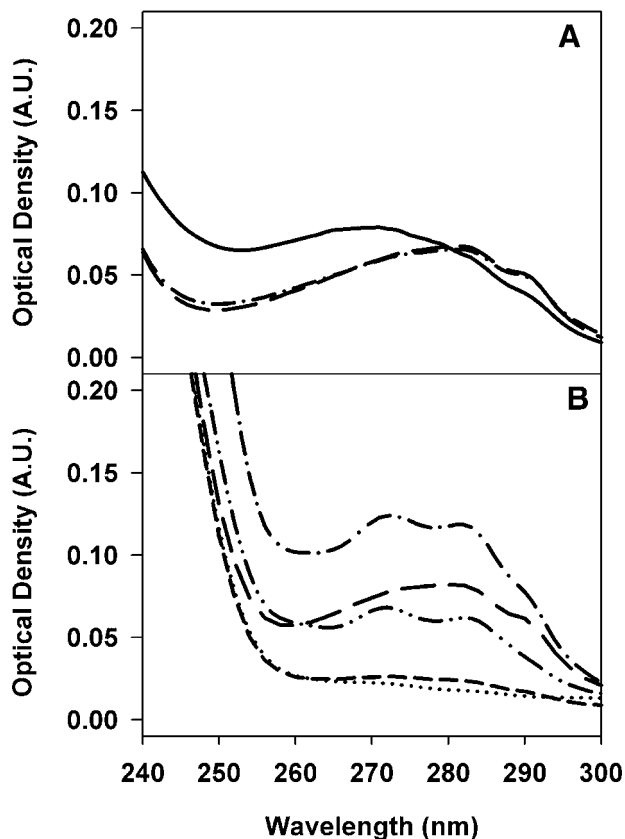


Figure 3. UV spectra (A) with background subtraction of purified Epogen (—), purified Eporex (---), and purified Eporex post peak (— · —) and (B) without background subtraction of commercial Eporex 10000 (1mL at 10000 IU/mL, - · - · -) and commercial Eporex 1000 (0.5 mL at 2000 IU/mL, - - - -), and Epogen bulk formulated in our prepared polysorbate Eporex formulation at the same concentrations (10000 IU/mL - - -, and 2000 IU/mL, - · - · -) and commercial Eporex formulation blank (· · · · ·). The second set of samples exhibited the same results as those shown (data not shown).

Eporex samples used for subsequent CD and fluorescence experiments were compared. As shown in Figure 3A, UV absorbance spectra for purified Epogen and purified Eporex are nearly identical. Similar data was seen without background correction (data not shown). Figure 3B shows absorbance spectra without background correction of commercial Eporex at 10000 and 2000 IU/mL, and Epogen bulk formulated in the reported Eporex commercial formulation buffer at the same concentrations. In contrast to the purified proteins the absorbance spectra of the commercial Eporex and the corresponding formulated Epogen are not identical. The commercial Eporex has a higher absorbance and contains an

additional absorbing species with a maximum at 273 nm. As previously indicated the post peak on the lagging edge of the main Eporex peak was purified during the anion-exchange chromatography of the 2nd commercial lot. The fraction contained some Eporex, which may have been from the main peak, along with a chemical species having a UV spectrum distinct from that of Eporex, but with an absorbance maximum at 271 nm. In later experiments we were able to chromatographically separate the unknown chemical species and determine that it was not composed of protein. The unknown nature of the species made it impossible to exactly replicate the Eporex formulation in the syringes and we were therefore unable to correct for the background in the spectra. Additionally, the effect of the species on the biophysical analysis could not be determined preventing us from comparing the structure of the Epoetin alfa in its commercial formulation to that of Epogen bulk in the Eporex formulation. These unknown compounds from the purified Eporex post peak were unlikely contaminated in the purified Eporex main peak since the overlapping fractions were discarded. Comparison of the second derivative UV spectra of purified Eporex and purified Epogen showed no discernable differences between 275 and 280 nm, a region in which contamination from the post peak would have interfered.

