The Influence of Sucrose, Dextran, and Hydroxypropyl-β-cyclodextrin as Lyoprotectants for a Freeze-Dried Mouse IgG_{2a} Monoclonal Antibody (MN12)

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The influence of lyophilization on the stability of a monoclonal antibody (MN12) was investigated. MN12 was freeze-dried in different formulations [without lyoprotectant or in the presence of sucrose, dextran, or hydroxypropyl-β-cyclodextrin (HPβCD)] and under varying conditions (with or without secondary drying). Subsequently, the monoclonal antibody was stored for 18 or 32 days at various temperatures (4, 37, or 56°C). For comparison, solutions of MN12 were stored under the same conditions. Regardless of the lyoprotectant used, precipitation and a concomitant reduction of the antigen-binding capacity by about 10% were observed upon reconstitution of lyophilized MN12. HPβCD proved to be the most effective stabilizer to prevent degradation of lyophilized MN12 during storage. Compared with MN12 solutions, HPβCD-containing lyophilized MN12 cakes were more resistant to heat-induced charge alterations and loss of antigen-binding capacity.

KEY WORDS: monoclonal antibody; lyophilization; freeze-drying; stability; formulation; cyclodextrin.

INTRODUCTION

Monoclonal antibodies (MAbs)[5] belong to the new class of protein pharmaceuticals being introduced through recent advances in biotechnology. Proteins pose novel formulation problems, because of the limited stability of many of these compounds in aqueous solution (1–4). Protein instability is caused by both chemical degradation reactions and physical processes (1,3–5).

Although production, purification, and characterization of MAbs have been described extensively, little information is available on the stability of MAbs upon storage (2,6–9). Previous experiments have shown that two purified MAbs (MN12 and WT31) are susceptible to degradation when stored at elevated temperatures or extreme pH values in aqueous solution (2).

Lyophilization, or freeze-drying, is commonly used in the manufacture of protein products that are insufficiently stable in aqueous solution (10). For instance, pH-induced and/or temperature-induced hydrolysis and deamidation reactions may be retarded when a protein is stored in a lyophilized state. Besides, lyophilized products are less prone to shear-induced denaturation and precipitation during transport. Freeze-drying process parameters and formulation of a protein can largely determine in-process degradation and stability of the freeze-dried product. Secondary drying has, in particular, an influence on the residual moisture content of the dried cake, which is believed to be a critical parameter for product stability (10–12). In general, lyoprotectants are added to the formulation to stabilize the protein both during freeze-drying process and on storage (3,4,13).

In this study, the effect of freeze-drying on the stability of a mouse $IgG_{2a,\kappa}$ MAb (MN12) was investigated. Various analytical methods, including enzyme-linked immunosorbent assay (ELISA), gel permeation chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing (IEF), were used to determine changes in physicochemical properties of the MAb (2). The influence of the freeze-drying process and storage for 18 days at 56°C on the stability of MN12 lyophilized in the presence of sucrose, dextran (MW 10,000), or hydroxypropyl-β-cyclodextrin (HPβCD) was investigated. The stability of MN12 lyophilized with HPβCD, with or without secondary drying phase, was also compared with MN12 in solution stored for 32 days at different temperatures (4, 37, 56°C).

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade. Sucrose (Sigma Chemical Company, St. Louis, MO), dextran (Pfeiffer & Langen, Dormagen, Germany), and HPβCD (a gift from Dr. J. Mesens, Janssen Pharmaceutica, Beerse, Belgium) were used as lyoprotectants. The hybridoma cell line MN12H2 was kindly provided by Dr. J. T. Poolman (Laboratory for Bacterial Vaccines, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). This cell line produces a mouse $IgG_{2a,\kappa}$ MAb (MN12) with a molecular mass of about 150 kDa and an isoelectric point pattern from 7.8 to 8.5, which is directed against the class 1 outer membrane protein of meningococcal strain H44/76, subtype P1.16 (2). *In vitro* cultivation of the hybridoma cell line and purification of the MAb are described elsewhere (14). Purified MN12 was stored in aliquots at −70°C. Before use it was thawed and extensively dialyzed against 10 m*M* Tris/HCl, pH 7.4 (Tris), at 4°C.

