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Review Paper

Immunogenicity of therapeutic proteins. Part 1: Impact of product handling

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Abstract

The patents of first-generation biopharmaceutical proteins are expiring, creating opportunities for biosimilar products. Unlike conventional generic pharmaceuticals, the development of biosimilar products is far more complex and requires more than a simple demonstration of pharmacological bioequivalence to establish efficacy and safety. The main concern with biosimilar products, as for any therapeutic protein, is immunogenicity and with it the potential for serious clinical sequelae. In the absence of adequate predictors of immunogenicity outside the clinical trial setting, biosimilar products should be evaluated in the same way that any novel pharmaceutical is evaluated. Herein, the factors involved in breaking host tolerance following administration of a therapeutic protein are discussed. The impact of product handling on immunogenicity is considered in the context of some hard-fought lessons that have helped to shape the current era of biopharmaceutical manufacturing, packaging, distribution, storage, and quality assurance.

Keywords:

Biopharmaceutical; Therapeutic protein; Biosimilar; Immunogenicity; Neutralizing antibody; Epoetin; PRCA

1. Introduction

First-generation therapeutic proteins or biopharmaceuticals have had a major impact on the treatment of many diseases since their introduction in the late 1980s. Manufactured using recombinant DNA technology and hybridoma techniques, biopharmaceuticals have been variously applied to correct acquired or inherited deficiencies in native protein expression, alter disease processes, and protect against disease (Nihalani et al., 2002; Schellekens, 2002). To date, biopharmaceuticals have become an important part of the therapeutic armamentarium in a diverse range of indications, including growth disorders, diabetes, viral hepatitis, multiple sclerosis, cancers, and anemia. Current growth in the use of biopharmaceuticals is set to increase, with nearly half of all newly approved pharmaceuticals predicted to be of biotechnological origin by 2010 (Schellekens, 2005).

The patents of several first-generation biopharmaceuticals, such as interferons, growth hormone, insulin, and erythropoietins, have either recently expired or will soon expire in European and US markets, creating demand for biosimilar versions of these products as well as next-generation products (Schellekens, 2004). Whereas regulatory approval of conventional, low-molecular-weight generic pharmaceuticals is contingent on the relatively straightforward demonstration of physicochemical similarity and pharmacological bioequivalence with the innovator compound, the concept of generic pharmaceuticals gaining a marketing license on limited documentation cannot be extrapolated to off-patent biopharmaceuticals. In contrast to low-molecular-weight, chemically synthesized drugs, biopharmaceuticals are much larger proteins with a complex, three-dimensional structure, and a similarly complex manufacturing process that may include numerous extraction, purification and concentration steps involving protein denaturation and renaturation (Schellekens, 2004). Moreover, current analytical methods used to characterize biosimilar products and compare them with the innovator product are inadequate to predict efficacy and safety (Biotechnology Industry Organization [BIO], 2004; Nihalani et al., 2002; Schellekens, 2005). The argument put forward by the regulatory authorities in Europe is that full clinical trial data should therefore be required to demonstrate safety and efficacy of the biosimilar product together with a commitment to conduct extensive postmarketing surveillance until such time as there are robust analytical methods to establish predictive comparability (Schellekens, 2005).

The complexity involved with establishing equivalence between innovator and follow-on biopharmaceuticals is reflected in a more stringent approval process for biosimilar products than for conventional, low-molecular-weight pharmaceuticals. The new medicines legislation of the European Medicines Agency (EMA) has issued general and product-class-specific guidelines for the evaluation and approval of biosimilar products in Europe (Committee for Medicinal Products for Human Use [CHMP], 2005, 2006a,b,c,d,e,f). The guidelines require an extensive comparability exercise to demonstrate similar quality, safety, and efficacy between a new or modified product and the reference product (CHMP, 2006a,b). The extent of any trials needed for approval of biosimilar products is determined by the complexity of the molecular structure of the biopharmaceutical agent, any changes in the manufacturing process, and the impact of any differences in quality, safety, and efficacy of the product. In order to detect rare immune reactions, regulatory approval also requires a pharmacovigilance plan to be in place when the marketing authorization is granted, and postmarketing surveillance when the product is dispensed (CHMP, 2006b). The US Food and Drug Administration, which is yet to publish any guidelines on approval of biosimilar products, is currently undergoing a public consultative process (BIO, 2004).