Secondary and Tertiary Structure of Epoetin Alfa: Spectroscopic Comparisons

Secondary and tertiary structures of purified Epogen and purified Eporex were compared using the optically matched samples described in the preceding section. Far-UV circular dichroism spectra for all samples showed that Epoetin alfa adopts an α -helix conformation, as evidenced by two minima at 208 and 222 nm (Fig. 4A).¹⁸ Mean residue ellipticities for purified Epogen and Epogen bulk at both minima were similar and lower than those for purified Eporex (see Tab. 2). Fraction helix calculated based on MRE at 208 nm²¹ for purified Epogen and Epogen bulk were approximately 5% higher than that for purified Eporex. These results indicate that the purification process did not alter the secondary structure of Epoetin alfa in Epogen and that the Epoetin alfa in purified Eporex is less helical than that in purified Epogen.

The local environment of the Trp residues was examined using near-UV CD (Fig. 4B and Tab. 2)

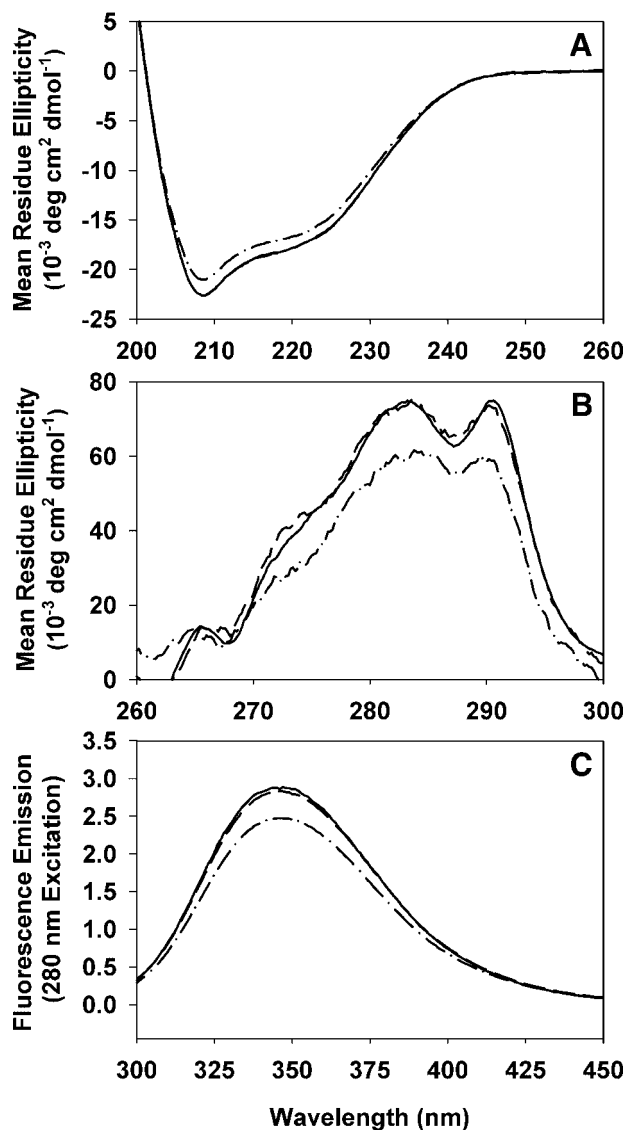


Figure 4. (A) Far-UV CD spectra of Epogen bulk (—), purified Epogen (---), and purified Eprex (- · -). (B) Near-UV CD of Epogen bulk (—), purified Epogen (---), and purified Eprex (- · -). (C) Tryptophan emission spectra (280 nm excitation) of Epogen bulk (—), purified Epogen (---), and purified Eprex (- · -). The spectra from both sets are similar and only those from the first set are shown.

and fluorescence spectroscopy (Fig. 4C and Tab. 2). The MRE for Epogen bulk and purified Epogen were nearly identical while the MRE of purified Eprex showed a decrease between 280 and 295 nm indicative of a change in the local Trp environment compared to Epogen. When Trp residues in Epoetin alfa are excited at 280 nm, the combined fluorescence emission spectrum from Trp51 and

Trp64 has a maximum at 346 nm. As Epoetin alfa unfolds or adopts alternate conformations, the environments surrounding the Trp residues change, causing the emission intensity at 346 nm to change or the emission maximum to shift to a different wavelength.¹² While emission maxima (346 nm) of Epogen bulk and purified Epogen have the same intensity indicating equivalent molecular environments, the fluorescence intensity of purified Eprex is lower, indicating that the environments surrounding the Trp residues are different. There was no discernible difference in the fluorescence peak maxima between the purified Epogen and purified Eprex. The lower intensity or quantum yield is indicative of either greater exposure to the solvent or a change in the local polarity of the Trp environment in purified Eprex due to a change in the protein structure. The change in Trp environment was also observed by near-UV circular dichroism (Fig. 4B).