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[5] Abbreviations used: MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPβCD, hydroxypropyl-β-cyclodextrin; Tris, 10 m*M* Tris/HCl, pH 7.4; P, lyophilized including freezing and primary drying phase; PS, lyophilized including freezing, primary, and secondary drying phase; SOLV, in solution.

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Lyophilization and Sample Treatment

MN12 solutions consisted of 1.0 mg/ml purified MN12 in Tris as such or with 5% (w/v) lyoprotectant. The solutions were filtered through a filter with 0.2-μm pores (Minisart NML, Sartorius GmbH, Göttingen, Germany) and filled in aliquots of 0.5 ml into 3-ml sterile glass vials (diameter, 15 mm; length, 36 mm; Müller & Müller, Holzminden, Germany). Sterile bromobutyl stoppers (13 mm, Helvoet Pharma, Alken, Belgium) were partly inserted into the vials and the vial contents were kept for 30 min on a freeze-dryer (Leybold Heraeus GT4, Cologne, Germany) shelf, which had been precooled to −40°C. Primary drying was performed by keeping the samples for 48 hr at a vacuum of 8 Pa, a condenser temperature of −60°C, and a shelf temperature of −38°C. Secondary drying was carried out by elevating the shelf temperature at a rate of 10°C/hr to a maximum of +20°C during variable periods of time (see below); this was accompanied by a reduction in chamber pressure to 5 Pa. Lyophilization was terminated by stoppering the vials under vacuum. Before storage, aluminium seals were crimped on the vials.

Lyophilized samples were reconstituted with 0.5 ml distilled water. Next 1 μl of a 10% (w/v) sodium azide solution was added as conservant. The samples were centrifuged for 10 min at 10,000*g* to remove precipitates, if any. The clear supernatants were kept at 4°C prior to analysis.

Experiment A. In one freeze-drying cycle, MN12 was lyophilized with 5% (w/v) sucrose, dextran, or HPβCD. Secondary drying either was not performed (P) or lasted 8 hr (PS). After lyophilization, part of the samples was immediately reconstituted (t = 0); the other vials were stored for 18 days at 56°C.

Experiment B. In one freeze-drying cycle, MN12 was lyophilized with 5% (w/v) HPβCD. Secondary drying either was not performed (P) or lasted 18 hr (PS). After lyophilization, a number of samples was immediately reconstituted (t = 0); the other lyophilized samples and the MN12 samples in solution (SOLV) were stored for 32 days at 4, 37, or 56°C. For each experimental data point, samples were prepared in triplicate. A freshly prepared solution of 1.0 mg/ml MN12 in Tris + 0.02% (w/v) sodium azide was used as reference.

Analytical Methods

Residual moisture contents were determined by Karl-Fischer titration, according to document number 93N-RV-02 of the Foundation for the Advancement of Public Health and Environmental Protection (Bilthoven, The Netherlands). After the freeze-dried material had been dissolved under vacuum in Coulomat A (Riedel-de Häen AG, Seelze, Germany), the amount of water was measured using a CA-05 moisture analyzer (Mitsubishi Chemical Industries Ltd., Tokyo).

Antigen-binding capacity was determined by an antigen-specific ELISA as described elsewhere (2). Recoveries of antigen-binding capacity per sample volume are expressed as percentage of that of the reference. Coefficients of variation of three repeated ELISAs were typically less than 10%.

Gel permeation chromatography was performed on a prepacked Superose HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden) with detection at 280 nm as described previously (15).

SDS-PAGE was carried out under reducing and nonreducing conditions with a PhastSystem and PhastGel Homogeneous 12.5 gels (Pharmacia). Low molecular weight standards (Cat. No. 161-0304, BioRad Laboratories, Richmond, CA) were used to determine the apparent molecular weights.

