

Research review paper

Immunogenicity of therapeutic proteins. Part 2: Impact of container closures

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Abstract

Immunogenicity as a potential consequence of therapeutic protein administration is increasingly being scrutinized in the biopharmaceuticals industry, particularly with the imminent introduction of biosimilar products. Immunogenicity is an important safety aspect requiring rigorous investigation to fully appreciate its impact. Factors involved in product handling, such as storage temperature, light exposure, and shaking, have been implicated in immunogenicity, while container closure systems are no less important. Intended to provide a stable environment for the dosage form, container closures may also interact with a product, affecting performance and potentially enhancing immunogenicity. Glass surfaces, air–liquid interfaces, and lubricants can mediate protein denaturation, while phthalates in plastics and latex rubber are sources of extractables and leachates that may contaminate a product, causing allergic reactions and increasing immunogenicity. The manufacture of therapeutic proteins therefore requires rigorous safety evaluations not just in the context of the product, but also product containment.

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1. Introduction

Immunogenicity in the context of biopharmaceuticals is a potentially serious and largely unpredictable outcome of the administration of protein therapies derived from recombinant DNA and hybridoma technologies (Ryff and Schellekens, 2002). The induction of high neutralizing and non-neutralizing antibody titers in response to therapeutic proteins has been associated with reduced or abrogated efficacy, and adverse immunologic events such as allergy, anaphylaxis, and serum sickness (Koren et al., 2002; Schellekens, 2002, 2004). Of greatest concern is the potential for a neutralizing antibody response that nullifies expression of the native protein, resulting in serious iatrogenic morbidities, such as occurred from 2000 to 2002 with erythropoietin-induced pure red cell aplasia (PRCA) in patients with chronic kidney disease, and thrombopoietin-induced thrombocytopenia in healthy volunteers and cancer patients (Casadevall et al., 2002; Li et al., 2001). Thus, immunogenicity is an important aspect of the safety of biopharmaceuticals that requires rigorous investigation in order to fully understand its likely clinical impact (Koren et al., 2002; Schellekens, 2002).

The majority of biopharmaceuticals are homologs of human proteins for which the main mechanisms of immunogenicity relate to extrinsic factors, such as route of administration, frequency of dosing, formulation, and impurities, and intrinsic factors, such as tertiary structure, complexity, and degradation of the protein (Schellekens, 2004). Patient-related factors can also determine immunogenicity and include immune status, nature of the disease, genetic background, and concomitant medications (Antonelli and Dianzani, 1999; Schellekens, 2002). The issue of immunogenicity is therefore complex and is increasingly being scrutinized by regulators, particularly with the imminent expiry of innovator biopharmaceutical patents and the entry of biosimilar products into European and US markets (Schellekens, 2004). In the absence of suitable methods for predicting immunogenicity outside the clinical trial setting, verification of biosimilar products will require more than just a demonstration of pharmacological bioequivalence, which is sufficient for manufacturers of conventional, low-molecular-weight generic pharmaceuticals (Schellekens, 2005).

Product handling, the focus of the first article in this series, has been shown to impact on the immunogenicity of biopharmaceutical products. Sources of immunogenicity relating to product handling include product preparation, storage temperature, exposure to light, and shaking (Koren et al., 2002; Schellekens, 2002). Examples from the literature indicate that these and other

factors have variously contributed to the immunogenicity of monoclonal antibodies, interferon alpha, insulin, and erythropoietin, among others (Gregory et al., 1991; Hochuli, 1997; Kroon et al., 1992; Lam et al., 1997; Sharma et al., 2004). In addition, container closure systems used in packaging pharmaceuticals can also have an impact on immunogenicity and are the focus of this second article of three on the immunogenicity of therapeutic proteins.

2. Impact of container closures on product performance and immunogenicity

The main function of container closure systems is to offer protection of the dosage form against factors that can promote degradation over the shelf-life of the product (Food and Drug Administration (FDA), 1999). Such factors include exposure to light or reactive gases, loss of solvent, absorption of water vapor, and contamination by filth or microorganisms. In addition to providing a stable environment for the dosage form, container closures are also required to be compatible with the product, and packaging components must be safe. Factors that can influence the safety of packaging components include potential contaminants in glass, type of container used (vial or syringe), glass and air interfaces, use of lubricants, and materials used in syringe hubs.