Thermal Unfolding and Thermal Reversibility Monitored by Far-UV Circular Dichroism Spectroscopy

Monitoring the structural response of the protein to temperature stress can magnify differences in protein structures. When Epogen bulk, purified Epogen, and purified Eprex were heated from 0 to 100°C, their thermal unfolding curves (Fig. 5A) were similar with unfolding initiating at approximately 45°C and T_m values of 57°C. At 100°C, all proteins still maintained some helical structure (38.5% of the original content based on MRE at 208 nm) as shown by far-UV CD spectra (Fig. 5B). However, the similarities ended when comparing the thermal reversibility after heating. Both Epogen bulk and purified Epogen showed almost complete secondary structure reversibility (Fig. 5C) as evidenced by the superimposition of the spectra before and after heating. In contrast, purified Eprex showed only partial reversibility. Helical conformations were retained, but only at 80%–85% of the preheated content. The loss of helix could be attributed to many factors, such as thermal-induced aggregation or kinetic traps upon refolding. Analysis of the samples by AUC-SV showed only minor increases in high molecular weight species and no change in s values while no difference was observed for light scattering by absorbance spectroscopy indicating there was no change in the solubility of the protein.

To confirm that the thermal unfolding reaches equilibrium at each temperature, three additional

Table 2. Relative Ellipticity at 208 and 222 nm, Fraction Helix, and Fluorescence Emission at 346 nm of Samples Shown in Figure 4

Protein	CD Mean Residue Ellipticity (deg cm ² /dmol)		Fraction Helix	Fluorescence Emission at 346 nm
	222 nm	208 nm		
Purified Epogen	-17198 ± 264 (-17375 ± 312)	-22457 ± 365 (-21971 ± 357)	0.636 ± 0.010 (0.620 ± 0.010)	2.829 ± 0.003 (2.867 ± 0.012)
Epogen Bulk	-17203 ± 208	-22493 ± 306	0.638 ± 0.009	2.860 ± 0.027
Purified Eprex	-16163 ± 157 (-16272 ± 266)	-20950 ± 176 (-20552 ± 298)	0.584 ± 0.005 (0.571 ± 0.008)	2.471 ± 0.063 (2.460 ± 0.027)

The numbers in parentheses are from the second set of samples. Data are the average of three independent measurements with standard error.

temperature ramping experiments were conducted: (1) step-wise heating at 2°C/step with 30 s equilibration time per step from 0 to 100°C, (2) continuous heating at 2°C/min from 0 to 100°C, and (3) continuous heating at 0.33°C/min from 40 to 85°C. The unfolding profiles for all samples (raw signal vs. temperature) remained the same regardless of the temperature ramping program (data not shown). Purified Epogen was fully reversible under all conditions tested; the degree of reversibility for purified Eprex improved to approximately 95% when the samples were heated to only 85°C. The results indicate that the final temperature and not the ramp rate affected the final refolded structure.

Isoforms and Carbohydrate Analysis

Epogen bulk, commercial Eprex, and their corresponding purified proteins were characterized for sialic acid content by CZE and glycan structure by LC analysis. As shown in the CZE electropherogram in Figure 6A, isoform types and distributions for all samples appeared similar. Therefore, the sialic acid content in all the samples should be the same. Carbohydrate analysis was used for quantitation of the *bi*-, *tri*-, and *tetra*-antennary carbohydrate structures on Epoetin alfa. The glycan structures of the proteins were enzymatically released, separated by ion-exchange chromatography and quantitated as shown in Figure 6B. Interestingly, only commercial Eprex showed a different pattern compared to those of Epogen bulk, purified Epogen, and purified Eprex. Commercial Eprex contains an additional peak around 75–76 min that was removed by anion-exchange chromatography. It is possible this additional peak is the same as that in the purified Eprex post peak described above or a

totally different type of impurity. Future analysis of these peaks will be conducted. It is important, however, to point out that both purified Epogen and Eprex had the same percentages of *bi*, *tri*, and *tetra*-antennary carbohydrate structures.

