



Research review paper

Advances in liquid formulations of parenteral therapeutic proteins

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ABSTRACT

Liquid formulation of therapeutic proteins is a maturing technology. Demand for products that are easy to use in the clinic or that are amenable to self-administration make a ready to use liquid formulation desirable. Most modern liquid formulations have a simple composition; comprising a buffer, a tonicity modifier, a surfactant, sometimes a stabiliser, the therapeutic protein and water. Recent formulations of monoclonal antibodies often use histidine or acetate as the buffer, sucrose or trehalose as the tonicity modifier and polysorbate 20 or 80 as the surfactant with a pH of 5.7 $\pm$ 0.4. The mechanisms for the behaviour of excipients is still debated by academics so formulation design is still a black art. Fortunately, a statistical approach like design of experiment is suitable for formulation development and has been successful when combined with accelerated stability experimentation. The development of prefilled syringes and pens has added low viscosity and shear resistance to the quality attributes for a successful formulation. To achieve patient compliance for self-administration, formulations that cause minimal pain and tissue damage is also desirable.

1. Introduction

Parenteral therapeutic proteins are currently used to treat a wide variety of human ailments ranging from rheumatism, macular degeneration, a range of cancers and to provide treatment for patients' after chemotherapy. The first therapeutic protein to be developed was insulin for the treatment of diabetes mellitus. This product has a long history since the discovery by a team in Toronto in the 1920s that an extract from bovine pancreas could be used to treat diabetic patients (Rosenfeld, 2002). The challenge for the early pioneers of commercial insulin development at Eli Lilly and Company was to produce a sufficiently pure product in a formulation that was suitable for injection into patients and had an adequate shelf life. The insulin formulations that have evolved since the 1920s have elements that are quite sophisticated. The use of zinc ions converts the insulin to a stable but inactive form, this enabled formulations to be developed with varying rates of release of the active ingredient (Teska et al., 2014). The modern recombinant insulin product Humulin® N is a good example. It contains protamine sulphate, glycerol, dibasic sodium phosphate, meta-cresol, phenol, zinc oxide (adjusted to provide 0.025 mg/mL zinc ion), and water for injection adjusted to a pH of 7.0 to 7.5 (Humulin®N package insert). Apart from the phosphate which controls the pH value, the other components zinc, protamine sulphate, glycerol, meta-cresol and phenol improve stability, modulate the release of the active ingredient (meta-cresol and phenol also act as antimicrobial preservatives).

Most modern parenteral therapeutic protein formulations are not

very complex. Humira® for example which is a monoclonal antibody product released in 2002 to treat rheumatoid arthritis has a simple formulation. It contains a citrate-phosphate buffer to control the pH, mannitol and NaCl to set the osmotic pressure, and polysorbate 80 to prevent product loss due to adhesion to surfaces or air-water interfaces (Humira® package insert). Even more sophisticated products like Enbrel® which is a synthetic designer protein comprised of a tumour necrosis factor receptor 2 fused to the Fc domain of IgG1, has a simple formulation comprising a phosphate buffer, sucrose and NaCl to set the osmotic pressure, and the "stabiliser" arginine (Enbrel® package insert). A successful formulation not only ensures an efficacious treatment for the life of the product (usually 2 years at 4°C) but also does not result in unwelcome side effects or adverse reactions in the patients. Reassuringly, products like Humira® and Enbrel®, despite their active ingredient's size and complexity, have proven successful stable products despite the simplicity of their formulations.

The challenge to contemporary formulations scientists is to produce stable efficacious products despite the imperfect understanding of the interaction between protein therapeutics and the simple cosolutes used as excipients in liquid formulations. One strategy to formulation development is to apply quality by design principles and use a statistically driven screening approach. This paper is designed to provide the reader with our current knowledge of formulation design, the most suitable excipients and their function, the different types of storage trials and the appropriate analytical tests. The paper will also detail what is known about excipients and their mode of operation.

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2. Materials used as excipients

The ideal therapeutic product is efficacious, stable, causes no adverse side effects, is easy to use and causes minimal pain or long-term tissue damage. The formulation scientist can play an important role in creating a product that is close to this ideal.

The product not only has to survive storage at 5 °C +/− 2 °C for at least two years, it should be rugged enough to survive “unplanned” excursions from the specified storage conditions such as higher temperature, freezing and thawing and light exposure. It should also be able to survive shear associated with dispensing, mechanical agitation during transport and during injection into the patient. The tests to assess these requirements are discussed in section 4 of this paper. The formulation needs to be physically compatible with device used for its delivery into the patient. The viscosity of the formulation has to be sufficiently low to be able to easily pass through a hypodermic needle which are increasingly narrow bore to reduce pain. The formulation needs to ensure the active ingredient does not accumulate at interfaces (either solid-liquid or liquid gas interfaces). The formulation ideally causes minimal stimulation of the neurons of the peripheral nervous system responsible for pain and cause minimal tissue damage during administration of the product. The formulation scientists, the designers of the delivery device, the protein engineers and clinical pharmacologists all play a key role creating a successful therapeutic protein formulation.

2.1. Buffers

The effect of the acidity of a liquid formulation (its pH) has a direct impact on a therapeutic protein. The protein's amino acid side chains (lysine, arginine, histidine, aspartate and glutamate) are usually charged at physiological pH values. The charges of the side chains will alter as the pH is varied. There will be a pH that is optimal for maximising protein solubility, for maximising thermal stability and for reducing chemical instability. These pH values are not necessarily the same. There are common excipients that are used to stabilise the pH of the formulation (Table 1). Their suitability is dictated by the pH that is required.

The therapeutic protein has some buffering capacity in its own right. At higher protein concentrations over 50 mg/mL, the protein's buffering capacity may be sufficient to hold the pH at the desired value. 50 mg/mL of a monoclonal antibody is equivalent to 6 mM citrate or 14 mM histidine buffers (Karow et al., 2013). Where the protein's buffering capacity is insufficient an excipient with suitable buffering capacity is used in the formulation.

