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Optimization of Anti-Rh D Immunoglobulin Stability in the Lyophilization Processes

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Abstract

Objective
Anti-Rh D IgG is used for the prevention of anti-D antibody production in Rh^- individuals who have been exposed to Rh^+ red blood cells. The stability of IgG preparations as a solution is low, with a shelf life of a year or more. Formulation of anti-Rh D IgG as a lyophilized preparation would decrease its degradation rate and increases its shelf life. The objective of this study was to formulate the anti-Rh D as a lyophilized preparation using different formulations and optimize the lyophilization processes.
Materials and Methods
The effect of various formulations on the stability of anti-Rh D was evaluated using accelerated stability test. In this method the amount of transmittance (T %) at 585 nm for the lyophilized preparations had inverse relationship with aggregation of anti-Rh D. To improve stability, the most stable formulation was selected and different concentrations of sucrose in the presence of sodium-potassium phosphate buffer 25 mM pH 7.5. Then, the bioactivity was determined, using the ELAT test and also, the amount of moisture measured in this formulation.
Results
Among different formulations, the one with anti-Rh D 5 mg/ml, tween 80 0.1%, glycine 0.15 M, manitol 7% and sucrose 60 mM in sodium-potassium phosphate buffer 25 mM pH 7.5 was the most stable formulation ($P<0.05$). The result of biological test of ELAT showed that bioactivity of more than 93% meets the requirement set by British Pharmacopoeia. The amount of moisture measured in this formulation was less than 3%.
Conclusion
It was concluded that this formulation could be introduced as a candidate for the formulation of anti-Rh D in a lyophilized dosage form.

Keyword: Anti-Rh D IgG, Bioactivity, Formulation, Lyophilization, Protein stability

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Introduction

Anti-Rh D immunoglobulin G (IgG) preparations have been used successfully to prevent the rhesus sensitization of Rh-negative following exposure to rhesus-positive red blood cells and thus to prevent Anti-Rh D related diseases such as hemolytic disease of the newborn (1). Many protein preparations, in particular, immunoglobulins are unstable in very dilute or highly concentrated solutions and tend to form aggregates and particulates in these solutions. This instability is often increased when the protein preparation is stored for long time, or shipped (2). It is known that the formation of aggregates (oligomers and polymers) in intravenous IgG preparations (IgGIVs) can activate the complement system, which is associated with the risk of anaphylactic reactions. Furthermore, the presence of dimmers in the IgGIVs is correlated with arterial pressure drops *in vivo* (3). Therefore, it is important to dry them in order to prevent degradation. But drying always causes some loss of activity or other damage. Lyophilization, also called freeze-drying, is a method of drying without using increased temperatures that significantly reduces such damage. Nevertheless, the lyophilization process induces conformational instability in many proteins such as immunoglobulins. Then, the stabilization of lyophilised immunoglobulin requires the addition of compounds in order not only to obtain undenaturated Ig compositions suitable for therapeutically use, but also Ig compositions with increased storage stability (4). Recently, numerous studies have been conducted in the area of protein lyophilization technology, and instability/stabilization during lyophilization and long-term storage (5). The effect of different parameters on IgG stability during lyophilization was also determined in these studies. Formulation with various excipients, such as sucrose and trehalose, suitable buffer and species processing conditions has proven to be effective in the stabilization of freeze-dried immunoglobulins (6- 8).

The aims of this study were to formulate the anti-Rh D IgG as a lyophilized preparation using various buffers and stabilizing agent also, to optimize the lyophilization processes using accelerated method.

Materials and Methods

Anti-D Immunoglobulin preparation

Liquid preparation was obtained from one batch of anti-Rh D, purified at Avicenna Research Institute, Mashhad University of Medical Sciences, using ion exchange chromatography as described previously (9). Briefly, the method includes adsorption of plasma proteins (from Rh-negative pregnant women containing a high titer of anti-Rh D) onto DEAE-sephadex A50 except anti-Rh D IgG, washing of the gel and elution of the anti-Rh D IgG with 25 mM phosphate buffer pH 7.5. The eluted anti-Rh D was then concentrated by an ultra filtration system (Minitan system/Millipore).