IEF was performed with the same system using PhastGel IEF 3-9 gels (Pharmacia). The samples were routinely applied at the anodic side of the gel. The pH gradient over the gels was monitored with an IEF calibration kit (Cat. No. 17-0471-01, Pharmacia).

SDS-PAGE, IEF, and silver staining were carried out according to the manufacturer's instructions.

RESULTS

Experiment A

After reconstitution of lyophilized samples, all solutions were turbid. The residual moisture content of the samples immediately after lyophilization did not vary substantially between the different lyoprotectants used. The MN12 samples contained 1–2% (w/w) and 3–4% (w/w) residual moisture, respectively, with or without secondary drying. Samples that were reconstituted immediately upon lyophilization contained about 90% of the antigen-binding capacity of nontreated MN12, as shown in Fig. 1. Addition of lyoprotectants had no measurable effect on the in-process loss of MN12 during freeze-drying. In contrast, the additives had a dramatic influence on antibody stability during storage. When no lyoprotectant was added or in the presence of sucrose, nearly no antibody was detected by ELISA in the lyophilized samples stored for 18 days at 56°C (see Fig. 1). A moderate recovery of about 30% was obtained when dextran was added. HPβCD was the most effective stabilizer for MN12 under the storage conditions used: the antigen-binding recovery was approximately 70% (Fig. 1). Secondary drying did not have a substantial effect on recovery of antigen-binding activity.

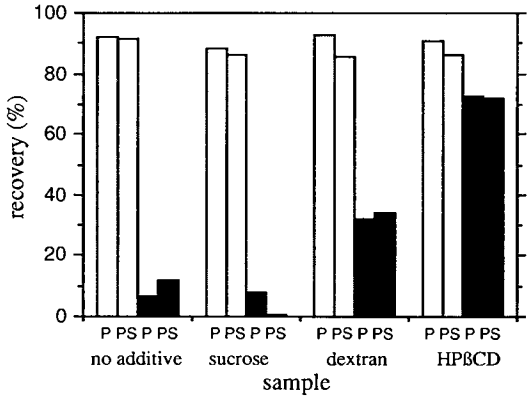

Fig. 1. Recoveries of antigen-binding capacity of MN12 in Tris as such (no additive) or with 5% (w/v) lyoprotectant (sucrose, dextran, or HPβCD) lyophilized without (P) or with (PS) secondary drying, immediately after lyophilization (□) or after incubation for 18 days at 56°C (■). The bars represent mean recoveries according to two or three repeated ELISAs.

Aggregation and/or fragmentation was observed with gel permeation chromatography after storage of MN12 samples lyophilized without lyoprotectant and in the presence of either sucrose or dextran. The occurrence of soluble aggregates in these samples was confirmed by SDS-PAGE. IEF revealed an acidic shift in the isoelectric point (pI) of the dextran-containing sample. IEF patterns were no longer observed after storage of MN12 lyophilized as such or with sucrose. Apart from an additional 100-kD band of low intensity in SDS-PAGE under reducing conditions, no structural changes were observed after storage of MN12 freeze-dried with HPβCD (not shown).

Experiment B

On the basis of the above-mentioned results, HPβCD was selected to investigate the effect of freeze-drying process parameters and storage conditions on the stability of lyophilized MN12. Further, a comparison was made between the stability of lyophilized MN12 and that of MN12 in solution.

Table I shows the residual moisture contents. Immediately after freeze-drying, the MN12 samples contained about 1% (w/w) and 3% (w/w) residual moisture, respectively, with or without secondary drying. Storage of the vials resulted in an increase in water content, which was more pronounced at elevated incubation temperatures.

The MN12 solutions remained clear when stored for 32 days at 4 and 37°C but contained precipitated material when stored at 56°C. All lyophilized samples were slightly turbid after reconstitution. The freeze-drying process caused a 10% loss of antigen-binding capacity of MN12 (see Fig. 2). After storage at 4 and 37°C MN12 solutions and lyophilized samples showed comparable recoveries of circa 90 and 80%, respectively. However, incubation of MN12 solutions at 56°C resulted in a virtually complete loss of antigen-binding capacity, whereas the lyophilized samples still contained about 70% antibody activity (Fig. 2).