Container closures can interact with the dosage form to alter the quality of a product. Arsenic, for example, is a potential contaminant in glass; therefore the pharmacopoeia has arsenic limits for glass containers. The choice of container closure is important because those that interact with the product contents can affect the activity and immunogenicity of the dosage form, with denaturation products such as aggregates implicated in the immunogenicity of therapeutic proteins (Cleland et al., 1993; Koren et al., 2002). Vials have simpler container closure compared with syringes. Vials consist of a glass surface and a vial stopper that has a coated surface to prevent leachables and extractables. In contrast, syringes have multiple surfaces and materials that can interact with the product and alter product quality. Hydrophobic surfaces and lubricants used in prefilled syringes can mediate protein denaturation (Jones et al., 2005; Mizutani, 1980; Treuheit et al., 2002). For example, glass and air interfaces are hydrophobic surfaces that can mediate protein denaturation and aggregation, and silicone oil is a lubricant used to coat the syringe barrel and syringe plungers that facilitates injection but also provides a surface on which proteins can denature and aggregate. Interactions between container closures and the formulated product can also cause extractables

and leachables, which also have the potential to affect the immunogenicity of the product (Hill et al., 2003; Jenke et al., 2005; Primeau et al., 2001; Sharma et al., 2004). Organic compounds involved in vulcanization or plasticization can leach into the product from uncoated rubber surfaces, and residual levels of metals from wire used to create the syringe hubs in glass syringes can also interact with the product.

The use of prefilled syringes has increased because it offers many advantages over conventional systems for parenteral injections (drugs packaged in ampoules or vials). Best of all, it provides a single, “ready-to-use” package. For safety assessments, most of the attention is devoted toward the glass surface and the siliconization process for prefilled syringes. However, as a part of the PRCA investigation, leachates (phenolic derivatives commonly used as curing agents) extracted by polysorbate 80 from uncoated rubber syringe stoppers were identified, which highlights the importance of materials used in syringe hubs. According to a report presented at the Parenteral Drug Association (PDA) Scientific Forum, pharmaceutical containers and closures are no less important than the contents, as evidenced by these and other quality assurances of packaging components imposed by regulatory agencies as part of the drug approval process (Adlaf, 2001).

2.1. Effect of container closures on biologic activity

The use of fused glass ampoules as containers for lyophilized biological standards and reference materials

is standard practice among biopharmaceutical manufacturers to ensure product stability by preventing gas exchange with the external environment and preserving low residual moisture levels. However, filling procedure, speed of filling, and ease of opening and sampling are drawbacks compared with the use of capped vials. A study conducted using alkaline phosphatase as a model for the stable storage of freeze-dried biological standards compared the relative activity of the enzyme when stored at a range of temperatures and humidities in vials with untreated stoppers versus treated stoppers and ampoules (Ford and Dawson, 1994). Although alkaline phosphatase activity was similar immediately after freeze-drying in vials and ampoules, activity subsequently declined over a 12-month storage period at variable and fixed temperatures, with the greatest loss of activity occurring in vials with untreated stoppers and at fluctuating temperatures rather than a fixed temperature of 37 or 45 °C (Fig. 1). Moisture levels were highest in vials with untreated stoppers, intermediate in ampoules, and lowest in vials with stoppers treated with industrial methylated spirits then air dried and heat sterilized. The key findings of this study were that the source of the water appeared to be from the stoppers rather than the external environment, making the use of vials feasible in place of ampoules provided the stoppers are appropriately treated before use (Ford and Dawson, 1994).