Optimizing the physical stability of lyophilized anti-D using different stabilizers

To study the effect of media composition on the stability of anti-Rh D, different stabilizers were added to the above anti-Rh D solution as shown in Table 1. One ml of these liquid anti-Rh D formulations were freezed at -20 °C for 1-3 hrs (to ensure complete freezing of sample) and then lyophilized (condenser temperature: -52 °C, pressure 0.041 mBAR) (Missouri/Labconco). The effect of various formulations on the stability of anti-Rh D was evaluated using accelerated stability method as described by Fernandez and Lundblad (10). This method briefly consists of the measurement of the transmittance of a sample before and after incubation at 57 ºC for 4 hrs. The change in the degree of opalescence was measured in percent age, at T580 nm by the following formula:

$$\%\Delta T = (T_0h - T_4h / T_0h) \times 100$$

where T_0 h is the transmittance at the beginning of the experiment and T_4h is the transmittance after 4 hrs.

Then, samples were reconstituted with distilled water and the amount of changes of transmittance at 580 nm was measured. The most stable formulation was selected (O formula) for further studies. To improve the

stability, this formulation was dialyzed against sodium-potassium phosphate buffer 25 mM pH 7.5 and the amount of changes of transmittance at 580 nm were remeasured. Furthermore, different concentrations of sucrose in the presence of sodium-potassium phosphate buffer 25 mM pH 7.5 were added as lyoprotectant, according to Table 2.

Table 1. Components of different formulations prepared for lyophilization of Anti-D preparations.

Formulation	Stabilizing agent in sodium acetate (0.01M pH 5.5)
A	Whithout adjuant
B	Tween 80 0.1%, Gly 0.15 M
C	Tween 80 0.01% , Gly 0.15 M
D	Tween 80 0.1% , Gly 0.3 M
E	Tween 80 0.01%, Gly 0.3 M
F	Tween 80 0.1%, Manitol 4%
G	Tween 80 0.1%, Manitol 7%
H	Tween 80 0.01%, Manitol 4%
I	Tween 80 0.01%, Manitol 7%
J	Gly 0.15 M, Manitol 4%
K	Gly 0.15 M, Manitol 7%
L	Gly 0.3 M, Manitol 4%
M	Gly 0.3 M, Manitol 7%
N	Tween 80 0.1%, Gly 0.15 M, Manitol 4%
O	Tween 80 0.1%, Gly 0.15 M, Manitol 7%
P	Tween 80 0.01%, Gly 0.15 M, Manitol 4%
Q	Tween 80 0.01%, Gly 0.15 M, Manitol 7%
R	Tween 80 0.1%, Gly 0.3 M, Manitol 4%
S	Tween 80 0.1%, Gly 0.3 M, Manitol 7%
T	Tween 80 0.01%, Gly 0.3 M, Manitol 4%
U	Tween 80 0.01%, Gly 0.3 M, Manitol 7%

Table 2. Formulations containing different concentrations of sucrose.

Stabilizing agents in sodium-potassium phosphate buffer (0.025 M pH 7.5) in the presence of different concentrations of sucrose (mM)
Tween 80 0.1%, Gly 0.15 M, Manitol 7%, Sucrose 30 mM
Tween 80 0.1% , Gly 0.15 M, Manitol 7%, Sucrose 60 mM
Tween 80 0.1%, Gly 0.15 M , Manitol 7%, Sucrose 90 mM

At this stage, the effects of stabilizing agents on the stability of anti-D preparations were also monitored by the accelerated test described above.

Evaluation of the biological activity of different formulations of Anti-D

The biological activity of anti-Rh D was quantified by an enzyme-linked antiglobulin test (ELAT) (11).

