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Stabilization of Botulinum Toxin Type A during Lyophilization

MICHAEL C. GOODNOUGH[1] AND ERIC A. JOHNSON[1,2]*

Departments of Food Microbiology and Toxicology[1] and Bacteriology,[2] Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706

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Botulinum toxin for medical use is diluted to very low concentrations (nanograms per milliliter); when it is preserved by lyophilization, considerable loss of activity can occur. In the present study, conditions that gave >90% recovery of the toxicity after lyophilization of solutions containing 20 to 1,000 mouse 50% lethal doses per ml were found. Toxicity was recovered upon drying 0.1 ml of toxin solution when the pH was maintained below 7 and bovine or human serum albumins were used as stabilizers. Various other substances tested with albumin, including glucose, sucrose, trehalose, mannitol, glycine, and cellibiose, did not increase recovery on drying.

Crystalline botulinum toxin type A produced in 1979 by E. J. Schantz (8, 10) is being used medicinally in the treatment of hyperactive muscle movement disorders (1, 4, 8, 11). Treatment involves injection of nanogram quantities (1 ng is equal to 30 mouse 50% lethal doses [30 U]) of the toxin directly into the hyperactive muscles (1, 4, 11). The toxin inhibits the release of acetylcholine from the motor neuron across the synaptic junction, causing a decrease in the hyperactivity of the injected muscles. Botulinum toxin injections have provided profound relief to thousands of individuals who suffer from dystonias and other hyperactive muscle disorders that were previously untreatable by other means (4, 11).

Crystalline type A botulinum toxin is a high-molecular-weight protein aggregate of about 900,000 M_r composed of two or three neurotoxin molecules (~150,000 M_r) noncovalently bound to nontoxic proteins that have an important role in the stability of the toxin unit. High-quality crystalline type A toxin produced from the Hall A strain has a specific toxicity in mice of $\geq 3 \times 10^7$ U/mg, an A_{260}/A_{278} ratio of less than 0.60, and a distinct pattern of banding on gel electrophoresis (8). Currently, for commercial preparation as a drug, a sufficient quantity of toxin is diluted and lyophilized in a solution (pH 7.3) containing sodium chloride and human serum albumin (HSA) as bulking agents. Each vial of the commercial toxin product is reported to contain 100 U of toxin, 0.5 mg of HSA, and 0.9 mg of sodium chloride. HSA is present as an excipient to stabilize the toxin during drying and to prevent the toxin from adhering to the glass walls of the vial. However, during dilution, compounding, dispensing, and lyophilization, a substantial loss of activity may occur because of the fragility of the toxin molecule. Botulinum toxin produced in the United States (Oculinum) has been reported to contain 2.5 U/ng (5, 7, 12), which is considerably less than the toxicity of crystalline toxin (~30 U/ng). Part of this loss in toxicity occurs during lyophilization. In this study we found that conditions including the pH and the use of excipients during the lyophilization procedure affect the recovery of toxicity.

Crystalline botulinum type A toxin was prepared as previously described (1, 8). The procedure used was identical to that used by Schantz for the manufacture of the current commercial product. The toxin preparations used in the present study were crystallized twice and had an A_{260}/A_{278} ratio of 0.53, indicating that there was very little contaminating nucleic acid present (8). The specific toxicity of the toxin dissolved in 50 mM sodium phosphate (pH 6.8) was 28.4 U/ng as calculated by the method of Schantz and Kautter (9). The crystalline toxin suspension was kept at ca. 4.0 mg/ml. For drying experiments, a sample of toxin crystals was diluted 10-fold in 50 mM sodium phosphate buffer (pH 6.8) and held at room temperature for ~3 h with occasional gentle mixing by inversion to dissolve the crystals. This solution was further diluted 1:100 in 30 mM sodium phosphate–0.2% gelatin (pH 6.2) (gel-phosphate), and the toxin titer was approximated by determining the time to death after intravenous injection by the method of Boroff and Fleck (2). The original 1:10 dilution was then diluted to the desired level of toxicity (usually 1,000 U/ml) in a solution containing the stabilizing compounds to be tested (see Table 1 for the compounds and concentrations tested). The pHs of the solutions were adjusted with 1 N NaOH and 1 N HCl. The toxicity of the solutions to be lyophilized were determined by diluting the toxin-excipient combinations in gel-phosphate and injecting 0.5 ml of each dilution intraperitoneally into 18- to 22-g white mice.

For lyophilization, aliquots (usually 0.1 ml) of the toxin solutions were pipetted into 0.5-dram (ca. 1.85-ml), screw-cap glass vials with rubber-lined closures (Fisher Scientific, Pittsburgh, Pa.). The solutions in glass vials were usually instantly frozen in a bath of liquid nitrogen (−200°C) or in some experiments by placing them in a freezer at −20°C or −70°C. The loosely capped vials were then placed into a 150-ml lyophilization flask that had been precooled in liquid nitrogen. The lyophilization flask was partially immersed in liquid nitrogen and connected to a Virtis Freezemobile 12 lyophilizer (Virtis Co., Inc., Gardiner, N.Y.). The liquid nitrogen was maintained in contact with the lyophilization flask until the pressure dropped to approximately 30 millitorrs (ca. 4.0 Pa). The liquid nitrogen jacket was then removed, the flask and contents were allowed to come to room temperature (ca. 3 to 4 h), and the drying procedure was continued for 12 to 18 h. The pressure was maintained at or below 20 millitorrs for the remainder of the cycle. Condensor temperature on the lyophilizer was constant at ca. −60°C. After the drying cycle was completed, the lyophilized toxin vials were removed from the flask, tightly

* Corresponding author.

