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Research Paper

Interaction of Polysorbate 80 with Erythropoietin: A Case Study in Protein–Surfactant Interactions

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Purpose. The cause of antibody positive pure red cell aplasia associated with the subcutaneous administration of EPREX® to patients with chronic kidney failure has been determined to be due to the leaching of weakly adjuvant compounds from the uncoated rubber stoppers that were formerly used in prefilled syringes. Other researchers have suggested that polysorbate 80 micelles containing erythropoietin may be a causative factor. The purpose of this work was to repeat previously published studies in a more controlled manner and to define the precise nature of the interactions between polysorbate 80 and erythropoietin.
Methods. The contents of EPREX® prefilled syringes and laboratory-prepared, well-characterized formulations of EPREX® were analyzed by size exclusion chromatography. Fractions were analyzed for the presence of erythropoietin by ELISA. EPREX® formulations prepared with increasing amounts of polysorbate 80 were analyzed by light scattering.
Results. Well-controlled chromatographic studies showed that when EPREX® formulations containing no aggregate were analyzed by high-performance liquid chromatography, erythropoietin monomer could not be detected under the polysorbate 80 peak. Dimer and oligomers of erythropoietin coeluted under the polysorbate 80 peak as the molecular weights overlapped on the size exclusion chromatogram. Solution light scattering indicated that polysorbate 80 associates with erythropoietin in a defined stoichiometric ratio of 1:12.
Conclusions. Based on controlled studies, previous results suggesting that EPREX® contains micelle-associated erythropoietin were incorrect. As with other surfactants and proteins, polysorbate 80 associates with erythropoietin in a defined stoichiometric ratio.

KEY WORDS: EPREX®; erythropoietin; micelles; polysorbate 80; PRCA.

INTRODUCTION

From 1998 the reported incidence of pure red cell aplasia (PRCA) associated with erythropoietin (epoetinum alfa, EPO) used in patients with chronic kidney disease increased sharply (1–3) over the sporadically reported incidence of the previous 10 years (4) to a peak of 71 cases in 2002. Of the 262 cases of suspected erythropoietin-associated PRCA that have been reported, 217 had antierythropoietin antibodies and 201 of those cases were associated with the subcutaneous administration of EPREX® from prefilled syringes. An extensive technical investigation identified the multifactorial, putative cause of PRCA with the temporally associated formulation change that replaced human serum albumin with polysorbate 80 (Tween-80) and the use of uncoated rubber stoppers on the prefilled syringe plungers that resulted in the leaching of organic compounds possessing adjuvant properties from the uncoated rubber stoppers (5). Replacement of the uncoated rubber stoppers with Teflon-coated stoppers prevented the leaching of these organic compounds into the syringe contents and has resulted in the incidence of EPREX®-associated PRCA returning to basal levels. The decline in cases of PRCA also coincided with other measures taken by the company, in particular, changing the route of administration in patients with chronic kidney disease from s.c. to i.v. (6). During our investigations, a report was published by Hermeling *et al.* in which they reported on size exclusion high-performance liquid chromatography (HPLC) studies on EPREX® expressed from commercially purchased EPREX® prefilled syringes (7). From their studies, they postulated the existence of polysorbate 80 micelles presenting multiple erythropoietin molecules and suggested that ". . . at least a few epoetin molecules can be present in one micelle. This could lead to increased immunogenicity as a result of the presence of multiple epitopes exposed on the micellar surface."

Their evidence for the existence of polysorbate 80–erythropoietin micelles was based primarily on two observations. First was that in the size exclusion (SEC) HPLC,

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polysorbate 80 showed two peaks with molecular weights of approximately 255 and 670 kDa based on external molecular weight standards. Second, the collection of fractions corresponding to the polysorbate 80 peaks and subsequent analysis by ELISA and Western blot indicated the presence of trace amounts of erythropoietin-like material. Although our investigations had excluded polysorbate 80 as a causative factor in PRCA due to the inability of polysorbate 80 at concentrations up to ten times the amount of that found in EPREX® to stimulate an immune response to erythropoietin in animal models (data not shown), we sought to investigate the observations of Hermeling *et al.* (7) to determine the origin of the erythropoietin-like material identified in the polysorbate 80 region of the SEC-HPLC and to more fully characterize the nature of the interactions between erythropoietin and polysorbate 80. In addition to repeating the work of Hermeling *et al.* using commercially available prefilled syringes, we studied well-characterized laboratory preparations of EPREX® and used light scattering analysis of column eluates and erythropoietin–polysorbate 80 solutions to define the nature of protein–surfactant interactions.

MATERIALS AND METHODS

EPREX®

EPREX® is manufactured by Ortho Biologics, L.L.C. (Manati, Puerto Rico).

