Expert Opinion

Factors of importance for a successful delivery system for proteins

Marco van de Weert†, Lene Jorgensen, Eva Horn Moeller & Sven Frokjaer
†*The Danish University of Pharmaceutical Sciences, Biomacromolecules Group, Department of Pharmaceutics and Analytical Chemistry, Universitetsparken 2, 2100 Copenhagen, Denmark*

Protein pharmaceuticals have matured into an important class of drugs, now comprising one in three novel drugs introduced on the market. However, significant gains are still to be made in reducing the costs of production, ensuring proper pharmacokinetics and efficacy, increasing patient compliance and convenience, and reducing side effects such as immunogenicity. This review summarises these issues and provides recent examples of methods to reduce costs, alter pharmacokinetics and increase patient compliance. It also discusses the increasing interest in understanding immunogenicity in order to prevent failure of the protein drug or serious life-threatening side effects due to autoimmunogenicity.

Keywords: bioavailability, biotechnology, immunogenicity, pharmacokinetics, protein delivery, protein drugs, protein stability

Expert Opin. Drug Deliv. (2005) 2(6):1029-1037

1. Introduction

Over the last two decades, proteins have become an important new class of drug molecules. At present, approximately one in three new drug applications involve a protein as the active compound [1]. This resulted in > 100 marketed products in the US in 2004 [101], with probably a similar number marketed in the EU at present [1,2]. In addition, a total of 324 biotechnology products were reported to be in clinical development in 2004 [101], of which most contain a protein as the active compound. Most of these protein drugs are used for life-threatening and seriously debilitating diseases such as diabetes, cancer, rheumatoid arthritis, hepatitis and so on. The high activity and specificity of proteins compared with the more conventional, low molecular weight drugs often allows for a better treatment of these diseases. However, the production and delivery of these protein drugs is not without problems.

The main aim of any drug delivery system is to deliver the drug to the active site at the right time, at a therapeutically effective concentration, at the highest patient convenience/compliance, with the lowest possible side effects and at the lowest possible cost. This aim also applies to protein drugs. However, due to the unique character of proteins, the methods to achieve this aim often differ from those for low molecular weight drugs [3]. In this review the authors will focus on the unique properties of proteins in relation to drug delivery approaches.

2. Factors of importance for a successful protein drug delivery system

2.1 Protein drug production

The first major difference between low molecular weight drugs and protein drugs is the production of the active compound. Similar to low molecular weight drugs, most peptide drugs can be synthesised, with the upper limit for commercial

Ashley Publications
www.ashley-pub.com

applications ~ 30 amino acids in length [4]. Proteins, however, are either isolated from natural sources or are produced through recombinant DNA technology in a suitable expression system. At present, most protein drugs are produced in mammalian cell cultures [5-7], but other expression systems are actively being explored [8]. The choice of expression system not only has major effects on the final yield, but also on the quality of the protein. For example, most bacterial cell cultures are unable to glycosylate a protein and may sometimes be unable to fold the protein into its native conformation, whereas other systems may not yield the same glycosylation patterns as in humans. Whether such differences have clinical implications can not yet be predicted.

Protein production is a costly process and a major hurdle for a protein drug. For example, the estimated cost of goods for a protein manufactured at ~ 250 kg/year amounts to ~ €1250 per gram [9]. More importantly, setting up manufacturing capacity for such a protein requires an initial investment in the magnitude of €1 billion [9]. Investigations aiming to reduce these costs by improving yield and efficiency, eventually through the use of alternative expression systems, is ongoing.

The manufactured protein also needs to be isolated and purified, which adds additional costs. These costs may even be higher than those for the initial manufacture [10,11]. Thus, optimisation of this downstream processing is of prime importance to keep production costs as low as possible.

2.2 Protein administration

With the exception of local delivery for local treatment (for example, pulmonary delivery of recombinant human deoxyribonuclease in the treatment of cystic fibrosis), the administration of protein drugs for systemic delivery is, at present, either by injection or by infusion. The high molecular weight of proteins, combined with their hydrophilic and charged nature, renders transport through membranes very difficult. Moreover, various proteases are present at potential sites of administration, such as in the gastrointestinal tract, lung, buccal epithelium and skin. Finally, cells of the immune system located at the point of entry may absorb and eliminate the protein, thus reducing bioavailability and increasing the potential for an immune response (*vide infra*).

For many drug molecules, including some of the peptide drugs, a relatively low bioavailability may be acceptable, for example, when patient compliance or convenience is a major issue. However, protein drugs are very expensive. Thus, a significant decrease in bioavailability can make the application of the protein drug too costly. Despite these bioavailability problems, there is a significant research effort exploring alternative routes of administration [12]. The most promising routes for proteins seem to be the pulmonary, transdermal and nasal route. For example, recent advances in particle technology and inhaler design have allowed a bioavailability of ~ 10 – 20% for pulmonary-delivered insulin. The pulmonary route offers two advantages over the more conventional subcutaneous injections. First, the onset of action is faster, allowing for better postmeal control of glycaemic levels. Second, the patients prefer the pulmonary delivery over injections [13,14]. However, there has been some concern on potential local side effects, mainly a reduced diffusive capacity of the lung, as well as the observed increased anti-insulin antibody formation [15].

The current experience with pulmonary insulin may prove crucial for the further development of pulmonary delivery. If serious side effects remain absent, many more proteins will be considered for pulmonary delivery. On the other hand, for many such proteins a potential drawback of the pulmonary route could be the rapid absorption, resulting in high peak levels and associated side effects. These peak levels may be reduced by administering the required total dose in multiple steps. However, this may reduce the patient compliance and convenience. At present, many groups are investigating the use of carriers to obtain a more sustained delivery [16]. These carrier systems are still in their early infancy, and their suitability has not yet been proven in any clinical trials.