This article is the first in a three-part series on the immunogenicity of biopharmaceuticals, intended to illustrate the particular challenges faced by manufacturers of innovator and biosimilar products alike in delivering a quality product that is safe and effective. The present article focuses on sources of immunogenicity, and the influence and clinical impact of product handling on immunogenicity. The following two articles will address the role of container closures and manufacturing changes on the immunogenicity profile of the product.

2. Potential sources of immunogenicity

Immunogenicity was first identified following the introduction of therapeutic animal proteins, such as porcine and bovine insulin, previously used in the treatment of diabetes (Schellekens, 2002). Although recombinant technologies are now used to produce humanized versions of most biopharmaceuticals (in theory making them less immunogenic than nonhuman proteins), even recombinant proteins with identical genetic sequences to native human proteins have been shown to induce antibody responses (Pecses et al., 1996; Zang et al., 2000). Conversely, divergence from the native human sequence is also not predictive of immunogenicity, and may result in less

immunogenicity than molecules with a higher degree of homology to the native protein sequence (Kontsek et al., 1999; Melian and Plosker, 2001). In fact, almost all therapeutic proteins have been shown to induce antibody production in humans to some extent, but the incidence of immunogenic effects varies widely among products (Pendley et al., 2003; Ryff and Schellekens, 2002).

There are many factors involved in breaking host tolerance following administration of a biopharmaceutical product. These can be broadly categorized in terms of treatment-related factors, and processing factors associated with the manufacture, storage, and handling of the product. Looking firstly at the treatment-related factors, a person with an immune system that is impaired, for example, as a result of cancer, may be less likely to develop antibodies to a therapeutic protein than a person whose immune system has already been activated in response to infection (Antonelli and Dianzani, 1999). Most protein drugs are administered to patients with normal immune tolerance, but individual characteristics such as genetic background, concomitant medications, and nature of the disease may influence whether tolerance to a particular protein can be overcome (Schellekens, 2002). For example, patients with severe hemophilia A involving large deletions or non-sense mutations of the factor VIII gene are more likely to mount an antibody response to exogenous factor VIII than hemophiliacs with mild or moderate disease because the former group does not express functional factor VIII antigen and there is no opportunity for immune tolerance (Fakharzadeh and Kazazian, 2000). Immunogenicity may also be affected by chronic illnesses that influence protein distribution, such as chronic kidney or liver disease, and by drugs that stimulate or suppress the immune system. Other treatment variables with an important bearing on immunogenicity of biopharmaceuticals are route of administration, dosing schedule, and length of treatment. Subcutaneous and intramuscular administration of proteins is more immunogenic than intravenous, oral or aerosolized delivery (Schellekens, 2002, 2004), while risk of immunogenicity also increases with frequency of dosing and duration of treatment, without necessarily being linked to dose (Schellekens, 2004).

Sources of immunogenicity with respect to processing of the therapeutic protein begin with the type of product. Biopharmaceuticals of non-native microbial, plant or animal origin, such as bacterial streptokinase or salmon calcitonin, are foreign proteins that induce an immune response comparable to that of an antigenic epitope found in a vaccine (Grauer et al., 1995; Lawley et al., 2000; Schellekens, 2004). However, the majority of biopharmaceuticals are homologs of human proteins and in these products the main factors associated with immunogenicity in immunocompetent individuals are related to the manufacturing process rather than the protein (Schellekens, 2004). Biopharmaceuticals undergo numerous production steps during a complex manufacturing process, with each step potentially contributing to the characteristics of the end product. Thus, contamination arising from the manufacturing process, leaching from container closures, and improper storage and handling may all contribute to an impure product capable of stimulating the immune system (Koren et al., 2002; Schellekens, 2002).