This method briefly consists of using OR_1R_2 blood cells washed and resuspended to 10% w/v concentration. The Sample was added to 100 µl aliquot of erythrocyte suspension, 100 µl of serial dilutions of Anti-Rh D preparations and the standard Anti-Rh D (containing 10 µg/ml Anti-Rh D) and incubated at 37 °C for 1 hr. After incubation, alkaline phosphates conjugated goat anti-human IgG was added. The enzyme activity, which is directly proportional to the amount of anti-Rh D antibodies bound in the first step, was determined with a specific substrate giving a colored product. The absorbance values were measured at 405 nm.

Assay of residual moisture level

The contents of residual moisture were tested by Gravimetric Method (Loss on Drying) (12). The sample was dried in a 103 °C to 110 °C oven for about 1 hr and allowed to cool down to room temperature in desiccators. It is then weighed, and heated again for about 30 mins. The sample was left to cool down and weighed for the second time. The procedure repeated until a constant weight was reached. The water weight was determined by measuring the weight of soil sample, before and after drying. The water weight was the difference in the weights of wet and dry samples.

Statistical analysis

Statistical treatment of the results was performed by one way ANOVA test.

Results

Evaluation of different stabilizing agent on the stability of Anti-Rh D IgG

Anti-D was formulated in different stabilizers (Table 1) and tested for their effect on the stability of Anti-Rh D immunoglobulin.

The results of stability test indicated that the O formulations which contains glycine 0.15 M, tween 80 0.1% and manitol 7% had a better stability effect compared to other formulations (Figure 1).

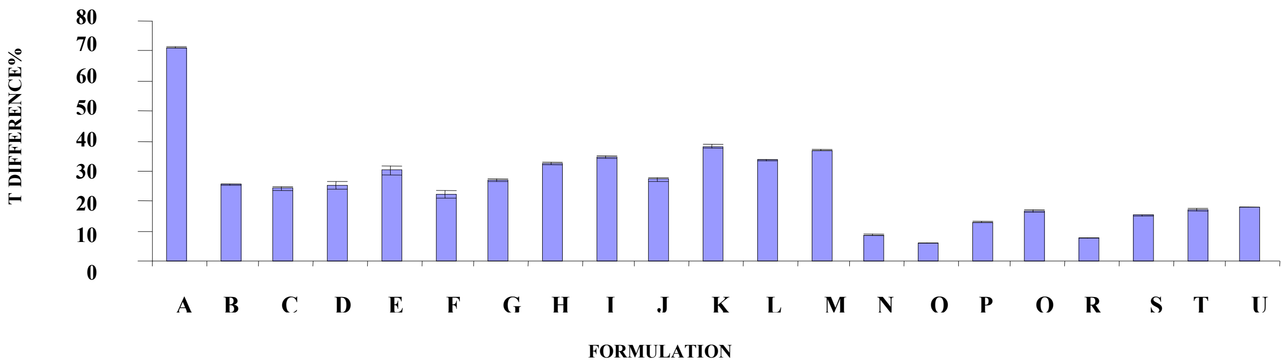

Figure 1. Percentage of transmittance difference at the beginning and end of the accelerated test in different formulations used for lyophilization of Anti-Rh D Mean±SD (n=3).

According to the results obtained for different formulations, the one which gave the best stability results (O formulation) with T % difference of 5.9±0.1 was dialyzed against sodium-potassium phosphate buffer 25 mM pH 7.5 and its stability was tested based on the mentioned test. The results showed that the percentage of transmittance difference was reduced to 3.9±0.2. Furthermore, to increase stability, different concentrations of sucrose were added to this formulation. Figure 2 shows the stability results using accelerated method.