The formulation for recombinant insulin product Humulin® N contained phosphate as the buffer which is compatible with the required pH range of 7.0 to 7.5. Humira® buffer was combination of phosphate and citrate, the two buffers being complementary extending the useful pH range from 3.1 to 8.2. The pH of the Humira® formulation was 5.2 so the choice of buffering system exceeded what was required. Enbrel® pH is 6.3 and was buffered using phosphate despite the required pH falling at the edge of phosphates useful range. Phosphate is particularly

useful at relatively neutral pH values between 6.2 and 8.2. Phosphate ions will interact with positively charged side chains and can have an unpredictable effect of thermal stability (Bye and Falconer, 2014). The concentrations used in biopharmaceutical formulations (<50 mM) is too low for the indirect “Hofmeister effect” to reduce protein solubility.

Citrate is a versatile buffer with a useful pH range between 2.1 and 7.4. Citrate is a component of the TCA cycle and is a normal intracellular component and is non-toxic. At pH values above 4.76 citrate is present as a divalent anion (at pH above 6.4 it is present as a trivalent anion) and has the potential to crosslink the positively charged side chains of a therapeutic protein especially if the protein has a net positive charge.

Succinate is another versatile buffer with a useful pH range between 3.2 and 6.7. Succinate is another component of the TCA cycle and is a normal intracellular component with no known toxicity. At pH values above 5.72, succinate is present as a divalent anion and has the potential to crosslink the positively charged side chains of a therapeutic protein and reduce its solubility.

Acetate with a pKa value 4.76 is a buffer for acidic pH values between 3.8 and 5.8. Acetate is a neutral buffer only weakly interacting with positively charged side chains of a therapeutic protein. Acetate is unlikely to provide any significant “stabilising” effects in its own right.

Histidine is a particularly useful buffer at pH values between 5.0 and 7.0. Histidine can also act as a stabiliser for some proteins (Platts et al., 2016) and has been linked to low viscosity formulations making it an attractive excipient (Chen et al., 2003).

The pH of a formulation can effect protein solubility and is kept away from the protein's pI value so the protein carries a charge and is electrostatically repelled from other proteins. Chemical reactions can be affected by pH. The pH range between 3 and 5 offers the region of optimum stability to asparagine deamidation (Gietz et al., 1998). Oxidation of methionine residues is relatively unaffected by pH (Peskin and Winterbourn, 2001).

2.2. Tonicity modifiers

The osmotic pressure of a therapeutic protein formulation should be close to being isotonic (approximately 290 mOsm/L). At osmotic pressures lower than 290 mOsm/L (hypotonic) red blood cells swell and can lyse. At osmotic pressures higher than 290 mOsm/L (hypertonic) red blood cells collapse as the water is drawn out of them. Isotonic formulations are desirable even for smaller subcutaneous injections as it can minimise tissue damage around the site of injection.

Commonly used tonicity modifiers used in excipients include NaCl, sucrose, trehalose, mannitol, glycerol and sorbitol. It is worth noting that the end osmotic pressure of a formulation is the sum of contributions from the active ingredient, the buffer and any other solutes in the final formulation, the tonicity modifier is used to bring the final osmotic pressure close to 290 mOsm/L.

2.3. Stabilisers

Some excipients are added to formulations as they have been observed to thermally stabilise proteins, improve their solubility or suppress their aggregation. These “beneficial” molecules are often referred to as stabilisers. Amongst commonly used excipients deemed to be stabilisers include arginine, histidine, glycine and methionine. Part of the complexity in discussing stabilisers is that the role they can play is multifactorial. The stabilisation may be effected by changing the physical properties of the protein altering its solubility or structural stability; it may also be chemical, reducing undesired reactions such as oxidation, hydrolysis or photo-degradation. The direct interactions between excipients and protein surfaces are driven by electrostatic forces (full charge, partial charge and pi interactions), hydrogen bonding, Van der Waals and water displacement (often called hydrophobic interaction). There is also an indirect interaction between

Table 1
Common excipients used for their buffering capacity.

Buffer	pKa ^a	Useful buffering range (pH)
Phosphate	2.16, 7.21 and 12.32	6.2–8.2
Citrate	3.13, 4.76 and 6.40	2.1–7.4
Succinate	4.21 and 5.72	3.2–6.7
Acetate	4.76	3.8–5.8
Glycine	2.35 and 9.78	1.4–3.4
Histidine	1.80, 6.04 and 9.33	5.0–7.0

^a Note, the definition of pKa is the negative decimal logarithm of the dissociation constant (Ka) of the acid in solution.

stabilisers and proteins variously explained as preferential hydration or competition for water resulting in salting-out proteins but stabilising their structures (Timasheff, 2002; Bye and Falconer, 2014). With many chemicals like the zwitterion glycine, the outcome from direct interaction is unpredictable and has been shown to have positive and negative impacts on different proteins structural stability (Platts and Falconer, 2015; Platts et al., 2016). The indirect interaction, at higher concentrations, is to stabilise the protein (presumably by competing for water needed to hydrate the unfolding protein). The positively charged amino acids, arginine and histidine are more predictable. Both have side chains with a low charge density group at the end (guanidinium for arginine and imidazole for histidine) and these are predictable, increasing protein solubility, decreasing structural stability and reducing viscosity. Arginine is widely claimed as a stabiliser for the reduction of aggregation but there are cases where arginine's ability to decrease a protein's structural stability can lead to aggregation (Cirkovas and Sereikaite, 2011). Methionine stabilises proteins by acting as a sacrificial chemical reacting with oxygen free radicals before they have an opportunity to react with the methionine side chains on the protein (Dion et al., 2018). Other amino acids like aspartate, isoleucine, leucine, glutamate and phenylalanine have been used in therapeutic protein formulations. Their effect on protein stability is difficult to predict and could only be determined by experimentation. The opportunity does exist for new and novel formulations to be discovered if a broader range of amino acid excipients are included at the early stages of formulation screening.