Major causes of instability in the processing of biopharmaceuticals relate to the alteration of a protein's three-dimensional structure, with protein aggregation, deamidation, and oxidation comprising the three most common pathways involved in protein degradation (Cleland et al., 1993). Protein degradation can produce novel antigenic epitopes not found in the parent molecule with the potential for increased immunogenicity (Koren et al., 2002), while biological activity and metabolic half-life may also be effected (Cleland et al., 1993). Protein aggregation typically involves the interaction of denatured protein molecules, with the solvent environment, temperature, surface interactions, and changes in the protein solution during freeze-drying all having an impact on denaturation (Cleland et al., 1993). The previous use of human serum albumin (HSA) as an excipient in the manufacture of interferon alpha-2a is an example of how a normally non-immunogenic stabilizing agent can cause protein aggregation and increased immunogenicity of the final product despite being used in the manufacture of other therapeutic proteins without adverse effect (Palleroni et al., 1997). Protein oxidation can occur both in solution and during freeze-drying, with potential consequences of reduced biological activity and immunogenicity when accompanied by protein conformational changes (Cleland et al., 1993). Oxidation may occur due to contamination with oxidants and from exposure to light during storage – with methionine, cysteine, histidine, tryptophan, and tyrosine residues the most susceptible (Li S et al., 1995). Deamidization can also occur during manufacture, although little is understood about the role of this in immunogenicity (Schellekens, 2002).

Other potential sources of immunogenicity are glycosylation, pasteurization, and inadequate postmanufacture product storage and handling. Glycosylation varies with the host cell-line and appears to be important given that non-glycosylated proteins can be more immunogenic than glycosylated proteins. Changes in the manufacturing process that result in glycosylation changes can affect tertiary structure, which although not always detectable by physicochemical methods could still cause a change in immunogenicity (Schellekens, 2002). Pasteurization may also affect tertiary

structure of a protein and with it the nature of any potential epitopes. The introduction of pasteurization into the manufacture of factor VIII to reduce the risk of viral transmission resulted in increased immunogenicity (Briet and Peters, 2001). Finally, improper storage and handling of a therapeutic protein as a result of high storage temperatures can lead to oxidation and aggregate formation, with the result that a protein may become more immunogenic (Hochuli, 1997).

3. Clinical consequences of immunogenicity

Immunogenic effects of therapeutic proteins have principally been defined by the detection of circulating antibodies using bioassays that measure neutralizing antibody (NAB) activity, and assays of binding activity that assess titers of both NABs and non-neutralizing antibodies (NNABs) (Schellekens, 2002). Incidence of response varies considerably between products, ranging from about 1% for innovator brand products of rituximab, alteplase, and interferon gamma-1b to up to 70% for muromonab-CD3, streptokinase, and agalsidase beta (Koren et al., 2002). These antibody responses mostly have little clinical significance, and may even enhance efficacy by protecting the agent from degradation (Schellekens, 2004). However, high antibody titers can reduce efficacy, abrogate therapeutic effect altogether, or neutralize the native protein, with the latter perhaps associated with the most serious clinical outcomes.

One high-profile example involving neutralization of the native protein occurred in normal human platelet donors treated with megakaryocyte growth and differentiation factor (MGDF), which resulted in severe long-term thrombocytopenia (Li J et al., 2001). A second example involved the development of NABs to erythropoietin in patients with anemia who subsequently developed pure red cell aplasia (PRCA), despite only a few prior reports of antibodies in the many thousands of patients already treated with erythropoietins (Casadevall et al., 2002; Koren et al., 2002). NABs have also been associated with reduced efficacy of factor VIII in hemophilia (Jacquemin and Saint-Remy, 1998), streptokinase in acute myocardial infarction (Rosenschein et al., 1991), denileukin difitox in T-cell lymphoma (Olsen et al., 2001), interferon alpha-2a in hepatitis C (Antonelli et al., 1996), interferon beta in relapsing-remitting multiple sclerosis (Sorensen et al., 2003) and, among several others, interleukin-2 and granulocyte-macrophage colony-stimulating factor (Prümmer, 1997; Ragnhammar and Wadhwa, 1996). Although NNABs bind to the protein but do not neutralize it, their ability to form immune complexes has been associated with decreased efficacy and accelerated clearance, while formation of NNABs to interferons reportedly precedes the development of NABs (Schellekens, 2002). A number of products have been associated with allergic reactions and other general immune effects, but these have typically involved impure preparations containing large amounts of foreign protein, and have become rare with the development of better products (Schellekens, 2002).

4. Examples of immunogenicity associated with product handling

There is no direct relationship cited in the literature between immunogenicity and product handling. However, several examples of the formation of degradants (protein aggregation, oxidation, deamidation, and truncation) are identified as a cause of immunogenicity. During product development, stress-testing studies are conducted to provide assurance that there are no changes in the identity, purity, and potency of the product. However, it depends upon several parameters, such as objectives, timing, selection of stress conditions, and extent of degradation and sensitivity of analytical methods.