The filling procedure can also have an effect on the activity of the protein. Orthoclone OKT[®]3 (muromonab-CD3), which has been marketed since 1986 for reversal of human kidney graft rejections, is filled in

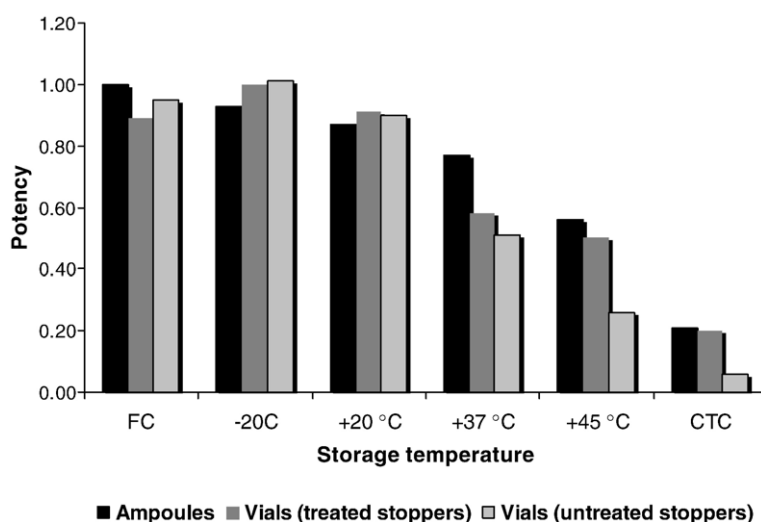


Fig. 1. Effect of temperature on activity of freeze-dried alkaline phosphatase relative to a frozen control following storage for 12 months in ampoules, vials with untreated stoppers, or vials with stoppers treated with industrial methylated spirits then air dried and heat sterilized (Ford and Dawson, 1994). CT, cycling temperature; FC, frozen control, -70 °C in ampoules.

ampoules under nitrogen to prevent degradation due to oxidation and deamidation. Flushing with nitrogen has been shown, like argon, to greatly slow the oxidative degradation of orthoclone OKT[®]3 and preserve the binding ability of the antibody (Rao and Kroon, 1993).

The nature of the container surface interface is an important consideration for product stability in relation to the individual protein itself. The biological activity of different enzymes has been shown to vary considerably on a single type of glass surface (Mizutani, 1980). Following absorption of horseradish peroxidase, alkaline phosphatase, and catalase onto a glass column for 1 week, the respective activities of the enzymes referenced to a porous glass column were 97%, 88%, and 63%. After absorption for 3 months, the respective biological activities were just 48%, 14%, and 2% of the total activity loaded onto the column. Insulin had almost full activity after absorption for 3 months, while activity was absent for calcitonin altogether. Although all proteins were in an active state when initially absorbed onto the glass surface, dissociation of subunits over time resulted in reduced activity of the more complex proteins, while activity for the smaller insulin peptide was preserved (Mizutani, 1980).

2.2. Effect of container closures on protein denaturation

The impact of surface-induced protein aggregation on product stability was first recognized over 50 years ago, with subsequent research revealing that protein conformational changes resulting in partial unfolded states are the most susceptible to aggregate formation (Treuheit et al., 2002). In this unfolded state, hydrophobic regions of the peptide otherwise buried in the native molecule are exposed to intermolecular protein interactions that originate at the interface between the air and liquid, which is a suitable denaturing surface for aggregation to occur. Agitation during transport and the use of high-pressure filling lines can increase the area of this boundary enhancing the potential for aggregation, while preservation of the native structure of the protein is more compatible with preventing aggregation. Thus, low protein concentration in a quiescent shelf-life setting is one strategy for preventing aggregation. Alternatively, aggregation induced by an air–water interface has unexpectedly been shown to decrease with higher protein concentration in conjunction with agitation, highlighting the complexity of protein aggregation reactions (Treuheit et al., 2002).

Metals used in creating syringe hubs can also influence product stability. For one therapeutic protein, increased levels of truncated product were attributed to

the leaching of metal ions from the syringe gasket, which activated a metalloprotease that co-eluted with the product (Rosenberg and Worobec, 2004). In another case, the protein was oxidized by tungsten metal ions from the syringe barrel, causing aggregation of the product (Rosenberg and Worobec, 2004).

In addition to the container surface and air–liquid interfaces already described, biopharmaceuticals frequently come into contact with chemical coatings, such as silicone oil, used to treat container surfaces in order to facilitate processing or prevent protein binding. Silicone oil has been implicated in the induction of protein aggregation, although evidence of large conformational changes or alterations in thermal stability in the presence of the lubricant is limited. The most likely explanation for aggregation is that the silicone oil directly effects intermolecular protein interactions or exerts an effect via a solvent (Jones et al., 2005).