2.4. Surfactants

Polysorbate is by far the most widely used surfactant in therapeutic protein formulation. There are two variants of polysorbate that are commonly used PS20 and PS80. They differ in molecular weight and there is a minor structural difference but their actions are very similar and in many cases are interchangeable. The alternative to the polysorbates are the poloxamers. These are often mentioned in patents on formulation but in reality infrequently replace polysorbates.

The surfactant can play an important role in reducing therapeutic protein interactions with the air-liquid and solid-liquid interfaces. Many proteins will associate with an air-liquid interface. The air surface is hydrophobic and the water hydrophilic generating force on hydrophobic side chains to move to the hydrophobic interface. Some protein's structures are minimally changed but some are partially unfolded and prone to form aggregates. The surfactant displaces the protein from an air-liquid interface reducing this source of instability. Solid-liquid interfaces such as syringe or vials walls are also a surface where proteins may associate. Adsorption to surfaces can reduce the therapeutic protein concentration and reduce the effective dose of the product. Again the surfactant displaces the protein from the interface reducing this source of loss.

Polysorbates have been associated with peroxide contamination (Ha et al., 2002) which in turn can lead to chemical modification of the therapeutic protein. The chemical process used to manufacture polysorbate results in an impure product and batch to batch variation. There are also reports of polysorbates degrading with time releasing fatty acid particulates into the product (Tomlinson et al., 2015). Despite this cautionary note, polysorbates are used in the majority of liquid therapeutic protein formulations and their benefits outweigh the disadvantages.

2.5. Divalent cations, DTPA and EDTA

The addition of divalent cations to a formulation is beneficial for specific proteins. The use of zinc ions in insulin formulations has a precise reason, the formation of inactive tetramers. Some enzymes benefit from the addition of specific cations. In many cases divalent cations are deleterious to stability. When a divalent cation interacts

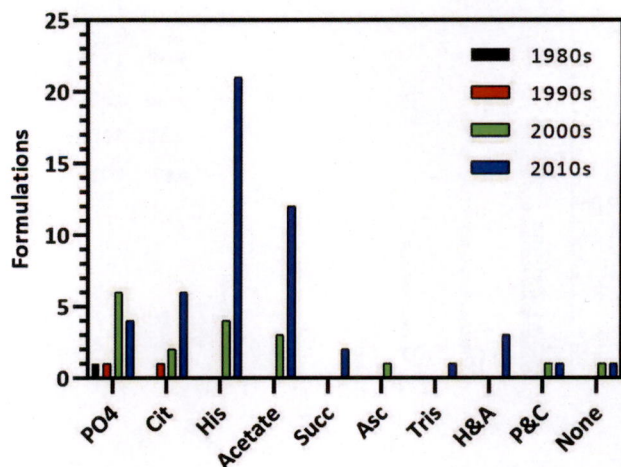


Fig. 1. The buffers used in therapeutic monoclonal antibody liquid formulations separated by decade demonstrating the rise of histidine and acetate as preferred buffers in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). Note, PO4 is phosphate, Cit is citrate, His is histidine, Succ is succinate, Asc is ascorbic acid, Tris is trisaminomethane, H & A is histidine and acetate, and P & C is phosphate and citrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with a negatively charged side chain on a protein's surface it changes the local charge, this can destabilise the protein's structure. It can also aid in crosslinking negatively charged side chains reducing the proteins solubility.

Where divalent cations are undesirable a chelating agent such as DTPA and EDTA can be used to remove any residual divalent cations that may have been used in the manufacturing process. These chelating agents are also useful to remove metal ions which catalyse the formation of oxygen free radicals.

3. Trends in monoclonal antibody formulation

The frequency of the use of the buffers, tonicity modifiers and surfactants is shown in Figs. 1, 2 and 3, respectively. The early Mab formulations tended to use phosphate as the buffer and sodium chloride as the tonicity modifier but recently these excipients have been displaced. Between 2016 and 2018 the formulations pH values have been 5.7 $\pm$ 0.4 ($n = 22$), which is a tight cluster. This has driven the selection of buffer to those compatible with this pH range. Recent Mab formulations are using histidine or acetate as the preferred buffer and sucrose and trehalose as the tonicity modifier. The surfactant that is widely used in formulations is polysorbate 80 followed by the closely related polysorbate 20. Reduction of oxygen free radical generation is reduced by the addition of the metal ion chelators. EDTA is present in the formulations of Campath®, Arzerra®, Lemtrada®, Opdivo and Ajoyv™; and DTPA is present in the formulations of Vervoy® and Zinplava™. The sacrificial excipient methionine to minimise oxygen free radical damage is present in the formulations of Stelara®, Cosentyx™ and Crysvita®.

There are examples where formulators have deviated from the use of common amino acid excipients (glycine, histidine and arginine). Hemlibra™ which is a humanized bi-specific IgG4, used a combination of histidine, arginine, aspartate with the surfactant poloxamer 18,8 which stands out from the other formulations as a unique combination of excipients. Siliq™ which is a human IgG2/κ, used a combination of glutamate, proline and polysorbate 20, which is also an innovative formulation.