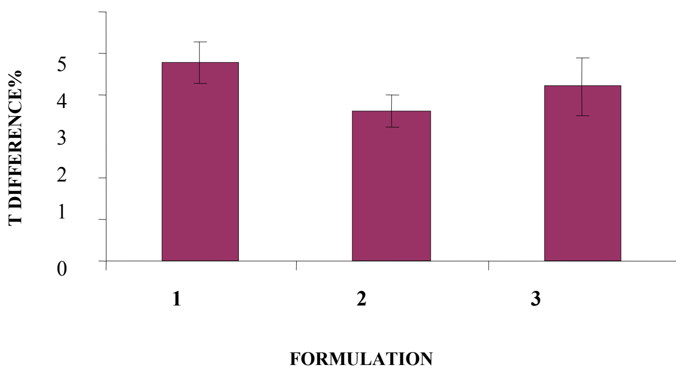

Figure 2. Percentage of transmittance difference at the beginning and end of the accelerated test for formulations O with different concentration of sucrose (formulation 1:30 mM, formulation 2:60 mM and formulation 3: 90 mM sucrose) Mean±SD (n=3).

The results also, demonstrated the highest T% difference of these formulations, before and after lyophilization as 4.8%, which was less than the maximum allowed (5%) (10). However, formulation 2 containing 60 mM sucrose had the least T% difference.

Evaluation of biological activity of the formulation by ELAT test

The immunological activity of formulation containing glycin 0.15 M, tween 80 0.1%, manitol 7% in sodium-potassium phosphate buffer 25 mM pH 7.5 with different concentrations of sucrose was determined by ELAT test. Standard curve ELAT test. The result is shown in Figure 3 (In standard cure ELAT test, R^2=0.9979). Formulation containing sucrose 60 mM had more than 93% biological activity. According to British Pharmacopoeia, the biological activity should not be, less than 90% and more than 11% (13).

Determination of moisture content of formulation

For most dried pharmaceuticals products, the level of residual moisture should be low, usually between 1.0 percent to 3.0 percent. The amount of moisture was measured in the

formulations containing different concentrations of sucrose. As the result in Table 3, shows there is no significant difference in the level of moisture for different studied formulations.

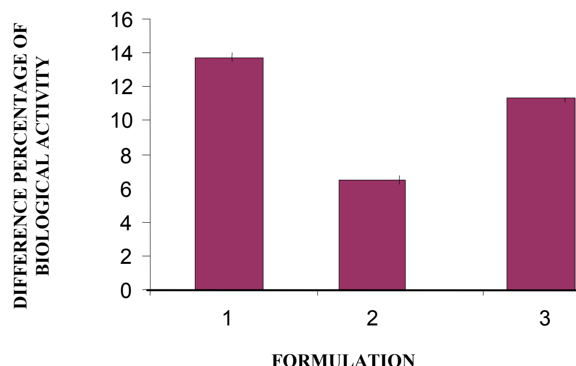

Figure 3. Result of ELAT test for formulation with different concentrations of sucrose (formulation 1:30 mM, formulation 2:60 mM and formulation 3:90mM sucrosr) Mean±SD (n=3).

Table 3. The amount of moisture in the different formulations containing sucrose.

Different concentrations of sucrose(mM)	The amount of moisture (%)
30	1.8
60	1.3
90	1.7

Discussion

The aims of this study were to formulate the anti-Rh D IgG as a lyophilized dosage form for intravenous use and to optimize the lyophilization process, which depends on the multiple parameters, such as, surface area, thickness and eutectic point of the sample, temperature of the condenser and vacuum pressure. In lyophylization, the conditions with highest area and lowest thickness would be optimum, but for the sample with low volume (a few ml) the influence of these factors can be neglected. In our study, as the volume of the sample was 1ml, these factors were not taken into consideration.

Usually, during lyophilization process the condenser temperature should be at least 10 to 15 °C lower than the eutectic point of the sample. The eutectic temperature of the biological solution is usually between -10 to -25 °C. As the condenser temperature in the current study was -52 °C, this condition met the optimum requirement.