Gel permeation chromatography showed MN12 recoveries comparable to those found by ELISA (data not shown). The retention time of the monomeric IgG peaks of the MN12 solutions and the lyophilized and reconstituted MN12 samples after storage was not significantly different from that of the reference. Lyophilized samples that were not stored contained no detectable levels of soluble aggregates or fragments. In the MN12 solutions stored at 37°C, soluble aggregates were detected (see Table II). The lyophilized samples only showed aggregation when stored at 56°C. Fragmentation was observed in samples lyophilized without secondary drying after storage at 56°C.

Table I. Effect of Storage Temperature on the Residual Moisture Content of Lyophilized MN12[a]

Storage time (days)	Temperature (°C)	P[b]	PS[b]
0	—	2.8 ± 0.1	1.1 ± 0.1
32	4	3.3 ± 0.2	1.5 ± 0.1
32	37	4.0 ± 0.1	2.5 ± 0.2
32	56	4.1 ± 0.1	3.0 ± 0.1

[a] The original solutions contained 1.0 mg/ml MN12 in Tris buffer with 5% (w/v) HPβCD. P, MN12 lyophilized without secondary drying; PS, MN12 lyophilized with secondary drying.

[b] Expressed as percentage (mean ± standard deviation; $n = 3$) of the total weight of the lyophilized cake.

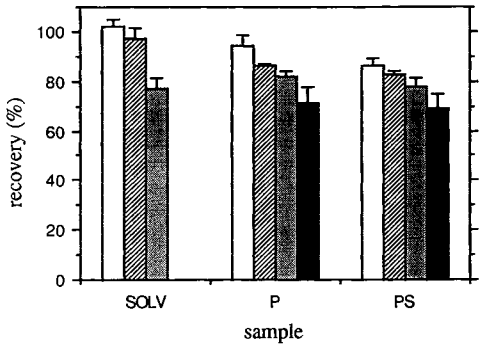

Fig. 2. Recoveries of antigen-binding capacity of MN12 in Tris with 5% (w/v) HPβCD as solution (SOLV) or lyophilized without (P) or with (PS) secondary drying, at $t = 0$ (□) or after incubation for 32 days at 4°C (▨), 37°C (▩), or 56°C (■). The bars represent mean recovery + standard deviation of the triplicate samples according to ELISA.

Figure 3 shows the results of SDS-PAGE. MN12 samples lyophilized without secondary drying exhibited SDS-PAGE patterns (not shown) similar to those of MN12 samples lyophilized with secondary drying (Fig. 3, lanes 5–7). Most samples had SDS-PAGE patterns similar to those of the reference: a 50-kD (heavy chain) and a 27-kD (light chain) band for reduced samples and a 150-kD band with a shadow band of 137 kD for nonreduced MN12. The origin of the 137-kD band is unclear but may be due to sample treatment. Under reducing conditions an additional band of 100 kD was detected for samples lyophilized with HPβCD and stored for 32 days at 56°C (Fig. 3, lane 7a). For the MN12 solutions no signal was detectable after storage at 56°C because the protein content was too low (Fig. 3, lane 4). The reference, the samples stored in solution at 4°C, and the lyophilized samples were focused as six separate bands ranging from pH 7.8 to pH 8.5 in IEF (Fig. 4, lanes 1, 2, 5–7). MN12 samples lyophilized without secondary drying exhib-

Table II. Effect of Storage Temperature on the Aggregation State of MN12 Stored in Solution and in a Lyophilized State as Determined by Gel Permeation Chromatography[a]

Storage time (days)	Temperature (°C)	SOLV[b]	P[b]	PS[b]
0	—	0/0/0	0/0/0	0/0/0
32	4	1.1/0/0	0/0/0	0/0/0
32	37	3.8/5.0/0	0/0/0	0/1.1/0
32	56	ND[c]	0/1.9/1.6[d]	0/8.0/3.7

[a] The solutions contained 1.0 mg/ml MN12 in Tris buffer with 5% (w/v) HPβCD. SOLV, MN12 solution; P, MN12 lyophilized without secondary drying; PS, MN12 lyophilized with secondary drying.