The transdermal route is easily accessible, but some method to disrupt the skin barrier is required [12,17]. Potential methods include the use of iontophoresis [18], ultrasound [19], transferosomes [20] or microneedles [21]. The biggest concern for transdermal delivery of a protein is the large amount of immunocompetent cells present in the skin, which are activated by the skin damage required to deliver the protein. Moreover, the delivery capacity may be too small for some proteins. The clinical data on this route of administration is still too limited to determine its ultimate feasibility for protein delivery, but preclinical studies show that bioavailabilities of ≥ 50% may be possible.

For selected peptide drugs, nasal delivery has shown to be commercially viable. Desmopressin (Minirin®, Ferring Pharmaceuticals) is available as both an oral tablet as well as a nasal spray. However, even for this relatively stable peptide, the bioavailability is only around 0.1% for the oral route, and 3 – 5% for the nasal route. Salmon calcitonin (Miacalcin®, Novartis Pharma) is also available as a nasal spray, with a similarly low bioavailability of 3% reported by the company. A considerable amount of research is aimed at improving these low bioavailabilities for peptides by advanced formulation strategies [22]. Some of these may also be useful for proteins but clinical data are scarce. Recently, Phase I clinical studies were published on the nasal delivery of insulin [23], while Phase II studies on the same system were reported by Bentley Pharmaceuticals at the June 2005 Annual Meeting of the American Diabetes Society. Reportedly, the bioavailability of intranasal insulin delivery was 15 – 20%, which makes this as good as, or even better than most of the pulmonary delivery systems for insulin.

The nasal route also provides the possibility to bypass the blood–brain barrier and achieve some level of brain targeting. Proof-of-concept has been achieved in cognition in memory-impaired older adults using insulin [24], as well as for the delivery of IFN-β to the CNS [25].

Table 1. Common degradation pathways for proteins.

Degradation pathway	Comments
Oxidation	Mainly at Met, His, Trp, Cys residues
Deamidation	Mainly at Asn residues, sometimes Gln residues
Proteolysis	Mainly at Asp-X linkages
β-elimination	
Disulfide scrambling	May result in misfolding or covalent aggregation
Racemisation	
Aggregation	Implicated in immunogenicity development
Adsorption	Loss of protein but also nucleus for bulk aggregation
Precipitation	
Denaturation	

For insulin the feasibility of the oral route has been demonstrated with modified insulin, hexyl-insulin-monoconjugate-2, developed by Nobex Corp. An oral bioavailability of 5% was shown [15] as well as a clinically relevant activity [26]. A potential advantage of this route for insulin is the reduced systemic insulin concentration compared to subcutaneous injections [26]. Generex Biotechnology Corp. has shown promising results for the buccal delivery of insulin using a specially designed spray and undisclosed absorption enhancers, termed Oralin [27]. A bioavailability of 7 – 8% was reported, as well as a more rapid onset of action compared with subcutaneous injections [27].

The studies on orally delivered insulin show considerable promise, but the bioavailability through this route is still much lower than, for example, the transdermal or pulmonary route. Moreover, for insulin the hepatic first-pass metabolism may be a minor problem, as the liver is the main target organ [28]. This may not be the case for other proteins that are metabolised in the liver, such as monoclonal antibodies, epidermal growth factor and tissue-plasminogen activators [29]. In the future it will have to be shown whether the oral route is feasible for protein drugs other than insulin and vaccines [30]. At present, a number of companies are testing proteins such as human growth hormone (Emisphere Technologies) and IFN-α-n3 (Alferon LDO®, Hemispherx Biopharma) for oral delivery, but clinical data on bioavailability is not yet available.

Although alternative routes are being explored, much research effort is also aimed at improving compliance towards and convenience of the actual injection systems. These improved injection systems are especially of importance for self-administration. For example, injection pens allow for accurate delivery of the required amount of protein using hyperfine needles, and are common practice for the delivery of proteins such as insulin, (pegylated) IFN-α_{2a} and $_b$ and human growth hormone. The pen systems are both aesthetic as well as easy to use, whereas the hyperfine needles reduce the pain of injection and patient anxiety [15,31]. Improving patient compliance by designing more patient-friendly injection systems has now become standard for the delivery of many protein drugs, and will allow more home-based care in the near future.

For patients with an extreme fear of needles even hyperfine needles may be an unsurpassable barrier. In such cases, the use of jet-injectors can be considered, even though these do not provide any advantage in terms of pain or discomfort [15]. These devices use a pressurised system to propel a stream or spray of solution through the skin. Thus, they also reduce the risk of needle-stick for healthcare personnel. Their use is still very limited but will probably increase in the near future.

2.3 Protein stability and formulation

The most common route of administration requires that the protein is in solution. However, proteins are susceptible to a wide variety of chemical and physical degradation mechanisms, which means that they are often unstable in solution [32-36]. Table 1 summarises the most common degradation pathways. Analysing these mechanisms is not an easy task and often requires the use of many different analytical techniques [36-39]. These include a large variety of chromatographic and spectroscopic techniques, as well as other techniques such as differential scanning calorimetry. However, not every technique can be used for each formulation. Moreover, it is impossible to fully characterise all the degradation products of a protein [3]. Thus, the protein formulation challenge is also an analytical challenge.

The susceptibility to the individual degradation mechanisms differs significantly between proteins, even structurally similar ones. For example, the single amino acid substitution Ile→Val at the A10 position in insulin increases the lag time for fibrillation by a factor of 2 [40]. Such differences are, at present, difficult to predict and usually must be determined experimentally.

