Stability of botulinum neurotoxin type A, devoid of complexing proteins

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Abstract: Botulinum toxin type A is a complex composed of the biologically active neurotoxin, several hemagglutinins and other nontoxic proteins. After intramuscular injection these complexing proteins do not have any therapeutic effect. However, they protect the neurotoxin from harsh environmental conditions, e.g., low intragastral pH after oral ingestion. NT201, a BoNT/A drug product devoid of complexing proteins was tested in real-time and accelerated stability studies. NT201 was found to be stable without refrigeration for 48 months and even not affected by short-term temperature stress up to 60°C, demonstrating that complexing proteins are not required for the stability of BoNT/A preparations.

Keywords: botulinum neurotoxin type A; BoNT/A; neurotoxin; stability; complexing proteins; temperature stress; ICH guideline.

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1 Introduction

Botulinum neurotoxins are produced by *Clostridium botulinum*, a gram-positive anaerobic bacterium, and are the most potent poisons known (Hambleton, 1992). With its widespread therapeutic use in more than 100 indications, comprising neurologic (Benecke et al., 2005; Jankovic and Brin, 1991; Dolly et al., 2009; Roggenkamper et al., 2006; Wohlfarth et al., 2007) as well as aesthetic indications (Carruthers, 2002), botulinum neurotoxin type A is quite unique.

Botulinum neurotoxin type A (BoNT/A) is isolated from *C. botulinum* as a high molecular weight complex of approximately 900 kDa, which is composed of the biologically active 150 kDa neurotoxin as well as several hemagglutinins and other non-toxic proteins of bacterial origin (Schantz and Johnson, 1992). If the neurotoxin is ingested, the complexing proteins confer stability to the 900 kDa complex under the harsh pH conditions during gastric passage (Chen et al., 1998). Although these complexing proteins do not have any therapeutic effect after intramuscular injection, it has been thought that they might be required for sufficient stability of botulinum neurotoxin preparations (Schantz and Johnson, 1992).

While much is known about the mechanism of action of botulinum neurotoxins (Black and Dolly, 1986; Dong et al., 2006; Mahrhold et al., 2006; Schiavo and Montecucco, 1997; Koriazova and Montal, 2003), the stability of botulinum neurotoxins used in pharmaceutical preparations is not completely clear. Sesardic et al. (2003) conducted a stability study with three different lyophilised BoNT/A preparations, including one investigational product without complexing proteins. The long-term stability profile of the preparation without complexing proteins was comparable to an identical formulation of BoNT/A with complexing proteins at storage temperatures of 4°C and 20°C. However, the excipients of the tested non-complexed neurotoxin preparation were significantly different from NT201, the product tested in the study presented here. Furthermore, long-term/real-time stability data for pharmaceutical preparations of botulinum neurotoxin without complexing proteins have not been published so far.

NT201 is a botulinum neurotoxin type A preparation, which, in contrast to other commercially available BoNT/A preparations, only contains the pure 150 kDa neurotoxin devoid of complexing proteins (Eisele, 2010). In this paper, we assess the stability of a complex protein-free BoNT/A drug product (NT201; marketed in the EU as Xeomin®/Bocouture®) in comprehensive real-time and accelerated stability studies.

2 Methods

The neurotoxin content per vial was determined by a validated Enzyme-Linked Immunosorbent Assay (ELISA, in-house method, unpublished) as follows. A rabbit

anti-BoNT/A antibody is immobilised on a 96-well plate to capture neurotoxin molecules, which are then detected by a primary guinea pig anti-BoNT/A antibody, followed by a secondary anti-guinea pig IgG monoclonal antibody conjugated with peroxidase. After addition of the chromogenic substrate o-phenylenediamine dihydrochloride, the absorbance at 490 nm is measured with a plate photometer. The neurotoxin content of the sample is calculated from a calibration function with a BoNT/A in-house reference standard.

The protein content, i.e., the content of the excipient Human Serum Albumin (HSA), was determined by HPLC analysis in accordance with an in-house validated method as follows. The content of 1 vial of NT201 is quantitatively reconstituted in 500 µL eluent (Phosphate Buffered Saline (PBS)) and applied onto a size exclusion HPLC column at a flow rate of 1.0 mL/min at room temperature. A calibration function is established by application of six different HSA concentrations. The absorption of the eluent is measured at 213 nm, and the peak areas are integrated to calculate the amount of protein in each calibrator of the sample. The protein content of the NT201 sample is then calculated from the calibration function.