[b] Aggregation is expressed as percentage of the total peak area for each of the triplicate samples.

[c] Not determined because the signal-to-noise ratio was too low.

[d] Fragments also detected: 0/5.1/4.8%.

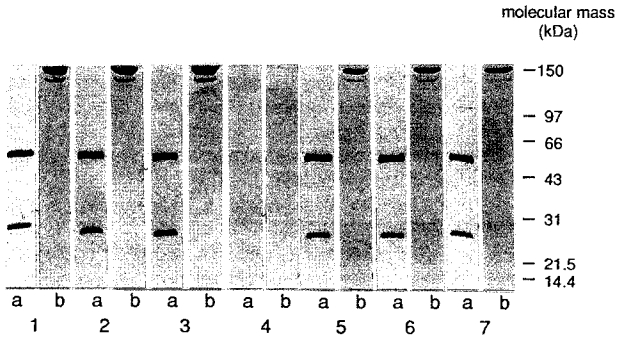

Fig. 3. Silver-stained SDS-PAGE patterns of MN12 in Tris with 5% (w/v) HPβCD as solution (lanes 2–4) or lyophilized with secondary drying (lanes 5–7) after incubation for 32 days at 4°C (lanes 2,5), 37°C (lanes 3,6), or 56°C (lanes 4,7) and reference (lane 1). Electrophoresis was performed under reducing (a) and nonreducing (b) conditions.

ited IEF patterns (not shown) similar to those of MN12 samples lyophilized with secondary drying (Fig. 4, lanes 5–7). For MN12 solutions that were incubated at 37°C, an acidic shift was observed (Fig. 4, lane 3). The protein content of the MN12 solutions stored at 56°C was too low to give a detectable IEF pattern (Fig. 4, lane 4).

DISCUSSION

Both the freeze-drying process and reconstitution can have damaging effects on protein structure and function. Aggregation of proteins during the freeze-drying process, often leading to insoluble protein, is a common observation. Moreover, degradation may occur during storage of the product (13). For instance, increased levels of aggregation of growth hormones and prolactins and inactivation of enzymes have been reported (3,10,13,16–18). Some adverse effects can be reduced by additives. For instance, turbidity of lyophilized and reconstituted interleukin-2 solutions can be prevented by the addition of HPβCD (19).

In the present study, the freeze-drying process of MN12

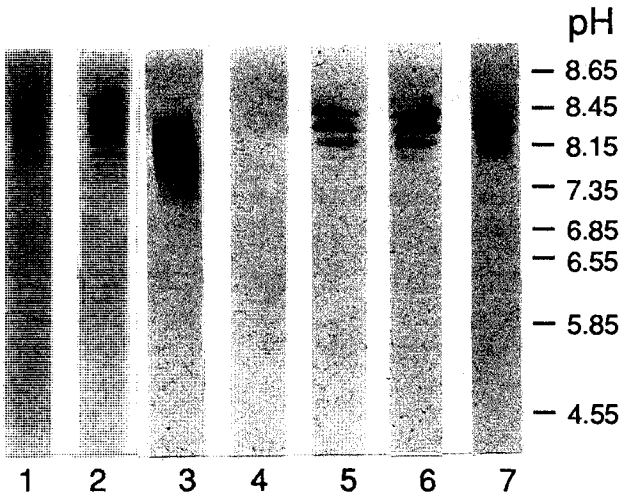

Fig. 4. Silver-stained IEF patterns of MN12 in Tris with 5% (w/v) HPβCD as solution (lanes 2–4) or lyophilized with secondary drying (lanes 5–7) after incubation for 32 days at 4°C (lanes 2,5), 37°C (lanes 3,6), or 56°C (lanes 4,7) and reference (lane 1).