DISCUSSION

Numerous research labs have demonstrated that proteins with equivalent amino acid sequences should fold into the same tertiary structure.^{22–26} While the Epoetin alfa from Epogen and Eprex have the same amino acid sequence, we detected differences in their biophysical characteristics. This study outlined an approach to compare biophysical characteristics of the same protein produced by different laboratories or manufacturers, and formulated with different solution components. The study was not designed to determine superiority of one product to another. The biophysical techniques used in this study indicate that Epoetin alfa from Eprex is not thermally reversible under extreme conditions but is reversible under less severe conditions while Epogen was reversible under all the conditions tested. Eprex is more expanded in solution by approximately 0.1 nm, and shows differences in CD and fluorescence spectra compared to those of Epoetin alfa of Epogen. The differences in far-UV CD suggest that purified Eprex contains approximately 5% less α -helix than Epogen, as determined by the mean residue ellipticity at 208 nm. A measure that for erythropoietin correlates directly with the α -helix content.²¹ The decrease in the near-UV CD signal indicates a loss of optical activity that is likely tied to an increase in the motion or flexibility of the Trp residue since fluorescence analysis showed a

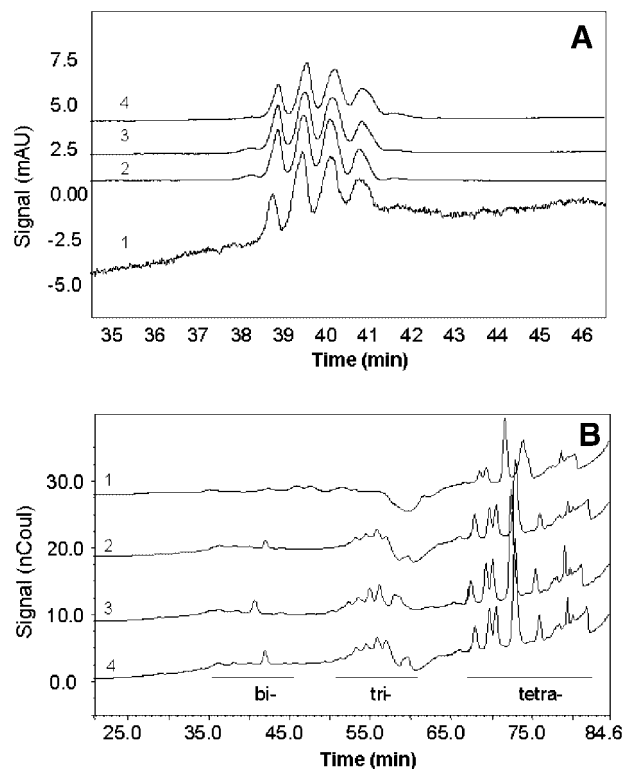
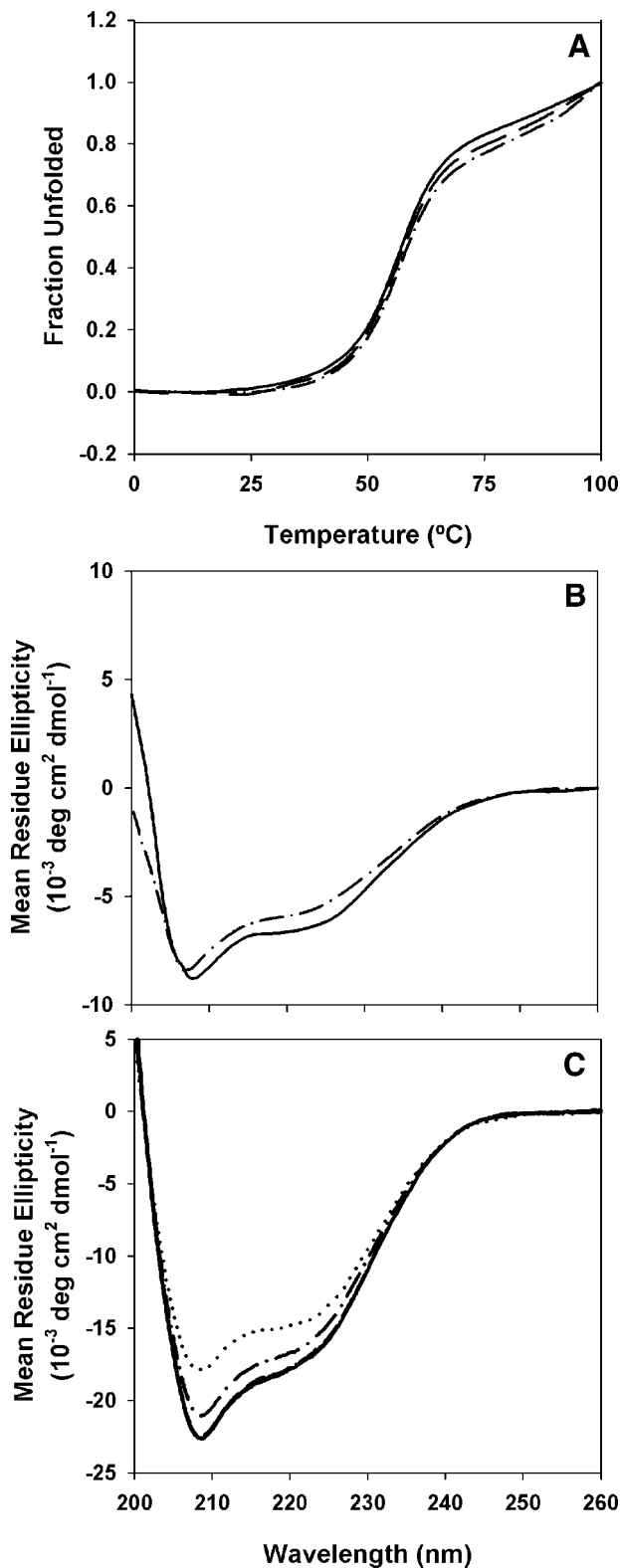