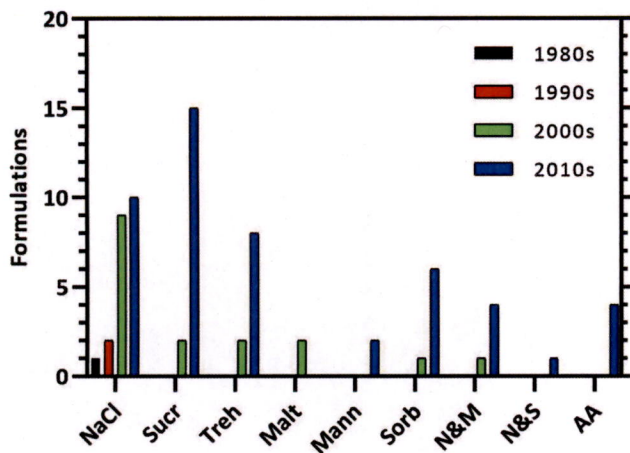


Fig. 2. The tonicity modifiers used in therapeutic monoclonal antibody liquid formulations separated by decade demonstrating the rise of sucrose and trehalose as preferred tonicity modifiers in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). Note, Sucr is sucrose, Treh is trehalose, Malt is maltose, Mann is mannitol, Sorb is sorbitol, N&M is sodium chloride and mannitol, N&S is sodium chloride and sucrose, and AA is amino acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

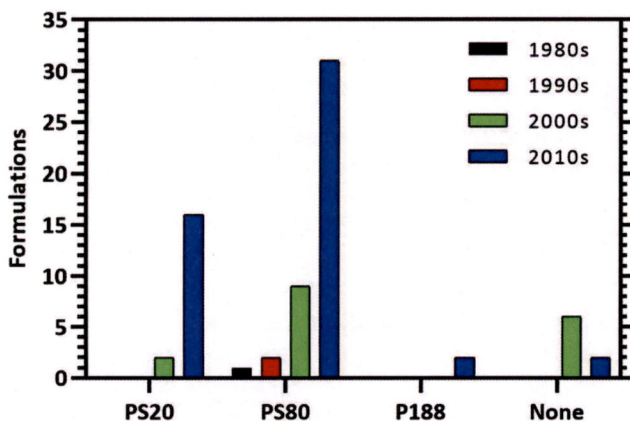


Fig. 3. The surfactants (and albumin) used in therapeutic monoclonal antibody liquid formulations separated by decade demonstrating the continued dominance of polysorbate 20 and 80 as preferred surfactants in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). PS20 is polysorbate 20, PS80 is polysorbate 80 and P188 is poloxamer 188. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Trends in protein formulations other than Mabs

The frequency of the use of the buffers, tonicity modifiers and surfactants is shown in Figs. 4, 5 and 6, respectively. Being a diverse range of proteins the formulations have a wide pH range of 6.1 $\pm$ 1.2. Recent non-Mab formulations use phosphate or acetate as the preferred buffer and sodium chloride, sorbitol and mannitol as the tonicity modifier. Formulations without a surfactant are also frequent in non-Mab formulations.

Retacrit™ which is epoetin alfa-epbx, an Epogen biosimilar, the formulation used a combination of amino acids in its formulation including glycine, isoleucine, leucine, glutamate and phenylalanine. While the motivation may have been to create a unique formulation evading existing patents on epoetin formulation, it does show that a diverse range of amino acids are available for use as excipients.

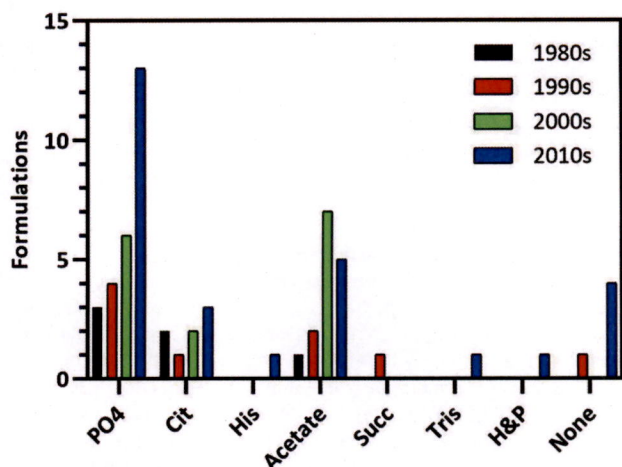


Fig. 4. The buffers used in therapeutic protein (other than monoclonal antibody and insulin) liquid formulations separated by decade demonstrating the rise of phosphate and acetate as preferred buffers in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). Note, PO4 is phosphate, Cit is citrate, His is histidine, Succ is succinate, Tris is trisaminomethane, and H&P is histidine and phosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

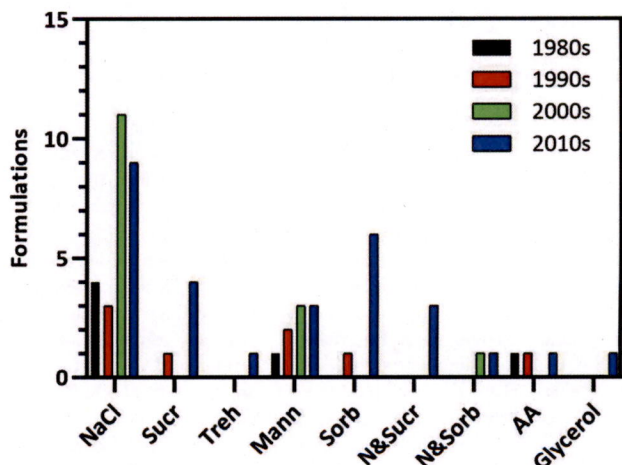


Fig. 5. The tonicity modifiers used in therapeutic protein (other than monoclonal antibody and insulin) liquid formulations separated by decade demonstrating the rise of sodium chloride as preferred tonicity modifiers in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). Note, Sucr is sucrose, Treh is trehalose, Mann is mannitol, Sorb is sorbitol, N&Sucr is sodium chloride and sucrose, N&Sorb is sodium chloride and sorbitol, and AA is amino acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Reduction of oxygen free radical generation is reduced by the addition of the metal ion chelators. EDTA is present in the formulations of ONTAK and Anakinra. The sacrificial excipient methionine to minimise oxygen free radical damage is present in the formulations of Mircera and Adlyxin. Adlyxin's formulation also contains metacresol and glycerol which are more commonly found in insulin formulations. Adlyxin's active ingredient is a 44 amino acid peptide which is a GLP-1 receptor agonist, it is supplied as a prefilled pen and is used to stimulate insulin synthesis in patients with type 2 diabetic mellitus. The similarity with insulin formulations is not unexpected as the manufacturer also makes series of insulin products.