The sucrose content in the drug product NT201 was determined as follows: sucrose is hydrolysed with β-fructosidase (invertase). In the presence of adenosine-5′-triphosphate (ATP), the resulting product D-glucose is phosphorylated with hexokinase into D-glucose-6-phosphate. In the presence of NADP, the D-glucose-6-phosphate is then oxidised to D-gluconate-6-phosphate by glucose-6-phosphate-dehydrogenase (G6P-DH), resulting in the formation of reduced Nicotine Amide Adenine Dinucleotide Phosphate (NADPH). The amount of NADPH, which is stoichiometrically equivalent to the amount of D-glucose, is quantified by light absorbance at 340 nm. The sucrose content is then calculated from the D-glucose concentration.

Biological activity was determined by a validated mouse LD_{50} assay, according to the monograph "Botulinum toxin type A for injection" of the European Pharmacopoeia, which is also used for release testing of NT 201. Alternatively, the mouse Hemidiaphragm Assay (HDA) was used for determination of biological activity (Göschel et al., 1997).

Proteolytic light chain activity was determined by a non-validated SNAP-25 cleavage assay as follows: a microtiter plate is coated with a 16-mer synthetic peptide resembling the natural substrate SNAP-25. After reductive cleavage of the disulphide bond between heavy and light chain of NT201 drug substance, various amounts of NT201 treated as described are added. The resulting cleavage of the substrate peptide is then detected with a rabbit antibody, which specifically recognises the newly generated C-terminus of the cleaved substrate peptide bound to the plate but not the uncleaved peptide. The amount of bound anti-SNAP-25-antibody, which is proportional to the amount of cleaved substrate peptide, is then detected by a secondary goat anti-rabbit IgG antibody conjugated with peroxidase. After addition of o-phenylendiamine as a chromogenic substrate, the absorbance at 490 nm is measured, and the proteolytic activity is calculated from a calibration function with a NT201 in-house reference standard.

3 Stability testing

Three individual batches of NT201 were each tested at four different storage conditions as defined in the ICH Q1A(R2) guideline (ICH Expert Working Group – International

Conference on Harmonisation of Technical Reuquirements for Registration of Pharmaceuticals for Human Use, 2003) on stability testing of drug products: For the long-term (real-time) testing, samples were stored at 5°C (refrigerator storage) as well as 25°C/60% r.h. (storage at ambient conditions in moderate climate regions) for up to 48 months. An additional long-term stability study at 30°C/65% r.h. (representative for storage at ambient conditions in subtropical regions) was started on three individual batches of NT201 after the initial stability testing programme, and the currently available data cover a period of 18 months. Furthermore, an accelerated study at 40°C/75% r.h. covering a period of 6 months has been completed.

In an additional stress stability study, samples from one batch of NT201 were stored at temperatures ranging from 45°C to 80°C for up to 6 months. The samples were tested with fully validated or compendial analytical methods (except SNAP-25 cleavage assay), which are also used for release testing of NT201.

4 Results

4.1 Long-term (real-time) stability studies of NT201

The results of long-term (real-time) stability studies with three individual batches of NT201 tested at four different storage conditions are shown in Figures 1–4. The long-term (real-time) stability data collected over 48 months at storage conditions of 2–8°C (refrigerator storage) and 25°C/60% r.h. (storage at ambient conditions) are shown in Figures 1 and 2. No significant differences were observed between storage at 2°C and 8°C or storage at ambient conditions (Figures 1 and 2), as all test parameters display a linear course over time. The same consistency of results over the whole storage period is found in the studies conducted at 30°C/65% r.h. and 40°C/75% r.h. (Figures 3 and 4).