Figure 6. (A) Capillary zone electropherograms of (from bottom): (1) commercial Epex 10000, (2) Epogen bulk, (3) purified Epogen, and (4) purified Epex. (B) Chromatograms showing carbohydrate structural analysis of (from bottom): (1) commercial Epex 10000, (2) purified Epex, (3) Purified Epogen, and (4) Epogen bulk.

decrease in intensity without a change in the emission maxima. We can now interpret that as an increase in flexibility of the Trp without a major change in the exposure of the Trp to the solvent as indicated by a lack of difference in the 2nd derivative UV absorbance (data not shown).

There are many possible causes for the observed differences between Epogen (bulk and purified)

Figure 5. (A) Thermal unfolding profile of Epogen bulk (—), purified Epogen (---), and purified Epex (-·-·). (B) Overlays of Epogen bulk (—), purified Epogen (---), and purified Epex (-·-·) at 100°C. (C) Overlays of Epogen bulk (—), purified Epogen (---), and purified Epex (-·-·) at 25°C before thermal unfolding and Epogen bulk (-·-·-·), purified Epogen (- - -), and purified Epex (· · · · ·) at 25°C after thermal unfolding. The unfolding profiles and spectra from both sets are similar and only those from the first set are shown. The second set of samples exhibited the same results (not shown).

and purified Eprex. The purification procedure used to remove the formulation components may have directly or indirectly affected the structure. Biophysical comparisons of the unmanipulated Epogen bulk and purified Epogen bulk previously stored for 1 week in a formulation similar to that used for Eprex demonstrated that the purification procedure did not change the conformational integrity of the Epogen, albeit an increase in the level of aggregation was observed by AUC-SV. Without a reference standard for the Eprex bulk we cannot demonstrate that the purification procedure did not alter the Eprex conformation, but can only infer it from the data with Epogen bulk. Alternatively, stress related to product handling may result in protein unfolding. The Eprex used in these studies was purified from material purchased from the market and out of the control of the manufacturer. The formulation or unspecified components in the container closure, in conjunction with common stress mentioned previously may also modify the protein during storage. The possibility also exists that a difference in the integrity of the bulk materials itself may cause differences in the biophysical characteristics. It is also feasible that these possibilities work in concert resulting in the redistribution of native ensembles to different minima of the Epoetin alfa's folding energy landscape.²⁷