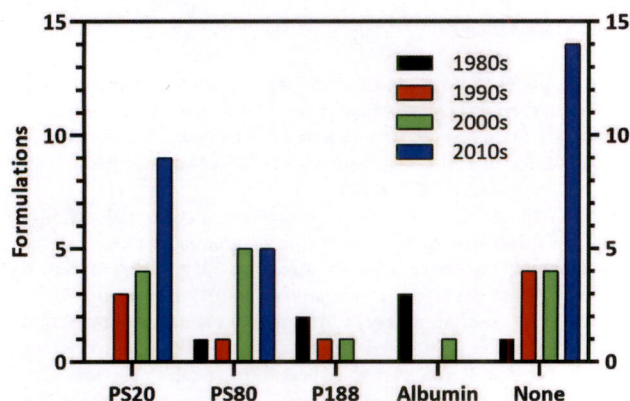


Fig. 6. The surfactants (and albumin) used in therapeutic protein (other than monoclonal antibody and insulin) liquid formulations separated by decade demonstrating the continued dominance of either no surfactant or polysorbate 20 in recent formulations. The formulations from the 1980s (black), 1990s (red), 2000s (green) and the 2010s (blue). PS20 is polysorbate 20, PS80 is polysorbate 80 and P188 is poloxamer 188. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

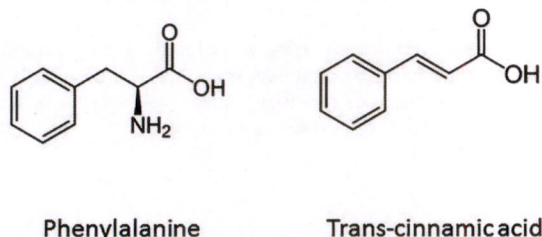


Fig. 7. Phenylalanine, the substrate for phenylalanine ammonia lyase, and trans-cinnamic acid product of phenylalanine deamidation by the lyase and used as an excipient in Palynziq™.

An example where a product specific stabiliser is used in the formulation is Palynziq. Palynziq is a treatment containing the recombinant enzyme phenylalanine ammonia lyase (rPAL) conjugated to N-hydroxysuccinimide (NHS)-methoxypolyethylene glycol (Palynziq Package Insert). rPAL is used for the treatment of phenylketonuria where the patient does not have the capability to break down phenylalanine which accumulates in the blood and brain causing a range of mental disorders. A non-standard formulation was used for Palynziq which uses tromethamine (Tris) for pH control (pH 7.0) and sodium chloride for tonicity adjustment. The interesting inactive ingredient is the trans-cinnamic acid. trans-Cinnamic acid is the product of deamidation of phenylalanine catalysed by rPAL (Levy et al., 2018). Use of the excipient trans-cinnamic acid stabilises rPAL by associating with the active site without being chemically modified. This is an example of stabilising a protein using a small molecule that can associate with the active site due to its structural similarity to the enzymes substrate phenylalanine (see Fig. 7). The strategy of stabilising proteins by the addition of a molecule that can associate with the catalytic site or a molecule that can associate with a specific binding site on the therapeutic protein has much promise.

5. Risks associated with parenteral therapeutic protein formulations

When undertaking a risk assessment of a parenteral therapeutic protein product the historic data is useful for highlighting several of the risks associated with this type of product.

5.1. Aggregation

Aggregation can be a serious problem in therapeutic proteins. At its least serious aggregation can result in a loss of efficacy of the therapeutic as the active ingredient is lost. Aggregates or particles of the active ingredient can be detected by the immune system in the patient and elicit an immune response (Moore and Leppert, 1980; Baert et al., 2003; Rosenberg, 2006). If the cell-mediated immune system is triggered there can be site-of-injection swelling and pain and in rare cases systemic hypersensitivity reactions including anaphylactic reactions. Aggregates can also trigger the acquired immune system generating antibodies against the active ingredient. Where the active ingredient is an endogenous protein such as erythropoietin, the antibody can eliminate endogenous protein and lead to serious side effects (in erythropoietin case the undesired side effect is red cell aplasia) (Casadevall et al., 2002; Bennett et al., 2004; Boven et al., 2005). In the early days of monoclonal antibody treatments with murine Mabs, patients developed a response to murine antibodies referred to as the HAMA (human anti-mouse antibody) response (Kricka, 1999). The presence of HAMA antibodies can reduce the efficacy of the treatment and generate false positives in immunological diagnostic tests that use murine Mabs. The presence of aggregated human or humanized mouse monoclonal antibodies particles in a therapeutic product is undesirable due to the unpredictable potential immunogenicity risk (Singh et al., 2010; Joubert et al., 2012). Analysis for the presence of antibodies against the active ingredient in patients is a valuable component in clinical trials.

Aggregates or particles of the active ingredient can be detected using a diverse range of assays and are easily studied. The prevention of aggregation, however, is not simple due to it being the observable effect that can be caused by a variety of changes (Chi et al., 2003). The causes of aggregation can include structural change to the protein such as complete thermal unfolding, partial thermal unfolding and partial unfolding induced at air-water interfaces. Protein solubility can play a role as reversible protein precipitation can be followed by irreversible compaction. Chemical modification can also lead to aggregation where the chemical change alters the protein structural stability, solubility or where the chemical modification can cause crosslinking between protein molecules (this includes bonding between cysteine residues and between tyrosine residues).