2.3. Effect of container closures on extractables and leachables

In the FDA guidance document on container closure systems for packaging pharmaceuticals and biologics, those products with the highest level of concern regarding route of administration and the greatest likelihood of packaging component–dosage form interactions are inhalation aerosols and solutions, injections, and injectable suspensions (FDA, 1999). Given that biopharmaceuticals are typically injectable suspensions, and therefore fall into the category with the highest concern about packaging, biopharmaceutical manufacturers need to make considerable efforts to ensure their products are adequately packaged. Compounds that are extracted or leached from container closure systems are particularly concerning because they have many potential sources and factors affecting them. Consequently, testing needs to be conducted with the individual product and specific extractables and leachables in mind.

Sources of compounds that are extracted or leached from container closures include rubber stoppers, PVC tubing used in respiratory therapy, and plastic materials used in medical items, such as solution containers, transfusion sets, transfer tubing, and medical devices (Hill et al., 2003; Jenke et al., 2005; Primeau et al., 2001). Commonly identified compounds from these products include latex allergens, antioxidants, and plasticizers, with the latter added to PVC tubing to act as a lubricant between the polymer chains to improve flexibility and durability (Hill et al., 2003). Diethyl phthalate (DEP) and di-(2-ethylhexyl) phthalate (DEHP), among other phthalates, are routinely used as plasticizers, while

the antioxidants *p*-nonylphenol (*p*-NP) and butylated hydroxytoluene (BHT) are also used in plastics manufacture, including plastics with medical applications (Hill et al., 2003).

The use of natural rubber container closure systems has led to the induction of allergic reactions in individuals with a latex allergy receiving medications from such containers (Primeau et al., 2001). Natural latex allergy is a recognized health problem, with a prevalence of 1–6% in the general population, while health care workers are especially vulnerable. In one study investigating the impact of latex rubber container closures on release of allergenic proteins, the concentration of latex allergens in natural rubber vial closures was sufficient to elicit positive intradermal skin reactions in some individuals with latex allergy (Primeau et al., 2001). Importantly, the latex allergens were released into solution following direct contact with the vial closures during storage and not as a result of container puncture for the purpose of delivering the dosage form. Moreover, synthetic closures did not result in positive skin tests in volunteers with latex allergy, indicating that the use of latex-free synthetic rubber container closures is a desirable goal.

The use of phthalates in plastic products is widespread. In the building industry, DEHP has been identified as the major plasticizer exposure in households where its inhalation has been linked with the development of airway inflammation and asthma (Oie et al., 1997). Concerns over phthalate use in the health sector are based on the potential for direct contact of plastic materials with biologic products resulting in extractables in the finished product. Such interactions are well

documented (Jenke et al., 2005). Of the many compounds that leach into the bore of medical tubing used in respiratory therapy, DEP, DEHP, BHT, and *p*-NP were found in sufficiently high concentrations to warrant concern for patients receiving oxygen on a long-term basis, those with allergies to such compounds, and those with asthma (Hill et al., 2003). Thus, while the role of plasticizers in enabling medical plastics to perform a variety of clinical applications is accepted as being sufficiently important to retain these compounds, the trade-off may be a variety of chronic health effects (Hill et al., 2001).

A further effect of extractables and leachables is their ability to modulate immune responses. In a subcutaneous injection experimental model, degradation products from commonly used phthalate plasticizers such as DEHP were shown to produce adjuvant activity (Larsen et al., 2001). Although the adjuvant effect varied according to the individual phthalates investigated (Larsen et al., 2002), this finding does indicate the need to include adjuvant properties in any risk assessment of phthalates.