TABLE 1. Effect of excipients on recovery of toxicity after lyophilization

Excipient(s)[a]	Toxin concn[b]	pH	% Recovery[c]
Sodium phosphate (50 mM)	50, 100, 1,000[d]	5.0, 6.0, 6.8	<10
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)	20	6.4	10
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)–mannitol (100 mM)	20	6.4	20
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)–trehalose (100 mM)	20	6.4	<10
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)–sucrose (100 mM)	20	6.4	10
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)–glucose (100 mM)	20	6.4	10
BSA (5.0 mg/ml)–sodium chloride (9.0 mg/ml)–cellibiose (100 mM)	20	6.4	10
BSA (9.0 mg/ml)	100, 1,000	6.4	88, 75
BSA (9.0 mg/ml)–sodium citrate (50 mM)	100, 1,000	5.0	>90, >90
BSA (9.0 mg/ml)–sodium phosphate (50 mM)	100, 1,000	5.5	>90, >90
BSA (9.0 mg/ml)–sodium phosphate (50 mM)	1,000	7.3	60
HSA (9.0 mg/ml)	100, 1,000	6.4	>90, >90

[a] All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.
[b] Mouse intraperitoneal lethal doses per vial before lyophilization.
[c] (Number of mouse lethal doses after lyophilization/number mouse lethal doses before lyophilization) × 100.
[d] Each toxin concentration was tested at each pH designated.

capped, and held at room temperature for up to 3 days until the assay.

Lyophilized preparations were usually reconstituted in 1.0 ml of distilled water. The use of 0.85% saline as a diluent gave equivalent results. The white cake dissolved immediately and was mixed by gentle inversion of the vials. The resulting solution was transparent and contained no particulates. This solution was titrated by the method used for the prelyophilization solution. The percent recovery (calculated as number of mouse intraperitoneal lethal doses per vial after lyophilization divided by number of mouse intraperitoneal lethal doses per vial before lyophilization × 100) represent averages of trials done in at least duplicate. The variation in independent assays was ca. ±20%, as reported earlier by Schantz and Kautter (9).

We initially determined whether the recovery of active toxin was dependent on the freezing temperature. Freezing at −20 or −70°C resulted in slight losses of activity (75 to 90% recovery) compared with >90% recovery with freezing at −200°C in liquid nitrogen (data not shown). Previous studies in our laboratory also showed no detectable inactivation (≤20%) of type A crystalline toxin (10^4 U/ml) during repeated freezing and thawing at −20°C in any of several buffers, including 50 mM sodium phosphate (pH 6.2 to 6.8), 50 mM sodium succinate (pH 6.0), and 50 mM sodium citrate (pH 5.5) (13). However, freezing in acetate buffer results in irreversible loss of toxicity (10, 13).

The conditions used for lyophilization in this study had a marked effect on the recovery of toxin. The most critical factor that contributed to recovery of active toxin was the omission of sodium chloride from the solution for lyophilization; this omission resulted in recovery of >90% of the starting toxicity. Recovery of toxin was also increased by the addition of HSA or bovine serum albumin (BSA) as a bulking agent (Table 1). Solutions of BSA or HSA at 9.0 mg/ml had an unadjusted pH of 6.4, at which full recovery of activity was obtained. Full recovery of toxin activity was also obtained when the pH was adjusted to 5.0 or 5.5, and a slight loss of 20 to 30% was found at pH 7.0 to 7.3 (Table 1). In most experiments, 0.1 ml of solution was lyophilized, whereas drying 0.5 ml (with a reduction in serum albumin concentration to 1.8 mg/ml to maintain a postlyophilization concentration of 0.9 mg/ml after reconstitution) gave slightly lower recoveries (60 to 80% of the initial toxicity; data not shown), possibly because of supercooling during lyophilization.

The results of this study indicate that full recovery of type A botulinum toxin can be obtained after lyophilization. It has been reported that commercially available type A botulinum toxin (Oculinum) has 2.5 U/ng, indicating that considerable loss of activity occurs during dilution and drying. Alternatively, the crystalline starting material used for Oculinum (toxin batch 79-11) prepared in 1979 could have a much lower specific activity due to degradation during aging. Inactivated toxin could act as a toxoid and lead to antibody formation and patient immunity. In the United States, a number of patients (>20) treated for torticollis have developed antibody-mediated immunity to toxin and become refractory to treatment (4, 6). Our results suggest that the amount of toxoid formed during preparation could be decreased by elimination of NaCl and use of the proper excipients, pH, and drying cycle.

Although HSA is currently used in commercial practice, it may be desirable to use another protein or low-molecular-weight excipients (3) for toxin stabilization, since certain unknown contaminants may be present in human blood proteins. Other proteins, e.g., gelatin, may also contain pyrogens or other substances that could cause adverse reactions upon injection into patients. Further work is being carried out in our laboratory to optimize procedures for production, purification, and stabilization of type A and other serotypes of botulinum toxin.

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