5.2. Loss of efficacy

The structural or chemical modification that cause aggregation, can also create a soluble inactive form of the therapeutic protein. Whilst this outcome does not have the adverse side effects observed with aggregation, the treatment is less efficacious. Adsorption of the therapeutic protein can also reduce the concentration of the active ingredient and results in a loss of efficacy of the treatment. Chemical modification of the protein around its binding or catalytic site can reduce the efficacy of an otherwise intact protein (Bertolotti-Ciarlet et al., 2009). Unfolding followed by refolding to inactive but soluble form can also reduce efficacy without changing the proteins concentration.

5.3. Pain of injection and tissue damage

Most formulation work on therapeutic proteins has focused on their structural stability, solubility, and chemical stability. Pain and tissue damage upon injection of the product into the patient is less well studied despite it adversely affecting patient compliance and the acceptance of the product in the market. Many therapeutic proteins are administered subcutaneously and is associated with pain.

Pain receptor activation is dependent on ion channels at the ends of the neurons. These receptors have the capacity to detect chemical, mechanical and thermal changes. One capability of pain receptors is to detect the chemicals released by tissue damage (Rang et al., 1991; Bevan and Geppetti, 1994). From a formulation point of view this

creates a challenge as pain activation is complex.

An obvious response is to minimise tissue damage through the use of narrow needle sizes and reducing the injection volume. Published work on the role of formulation on pain of injection has focused on erythropoietin where some studies have suggested a citrate buffer was associated with greater pain than a corresponding histidine buffer (Laursen et al., 2005). Studies with citrate buffer and phosphate buffer with no active ingredient also showed the same trend (Veys et al., 1998; Yu et al., 1998). The choice of tonicity modifier may also play a role in pain of injection. One study suggested that formulations containing mannitol caused less pain than an equivalent containing sodium chloride (Tanikawa and Iida, 2004).

5.4. Incompatibility with delivery devices

To minimise pain of injection and tissue damage narrow bore needles are increasingly used. During injection relatively high velocities are attained and the therapeutic protein can experience quite high shear. The protein should be rugged enough to sustain minimal structural damage on injection which could lead to post-injection aggregation.

The materials in delivery devices that are in product contact should not negatively affect the therapeutic protein. Glass or polymer used to make the container provides a surface where the therapeutic protein can be adsorbed and where forces on the protein can lead to structural damage. Interaction with surfaces can be minimised by the choice of hydrophilic materials where binding is less favoured and by the use of excipients like albumin or polysorbate than compete for the surface, displacing the protein. Traces of oil from the delivery device's manufacturing process (used as lubricants or for mould release) can carry over to the end product and if present can interact with the therapeutic protein creating conditions where aggregation can take place.

Measurement of the viscosity of a formulation is important for treatments where the drug is delivered to the patient using narrow bore needles. Where the viscosity is too high the liquid will require a higher force to create the desired flow than can be supplied manually by the patient or clinician. A specification for the viscosity should be set, based on the inner dimensions of the needle and the force a patient can easily exert.

6. Stability studies

Stability studies fulfil two functions in therapeutic protein development. Firstly, they provide evidence used to establish the shelf-life of the product and are an important part of regulatory submissions. Secondly, stability studies can be used to determine the combination of excipients comprising the formulation with stability and ruggedness to make a reliable effective therapeutic product.

6.1. Long-term stability

The definitive stability trial to verify the shelf life of the product is to store the therapeutic protein formulation at the recommended temperature ($5^{\circ}\text{C} \pm 2^{\circ}\text{C}$). For most therapeutic proteins at least 2 years' shelf life is required to make it a viable product. This type of study is an intrinsic part of regulatory submissions and is used to establish the products shelf life. The standards ICH Q1A and ICH Q5C provides guidance on stability testing required for regulatory submissions.

Long-term stability trials are not suitable for screening different formulations due to long time scale so accelerated stability, freeze thaw, and light exposure testing have been created to provide quicker methods for determining formulations with a high probability of success.

6.2. Accelerated stability

Elevated temperatures are common unplanned excursions from the specified storage conditions either during transport or in the clinic. Accelerated stability studies can be a valuable component in regulatory submissions. For this purpose, relatively long time scales are used, $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 6 months (ICH Q1A).

From a formulation scientists' perspective, accelerated stability studies are invaluable as some elements of protein instability occur more quickly as the temperature is raised (i.e. they follow Arrhenius behaviour). These reactions include protein unfolding (partial or complete) and the hydrolysis reactions, deamidation and fragmentation of the peptide backbone. Accelerated stability studies at temperatures higher than the preferred storage temperature enables processes that may take years at the refrigerated storage conditions to be studied in a few weeks. Reactions that don't follow Arrhenius behaviour (i.e. they are faster at lower temperatures) include oxidation reactions, precipitation, adsorption to surfaces and photo-degradation. The results of accelerated stability studies are not predictive of these reactions at refrigeration storage. Oxygen is more soluble in water at lower temperatures which affects the rate of oxidation reactions. In the case of precipitation and adsorption to surfaces, the reduced kinetic energy at lower temperatures enables weak interactions to occur that are not seen at higher temperatures. Photo-degradation is also independent of the storage temperature.

The screening formulations using accelerated stability studies is widely used by biopharmaceutical companies and is easy to standardise. For screening purposes conditions such as $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 weeks or $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 4 weeks are suitable.