Size Exclusion Chromatography

Size exclusion HPLC was done using Superdex S-200 HR 10/30 (Amersham Biosciences, Piscataway, NJ) or TSK G3000 (Tosohaas, Montgomeryville, PA) size exclusion columns on a Waters 2695 Alliance system (Milford, MA, USA) equipped with a photodiode array detector, a Wyatt Dawn EOS light-scattering detector (Santa Barbara, CA, USA) and a Wyatt Optilab DSP refractometer or an Agilent 1100 system (Wilmington, DE, USA). The columns were eluted with a mobile phase consisting of 50 mM sodium phosphate buffer (pH 6.8) containing 300 mM sodium chloride at flow rates of 0.33 or 0.5 mL/min. Column eluates were monitored at 214, 220, or 280 nm.

Preparation of Polysorbate 80–Erythropoietin Solutions

Polysorbate 80 in EPREX® buffer

For light-scattering studies, a stock solution (twice the concentration in EPREX®) was prepared containing 2.32 g of monobasic sodium phosphate monohydrate, 4.46 g of dibasic sodium phosphate dehydrate, 8.383 g of sodium chloride, and 10 g glycine in 1 L of water and filtered through a 0.2-μm filter. A second stock solution was prepared of 0.4% polysorbate 80 in water and filtered through a 0.2-μm filter. Five milliliters of the buffer stock solution was added to a cleaned scintillation vial. Aliquots of the polysorbate 80 stock solution and sufficient water to make a final volume of 10 mL were added to the scintillation vial to prepare concentrations of polysorbate 80 of 0.0, 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008, 0.01, 0.02, 0.03, 0.05, 0.1, and 0.2%. The outside of the scintillation vials were washed with water and ethanol and then dried with nonlinting lens tissue.

Polysorbate 80 in 0.25 mg/mL EPREX®

A stock solution (twice the concentration in EPREX®) was prepared containing 0.232 g of monobasic sodium phosphate monohydrate, 0.446 g of dibasic sodium phosphate dehydrate, 0.8383 g of sodium chloride, 1.0 g glycine, and 50 mg epoetinum alfa in 100 mL of water and filtered through a 0.2-μm filter. A second stock solution was prepared of 0.4% polysorbate 80 in water and filtered through a 0.2-μm filter. Five milliliters of the buffer stock solution was added to a cleaned scintillation vial. Aliquots of the polysorbate 80 stock solution and sufficient water to make a final volume of 10 mL were added to the scintillation vial to prepare concentrations of polysorbate 80 of 0.0005, 0.001, 0.002, 0.004, 0.006, 0.008, 0.01, 0.02, 0.03, 0.05, 0.1, and 0.2%. The outside of the scintillation vials were washed with water and ethanol and then dried with nonlinting lens tissue. For HPLC studies, solutions were prepared according to the EPREX® batch record except that a stock solution of 1% polysorbate 80 in water was used and diluted to the desired polysorbate 80 concentrations.

Preparation of Heat-Induced Erythropoietin Dimer/Oligomer

Purified bulk erythropoietin (Ortho Biologics) was diluted to 1 mg/mL in 20 mM citrate/100 mM NaCl (pH 7.0). Seven milliliters of the solution in 15 mL polypropylene conical tubes was heated at 55°C in a water bath for 14 days. The appearance of dimer/oligomer was monitored by gel permeation chromatography. The heat-aggregated erythropoietin used in this study had a total of 56% dimer/oligomer.

Determination of Erythropoietin by ELISA

Human erythropoietin-specific immunoassay kits were purchased from Quantikine IVD (R&D Systems, Minneapolis, MN, USA) Reference standard erythropoietin from Ortho Biologics was used as a standard. One hundred microliters of assay diluent (supplied by the manufacturer) was added to each well of a microplate that has been previously coated with a murine monoclonal antibody against erythropoietin. Eighty microliters of dilution buffer (supplied by the manufacturer) and 20 μL of each fraction were added to a well. The plates were incubated for 1 h at room temperature under constant orbital shaking. The contents were discarded and the wells carefully tapped dry on a tissue. A solution of horseradish peroxidase-conjugated rabbit polyclonal antierythropoietin antibodies was added to each well and the plate was incubated on an orbital shaker at room temperature for 1 h. The contents were discarded and the wells washed four times with 400 μL of the supplied wash buffer. After the last wash, the plate was tapped dry on a tissue. Two hundred microliters of substrate solution was added to each well. After incubation for 20 to 25 min, 100 μL of stop solution was added to each well and the absorbance was read on a microplate reader at a 450 and 595 nm (reference wavelength).