Moreover, the conditions that reduce one degradation mechanism may promote another route of degradation, or result in other undesirable effects on, for example, side effects or solubility. For example, many chemical degradation reactions are slowest around pH 5, but many proteins have an isoelectric point in the same range. Thus, formulation at pH 5 reduces the solubility. This is, for example, a major issue for the peptide drug glucagon, which has to be formulated at pH 2.5 – 3 in order to achieve adequate solubility.

Finally, stabilisation approaches that work for one protein may actually promote the same degradation mechanism in another protein. The addition of preferentially excluded additives, such as sucrose, is generally used to increase physical stability, but may also reduce chemical degradation reactions such as methionine oxidation. The latter was observed for subtilisin [41], but, surprisingly, an increased oxidation rate was observed for recombinant Factor VIIa [42].

The complexity outlined above makes protein formulation a considerable challenge [3,33,36], as achieving the most stable formulation often requires a unique approach for each

protein. In some cases solutions may be found in the way nature itself stabilises proteins. In other cases, the solutions come from screening a wide range of potential additives. Fortunately, with the increase in the number of protein products the research into protein stabilisation and formulation has increased accordingly. This has resulted in a number of rational stabilisation approaches [36,43,44]. These can be summarised as follows: first, most chemical degradation reactions are minimised at pH values around 5 – 6. Second, protein adsorption can be minimised by the addition of, usually non-ionic, surfactants. Third, specific ligands often increase protein physical stability, which may also positively affect the chemical stability.

If the above-mentioned principles fail to yield a protein formulation that is stable for at least 2 years and can withstand the stresses of shipping, a dried product must be prepared. In fact, most protein drugs are provided as freeze-dried solids for reconstitution [44,45]. Freeze drying, or lyophilisation, is in itself a stressful process for proteins, and may result in physical degradation [45-52]. Over the last few years the critical process and formulation parameters have been studied in detail, allowing for rational design of the lyophilisation process [43,45,53]. Typically, the formulation principles for protein solutions also hold for lyophilised protein products. However, the use of so-called cryo- and/or lyoprotectants is imperative to obtain a stable product. These protectants prevent physicochemical degradation, both in the freezing and drying stages of the process. Moreover, using a proper lyophilisation cycle design they yield a good cake that is easily rehydrated.

Freeze drying is a costly process, as it is a slow and energy-intensive process. Thus, alternative drying strategies, such as spray drying and supercritical fluid drying, are actively being explored [54,55]. These alternative strategies are also of importance for dry powder inhalation administration, where particle characteristics are of extreme importance to achieve proper delivery in the deep lung [56]. Spray freeze drying [54,57] and spray freezing into liquid [58], modifications of the freeze-drying process, can also be used to prepare protein particles for inhalation, but still involve the slow drying stage of the frozen solution.

2.4 Pharmacokinetics

The serum half-life of proteins differs greatly, and may range between a few minutes to several weeks [29]. In general, the half-life is closely connected to the physiological function of the protein. For example, insulin has a half-life of ~ 4 min, and should only be present at high concentration in the blood for a short period of time to regulate the uptake of carbohydrates after meals. Alternatively, vascular endothelial growth factor (VEGF), a potent angiogenic factor with a half-life of ~ 8 min, should only be present at a functional concentration close to its site of action [59]. High sustained concentrations throughout the body of these proteins may cause significant and undesired side effects. In contrast, human growth hormone has a serum half-life of ~ 13 – 19 min [60], but it is usually present at sustained, albeit fluctuating, concentrations in the body.

In a number of cases it may be of interest to alter the pharmacokinetics of the protein drug. These altered pharmacokinetics may improve patient compliance and/or effectivity of the protein. Again, insulin provides several good examples. The subcutaneous route of administration, necessary for self-administration, results in a slow onset of action. Patients need to administer these insulin formulations well before a meal, requiring strict planning. To improve the treatment, more rapidly absorbed insulin mutants have been engineered [6], which can be administered right before the meal, thus improving patient compliance and convenience. Long-acting insulins have also been developed, in order to achieve long-term basal insulin levels with one single injection. These include insulin mutants, insulin–protamine or insulin–zinc complexes, and conjugated insulin [6,61].

Altered pharmacokinetics may be obtained in several different ways, some of which are already indicated above. The simplest method is to alter the site of administration or the site of injection. For example, intravenous injections result in much faster elimination of the protein drug from the body than subcutaneous, intramuscular or intraperitoneal administration, in which the pharmacokinetics are mainly determined by the absorption rate from the site of injection. For subcutaneous injections, the absorption is mainly through the lymphatic system, which may be exploited to target a protein drug to, for example, the lymph nodes [29]. The subcutaneous route is also much more accessible to self-administration than intravenous injections.

An alternative method is to slow down the absorption or clearance kinetics, for example, through complexation or conjugation. Complexation has so far been used mainly for insulin, whereas conjugation has become a standard method for many proteins. Novo Nordisk has recently marketed a long-acting basal insulin formulation, Levemir®, which contains an acylated insulin. The acylation not only reduces the absorption rate from the subcutaneous depot but also increases the circulation time in the blood by binding to serum albumin [61], resulting in a serum half-life of ~ 14 – 15 h.