The problem of immunogenicity to therapeutic proteins has been around a long time, having as its origin the use of small proteins, such as insulin, which were initially derived from different animal species. The presence of insulin-binding antibodies in the sera of diabetic patients was first reported in 1956, with subsequent reports further suggesting that the species of insulin used and the form in which it was administered affected antibody titers (Chance et al., 1976). With the development of purification techniques, such as gel filtration and ion-exchange chromatography, many impurities present in insulin preparations could be removed, permitting comparison between formulations. Using these techniques, Chance et al. (1976) demonstrated that porcine insulin was less immunogenic than bovine insulin. Chance and colleagues also demonstrated that "single component" porcine insulin, detected as a single band by polyacrylamide gel electrophoresis following extensive purification from conventional crystalline insulin, was less immunogenic than either the conventional preparation or "single-peak" insulin, which contained small amounts of impurities following a less rigorous purification protocol.

Although the insulin now used in the treatment of diabetes is predominantly recombinant human insulin, a more recent study illustrates the potential for immunogenicity resulting from real-life

handling and storage conditions (Gregory et al., 1991). During storage in solution, insulin transformation products (ITPs) are formed comprising deamidated insulins, covalent dimers, and oligomers, the consequences of which are a potential loss of therapeutic effect and immunogenicity. In this study of 12 diabetes patients assigned to treatment with either human or bovine insulin, storage of vials at higher temperatures such as in a breast pocket or exposure of vials to sunlight resulted in more rapid ITP formation than would normally occur if the vials had been adequately stored. Interestingly, human insulin was more susceptible to ITP formation than was bovine insulin at higher temperatures, but this may have been due more to a difference in formulation than a species difference, with glycerol and sodium chloride used as the isotonic stabilizer in human and bovine insulin, respectively (Gregory et al., 1991).

Oxidation of methionine is one of the major degradation pathways in many protein pharmaceuticals. Methionine residues in proteins are susceptible to oxidation, resulting in the formation of methionine sulfoxide and, under extreme conditions, sulfones. In liquid formulation, recombinant humanized monoclonal antibody (rhMAB) HER2 was shown to undergo oxidation when exposed to intense light and elevated temperature. The temperature-induced oxidation resulted from the formation of free radicals, and the light-induced oxidation was through a single oxygen pathway. The presence of antioxidants such as methionine and thiosulfate in the formulation prevented the temperature-induced oxidation (Lam et al., 1997). It is therefore important to select antioxidants in the formulation, and to avoid light and temperature exposure.

Degradation of the monoclonal antibody muromonab-CD3 (Orthoclone OKT[®]3) was reported to occur as a result of deamidation at specific asparagine residues, and oxidation of a cysteine and several methionines. A unique chain cross-linked degradation product was also described. The relative distribution of degradation products was affected by changes in the storage conditions of the antibody (Kroon et al., 1992). Therefore, storage and handling conditions should be followed as recommended to reduce the risk of immunogenicity.

Erythropoietin-induced PRCA was rare prior to 1998, but a large increase in the number of cases was subsequently observed in chronic kidney disease patients who were treated for anemia with one specific epoetin alfa formulation (Eprex[®]) marketed outside the USA, which reached a peak in 2002 (Lim, 2005). The formulation in question was associated with the production of anti-erythropoietin NABs that were elicited following subcutaneous administration of the biopharmaceutical (Casadevall et al., 2002). While the immunologic basis for the response has not been elucidated, a product formulation change in Europe in 1998 involving the substitution of HSA for polysorbate 80 and glycine coincided with an immediate increase in the reported incidence of PRCA (Haselbeck, 2003).

Technical investigations initially focused on analyzing the epoetin alfa molecule for signs of product degradation, alteration, or the introduction of impurities. While none of these analyses were able to demonstrate any alteration in the protein compared with historical data, experimental high-performance liquid chromatography revealed the presence of phenolic derivatives (commonly used as curing agents in the manufacture of rubber stoppers) in Eprex[®] prefilled syringes (Sharma et al., 2004). These leachates were found in placebo syringes as well as syringes containing the active product, indicating the leachates were not protein related. Moreover, the leachates were only found in association with polysorbate 80 formulations of Eprex[®] or placebo in prefilled syringes with uncoated rubber stoppers, and not in HSA formulations of Eprex[®] with uncoated syringe stoppers or polysorbate 80 formulations with FluroTec[®]-coated syringe stoppers (Sharma et al., 2004). The concentration of the leachates in the uncoated prefilled syringes increased with storage time, even at the recommended storage temperature of 2–8 °C (Fig. 1). Interestingly, it was also observed that the amount of leachates detected in the product after exposure at 46 °C for 2 days was higher than in the control stored at 2–8 °C (Fig. 2). Thus raising the possibility that improper storage and handling contributed to the number of PRCA cases (DePaolis et al., 2005).