Determination of Molecular Weight by Light Scattering

A Wyatt Dawn EOS light scattering detector fitted with a holder for scintillation vials was calibrated with toluene and normalized with a solution of 5.0 mg/mL polystyrene (MW 25,000) in toluene according to the instrument operations manual. The outsides of the vials were rinsed with hexanes (HPLC grade) and dried with lens tissue just before readings were taken. The vials were placed in the sample holder and a reading taken for 1 min. The vial was then rotated in the holder and a second reading taken for 1 min. When the vial was rotated the signal was visually monitored to determine if there was a lower signal because scintillation vials are not of optical quality and imperfections in the glass can cause an artificially high signal. Three to five readings were taken for each sample. The first and last samples analyzed were buffer controls. For each solution, dn/dc values were calculated based on composition and using the experimentally determined values for the dn/dc of polysorbate 80 of 0.1266 and for erythropoietin of 0.1566. Data were analyzed from each reading and the lowest molecular weight from each sample was used.

RESULTS

EPREX® expressed from two lots of prefilled syringes was analyzed by HPLC and the results shown in Figs. 1 and 2. One batch was shown by an alternate, validated assay to contain 0.18% of dimer/oligomer (Fig. 1), whereas the second had no detectable levels of dimer/oligomer (Fig. 2). The chromatographic profiles (UV 220 nm) for these two preparations were identical under the chromatographic conditions used and were comparable to the chromatograms presented by Hermeling *et al.* (7). Fractions were collected from the column eluates post detector from 10 to 35 min and included the elution of polysorbate 80 (15–28 min) and erythropoietin (28–32 min). These fractions were analyzed for the presence of erythropoietin-like material using an ELISA that detects erythropoietin monomer, dimer, and oligomer. The results of the ELISA are shown in Figs. 1 and 2 (dashed lines) and indicate that erythropoietin-like material was detected eluting in the polysorbate 80 region of the chromatogram only when erythropoietin dimer/oligomer was shown to be present in the formulation by independent analysis (a manuscript describing the details of the HPLC method is in preparation). It can be noted that the erythropoietin-like material detected in the ELISA shown in Fig. 1 elutes on the trailing edge of the polysorbate 80 region and its elution pattern does not parallel that of either of the polysorbate 80-attributed peaks. The retention time is identical to where dimer/oligomer would elute in the absence of polysorbate 80. These results were replicated using laboratory-prepared EPREX® formulations free of dimer/oligomer and with small amounts of dimer/oligomer added (data not shown).

When erythropoietin is treated at 55°C for 14 days, appreciable quantities of the dimer/oligomer were formed. The size exclusion HPLC chromatogram for this material is shown in Fig. 3 overlaid with a chromatogram of EPREX®. The broad elution of the oligomer overlaps with much of the polysorbate 80 peaks; however, the dimer elutes on the trailing edge of the polysorbate 80 peaks, similar to the ELISA results shown in Fig. 1. Taken together, the data presented in Figs. 1–3 suggest that the presence of erythropoietin-like material detected eluting with polysorbate 80 by previous investigators is consistent with the presence of traces (0.1%) of erythropoietin dimer in the purchased EPREX® syringes used in their studies.

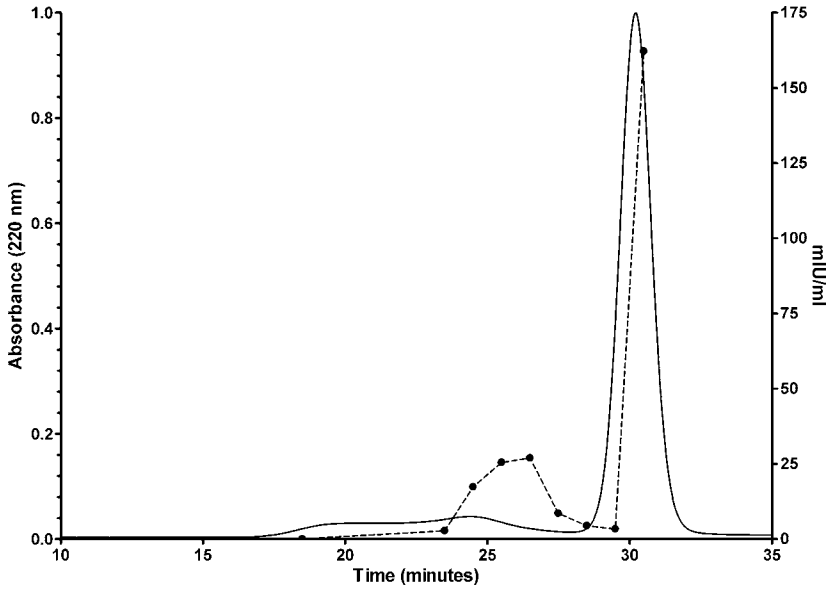

Fig. 1. Superdex HR 10/30 size exclusion HPLC of EPREX® containing 0.18% of erythropoietin dimer/oligomer (——) by an alternate chromatographic method and results from erythropoietin-specific ELISA on column fractions (- - - -). The ELISA detects monomeric, dimeric, and oligomeric erythropoietin. Erythropoietin positive material is detected in the region where polysorbate 80 elutes in this chromatographic system (16–27 min).