Several proteins conjugated to poly(ethylene glycol) (PEG) have also been marketed, such as PEG-Intron® (Schering-Plough), Pegasys® (Roche), Aralast® (Alpha Therapeutic Corp.), Oncaspar® (Enzon), Adagen® (Enzon), Somavert® (Pfizer) and Neulasta® (Amgen) [62]. PEG conjugation, or pegylation, generally exerts a number of positive effects, most notably a reduced kidney excretion and altered biodistribution due to an increase in molecular weight, a reduced degradation by enzymes and hydrolytic media, enhanced water solubility, reduced reticuloendothelial clearance, and reduced immunogenicity and antigenicity. These effects usually improve the therapeutic activity of the protein significantly, despite the fact that receptor binding is often diminished following pegylation [63]. For example, a once-weekly injection

of PEG-Intron generates the same clinical benefits as a three-times weekly injection of the non-pegylated IFN. Pegylation has now become routine and will probably be used for many other protein drugs that are still in development [62].

Sustained concentrations may also be achieved by implantable or injectable, preferably biodegradable, systems. Many of the systems currently being investigated involve a large variety of polymers [64]. Although most technological challenges have been solved, there are still a number of issues with regard to protein stability during encapsulation, storage and release [65]. Several methods have been suggested to overcome this instability [66], but so far only one protein-releasing injectable sustained release system has reached the market: Genentech's Nutropin Depot®, marketed in 1998 in the US. Nutropin Depot is a recombinant human growth hormone-releasing system, where the protein is entrapped in a hydrophobic matrix of poly(lactic-co-glycolic acid) (PLGA). Slow degradation of the matrix resulted in a slow release of the protein, sustaining therapeutic concentrations for ~ 4 weeks versus the 20 h for a subcutaneous injection of recombinant human growth hormone solution [67,68]. In 2004, the product was withdrawn from the market by Genentech, claiming 'the uncertainties and limitations in product supply required to meet future patient needs as well as the significant resources necessary for manufacturing' as the main cause.

Development of polymeric sustained release systems remains an active field of research. The current research focus is not only on improving protein stability in hydrophobic polymers but also on hydrophilic polymers. These hydrophilic polymers may not only be less detrimental to protein stability than PLGA, but the manufacturing procedure may also be less elaborate, and thus cheaper, compared with that of, for example, Nutropin Depot [67].

2.5 Immunogenicity

Any protein drug has the potential to invoke an immune response, that is, antibodies are formed against the protein. There are essentially four types of antibody response that may be recognised. The mildest of these is the formation of non-neutralising antibodies that seem to have little, if any, effect on the function of the protein [69]. The formation of sustaining antibodies, however, may reduce the clearance rate of the protein, thus altering the pharmacokinetics. This type of antibody response is rarely encountered but may have such a pronounced effect on the pharmacokinetics that one should monitor its occurrence.

A relatively more common, and also more problematic, immune response involves the formation of neutralising antibodies. These neutralising antibodies, when present at high titres, result in a fast clearance of the protein drug from the circulation, effectively cancelling the pharmacological effect [69-73].

The most severe, and potentially lethal, immune response concerns the formation of crossreactive antibodies [69-73]. These antibodies do not only bind to the protein drug but also to the endogenous protein, essentially yielding an autoimmune reaction. A recent well-known example includes the development of pure red cell aplasia (PRCA) in a subpopulation of patients treated with recombinant human erythropoietin (rhEPO) [74,75].

Box 1. Factors of importance for immunogenicity of protein drugs.

- Patient genetics
- Type of disease
- Protein origin, primary structure
- Route of administration
- Dosing frequency and amount
- Duration of treatment
- Formulation characteristics
- Degradation products
- Impurities

The causes for the development of an immune response are still poorly understood. For a considerable period of time, a difference in the primary structure, including post-translational modifications, between the protein drug and endogenous protein was considered to be the only risk factor. However, more recent research shows that there is a wide array of risk factors (Box 1), which may be associated to some extent [69,72,73]. The PRCA case mentioned above provides an excellent example, even though it involved only a low number of cases (< 300), and is described below in a historical perspective.

In 1998, Johnson & Johnson altered its rhEPO formulation Eprex® for marketing outside the US. The original formulation contained human serum albumin (HSA) as a stabiliser. Due to the concerns of using human blood-derived products in other products, HSA was replaced by polysorbate 80 (Tween 80) [74-76]. Shortly afterwards, the number of PRCA cases rose significantly and occurred almost exclusively in those patients treated with Eprex (Figure 1). The current hypothesis put forward, amongst others by the company itself, is that the presence of small organic contaminants leached from the rubber stopper of the syringe by Tween 80 caused the immune response [77]. These 'leachates' or 'leachables' may act as a danger signal or adjuvant in some patients. However, the presence of micelle-associated rhEPO has also been suggested as a possible risk factor [78].

When Eprex was identified as the main cause of the increased number of PRCA cases, several countermeasures were taken. These included the use of a coated stopper, stricter storage guidelines, as well as a change in administration route from subcutaneous to intravenous injection. Unfortunately, due to the combination of countermeasures it is difficult to ascertain the main cause of Eprex immunogenicity. The number of PRCA cases is still higher than before these countermeasures were taken, but this could also be due to a higher awareness of the disease.