Figure 1 Storage of NT201 at 5 ± 3°C over 48 months according to ICH Q1A (R2). Results of 3 different individual batches of NT201. Means as well as minimum and maximum values shown

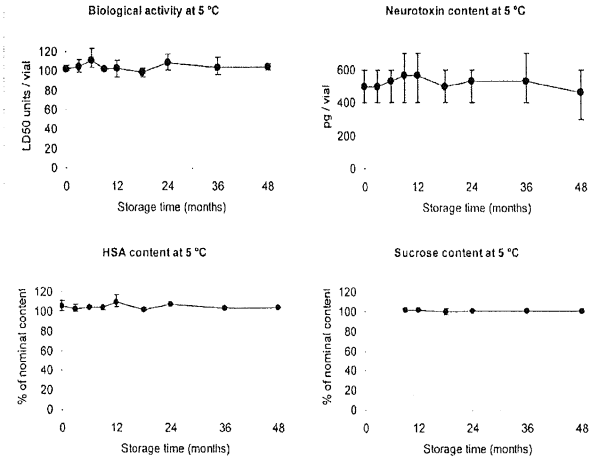


Figure 2 Storage of NT201 at 25 ± 2°C and 60 ± 5% relative humidity according to ICH Q1A (R2) guideline over 48 months. Results of three different individual batches of NT201. Means as well as minimum and maximum values shown. Sucrose content was included in stability testing from 9 months onwards

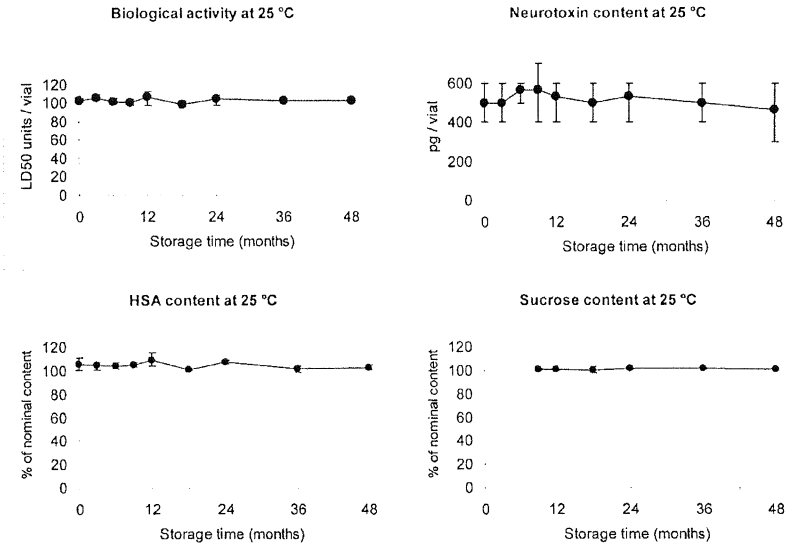


Figure 3 Storage of NT201 at 30 ± 2°C/65 ± 5% relative humidity according to ICH Q1A (R2) guideline over 18 months. Results of three different individual batches of NT201. Means as well as minimum and maximum values shown

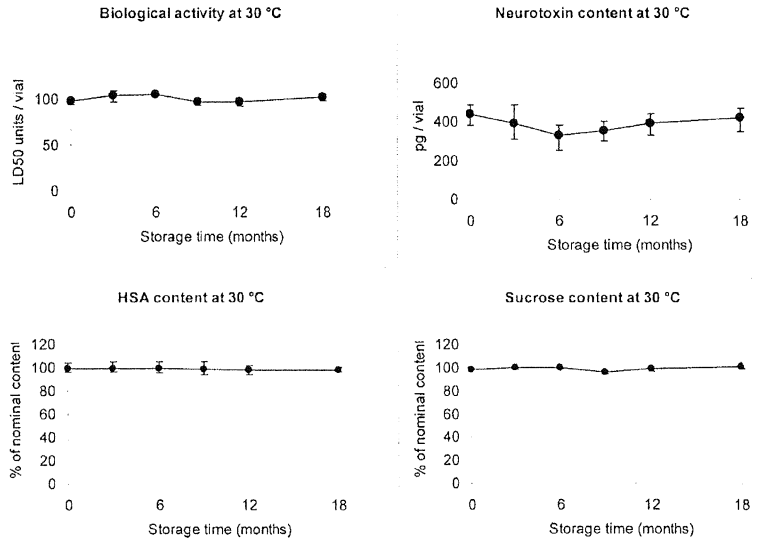
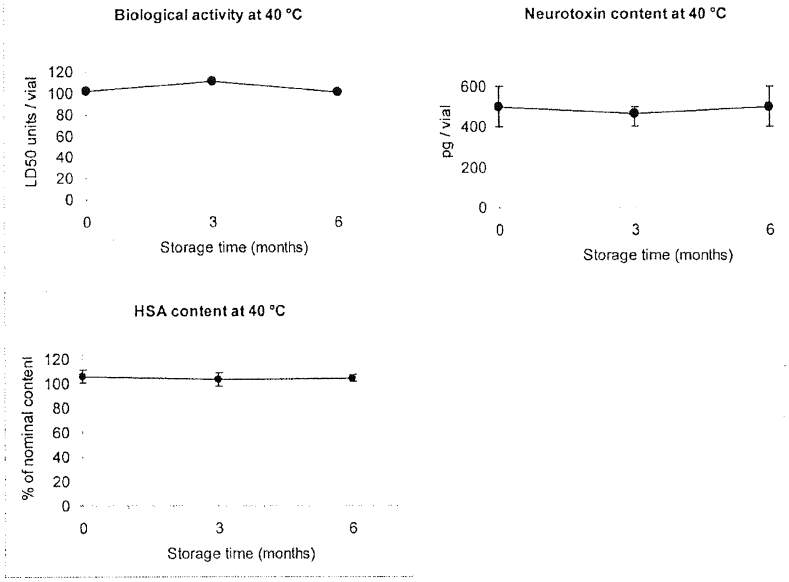