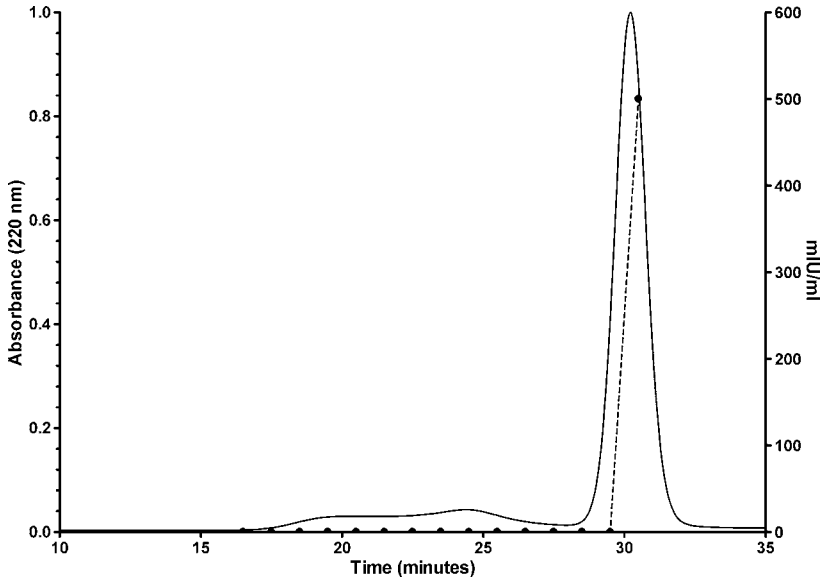

Fig. 2. Superdex HR 10/30 size exclusion HPLC of EPREX® (——) determined to be free of erythropoietin dimer/oligomer by an alternate chromatographic method and results from erythropoietin-specific ELISA on column fractions (- - - -). The ELISA detects monomeric, dimeric, and oligomeric erythropoietin. No erythropoietin positive material is detected in the region were polysorbate 80 elutes in this chromatographic system (16–27 min).

EPREX®-like formulations were prepared in which the polysorbate 80 concentration was increased from the 0.03% used in EPREX® to 0.3% to study whether increasing the polysorbate 80 concentration could initiate protein–micelle interactions. When identical volumes of these solutions were injected onto a Superdex HR 10/30 column, the profiles of the polysorbate 80 region changed, as shown in Fig. 4. As the concentration of polysorbate 80 increased, the ratio of the peak heights for the two peaks eluting at approximately 19 and 24 min changed, with the height of the 24-min peak increasing at a much more rapid rate. If protein-containing micelles are being formed, then increases in the micelle concentration should result in a concomitant decrease in the protein peak. Neither the height nor the area of the erythropoietin peak at

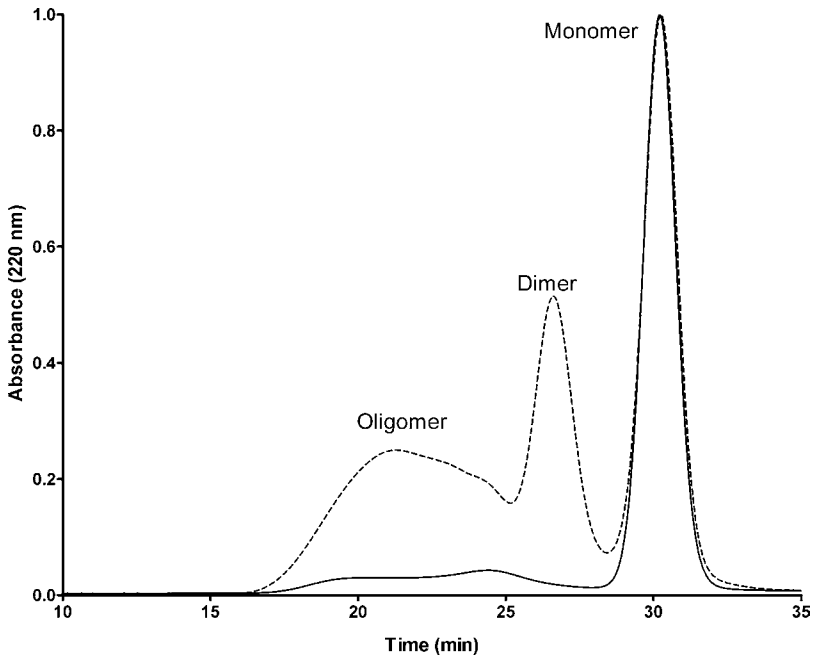

Fig. 3. Superdex HR 10/30 size exclusion chromatogram of EPREX® (——) and erythropoietin that was heat treated to create high levels of erythropoietin dimer and oligomer (- - - -). The chromatograms indicate that the dimer and oligomer of erythropoietin coelute with polysorbate 80 under these chromatographic conditions.