6.3. Freeze-thaw

Freezing may occur during shipping or storage as temperature gradients are common in refrigerated containers or rooms where sub-zero temperature can occur whilst trying to maintain the specified temperature (usually $5^{\circ}\text{C} \pm 2^{\circ}\text{C}$). The freezing process involves the crystallisation of water. As the ice crystals grow the active ingredient and the excipients are concentrated into the space between the ice crystals. At high concentrations and low temperatures proteins can interact and precipitate. The excipients can also crystallise. The presence of the ice and excipient crystals also provides a changing interface where significant forces can be applied to the protein and unfold or damage the protein's structure. The freeze thaw cycles can result in observable aggregation of the protein.

For screening purposes, 5 freeze thaw cycles between -80°C and 20°C are suitable study conditions and can be complete in a couple of days. Freeze thaw cycles are difficult to standardise. Rapid freezing using liquid nitrogen results in small ice crystals and shortened time scales for precipitation to occur and is not ideal. Slower freezing of the samples can result in larger ice crystals similar to those found during unplanned excursions from the specified storage conditions. Ice crystal formation at slower freezing rates is not uniform. Ice crystals need to nucleate before the crystal forms, so identical vials will freeze at different temperatures. Samples that stay liquid to lower temperatures then freeze to form small crystals and those that freeze close to freezing point form relatively large crystals. By repeating the freeze thaw cycle 5 times this variability should even out between the samples.

6.4. Light exposure

Low levels of oxygen and hydroxyl free radicals are present in liquid formulations. Background radiation from the earth or from the sun creates low levels of free radicals that can then react with the therapeutic protein. The two key reaction types are oxidation usually of methionine side chains, and hydrolysis which can fragment the peptide backbone or deamidate the asparagine or glutamine side chains. The

exposure of a protein formulation to light will generate free radicals in the amino acid residues, tryptophan, tyrosine, phenylalanine and cysteine which can react with oxygen causing chemical modification of the residues (Kerwin and Remmele, 2007; Pattison et al., 2012). Light exposure stability studies are effectively an accelerated chemical modification test. The standard ICH Q1B gives guidance for regulatory submissions and detail on the presentation of samples. For screening purposes 2000 watt hours per meter squared using an artificial daylight fluorescent lamp is suitable with the container placed (usually on its side) so maximum light can penetrate through to the product.

6.5. Mechanical stress

Mechanical shaking is another are common unplanned excursions from the specified storage conditions and can occur during transport or in the clinic. Mechanical shaking subjects the therapeutic protein to shear and can detect shear-susceptible proteins. Shaking also enables transfer of protein between the gas-liquid interface and the bulk liquid. Proteins susceptible to structural damage at the gas liquid interface can be detected using mechanical shaking that may be missed in long-term or accelerated stability studies. For screening purposes mechanical shaking for period of time (such as 48 h) at a set rate (100–200 rpm) is suitable. The samples should be studied in the container which will be used for long-term storage.

6.6. Additional testing of formulations

One aspect of therapeutic protein development that is often overlooked is the fate of the active ingredient after it is administered. On injection the protein can be exposed to high velocities passing through the needle and with it high shear which can damage the protein structure and cause loss of efficacy and in some cases aggregation. The protein whether delivered intravenously or subcutaneously also passes into environment different to the formulation buffer where the protein may respond by precipitating. This is used to advantage with slow release insulin formulations but for many therapeutic treatments this can be undesirable. Mimicking the conditions during administration of a therapeutic presents a technical challenge but warrants consideration. To detect damage to the protein due to shear experienced during injection a screen may constitute multiple passes through the needle to be used in administration followed by storage for a week at 20 °C. Determining whether the protein precipitates on injection either intravenously or subcutaneously can be achieved by injecting the formulation into a solution that mimics the conditions *in-vivo* followed by analysis to detect precipitation or aggregation.

For further information on accelerated testing of therapeutic proteins see the review Hawe et al., 2012.

7. Testing of critical quality attributes

7.1. Aggregation

Size exclusion chromatography (SEC) is the standard method for analysing for polymeric forms of the therapeutic protein and is used with a high-performance liquid chromatography (HPLC) or an ultra-performance liquid chromatography (UPLC) system. A pore size of 450 Å is ideal for separating large proteins such as IgGs from their polymeric forms. Smaller pore sizes are appropriate for smaller therapeutic proteins.

SEC has the disadvantage of only detecting small irreversible aggregates. The large aggregates are usually trapped in the guard column that protects the analytical column and are not observed. During the running of SEC the running buffer is often dissimilar to the formulation solution the protein is stored in and reversible aggregates can dissolve back to their monomeric state during the operation of SEC. The findings of SEC are excellent for screening studies if the main peak area is

measured as well as the peaks for any multimers so disappearance of the main peak can be interpreted as material lost as large aggregates or adsorbed to the container surfaces. Findings of SEC is best verified using a second technique such as asymmetrical flow field-flow fractionation (AF4), analytical ultracentrifugation (AUC), dynamic light scattering (DLS) or dynamic particle imaging systems (Liu and Shire, 1999; Liu et al., 2006; Gabrielson et al., 2007; Philo, 2008).

Turbidity measurement can also be used to detect particulates large enough to scatter light and is easy to use but has relatively low sensitivity and should not be relied on as a primary method for detecting aggregation. Light obscuration, nanoparticle tracking analysis and micro-flow imaging can be used to detect subvisible particles in formulations and determine the particle concentration and size distribution (Joubert et al., 2011). Detection of subvisible particulates in a formulation should be followed up with identification of the composition of the particulate using a technique like FTIR-microscopy as crystals or precipitates of excipients can be mistaken for aggregated therapeutic protein.

7.2. Fragmentation

Capillary electrophoresis sodium dodecyl sulphate (CE-SDS) is a standard method for analysing for fragmentation of therapeutic proteins (Salas-Solano et al., 2006; Rustandi et al., 2008). The system can be operated under non-reducing conditions maintaining the therapeutic protein structure and detecting fragments. It can also be operated under reducing conditions. This is particularly useful for heteromeric proteins like the IgGs where it can separate the protein into light chain, heavy chain and non-glycosylated heavy chain.