One of the major challenges in the future for a successful protein drug is to predict the possibility of an immune

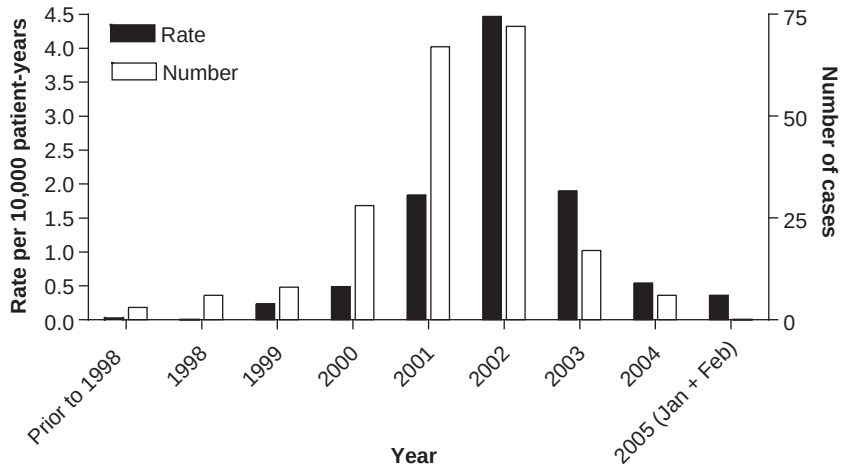

Figure 1. Rate of occurrence of rhEPO-related PRCA and the number of PRCA-patients that had received Eprex®. The latter constitute ~ 90% of all rhEPO treatment-related PRCA patients. The difference in the rate and number of patients is mainly due to a time difference in the reporting of the two numbers. Data are taken from [102] (latest access date July 2005).
PRCA: Pure red cell aplasia; rhEPO: Recombinant human erythropoietin.

response. Animal or *ex vivo* experiments are not yet predictive [69,70,72,73], although recently a transgenic mouse model was developed to study immunogenicity of human IFN-β [79]. Moreover, it may take months or years before an immune response is invoked. A significant problem is the poor standardisation of the antibody assays [80,81]. The poor standardisation is related both to the wide variety of assays, as well as the absence of proper standards. This makes it difficult for the regulatory agencies to establish clear guidelines. Thus, patients, especially those that need chronic treatment, will have to be continuously monitored for any immunogenicity development. A better understanding of how and which formulation factors may increase the risk for an immune response, as well as the development of good predictive *ex vivo* or animal models, will significantly reduce the chance of product failure due to a severe immune response after chronic treatment.

3. Conclusion

Protein drugs are slowly coming of age, providing improved treatment for several serious and life-threatening diseases. The focus turns more and more towards home treatment and self-administration, and various injection devices have been developed for this purpose. Alternative routes of administration for systemic delivery are also slowly reaching the market, further improving patient compliance and convenience. Some methods, mainly protein pegylation, to alter the protein pharmacokinetics have become standard strategies. However, there is still significant room for improvement in almost all areas of protein delivery, ranging from improved protein production to lower cost of goods to a better control of side effects, including immunogenicity.

4. Expert opinion

As the patent life of several biotechnology-derived protein drugs is running out, a discussion on the ability to produce generic biotechnology products has commenced. Such products may significantly reduce healthcare costs, as well as push the industry to further innovation to improve treatment and convenience.

In 2004 and 2005, the European Medicines Agency (EMEA) issued a number of draft guidelines to tackle the issue of biogenerics, or, rather, biosimilar products. The guidelines clearly indicate that these biosimilar products will have to show both safety and efficacy in clinical trials, but these trials are not necessarily as extensive as those for new drug applications. The extent of clinical trials will be evaluated on a case by case basis. There are, however, also draft guidelines for product classes such as recombinant human growth hormone, rhEPO, insulin and recombinant granulocyte-colony stimulating factor. With these guidelines, the EU is well ahead of the US, where the FDA guidelines essentially preclude biogenerics [82,83]. However, the FDA does allow 'process improvement' applications, where only limited clinical trials need to be carried out. The latter could also essentially be used for biogeneric products. There have been some developments since 2001, but there are still no guidelines available [83]. In September 2004, the FDA deferred a decision on the application for the biogeneric human growth hormone-containing product Omnitrope® (Sandoz), due to uncertainties regarding scientific and legal issues. In contrast, the Australian Therapeutic Goods Agency approved Omnitrope in October 2004.

The steps taken by the EMEA to set up guidelines for biosimilar products are to be commended and will hopefully be taken up within the International Conference on

Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, resulting in worldwide guidelines. An important issue remains: the potential of an immune response to the protein drug. The immune response to the novel Eprex formulation shows how a seemingly small factor, the leachates, can have major effects. Immunogenicity testing of any biosimilar product is obviously a clear prerequisite for any biosimilar product. However, the assays to determine an immune response are still poorly standardised. Without such standardised assays, discussions will remain not only on the efficacy, but also on the safety of the biosimilar product compared with its reference product. Thus, an important next step for the regulatory agencies will be to establish clear guidelines for immunogenicity assay development. Good starting points are already available in the scientific literature [80,81].

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. WALSH G: Pharmaceutical biotechnology products approved within the European Union. *Eur. J. Pharm. Biopharm.* (2003) 55:3-10.
2. WALSH G: Biopharmaceuticals: recent approvals and likely directions. *Trends Biotechnol.* (2005) (In press).
3. CROMMELIN DJ, STORM G, VERRIJK R, DE LEEDE L, JISKOOT W, HENNINK WE: Shifting paradigms: biopharmaceuticals versus low molecular weight drugs. *Int. J. Pharm.* (2003) 266:3-16.

• Gives a good comparison of the difference between protein drugs and low molecular weight drugs.

4. ANDERSSON L, BLOMBERG L, FLEGEL M, LEPSA L, NILSSON B, VERLANDER M: Large-scale synthesis of peptides. *Biopolymers* (2000) 55:227-250.

• Excellent review on the synthesis of peptides.

5. SCHMIDT FR: Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* (2004) 65:363-372.

• A brief but excellent review of the most common expression systems used today.