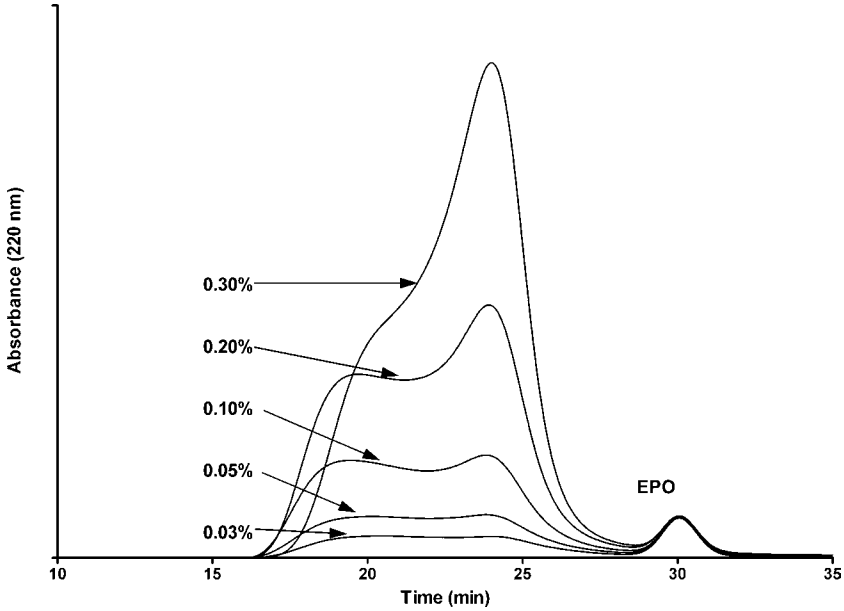

Fig. 4. Superdex HR 10/30 size exclusion chromatogram of EPREX®-like formulations containing from 0.03 to 0.3% polysorbate 80 and 10,000 IU/mL (0.083 mg/mL) erythropoietin showing that the peak shape changes with the amount of polysorbate 80 injected onto the column but the height and area of the erythropoietin peak remain constant and are independent of the concentration of polysorbate 80.

30 min decreased with increasing concentrations of polysorbate 80. The lack of decrease in the erythropoietin peak with increasing concentrations of polysorbate 80 is inconsistent with the hypothesis of the existence of stable higher-order polysorbate 80–erythropoietin structures.

In a similar experiment using a TSK G3000 size exclusion column, the effect of injection volume on peak shape was investigated. Volumes ranging from 5 to 90 μL of a 40,000-IU/mL (0.333 mg/mL) EPREX® formulation were injected and the column eluates monitored at 214 nm as well as by light scattering and refractive index. The absorbance chromatograms, normalized on the height of the erythropoietin peak, of these injections are shown in Fig. 5. With this column matrix, three distinct maxima can

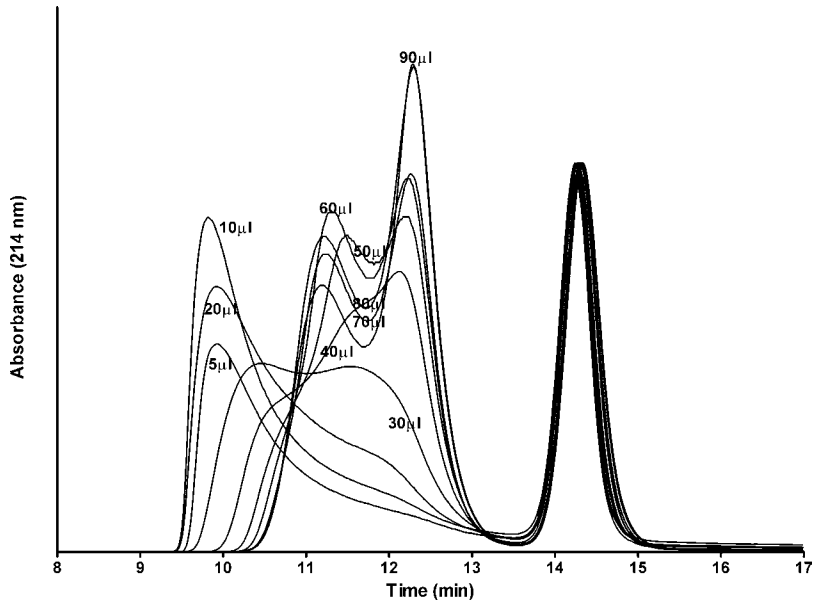

Fig. 5. TSK G3000 size exclusion chromatogram of EPREX® (40,000 IU/mL, 0.333 mg/mL) in which increasing volumes from 5 to 90 μL were injected. Chromatograms were normalized on the height of the erythropoietin peak The number of peaks, retention times of peaks, and relative peak areas vary depending on the volume of sample (amount of polysorbate 80) injected.