SEC is an alternative to CE-SDS for fragmentation analysis often using a smaller pore size than used for aggregation detection.

7.3. Unfolding

Differential scanning calorimetry (DSC) is used to determine the structural stability of a therapeutic protein in the liquid formulation. The instrument raises the temperature in the sample cell in a linear manner and measures the energy required. As the protein unfolds due to thermal denaturation the reaction is endothermic and the change in enthalpy of unfolding (ΔH) can be measured as it is the area under the curve on the thermogram. The temperature with maximum unfolding is usually very close to the mid-point temperature for unfolding (T_m). The kinetics of unfolding can be calculated from the data (Falconer, 2018) but in industry the T_m value is usually used as a benchmark to indicate thermal stability. A repeat scan of the sample is informative. If the protein refolds the unfolding reaction is reversible and suggests the protein is insensitive to thermal denaturation. Lack of refolding (seen as a flat second thermogram) indicates the protein is unable to refold or aggregates and is lost during thermal unfolding.

Differential scanning fluorimetry (DSF) is an alternative method to DSC and uses dye binding to the unfolded protein to detect the unfolding reaction (Lavinder et al., 2009; He et al., 2010). DSF has the advantage of being simple to operate and can be used in a multi-well format. It does rely on the presence of the dye having little or no effect on the thermal stability of the protein and is not suitable for detecting reversibility of unfolding.

7.4. Isoforms

There are two common methods of detecting changes in the charge of the therapeutic protein resulting from chemical reactions such as deamidation of asparagine and glutamine residues, and oxidation of histidine residues. Capillary isoelectric focusing (cIEF) (Anderson et al., 2012; Hosken et al., 2016) and cation exchange chromatography (CEX) (Rozhkova, 2009) are capable of separating the different charged species and are suitable for screening studies as well as regulatory

submissions.

Mass spectroscopy can be used to detect chemical modification where there is no change of charge by measuring the mass of trypsin digests of the therapeutic protein (Ji et al., 2009; Folzer et al., 2015). This can detect oxidation of methionine, photodegradation of tryptophan, tyrosine, phenylalanine and cysteine residues, and changes to the glycosylation of the protein.

7.5. Loss of efficacy

Measuring loss of efficacy is an important measure for a protein therapeutic. Testing different formulation in patient is usually impractical especially for screening formulations so an *in-vitro* assay is used. For some therapeutic proteins there are cell based assays available but are limited by their lack of precision. Alternative tests such as isothermal titration calorimetry, surface plasmon resonance or immune-assays (such as ELISAs) can be used to test binding to appropriate receptors, ligands or antigens and confirm the functionality of the protein.

8. Quality by design

Quality by Design (QbD) principles are appropriate to the development of liquid formulations of parenteral therapeutic proteins. The standard ICH Q8(R2) Pharmaceutical Development provides some guidance.

When undertaking the risk assessment for the product there are some generic risks associated with this type of product as outlined in section 3. Aggregation and loss of efficacy are risks encountered with a diverse range of therapeutic proteins. Pain of injection is of concern especially with products where patient self-administration is required, and incompatibility with the delivery device is a product specific risk especially for prefilled syringes and pens. The risk assessment needs to be bespoke to the specific product and conducted by a team with knowledge of the products manufacture, the supply chain, its use in the clinic and the pharmacology of the active ingredient.

The critical quality attributes (CQAs) for the product is determined during the risk assessment and must not only specify the CQAs but include the required specifications and the method of analysis for the attribute.

Selection of material attributes can include the excipients to be screened and their quality, the container materials includes all components that may come into product contact during storage (for vials this includes the container and the closure, for prefilled syringes the barrel and the seal on the plunger). Some materials are problematic, of special note is polysorbate which can carry contaminants that can generate free radicals. For companies wanting to develop novel formulations the selection of excipients can be quite broad at this stage widening the possibility to develop a formulation superior to the common combinations used in the past.

The selection of process parameters is not identical to a manufacturing process but can include unplanned excursions from the specified storage conditions to build ruggedness into the final product (see section 7). Fractional factorial experimentation is used in the elimination of unimportant material attributes and process parameters. This approach will eliminate excipients that don't contribute "stability" and ruggedness of the product. The ultimate use of multi-factorial experimentation to establish the design space for the formulation provides the traditional benefits of QbD of allowing flexibility in the future if change to the formulation is required and a sound knowledge of the product and the role of excipients and container materials in its stability and ruggedness.

9. Conclusion

Some manufacturers of Mabs seem to have settled on formulations

that are quite conservative and comprise histidine or acetate buffers, sucrose or trehalose tonicity modifiers and polysorbate 80 surfactant, such as Ilumya™, Aimovig™, Tremfya™, Renflexis, Imfinzi® and Cyltezo™ (all from 2017 to 18). Close scrutiny of the actual formulations used with Mabs show most differ from this combination in one or more ways, such as addition of an amino acid (arginine, asparagine, or methionine) or the substitution of polysorbate 80 with polysorbate 20.

The designer proteins such as Enbrel® and Eylea®, and recombinant proteins other than Mabs represent a challenge as they are broad population of protein structures and functionalities. It may be advantageous for formulators to broaden the pallet of excipients they use in their screening and apply partial factorial experimental design to eliminate ineffective excipients. This can be used to discover combinations of excipients that are effective but would not have been predicted based on their known functionality or on past formulations. The unusual formulations like Pfizer's Epogen biosimilar, Retacrit™ which contains phosphate, NaCl, glycine, isoleucine, leucine, glutamate, phenylalanine and polysorbate 20, could be discovered by this route.

There is also scope for selection of excipients with a specific binding capacity like trans-cinnamic acid to phenylalanine ammonium lyase active site. This type of binding interaction can often be highly stabilising and produce clever formulations with unique stability.

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