Figure 4 Storage of NT201 at 40 ± 2°C/ 75 ± 5% relative humidity according to ICH Q1A (R2) guideline over 6 months. Results of three different individual batches of NT201. Means as well as minimum and maximum values shown



All parameters are within the predefined specifications, demonstrating that NT201 has an excellent stability profile over at least 48 months, both at refrigerator or room temperature storage.

4.2 Short-term stress stability study

The results of the short-term (stress) stability studies with one batch of NT201 are shown in Figures 5 and 6. At a storage temperature of 60°C over a period of one month, the quality of NT201 remains unaffected (Figure 5). All parameters tested remain well within the shelf-life specifications of the product.

As expected, a complete loss of the biological activity of NT201 within five days can be observed at 80°C (Figure 6). However, the proteolytic activity is not reduced to less than one-third of the original value, and the rate of decline over time is slower than the total biological activity.

Taken together, the results from the long-term and accelerated stability studies have demonstrated the long-term stability of NT201 at ambient and elevated temperatures and have accounted for the shelf-life of three years without the requirement for refrigeration. Moreover, the results of the short-term temperature stress studies clearly show that NT201 is not adversely affected by short-term temperature stress between 45°C and 60°C for up to one month.

Figure 5 Storage of NT201 at 60 ± 2°C over 30 days. Mean results of five samples taken from one batch of NT201. SD values shown for biological activity and neurotoxin content

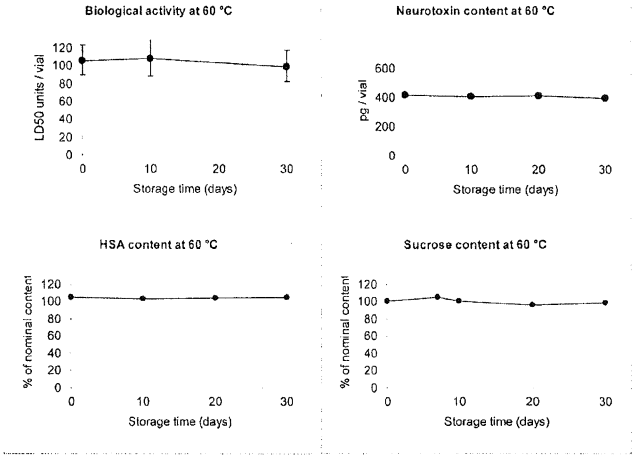
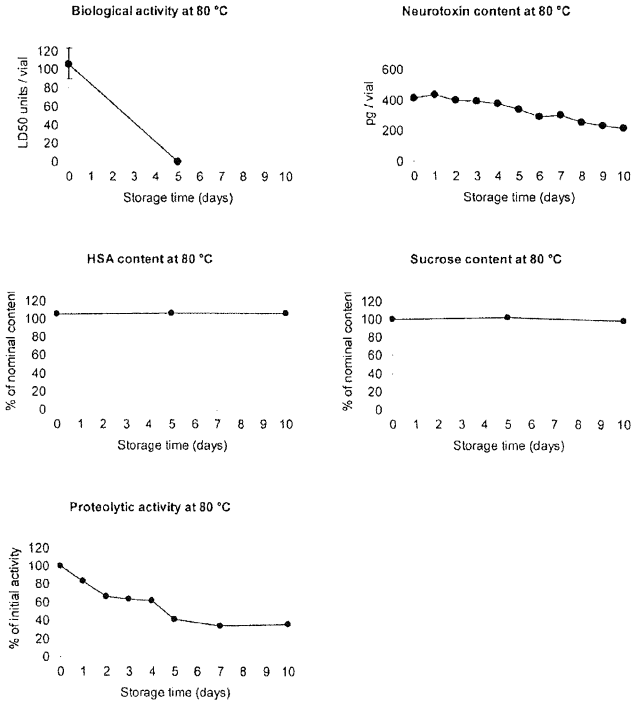