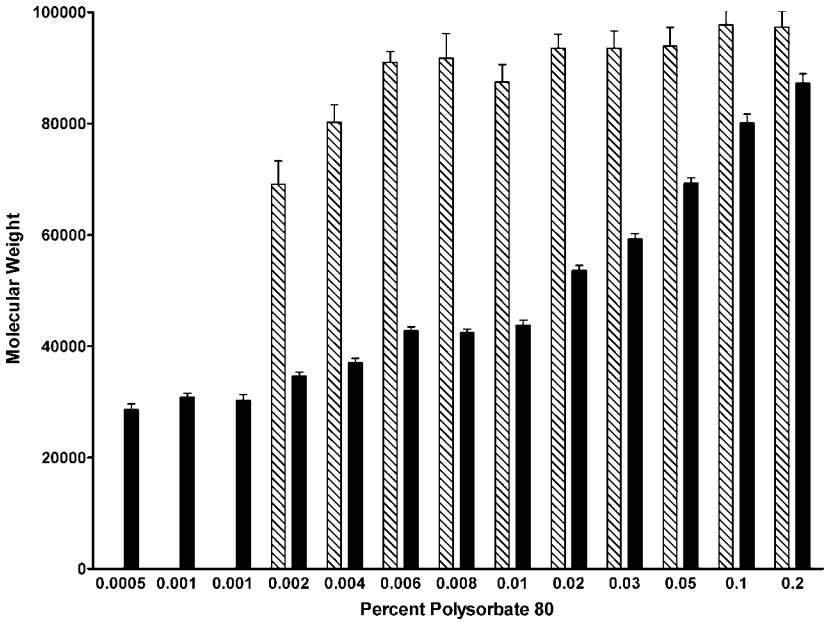

Fig. 6. The molecular weights as determined by light scattering of solutions containing increasing concentrations of polysorbate 80 in EPREX® buffer without erythropoietin (hatched bars) and in the presence of 30,000 IU/mL (0.25 mg/mL) erythropoietin (solid bars). In the absence of erythropoietin the CMC of polysorbate 80 in this buffer is between 0.001 and 0.002% and the molecular weight of polysorbate 80 micelles is 85,000–90,000 Da. In the presence of erythropoietin there is a change in the molecular weight pattern.

be observed, depending on the volume (amount of polysorbate 80) injected. Based on light-scattering analyses of the eluates, the molecular weight of the polysorbate 80 peaks vary from 150,000 to 1,000,000 Da, with the highest molecular weights corresponding to the smallest injection volumes (data not shown). The results from the size exclusion HPLC studies in which polysorbate 80 concentration, injection volumes, and column types (matrices) were varied strongly suggest that the multinode nature of polysorbate 80 elution is artifactual and may not be representative of solution interactions.

To investigate whether solution studies could be used to define the nature of the interaction of polysorbate 80 with erythropoietin, we prepared a series of solutions using the sodium chloride/glycine/phosphate buffer used in EPREX® in which the concentration of polysorbate 80 went from 0 to 0.2%. Solutions were prepared both without erythropoietin and with 30,000 IU/mL (0.25 mg/mL) erythropoietin. Analysis of the solutions without erythropoietin gave the molecular weights shown in Fig. 6 (hatched bars). Between 0.001 and 0.002% polysorbate 80, the molecular weight increases rapidly to ≈50,000 Da and reaches a plateau of 90,000–95,000 Da. This is consistent with the reported value for the critical micelle concentration (CMC) of polysorbate 80 of 0.017% and the micelle molecular weight of 87,000 ± 8,000 (8). Analyses of the solutions containing erythropoietin show a significantly different molecular weight pattern (Fig. 6, solid bars). In the absence of polysorbate 80, the mean molecular weight determined for erythropoietin was 29,000, similar to the 30,600 reported for the glycoprotein. As the polysorbate 80 concentration increased to 0.01% there was a gradual increase in the mean molecular weight to 44,000, followed by a more rapid rise in mean molecular weight to 87,000 at 0.2% polysorbate 80.