Retrospective case analyses supported the findings of technical investigations, with a substantial difference found in the incidence rates of PRCA in patients with subcutaneous exposure to product that was packaged in prefilled syringes with polysorbate 80 and uncoated rubber stoppers than in all other product forms (Boven et al., 2005). Furthermore, there was a temporal association between the number of reported cases of antibody-induced PRCA and the introduction and subsequent withdrawal of the suspect Eprex[®] formulation (Fig. 3). Additional preclinical studies demonstrated that the leachates acted as adjuvants to increase immunogenicity, while polysorbate 80 failed to demonstrate an adjuvant effect, thereby confirming the safety of polysorbate 80 as an excipient for therapeutic proteins when used with FluroTec[®]-coated syringe stoppers (Ryan et al., 2006). Conversion of all prefilled syringes with the polysorbate 80 formulated product to FluroTec[®]-coated stoppers occurred in April 2003, which, together with improved control of cold-chain distribution

and the recommendation to switch to intravenous administration in patients with chronic kidney disease, provided a suitable risk-mitigation strategy (Sharma et al., 2004).

Recombinant interferon alpha-2a is approved as therapy for a range of oncological and virological diseases where the reported incidence of antibodies in response to the biopharmaceutical varies from total absence to 61% (Kontsek et al., 1999). Immunogenicity of interferon alpha-2a is characterized by the development of NABs and subsequent resistance to the antiviral and antitumor effects of the protein, particularly when antibody induction occurs early during treatment and at high titers (Kontsek et al., 1999). Analyses of interferon alpha-2a clinical trials have provided evidence that NAB frequency is dependent on the source of interferon alpha-2a, which has prompted further investigations into technical aspects of its production and storage. One such investigation considered the influence of the production process on three-dimensional protein structure, chemical modification of the protein during storage, and the presence of contaminating forms of interferon alpha-2a (Hochuli, 1997).

While no changes were found in the tertiary protein structure as a result of the manufacturing process, an oxidized form of interferon alpha-2a was found in vials stored at ambient temperature that was subsequently shown to be more immunogenic in mice compared with the non-oxidized molecule. In contrast, very little of the oxidized molecule was formed when vials were stored at 4 °C, and immunogenicity did not increase for product stored at this temperature. Furthermore, at ambient temperature the oxidized molecule formed aggregates in the presence of HSA indicating that the use of HSA as an excipient may have contributed to the immunogenicity of this product (Hochuli, 1997). As a result of these findings, an HSA-free formulation was developed that is stored as a refrigerated lyophilizate (Ryff, 1997). Fig. 4 demonstrates how improvements in the production process of interferon alpha-2a associated with the incremental removal of HSA and introduction of storage under refrigeration contributed to a reduction in immunogenicity of the product in patients with chronic hepatitis C (Ryff, 1997).

5. Conclusion

Patents of innovator biopharmaceutical proteins are expiring, opening the way for biosimilar versions to enter US and European markets. Despite the publication of guidelines by regulators requiring a thorough characterization of any biosimilar product, immunogenicity remains a concern because there are no predictors for it outside of the clinical trial setting. We have shown here that product handling can have a major influence on the potential immunogenicity of a biopharmaceutical protein. Factors such as purification, exposure to light, cold-chain distribution, excipient, and route and frequency of administration can have a major influence on immunogenicity, with the possibility of serious clinical consequences. The production process involved in biopharmaceutical manufacture is complex; it is therefore imperative that manufacturers of biosimilar products are committed to achieving and maintaining the standards required for product quality, safety, and efficacy that are expected of any novel therapy. This requires a commitment not only to the production process, but also to product distribution and storage, patient and physician education (e.g. prefilled syringes), clinical trials, and ongoing pharmacovigilance. For these reasons, the biosimilar product should be evaluated as for any innovator product and have active pharmacovigilance, at least until new or improved methods become available for analyzing product characteristics, predicting immunogenicity, and measuring immune responses.