Figure 6 Storage of NT201 at 80 ± 2°C over 10 days. Mean results of five samples taken from one batch of NT201. SD values shown for biological activity and neurotoxin content



5 Discussion

Currently, there are several botulinum toxin drug products on the market. While much is known about the mechanism of action of botulinum neurotoxins (Montecucco and Schiavo, 1994; Simpson et al., 1994), the relative stability of botulinum neurotoxins used in pharmaceutical preparations is not very clear. To evaluate the stability of NT201, a botulinum neurotoxin type A free of complexing proteins, comprehensive real-time and accelerated studies according to ICH guidelines as well as short-term stress stability studies were carried out. It was found that NT201 is stable without refrigeration at 25°C, confirming that complexing proteins are not required for the stability of botulinum neurotoxin type A preparations.

The stress stability study results demonstrate that the quality of NT201 is not affected by storage at 60°C over a period of one month (Figure 5): All parameters tested remain well within the shelf-life specification of the product.

In contrast to the high stability at 60°C, a rapid decrease of the biological activity (as assessed by HDA and LD_{50} bioassay) can be observed at 80°C (Figure 6). The neurotoxin lost all biological activity within five days, with a faster reduction of the proteolytic activity during the first three days of storage. However, the proteolytic activity does not fall below one-third of the initial value, and the decline over time after two days is considerably slower than the total biological activity. Due to the highly modular structure of botulinum neurotoxin type A (Lacy et al., 1998), this observation indicates a higher temperature sensitivity of either the binding or the translocation domain (or of both domains) as compared to the light chain protease.

The neurotoxin content (ELISA) shows an even slighter, yet also continuous decrease over time. Considering that the decrease of activity correlates with loss of the native tertiary protein structure, the moderate signal reduction possibly indicates that the antisera used for capture and detection recognise predominantly linear or secondary structure epitopes and only to a lesser extent conformational epitopes constituted by the tertiary protein structure.

The surprising temperature stability of botulinum neurotoxin type A in NT201 is likely attributable to the formulation of the product. Especially HSA, one of the excipients used in NT201, is widely used as a stabilising agent for protein drugs, including botulinum neurotoxin type A (Goodnough and Johnson, 1992). HSA is itself a remarkably stable protein; it can be heated to 60°C for several hours and is stable as a liquid at room temperature over several years (Wang et al., 2005). Among other factors, the high intrinsic stability arises from the presence of 17 intramolecular disulphide linkages.

The data presented here contradict the hypothesis that the complexing proteins are required for sufficient stability of botulinum neurotoxin type A formulations (Schantz and Johnson, 1992).

6 Conclusion

NT201, a botulinum neurotoxin type A formulation, which only contains the pure 150 kDa neurotoxin without complexing proteins, can be stored safely without refrigeration. Moreover, NT201 shows a remarkable short-term stability at elevated

temperatures of up to 60°C, which demonstrates that even short intervals of temperature stress (e.g., during transportation in hot weather) have no detrimental effect on the quality of NT201. The study results also confirm that complexing proteins are not required for the stability of botulinum neurotoxin type A preparations. Nevertheless, it is highly recommended to store NT201 under the conditions described in the Summary of Product Characteristics (i.e., not above 25°C) at any time.

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