DISCUSSION

The previous analysis by Hermeling *et al.* (7) used EPREX® that was expelled from purchased prefilled syringes. For our investigations, we used two lots of EPREX® syringes, one shown to be free of dimer/oligomer and one containing 0.18% dimer/oligomer, and prepared two 40,000 IU/mL (0.333 mg/mL) EPREX® solutions using production-qualified excipients (polysorbate 80, glycine, sodium chloride, and mono- and dibasic sodium phosphate) and a lot of purified bulk erythropoietin drug substance with no detectable dimer/oligomer. One lot was spiked with heat-induced aggregate. Analyses were done by SEC-HPLC using the same column, elution buffer, flow rate, and UV detection as described by Hermeling *et al.* Column fractions were collected corresponding to the elution of polysorbate 80 and erythropoietin and these fractions were analyzed by ELISA. The sensitivity of the ELISA was such that 0.00025% of the total amount of erythropoietin injected could be detected in a single column fraction. Figures 1 and 2 show the chromatograms and corresponding ELISA of column fractions for EPREX® prefilled syringes containing 0.18% dimer/oligomer and dimer/oligomer free. Where erythropoietin dimer/oligomer was known to be present, erythropoietin-like material could be detected coeluting with the trailing edge of the second polysorbate 80 peak (Fig. 1); however, in the sample known to be free of dimer/oligomer, no erythropoietin-like material was found in the polysorbate 80 region of the chromatogram, suggesting that the previous studies may have

detected erythropoietin dimer that coelutes with polysorbate 80 under these chromatographic conditions. Erythropoietin is known to form a disulfide-exchanged dimer with time (9,10). A sample of erythropoietin was heated at 55°C for 14 days to accelerate formation of dimer and oligomer and this material was chromatographed using the same SEC-HPLC conditions previously used. Figure 3 shows the chromatogram superimposed with the chromatogram of EPREX®. The elution time of the dimer is consistent with the profile for the ELISA-detected material in Fig. 1. Given that the elution time of erythropoietin dimer is similar to the trailing edge of polysorbate 80, that erythropoietin is known to form trace amounts of dimer with time, and that erythropoietin-like material eluting with polysorbate 80 cannot be detected by a sensitive ELISA unless erythropoietin dimer is known to be present by an independent method, it can be reasonably concluded that the hypothesis of Hermeling *et al.* of the existence of polysorbate 80 micelles presenting erythropoietin on the surface was incorrect and made without benefit of controls involving erythropoietin dimer/oligomer.

If polysorbate 80 micelles containing erythropoietin were being formed in EPREX®, then increasing concentrations of polysorbate 80 would promote their formation. EPREX®-like formulations [10,000 IU/mL (0.083 mg/mL)] were prepared with polysorbate 80 concentrations from 0.03 (used in EPREX®) to 0.3% (10-fold higher). Identical volumes of these solutions were injected onto the SEC-HPLC (Fig. 4). There was no decrease in either the height or the area of the erythropoietin peak. If micelles containing erythropoietin were forming, there should be a concomitant decrease on the erythropoietin peak with increasing polysorbate 80 concentrations. The presumptive conclusion from this data is that there is no association of erythropoietin with polysorbate 80 micelles. Other researchers using analytical ultracentrifugation have reached the same conclusion (11).

There was a readily apparent change in the elution profile of polysorbate 80 with increasing polysorbate 80 concentrations. In a similar experiment, different volumes of 10,000 IU/mL EPREX® were injected onto a TSK G3000 SEC-HPLC column, giving the profiles normalized on the height of the erythropoietin peak (shown in Fig. 5). There was a significant change in the polysorbate 80 elution profile with increasing amounts of polysorbate 80 inject onto the column. The presence of multiple peaks on size exclusion columns with self-associating systems such as surfactants has been previously observed (12). The molecular weight of the polysorbate 80 peaks determined by Hermeling *et al.* (255 and 670 kDa) is significantly higher than that determined for polysorbate 80 micelles in solution. By analytical ultracentrifugation, the polysorbate 80 micellar molecular weight in water has been determined to be 87,000 ± 7,000 (13). Using light scattering we have obtained a value of 90,000–95,000 in EPREX® buffer (Fig. 6). Other researchers using size exclusion chromatography have obtained a molecular weight of 182,000 on a Sephadex G-200 column eluted with 0.15 M KCl (14), suggesting that surfactants such as polysorbate 80 elute as if they are a high molecular weight species on size exclusion chromatography columns and that these molecular weights are not representative of surfactant in solution. The behavior on size exclusion columns could be column matrix-induced association. Similar results were seen with polysorbate 20 (13,14).