Differences in the glycan structures of the proteins could contribute to the observed differences in the protein conformations. The glycan structure of Epoetin alfa is comprised of 3 N-linked and 1 O-linked glycan²⁻⁵ making up 40% of the overall molecular weight of the protein.²⁸ The N-linked glycans are composed of *bi*-, *tri*-, and *tetra*-antennary structures with sialic acid end capping giving rise to the charge variant isoforms.^{5,29} We observed identical levels of neuraminic acid isoforms and *bi*-, *tri*-, and *tetra*-antennary structures suggesting that gross changes in either the mass or structure of the glycans does not account for the protein structural differences. We cannot rule out the possibility that differences in the linkage and branching patterns of the glycans are giving rise to the observed biophysical differences. Studies by Toyoda et al.³⁰ suggested that the inner regions including the galactose residues of the highly branched N-glycans stabilize the protein conformation through the interaction with the hydrophobic surface areas made up of non-aromatic hydrocarbon groups. Linsley et al.²⁹ demonstrated that the branching patterns of the glycans were

complex with a high degree of heterogeneity. Possibly, differences in branching patterns could give rise to differences in intramolecular interactions of the N-glycans and the protein surface leading to the biophysical differences between Epogen and Eprex observed here.

For protein biologics, it is unknown how biophysical differences in protein structure, such as those described herein for Epogen and Eprex, affect shelf-life stability, clinical efficacy, and product safety, including immunogenicity. In a small number of patients, antibodies, which can cross-react with endogenous erythropoietin were produced against the cell-surface receptor-binding sites on the protein producing an immunologic disorder known as antibody-mediated pure red cell aplasia (Ab+PRCA).^{31,32} Ab+PRCA is an erythroid aplasia with a clinical presentation including severe anemia, low reticulocyte count, absent or reduced numbers of erythroid blast cells, low or undetectable levels of endogenous erythropoietin, and normal white cell and platelet counts.³³ Studies by Casadevall demonstrated that the antibodies were directed against a conformational epitope and not denatured protein or the glycan structures.³⁴ This raises the concern that differences in three-dimensional protein structure may alter the safety profile of protein biologics. Determining the clinical relevance of any biophysical differences in protein structure through robust clinical trials, which examine the safety and effectiveness of protein biologics that have identical amino acid sequences, is critical to providing optimal patient care.

Recently, the European Medicines Agency (EMA) issued draft guidelines for demonstrating biophysical and clinical comparability of bio-similar medicinal products (follow-on biologics) to those products currently on the market.³⁵ The EMA clearly states that the ultimate justification of any changes in biophysical structure must come with extensive clinical experience. The guidance on biophysical comparability raises a number of significant issues including identification of an appropriate reference standard and problems of direct comparison with and purification of a formulated protein. The current European Pharmacopoeia reference standard for EPO, Erythropoietin BPR, is a blend of Epoetin alpha and Epoetin beta compromising the utility of the reference standard in the context of analytical comparisons.

Often, components but not concentrations of the inactive ingredients in a formulated drug are

available making it difficult to exactly reproduce the formulation. Extractables from the containers, such as prefilled syringes, may be different for each product or change over time introducing additional unknown factors that could affect the protein conformation or analytical procedures. As described here the marketed Eprex contained an unknown impurity that interfered with the UV-absorbance (Fig. 3) and the glycan mapping (Fig. 6). The level of polysorbate can affect the protein structure (data not shown) and interferes with SEC analysis³⁶ making it necessary to remove prior to analysis. The purification procedure used here caused a slight increase in aggregation of the purified Epogen sample and presumably the same occurred during purification of the Eprex in which aggregation was also observed (Fig. 2). It is foreseeable the same will occur for a biosimilar manufacturer who will have to rely on in-house purification of the active substance from the commercially available drug product and will also not be able to definitively define the effect of the purification procedure on the protein.

To our knowledge this is the first study to systematically characterize the structural conformation of the same protein produced by different manufacturers. These types of findings could be important to establish guidelines for assessing biosimilar drugs currently in development by a number of companies. This work is also relevant because it is conceivable that conformational differences in research proteins may result in different properties, such as binding constant and aggregation rate. All these differences could impact the conclusions of any comparative research among different laboratories. Moreover these differences could impact clinical efficacy and safety.

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