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Figure legends

Fig. 1. The concentration of leachates increased with increasing product shelf life. Product in syringes with uncoated rubber stoppers stored at 2–8 °C. HPLC, high-performance liquid chromatography.

Fig. 2. The concentration of leachates increased when the product was exposed at 46 °C for 2 days compared with the control stored at 2–8 °C. Product in syringes with uncoated rubber stoppers. HPLC, high-performance liquid chromatography.

Fig. 3. Temporal association between the introduction and subsequent withdrawal of polysorbate 80 formulations of Eprex[®] with uncoated rubber stoppers and the incidence of pure red cell aplasia.

Fig. 4. Mean population anti-interferon alpha-2a antibody titers in patients with CHC treated from 1989 to 1994: (A) interferon alpha-2a (triple peak) stored as lyophilized powder, not refrigerated; 190 CHC patients treated by subcutaneous injection with 3 MIU of interferon alpha-2a three times weekly for 12 months; (B) interferon alpha-2a (triple peak) stored as lyophilized powder under refrigeration; 82 CHC patients treated by subcutaneous injection with 6 MIU of interferon alpha-2a three times weekly for 6 months; (C) interferon alpha-2a (triple peak) liquid formulation containing HSA, stored under refrigeration; 110 CHC patients treated by subcutaneous injection with 6 MIU of interferon alpha-2a three times weekly for 6 months; (D) interferon alpha-2a (single peak, ultrapure) HSA-free liquid formulation stored under refrigeration; 81 CHC patients treated by three times weekly subcutaneous injection with 6 MIU of interferon alpha-2a for 3 months followed by 3 MIU for 3 months; (E) interferon alpha-2a (single peak, ultrapure) stored as lyophilized powder under refrigeration; 74 CHC patients treated by three times weekly subcutaneous injection with 6 MIU of interferon alpha-2a for 3 months followed by 3 MIU for 3 months. CHC, chronic hepatitis C; HSA, human serum albumin; IFN, interferon; MIU, million international units.

[Adapted with permission from Ryff JC (1997)]