caused a slight reduction of the antigen-binding capacity by about 10%, regardless of the presence of different lyoprotectants. Upon reconstitution the MN12 solutions were turbid. In the MN12 samples reconstituted immediately after lyophilization, no structural changes were detected by gel permeation chromatography, SDS-PAGE, and IEF. When lyophilized MN12 was stored for 18 days at 56°C, however, the antigen-binding capacity was almost completely lost if no lyoprotectant was added. Thus, MN12 formed a good model to study the influence of process parameters and storage conditions on the stability of a lyophilized MAb. Human growth factor and ribonuclease A are proteins which can be freeze-dried without significant loss of activity. For these two proteins, excipients have also been reported to be crucial for the prevention of loss of activity during storage of the dry solid (3,13).

In aqueous solution sugars are known to stabilize native structure by preferential hydration of proteins and to accomplish cryoprotection during freezing (17). It is questionable if this mechanism accounts for lyoprotection, because in the lyophilized product most of the water has been removed. In the present study, sucrose and dextran were less effective stabilizers than HPβCD (Fig. 1). This is probably not caused by differences in the residual moisture content, because the measured water content of the lyophilized MN12 samples was essentially the same for the different lyoprotectants. Apparently, apart from preferential hydration, additional protection is needed during storage, Recently, in a review by Pikal (10,13) concerning freeze-drying process parameters and formulation of proteins, two general rules were presented: an additive (i) should have a high collapse temperature and (ii) should remain at least partially amorphous. HPβCD meets both requirements: it has a relatively high collapse temperature, which is probably close to the value of −9°C for α-cyclodextrin (20), and it is an intrinsically amorphous compound which has a good water solubility and a good complexation power (21,22). These properties of HPβCD could contribute to its stabilizing activity for lyophilized MN12.

HPβCD can be regarded as a cylinder with a hydrophillic outer surface and a hydrophobic internal cavity (23). The hydrophilic outside of the lyoprotectant gives the protein–HPβCD complex a higher degree of hydration and, therefore, promotes water structure. The most important requirement for the formation of a stable drug–HPβCD inclusion complex is the tight fitting, wholly or at least partially, of the guest molecule within the cyclodextrin cavity. The hydrophobic cavity of HPβCD may enclose amino acid side chains of the MAb MN12, thereby protecting them from degradation reactions. HPβCD has been reported to protect drugs against oxidation and gastric acid degradation (23).

Deamidation reactions probably cause the acidic shift in the pI pattern of MN12 (2). Deamidation of asparagine and glutamine residues is a widely prevalent degradation reaction of proteins in solution (1,9). The results of IEF indicate that storage in a lyophilized state causes retardation of deamidation reactions (Fig. 4). Apparently, the reduction in antigen-binding capacity of lyophilized MN12 (with about 20% after storage at 56°C; see Fig. 2) involves other reactions than deamidation.

When MN12 lyophilized with HPβCD was stored for 32

days, the residual moisture content of the dried cakes was increased compared to that at $t = 0$. This increase was more pronounced at higher storage temperatures (Table I). It has been reported that water can be absorbed by vial stoppers during storage, washing, or sterilization (13,24,25). Dry solid products tend to absorb moisture from rubber stoppers during storage. This transfer of water may adversely affect the freeze-dried product and is believed to depend largely on storage conditions, especially temperatures above ambient. Although the residual moisture content is considered a critical parameter for product stability, secondary drying did not have much influence on the stability of MN12.

In conclusion, lyophilization in the presence of HPβCD improved the shelf life of the MAb MN12, in particular at elevated temperatures. After removal of the aggregates by filtration, the MAb can be administered parenterally. Detailed toxicological studies have shown that HPβCD is tolerated as a parenteral carrier even at extremely high doses (19,23). MN12 freeze-dried in presence of HPβCD is more resistant to heat-induced charge alterations and loss of antigen-binding capacity, compared with MN12 stored in solution. However, degradation reactions observed at elevated storage temperatures are not necessarily the same as those occurring at lower temperatures, which may be detected after prolonged storage. Therefore, the behavior of MN12 in solution and in a lyophilized state upon long-term storage at 4°C still has to be assessed.

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