6. WALSH G: Therapeutic insulins and their large-scale manufacture. *Appl. Microbiol. Biotechnol.* (2005) 67:151-159.
7. WURM FM: Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotech.* (2004) 22:1393-1398.
8. ROY N, AGARWAL S: Therapeutic protein production – an overview. *Business Briefing: Future Drug Discovery* (2003):79-82.
9. WERNER RG: Economic aspects of commercial manufacture of biopharmaceuticals. *J. Biotechnol.* (2004) 113:171-182.
10. ROQUE AC, LOWE CR, TAIPA MA: Antibodies and genetically engineered related molecules: production and purification. *Biotechnol. Prog.* (2004) 20:639-654.
11. DESAI MA: *Downstream Processing of Proteins.* MA Desai (Ed.), Humana Press, Totowa, NJ, USA (2000).

•• This book gives detailed descriptions of all aspects of downstream processing, and is recommended to anyone working in the field.

12. CLELAND JL, DAUGHERTY A, MRSNY R: Emerging protein delivery methods. *Curr. Opin. Biotechnol.* (2001) 12:212-219.
13. HOLLANDER PA, BLONDE L, ROWE R *et al.*: Efficacy and safety of inhaled insulin (Exubera) compared with subcutaneous insulin therapy in patients with Type 2 diabetes. *Diabetes Care* (2004) 27:2356-2362.
14. MANDAL TK: Inhaled insulin for diabetes mellitus. *Am. J. Health Syst. Pharm.* (2005) 62:1359-1364.
15. CEFALU WT: Evolving strategies for insulin delivery and therapy. *Drugs* (2004) 64:1149-1161.

• The experience with insulin, well described in this review, may serve as inspiration for other protein pharmaceuticals.

16. CRYAN S-A: Carrier-based strategies for targeting protein and peptide drugs to the lungs. *AAPS J.* (2005) 7:E20-E41.
17. LEVIN G, GERSHONOWITZ A, SACKS H *et al.*: Transdermal delivery of human growth hormone through RF-microchannels. *Pharm. Res.* (2005) 22:550-555.
18. KALIA YN, NAIK A, GARRISON J, GUY RH: Iontophoretic drug delivery. *Adv. Drug Deliv. Rev.* (2004) 56:619-658.
19. MITRAGOTRI S, BLANKSCHTEIN D, LANGER R: Ultrasound-mediated transdermal protein delivery. *Science* (2005) 269:850-853.
20. CEVC G, GEBAUER D, STIEBER J, SCHÄTZLEIN A, BLUME G: Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic amounts of insulin across the intact mammalian skin. *Biochim. Biophys. Acta* (1998) 1368:201-215.
21. PRAUSNITZ MR: Microneedles for transdermal drug delivery. *Adv. Drug Deliv. Rev.* (2004) 56:581-587.
22. TÜRKER S, ONUR E, ÖZER Y: Nasal route and drug delivery systems. *Pharm. World Sci.* (2004) 26:137-142.
23. LEARY AC, STOTE RM, BREEDT HJ, O'BRIEN J, BUCKLEY B: Pharmacokinetics and pharmacodynamics of intranasal insulin administered to healthy subjects in escalating doses. *Diabetes Technol. Ther.* (2005) 7:124-130.
24. REGER MA, WATSON GS, FREY WH 2nd *et al.*: Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. *Neurobiol. Aging* (2005) (In press).
25. ROSS TM, MARTINEZ PM, RENNER JC, THORNE RG, HANSON LR, FREY WH 2nd: Intranasal administration of interferon beta bypasses the blood-brain barrier to target the central nervous system and cervical lymph nodes: a non-invasive treatment strategy for multiple sclerosis. *J. Neuroimmunol.* (2004) 151:66-77.
26. KIPNES M, DANDONA P, TRIPATHY D, STILL JG, KOSUTIC G: Control of postprandial plasma glucose by an oral insulin product (HIM2) in patients with Type 2 diabetes. *Diabetes Care* (2003) 26:421-426.
27. GUEVARA-AGUIRRE J, GUEVARA M, SAAVEDRA J, MIHIC M, MODI P: Oral spray insulin in treatment of Type 2 diabetes: a comparison of efficacy of the oral spray insulin (oralin) with subcutaneous (SC) insulin injection: a proof of concept

study. *Diabetes Metab. Res. Rev.* (2004) 20:472-478.