The vacuum pressure in the condenser conducts the water vapour flow to the condenser recipient. Suggested level of vacuum for optimum lyophylization, is regularly lower than 133×10^{-3} mBar. In this research, the vacuum pressure was 42×10^{-3} mBar.

The direct effect of protein concentration on stabilization against damage during freeze-drying has been reported by some investigators. The amount of protein in the lyophilized formulation is commonly 2-10 mg/ml, however, Anti-D IgG concentration in our preparations was 5mg/ml (2).

A lyophilized protein formulation may contains a buffer, bulking agent, tonicity modifier, buffer, surfactant, cryoprotectant and lyoprotectant (14- 16). Since proteins are very potent, small quantities are required in the pharmaceutical product. When solid concentrations of a formulation reach <2%, the resulting cake may have poor structural qualities and leave the container during the drying process. The addition of bulking agents such as manitol and dextran, strengthen cake structure. Manitol is commonly used as bulking agent with concentration of 5-10% when the protein concentration is 5 mg/ml (2). Manitol in addition to being a bulking agent can also adjust tonicity in the right concentration. In this study we used 4% and 7% monitol concentrations.

The term "buffer" encompasses those agents which maintain the solution pH in an acceptable range prior to lyophilization and may include succinate (sodium or potassium), histidine, phosphate (sodium or potassium), Tris (tris (hydroxymethyl) aminomethane), diethanolamine, citrate (sodium) and the like (7). In this Research, first we used sodium-acetate buffer (10 mM pH 5.5) and then selected samples were lyophilized in sodium potassium phosphate buffer (25 mM pH 7.5).

Cryoprotectant generally includes agents which provide stability to protein against freezing-induced stresses, presumably by being preferentially excluded from the protein

surface. Examples are polymers such as dextran and polyethylene glycol, polyol like manitol, sugars such as sucrose, glucose, trehalose, and lactose; surfactants such as polysorbates; and amino acids such as glycine, arginine, and serine. We used glysin in concentrations of 0.15 M and 0.3 M, manitol 4% and 7% as cryoprotective.

The term lyoprotectant includes agents that provide stability to the protein during the drying or dehydration process (primary and secondary drying cycles), presumably by providing an amorphous glassy matrix and by binding with the protein through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to maintain the protein conformation, minimize protein degradation during the lyophilization cycle and improve the long-term product stability. Examples include polyols or sugars such as sucrose and trehalose. Carbohydrates such as trehalose sucrose often used as lyoprotectant in concentration of 1-5% w/v (2). In the current study, sucrose was used in concentrations of 30, 60 and 90 mM.

Adsorption of surfactant between surfaces causes protein stability by minimizing interfacial denaturation. Polysorbat 20 and 80 in concentrations of 0.01%- 0.1 w/v are often used as surfactant (2, 8). In this study, we used polysorbate 80 (0.01%) as a surfactant.

Residual moisture has been the term used to describe the low level of surface water, usually from less than 1% to 5%, remaining in a freeze-dried biological product, after the bulk of the aqueous solvent has been removed during the freeze-drying process. The amount of moisture in the best formulation was less than 3%.

The stabilizers suitable for lyophilized forms of anti-Rh D IgG could be different from liquid anti-Rh D IgG compositions (6, 17).

As has been shown in this article, the formulation which contains anti-Rh D 5 mg/ml, glycine 0.15 M, tween 80 0.1%, manitol 7% and sucrose 20 mg/ml as stabilizing agent in sodium-potassium phosphate buffer (pH 7.5 25 mM) is suitable for the formulation of anti-Rh D IgG as lyophitized dosage form. Although the best buffer system for liquid formulation of anti-Rh D IgG was determined to be acetate buffer (pH 5.5, 10 mM) and glycine 0.3 M, however, polysorbate 80 0. 1% and manitol 7% were used to improve the stability of anti-D in this buffer (18).

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