Figure 1

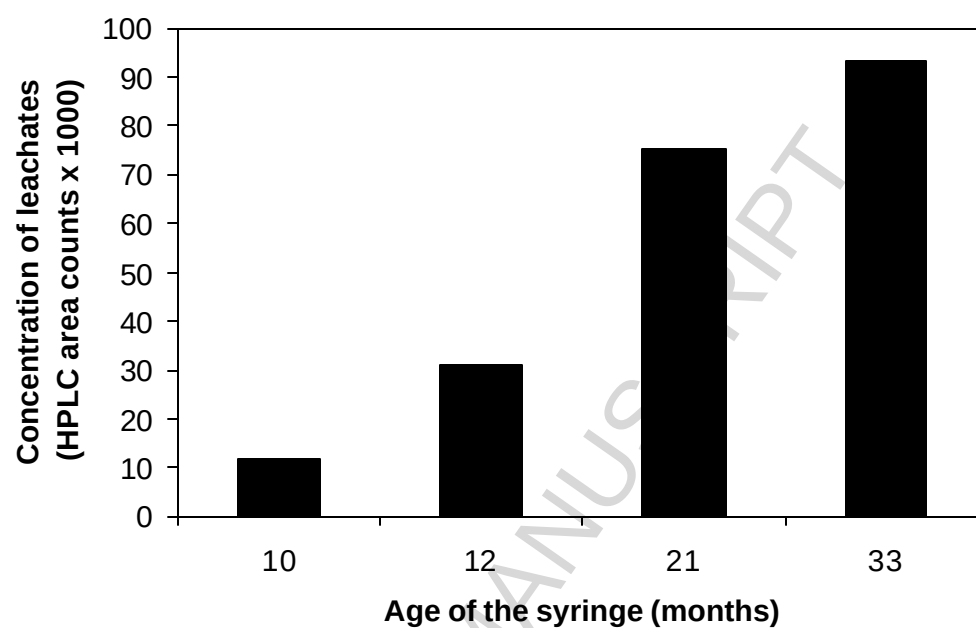


Figure 2

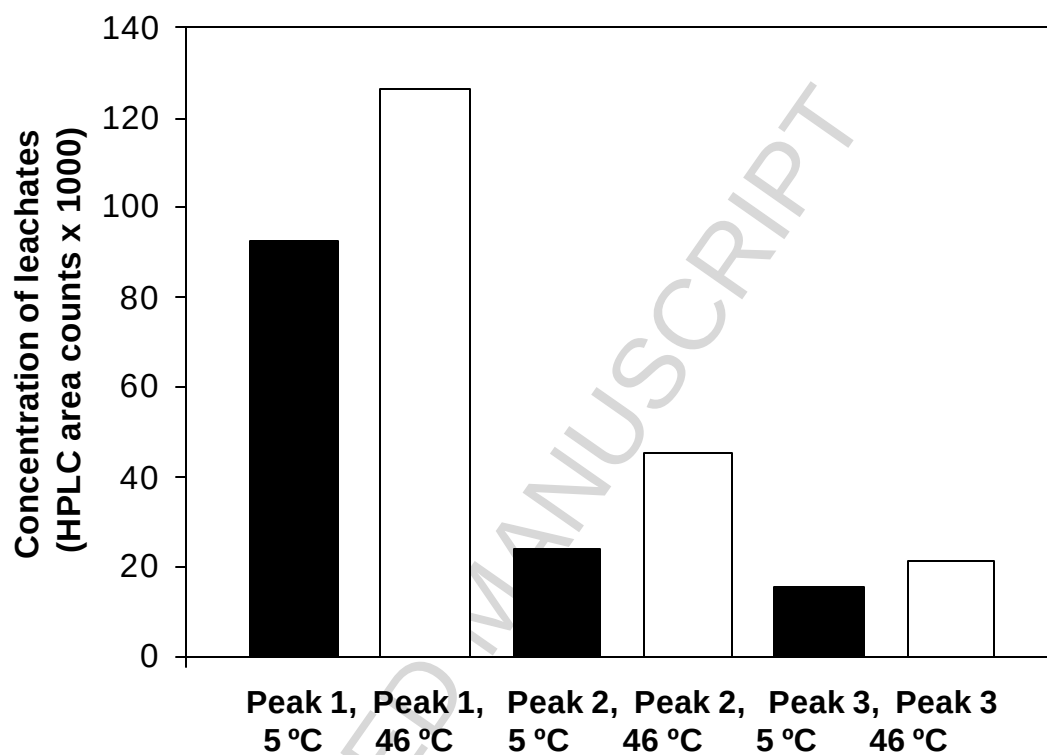


Figure 3

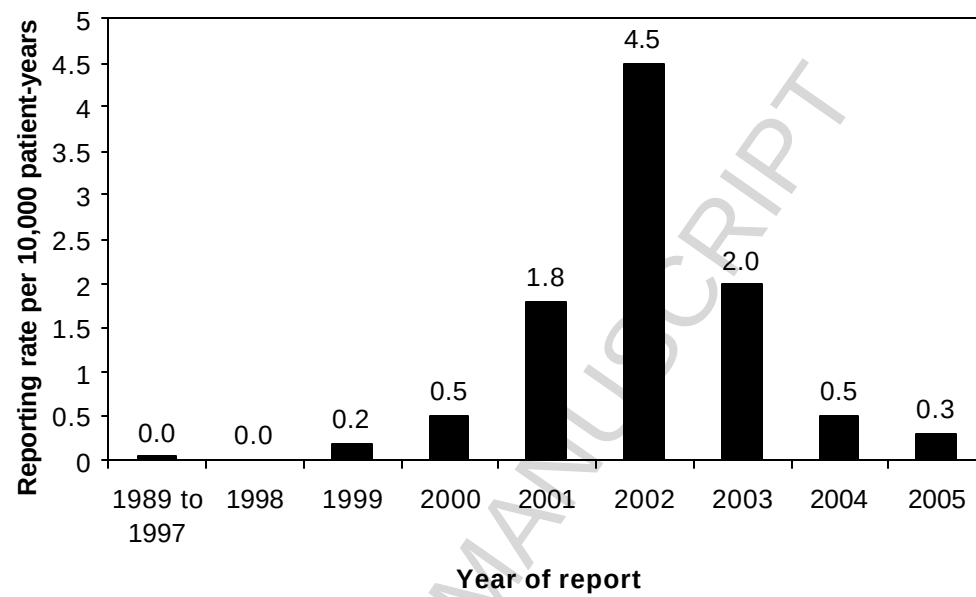


Figure 4