28. KRONES A, KIETZMANN T, JUNGERMANN K: Perivenous localization of insulin receptor protein in rat liver, and regulation of its expression by glucose and oxygen in hepatocyte cultures. *Biochem. J.* (2000) 348:433-438.
29. TANG L, PERSKY AM, HOCHHAUS G, MEIBOHM B: Pharmacokinetic aspects of biotechnology products. *J. Pharm. Sci.* (2004) 93:2184-2204.
30. FOSTER N, HIRST BH: Exploiting receptor biology for oral vaccination with biodegradable particulates. *Adv. Drug Deliv. Rev.* (2005) 57:431-450.
31. BOHANNON NJ: Insulin delivery using pen devices. Simple to use tools may help young and old alike. *Postgrad. Med.* (1999) 106:57-68.
32. ARAKAWA T, PRESTRELSKI SJ, KENNEY WC, CARPENTER JF: Factors affecting short-term and long-term stability of proteins. *Adv. Drug Deliv. Rev.* (2001) 46:307-326.
33. FROKJAER S, OTZEN DE: Protein drug stability: a formulation challenge. *Nat. Drug Discov.* (2005) 4:298-306.
- This review describes the intricate relationship between protein stability and formulation.
34. KRISHNAMURTHY R, MANNING MC: The stability factor: importance in formulation development. *Curr. Pharm. Biotechnol.* (2002) 3:361-371.
35. Ó FÁGÁIN C: Understanding and increasing protein stability. *Biochim. Biophys. Acta* (1995) 1252:1-14.
36. WANG W: Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* (1999) 185:129-188.
- A compilation of almost all published protein formulation research in the literature up to 1999.
37. REUBSAET JL, BEIJNEN JH, BULT A, VAN MAANEN RJ, MARCHAL JA, UNDERBERG WJ: Analytical techniques used to study the degradation of proteins and peptides: chemical instability. *J. Pharm. Biomed. Anal.* (1998) 17:955-978.
38. REUBSAET JL, BEIJNEN JH, BULT A, VAN MAANEN RJ, MARCHAL JA, UNDERBERG WJ: Analytical techniques used to study the degradation of proteins and peptides: physical instability. *J. Pharm. Biomed. Anal.* (1998) 17:979-984.
39. JISKOOT W, CROMMELIN DJ: *Methods for Structural Analysis of Protein Pharmaceuticals.* W Jiskoot, DJ Crommelin (Eds), AAPS Press, Arlington, VA, USA (2005).
•• An excellent compilation of almost all analytical techniques used to characterise proteins in formulations.
40. NIELSEN L, FROKJAER S, BRANGE J, UVERSKY VN, FINK AL: Probing the mechanism of insulin fibril formation with insulin mutants. *Biochemistry* (2001) 40:8397-8409.
41. DEPAZ RA, BARNETT CC, DALE DA, CARPENTER JF, GAERTNER AL, RANDOLPH TW: The excluding effects of sucrose on a protein chemical degradation pathway: methionine oxidation in subtilisin. *Arch. Biochem. Biophys.* (2000) 384:123-132.
42. SOENDERKAER S, CARPENTER JF, VAN DE WEERT M, HANSEN LL, FLINK J, FROKJAER S: Effects of sucrose on rFVIIa aggregation and methionine oxidation. *Eur. J. Pharm. Sci.* (2004) 21:597-606.
43. CARPENTER JF, MANNING MC: *Rational Design of Stable Protein Formulations – Theory and Practice.* JF Carpenter, MC Manning (Eds), Kluwer Academic/Plenum Publishers, New York, NY, USA (2002).
- Gives a good insight into the rational design of protein formulations.
44. WANG W: Lyophilization and development of solid protein pharmaceuticals. *Int. J. Pharm.* (2000) 203:1-60.
45. COSTANTINO HR, PIKAL MJ: *Lyophilization of Biopharmaceuticals.* HR Costantino, MJ Pikal (Eds), AAPS Press, Arlington, VA, USA (2005).
46. CARPENTER JF, PRESTRELSKI SJ, DONG A: Application of infrared spectroscopy to development of stable lyophilized protein formulations. *Eur. J. Pharm. Biopharm.* (1998) 45:231-238.
47. CHANG BS, BEAUVAIS RM, DONG A, CARPENTER JF: Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. *Arch. Biochem. Biophys.* (1996) 331:249-258.
48. COSTANTINO HR, CARRASQUILLO KG, CORDERO RA, MUMENTHALER M, HSU CC, GRIEBENOW K: Effects of excipients on the stability and structure of lyophilized recombinant human growth hormone. *J. Pharm. Sci.* (1998) 87:1412-1420.
49. DEPAZ RA, DALE DA, BARNETT CC, CARPENTER JF, GAERTNER AL, RANDOLPH TW: Effects of drying methods and additives on the structure, function, and storage stability of subtilisin: role of protein conformation and molecular mobility. *Enzyme Microb. Technol.* (2002) 31:765-774.
50. PASSOT S, FONSECA F, ALARCON-LORCA M, ROLLAND D, MARIN M: Physical characterisation of formulations for the development of two stable freeze-dried proteins during both dried and liquid storage. *Eur. J. Pharm. Biopharm.* (2005) 60:335-348.
51. REMMELE RL, STUSHNOFF C, CARPENTER JF: Real-time *in situ* monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. *Pharm. Res.* (1997) 14:1548-1555.
52. WEBB SD, GOLLEDGE SL, CLELAND JL, CARPENTER JF, RANDOLPH TW: Surface adsorption of recombinant human interferon-γ in lyophilized and spray-lyophilized formulations. *J. Pharm. Sci.* (2002) 91:1474-1487.
53. TANG X, PIKAL MJ: Design of freeze-drying processes for pharmaceuticals: practical advice. *Pharm. Res.* (2004) 21:191-200.
54. MAA Y-F, PRESTRELSKI SJ: Biopharmaceutical powders: particle formation and formulation considerations. *Curr. Pharm. Biotechnol.* (2000) 1:283-302.
55. JOVANOVIC N, BOUCHARD A, HOFLAND GW, WITKAMP G-J, CROMMELIN DJ, JISKOOT W: Stabilization of proteins in dry powder formulations using supercritical fluid technology. *Pharm. Res.* (2004) 21:1955-1969.
56. OKAMOTO H, TODO H, IIDA K, DANJO K: Dry powders for pulmonary delivery of peptides and proteins. *KONA* (2002) 20:71-83.
57. SONNER C, MAA Y-F, LEE G: Spray-freeze-drying for protein powder preparation: particle characterization and a

case study with trypsinogen stability. *J. Pharm. Sci.* (2002) 91:2122-2139.