2.4. Role of container closures in erythropoietin-induced PRCA

The container closure system used in a recombinant human erythropoietin (rHuEPO) marketed outside the USA (Eprex®) had a major impact on the immunogenicity of the product as a result of a change in formulation (Lim, 2005). Although a rare condition, the incidence of PRCA began to increase in 1998 in chronic renal failure patients receiving subcutaneous injections of rHuEPO, reaching a peak in 2002. Most cases were

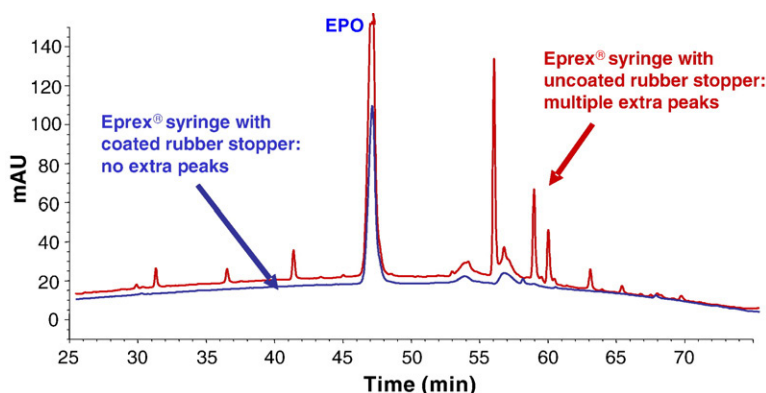


Fig. 2. Reverse-phase high-performance liquid chromatography chromatogram of: Eprex® from prefilled syringes with FluroTec®-coated rubber stoppers; and Eprex® from prefilled syringes with uncoated rubber stoppers. Profiles are of product formulated with polysorbate 80. The large peak at 47 min corresponds to epoetin alfa (EPO). The small peaks at 54 and 57 min are polysorbate 80. A series of extra peaks are present in the Eprex® prefilled syringes with uncoated but not with coated rubber stoppers. mAU, milliabsorbance unit. [Adapted with permission from European Journal of Hospital Pharmacy, 2004, volume 5, published by Pharma Publishing and Media Europe bvba, Belgium (Sharma et al., 2004).

associated with Eprex[®] use. The investigation to identify the cause of the increase in Eprex[®]-associated PRCA was discussed in relation to product handling in the previous article in this series. In the current article, the findings of the investigation are presented in relation to container closures.

As already discussed, plasticizers, antioxidants, and allergens can be leached from rubber in container closures, causing product impurities in the process. Following the sharp increase in PRCA cases, rigorous technical investigations by the manufacturer of Eprex[®] revealed the presence of leachates, extracted by polysorbate 80 from uncoated rubber syringe stoppers, as the most likely product-specific cause of the increased incidence of antibody-mediated PRCA (Sharma et al., 2004). The leachates were identified as phenolic derivatives, which are commonly used as curing agents in rubber stoppers, and were not associated with the earlier product formulation in which human serum albumin had been used as an excipient in place of polysorbate 80. Also relevant to the investigation, the use of FluroTec[®]-coated rubber stoppers in place of the uncoated stoppers failed to produce any peaks corresponding to the leachates using reverse-phase high-performance liquid chromatography (Boven et al., 2005) (Fig. 2).

Animal studies conducted to establish whether the leachates could act as adjuvants found that subcutaneous administration of ovalbumin together with leachates from uncoated rubber stoppers stimulated a stronger immune response than ovalbumin alone (Ryan et al., 2006). Moreover, when ovalbumin was substituted for epoetin alfa, the adjuvant effect of the leachates was associated with a reduction in hematocrit, consistent with symptoms of PRCA. The investigators concluded that the leachates were the most likely source of the increased immunogenicity associated with Eprex[®] and, as a result, uncoated rubber stoppers were replaced with FluroTec[®]-coated stoppers, thereby preventing the leaching of impurities into the product. The incidence of PRCA subsequently declined, returning to baseline levels (Sharma et al., 2004).

3. Conclusion

Container closure systems are a seemingly minor aspect of the production of biopharmaceuticals, but in fact are major factors able to influence the activity and immunogenicity of the product within the container. Interaction of the container contents with the container closure, whether during processing, storage or transport, can result in extractables and leachables contaminating the product. Compounds leached from plastic and rub-

ber container closure systems include those with allergenic and adjuvant properties, which in the context of therapeutic proteins can result in serious adverse clinical effects. This illustrates the need for careful examination of potential interactions between therapeutic protein formulations and their container closures in order to mitigate any risk associated with immunogenicity of a product. This applies to new innovative biopharmaceuticals, as well as biosimilar products.

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