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Letter to Editor

Reaction to the paper: Interaction of Polysorbate 80 with Erythropoietin: A Case Study in Protein–Surfactant Interactions

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Abstract. Micelle-associated epoetin is still a possible explanation for the upsurge of pure red blood cell aplasia associated with subcutaneous use of epoetin alpha in patients with chronic renal failure.

KEY WORDS: epoetin; Eprex; immunogenicity; micelles; polysorbate 80.

Dear Editor,

In a recent article (1), Villalobos *et al*. claim that our results suggesting that Eprex® contains micelle-associated epoetin (2) are incorrect. They base the claim on experiments failing to show a change in the monomeric epoetin peak with increasing polysorbate 80 concentrations in gel permeation chromatography (GPC) analysis. According to Villalobos and colleagues, uptake in micelles should lead to a decrease in the monomeric epoetin peak. Using the same GPC procedure, we have shown that less than 1% of epoetin coelutes with the polysorbate 80 micelles. This small amount will not be detectable as a significant decrease in the monomeric epoetin peak.

Villalobos *et al*. also claim that epoetin can be detected in the micelle fraction only if dimers/oligomers are present, and suggest that we have mistaken the micelle-associated fraction for disulfide-mediated dimers/oligomers. They disregard our observation that on removal of polysorbate 80 from the mobile phase, the amount of epoetin in the micellar fraction drops from ca. 0.7% to below 0.1% (2). A plausible explanation for our observation is a reduction of the micelle concentration during chromatography (because of continuous separation of micelles and monomers on the column, leading to reestablishment of the dynamic equilibrium between micelles and monomers) and, consequently, the fraction of micelle-associated protein. In contrast, our colleagues' theory of dimer/oligomer formation cannot explain our observation. Villalobos *et al*. do not observe an increase in the protein peak coeluting with the micelles when the micelle concentration is increased. We assume that the solutions they used were freshly made, which would be consistent with our findings that weeks of storage are necessary for polysorbate micelles to get loaded with protein (unpublished results).

We have also studied whether micelle association is unique for epoetin. We therefore performed similar experiments with recombinant human interferon alpha2b (rhIFNα2b). The lower molecular weight of rhIFNα2b (19.3 kDa) as compared to epoetin (30.4 kDa) allows the dimer peak of rhIFNα2b (ca. 1% of the total protein content) to be fully separated from the micellar fraction (Fig. 1). Formulations of rhIFNα2b with various concentrations of polysorbate 20 and polysorbate 80 showed protein in the micellar peak (Table I).

To support their claim that Eprex® does not contain micelle-associated protein, Villalobos *et al.* performed experiments with polysorbate concentrations up to 10-fold and protein concentrations up to 3-fold those present in the Eprex® formulation. However, the relevance of these studies for the polysorbate–epoetin interactions in Eprex® prefilled syringes is questionable.

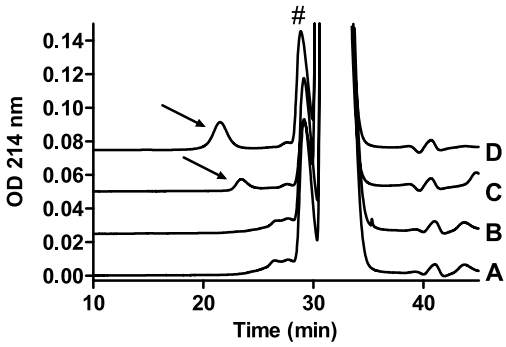

Fig. 1. GPC profile of the four rhIFNα2b formulations (see Table I for the compositions). The monomer peak of rhIFNα2b elutes at ca. 32 min. The micelle peaks of polysorbate 80 (elution time ca. 21 min) and polysorbate 20 (elution time ca. 23 min) are indicated by arrows. The dimer peak of rhIFNα2b (#) is clearly separated from the micellar fraction. Note that the mobile phase used for GPC analysis of rhIFNα2b slightly differed from the one used for epoetin, which led to a different shape of the micelle peak. This confirms the findings of Villalobos *et al.* (1) that the *apparent* hydrodynamic volume of polysorbate micelles on the GPC column is strongly dependent on the conditions used.

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Table I. Surfactant Concentrations and the Amount of rhIFNα2b in the Micellar Fraction of the rhIFNα2b Formulations Studied by GPC[a]

Sample	Surfactant	Concentration (% w/v)	Amount of rhIFNα2b in micellar fraction[b] (%)
Formulation A	None	Not applicable	<0.002[c]
Formulation B	Polysorbate 20	0.03 (4 × CMC)	<0.002[c]
Formulation C	Polysorbate 20	0.15 (20 × CMC)	0.11
Formulation D	Polysorbate 80	0.03 (20 × CMC)	0.14
Placebo C	Polysorbate 20	0.15 (20 × CMC)	<0.002[c]
Placebo D	Polysorbate 80	0.03 (20 × CMC)	<0.002[c]

[a] All formulations contained, next to surfactant, rhIFNα2b (0.5 mg/ml), mannitol (40 mg/ml), and sucrose (10 mg/ml) in 20 mM sodium phosphate, pH 7.4. GPC was performed as described before (2).
[b] Percentage of amount of rhIFNα2b applied to the GPC column; fractions were analyzed for rhIFNα2b by ELISA.
[c] Below detection limit.

Villalobos *et al.* claim that polysorbate 80 can be excluded as a causative factor in pure red cell aplasia (PRCA) because of the inability of the surfactant to stimulate an immune response to epoetin in animal models. In addition, they suggest to have identified organic leachates from uncoated rubber stoppers in combination with polysorbate 80 to be one of the putative causes of PRCA, referring to a previous paper (3). However, the leachates also failed to enhance the immune response against epoetin. Instead, their claim is based on a single experiment using an irrelevant antigen [ovalbumin, a foreign antigen that is intrinsically immunogenic in mice (4)] in combination with irrelevant leachate concentrations. We conclude that micelle-associated epoetin can be identified using the right analytical tools and conditions and is still a possible explanation for the upsurge of PRCA associated with subcutaneous use of epoetin alpha in patients with chronic renal failure.

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