58. YU Z, GARCIA AS, JOHNSTON KP, WILLIAMS RO 3rd: Spray freezing into liquid nitrogen for highly stable protein nanostructured microparticles. *Eur. J. Pharm. Biopharm.* (2004) 58:529-537.
59. CLELAND JL, DUENAS ET, PARK A *et al.*: Development of poly-(D,L-lactide-co-glycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis. *J. Control. Release* (2001) 72:13-24.
60. HANSEN TK: Pharmacokinetics and acute lipolytic actions of growth hormone – impact of age, body composition, binding proteins, and other hormones. *Growth Horm. IGF Res.* (2002) 12:342-358.
61. HAVELUND S, PLUM A, RIBEL U *et al.*: The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. *Pharm. Res.* (2004) 21:1498-1504.
62. PASUT G, GUIOTTO A, VERONESE FM: Protein, peptide and non-peptide drug PEGylation for therapeutic application. *Expert Opin. Ther. Patents* (2004) 14:859-894.

•• A very extensive review on almost all aspects of drug pegylation, including proteins.

63. CALICETI P: Pharmacokinetics of pegylated interferons: what is misleading? *Dig. Liver Dis.* (2004) 36(Suppl. 3):S334-S339.
64. ORIVE G, GASCÓN AR, HERNÁNDEZ RM, DOMÍNGUEZ-GIL A, PEDRAZ JL: Techniques: new approaches to the delivery of biopharmaceuticals. *Trends Pharmacol. Sci.* (2004) 25:382-387.
65. VAN DE WEERT M, HENNINK WE, JISKOOT W: Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm. Res.* (2000) 17:1159-1167.
66. BILATI U, ALLÉMAN E, DOELKER E: Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. *Eur. J. Pharm. Biopharm.* (2005) 59:375-388.
67. TRACY MA: Development and scale-up of a microsphere protein delivery system. *Biotechnol. Prog.* (1998) 14:108-115.

• A rare insight into the industrial production of an advanced delivery system.

68. CLELAND JL, JOHNSON OL, PUTNEY S, JONES AJ: Recombinant human growth hormone poly(lactic-co-glycolic acid) microsphere formulation development. *Adv. Drug Deliv. Rev.* (1997) 28:71-84.
69. SCHELLEKENS H: Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin. Ther.* (2002) 24:1720-1740.

•• Describes the important aspects of protein immunogenicity and its potential causes.

70. CHAMBERLAIN P, MIRE-SLUIS AR: An overview of scientific and regulatory issues for the immunogenicity of biological products. *Dev. Biol. Basel* (2003) 112:3-11.
71. FROST H: Antibody-mediated side-effects of recombinant proteins. *Toxicol.* (2005) 209:155-160.
72. HERMELING S, CROMMELIN DJ, SCHELLEKENS H, JISKOOT W: Structure-immunogenicity relationships of therapeutic proteins. *Pharm. Res.* (2004) 21:897-903.
73. SCHELLEKENS H: Bioequivalence and the immunogenicity of biopharmaceuticals. *Nat. Rev.* (2002) 1:457-462.
74. CASADEVALL N, ROSSERT J: Importance of biologic follow-ons: experience with EPO. *Best Pract. Res. Clin. Haematol.* (2005) 18:381-387.
75. EVENS AM, BENNETT CL, LUMINARI S: Epoetin-indcued pure red-cell aplasia (PRCA): preliminary results from the research on adverse drug events and reports (RADAR) group. *Best Pract. Res. Clin. Haematol.* (2005) 18:481-489.
76. SCHELLEKENS H: Immunological mechanisms of EPO-associated pure red cell aplasia. *Best Pract. Res. Clin. Haematol.* (2005) 18:473-480.
77. BOVEN K, STRYKER S, KNIGHT J *et al.*: The increased incidence of pure red cell aplasia with an Eprex formulation in uncoated rubber stopper syringes. *Kidney Int.* (2005) 67:2346-2353.
78. HERMELING S, SCHELLEKENS H, CROMMELIN DJ, JISKOOT W: Micelle-associated protein in epoietin formulations: a risk factor for immunogenicity. *Pharm. Res.* (2003) 20:1903-1907.
79. HERMELING S, JISKOOT W, CROMMELIN DJ, BORNÆS C, SCHELLEKENS H: Development of a transgenic mouse model immune tolerant for human interferon beta. *Pharm. Res.* (2005) 22:847-851.
80. MIRE-SLUIS AR, BARRETT YC, DEVANARAYAN V *et al.*: Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J. Immunol. Methods* (2004) 289:1-16.
81. GENG D, SHANKAR G, SCHANTZ A, RAJADHYAKSHA M, DAVIS H, WAGNER C: Validation of immunoassays used to assess immunogenicity to therapeutic monoclonal antibodies. *J. Pharm. Biomed. Anal.* (2005) 39:364-375.
82. DOVE A: Betting on biogenerics. *Nat. Biotech.* (2001) 19:117-120.
83. CHAMBERLAIN P: Biogenerics: Europe takes another step forward while the FDA dives for cover. *Drug Discov. Today* (2004) 9:817-820.

Website

101. http://www.phrma.org/newmedicines/biotech/
HOLMER AF: 324 biotechnology medicines in testing promise to bolster the arsenal against disease (2004).
102. http://www.jnjpharmarnd.com/company/n-casereports.html
Johnson & Johnson Pharmaceutical Research & Development update regarding erythropoietin antibody-positive pure red cell aplasia (July 2005).

Affiliation

Marco van de Weert[†1] PhD,
Lene Jorgensen PhD, Eva Horn Moeller PhD & Sven Frokjaer PhD
[†]Author for correspondence
[1]The Danish University of Pharmaceutical Sciences, Biomacromolecules Group, Department of Pharmaceutics and Analytical Chemistry, Universitetsparken 2, 2100 Copenhagen, Denmark
Tel: +45 35 306 186; Fax: +45 35 306 030;
E-mail: mvdw@dfuni.dk