To investigate the interaction between erythropoietin and polysorbate 80, we prepared 30,000 IU/mL EPREX®-like solutions containing concentrations of polysorbate 80 from 0 to 0.2% and used light scattering to determine the molecular weight. Light-scattering studies on solutions do not resolve individual species, but give a mean molecular weight and are, therefore, most useful when a solution contains a predominant species. The light-scattering results are shown

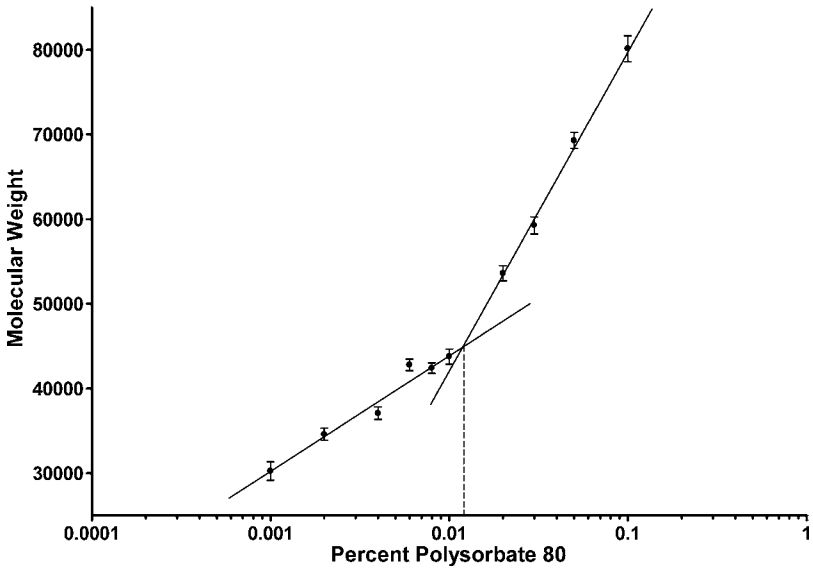

Fig. 7. Plot of the log of the polysorbate 80 concentration *vs*. the molecular weights (SD shown) as determined by light scattering of solutions containing increasing concentrations of polysorbate 80 in EPREX® buffer in the presence of 30,000 IU/mL (0.25 mg/mL) erythropoietin. Least squares analysis of the two phases of the plot intersected at 0.0125% polysorbate 80, corresponding to a ratio of one erythropoietin molecule to 12 polysorbate 80 molecules.

in Fig. 6. In contrast to polysorbate 80 in EPREX® buffer, which shows a CMC of between 0.001 and 0.002% [consistent with the literature value of 0.0014% (15)] and a micelle molecular weight of 90,000–95,000, these solutions showed a gradual increase in mean molecular weigh that appears to asymptotically approach the molecular weight in the absence of erythropoietin.

The nature of polysorbate 80–erythropoietin interactions can be inferred from a plot of the log of polysorbate 80 concentrations *vs*. the molecular weights from light scattering (Fig. 7). The plot is similar to that seen for a titration curve, with an equivalence (inflection) point at 0.0125% polysorbate 80. This corresponds to a ratio of 12 polysorbate 80 molecules to one erythropoietin molecule. This suggests a model in which polysorbate 80 interacts with the hydrophobic surfaces on erythropoietin and that surfactant monomer is in a dynamic equilibrium with surfactant-associated protein and polysorbate 80 micelles. At 0% polysorbate 80, the molecular weight determined by light scattering is 29,000, similar to the 30,600 of erythropoietin. In the presence of increasing concentrations of polysorbate 80, the increases in mean molecular weight are consistent with polysorbate 80 associating with the hydrophobic surfaces on erythropoietin. At the equivalence point, the calculated molecular weight of erythropoietin with 12 associated polysorbate 80 molecules is 46,440, similar to the 45,400 based on the simultaneous solution of the equations from the least square fit of the two phases of the experimental data shown in Fig. 7. Beyond the equivalence point, the concentration of additional polysorbate 80 is above the CMC and the formation of polysorbate 80 micelles would be expected.

The association of surfactants with proteins has been studied since 1968 when Pitt-Rivers and Impiombato demonstrated that surfactants are capable of associating with proteins (16) and that these interactions are through the hydrophobic surfaces of proteins (17). The interaction of surfactants with proteins in stoichiometric ratios not involving micelle formation is referred to as "mixed micelles" (18). There are several examples in the literature of proteins associating with surfactants in defined stoichiometric ratios where the number of surfactant molecules is below that necessary to form a micelle (19–23). The results of our studies on the interaction of polysorbate 80 with erythropoietin are consistent with other studies on surfactant–protein interactions. There are no documented instances of proteins incorporated into polysorbate 80 micelles.

The use of light scattering to determine the molecular weight of solutions with increasing concentrations of surfactant is a straightforward technique for determining the stoichiometry of surfactant–protein interactions. This method can be used to determine the minimum concentration of surfactant necessary to interact with the accessible hydrophobic surfaces of a protein. Nonionic surfactants such as polysorbate are known to have low affinity for protein hydrophobic surfaces (24). To maintain efficient protection of hydrophobic surfaces from the aqueous environment, an excess of surfactant beyond the equivalence point would be preferred.

The results of these studies clearly define the nature of the interaction between polysorbate 80 and erythropoietin. Polysorbate 80 associates with the hydrophobic surfaces of erythropoietin in a defined stoichiometric ratio.

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