The Botulinum Neurotoxin LD_{50} Test – Problems and Solutions

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Summary

Apart from the fact that the LD_{50} test is generally considered a procedure causing severe distress, which alone should result in its immediate deletion, it also conflicts with the EU wide ban on cosmetic testing in animals in the case of Botulinum Neurotoxin (BoNT) testing. The European Pharmacopoeia monograph allows for the use of three alternative methods provided they are validated. However these alternative assays are neither implemented by the manufactures of BoNT products nor are they enforced by the responsible authorities, e.g. by deleting the LD_{50} from the European Pharmacopoeia. The number of animals used for the testing of BoNT is not officially recorded. However, data from an undercover investigation allow the estimation that the number of mice used in LD_{50} tests for BoNT products is greater than 600,000 per year worldwide.

Keywords: BoNT testing, animal numbers, animal welfare, LD50, validation

1 Introduction

Botulinum Neurotoxin (BoNT) is produced by *Clostridium botulinum*. It inhibits signal transmission from nerve to muscle resulting in paralysis. It is the strongest toxin known today, the lethal dose for humans being 1 ng/kg when applied intravenously or subcutaneously and 3 ng/kg when inhaled (www.zet.or.at; www.friis-cosmetix.de).

Products containing BoNT are approved for the medical treatment of several diseases, i.e. cervical dystonia, torticolis, blepharospasm, hyperhydrosis, strabismus and migraine. Further, the products Botox Cosmetics®, Vistabel®, Bocouture® and Azzalure® are licensed for the temporary treatment of for example frown lines. Applications for other cosmetic purposes are carried out "off label", meaning that the product has not been approved for this treatment. In this case patients agree that the application of BoNT is performed at their own risk. This risk is not inconsiderable: systemic adverse reactions such as respiratory compromise as well as death after injection of BoNT have been reported (FDA, 2008; arznei-telegramm, 2007).

The potency of each batch of BoNT must be determined before release, no matter whether the batch is intended for medical or cosmetic purposes. This is still widely done using the classical mouse LD_{50} assay, although alternative methods are available and accepted by the *European Pharmacopoeia* subject to validation.

For determination of the lethal dose in the classical LD_{50} test, different doses of the toxin are injected intraperitoneally into groups of mice, and lethality is calculated for each group. This test strategy is associated with severe distress for the animals, which mostly die over the course of three to four days by asphyxiation. An investigation of the British Union for the Abolition of Vivisection (BUAV) showed that surviving mice were killed by cervical dislocation using a ball point pen or by carbon dioxide poisoning, a slow death (buav.org). Three animal-free testing methods may be used instead of the classical mouse assay, subject to their validation against it. However, legal regulations are insufficient to ensure that these non-animal methods are validated and implemented.

2 Animal numbers used for potency testing

Botulinum toxin A, which is most commonly used for both medical and cosmetic purposes, is manufactured by Allergan Inc., Ipsen Ltd., Merz-Pharma GmbH & Co. KgaA, and Galderma Ltd. In addition to these, companies in South Korea (Medy-Tox Inc.) and China (btxa) produce BoNT products mainly for the Asian market. The use of BoNT has increased rapidly in only a few years. The global sales of Botox® produced by market leader Allergan increased from $ 25 million in 1993 (Botrill, 2003) to $ 1.3 billion in 2008 (Allergan, 2008). According to

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Allergan's 2005 Annual Report, the cosmetic use of BoNT accounted for 43% and the medical use for 57% of sales (Allergan, 2005). In 2008 the turnover of Ipsen's product Dysport totalled € 142.5 million (Ipsen, 2008).

Approximately 100 mice are used to test each batch of BoNT product (Botrill, 2003). However, official numbers of animals used for testing BoNT products are not available, as the companies do not publish them. Some years ago an estimated 300,000 mice per year were subjected to this severe test worldwide (Bigalke, 2007). However, considering the increasing use of these products, especially in the cosmetic sector, reflected by the increasing sales of BoNT products, the number of animals used must also have increased substantially and must be estimated anew.

Only few assured indications of the number of animals used for the testing could be obtained so far. An undercover investigation by BUAV in 2009 in Wickham Laboratories, where the product Dysport® is tested, revealed that around 74,000 mice a year are subjected to the LD_{50} test. As stated in Wickham's own records, 989 mice used for BoNT testing died on only one day of the undercover investigation (www.buav.org). In addition, it could be determined that Merz used 34,000 mice to test BoNT products in 2008 (www.botox-tierversuche.de).

On the basis of Ipsen's sales of Dysport® in 2008 of € 142.5 million, for which 74,000 mice were used, and Allergan's net sales for the product line Botox® of $ 1.3 billion (approx. € 970 million), an estimation can be made of the number of animals used for testing by brand leader Allergan (approx. 504,000) and thus the total animal number worldwide may be estimated at more than 600,000 in one year. This estimation does not take into account possible differences between prices of the products and currency fluctuations. Also, neither further companies, such as the Asian ones, nor further BoNT products were considered, suggesting that this number is still underestimated.

3 Available alternative methods

A variety of alternative methods for the testing of BoNT products has been developed so far and three have been included as options in the *European Pharmacopoeia* monograph 01/2005:2113 subject to validation. Most are refinement or reduction methods and thus are not animal-free. Although animal-free methods must be the ultimate goal both for medical as well as for cosmetic BoNT testing, every measure resulting in less pain and distress to the animals or in lower animal numbers is desirable.

The three tests included in the *European Pharmacopoeia* monograph are:

1. The *in vitro* endopeptidase activity assay is an animal-free method that was developed by the British National Institute for Biological Standards and Controls (NIBSC), which has used it for several years, resulting in a reduction of animal numbers by 5,000 mice per year (Dr Hadwen Trust, 2006). It measures cleavage of the synthetic protein SNAP-25 by BoNT. Beyond the relevance for animal welfare it comprises economical advantages, as it is economy-priced and can be performed in only a few hours. Both Allergan and Ipsen are working on variations of the SNAP-25 test.
2. The *ex vivo* assay using the mouse phrenic nerve-diaphragm was developed at the German Medical School in Hannover, commissioned by manufacturer Merz. It uses electric stimulation of the excised nerve, which results in a contraction of the muscle. Depending of the concentration of BoNT, the contraction of the muscle is stronger or weaker.
3. The *in vivo* non-lethal mouse flaccid paralysis test is a refinement method by which the toxin is injected under the skin of one hind leg. It was validated by the National Institute for Biological Standards and Controls (NIBSC) (Straughan, 2006).

Further alternative assays include electrical stimulation of strips of rat intercostal muscles developed by Ipsen, where six test preparations may be prepared from a single rat (Straughan, 2006), several ELISAs that use antibodies to detect certain parts of the toxin and cell-based assays such as the murine neuroblastoma cell test, which Allergan is working on.

4 Ongoing efforts to implement alternative methods

So far, several attempts had been made to find strategies to abolish the LD_{50} assay for BoNT testing.

In 2006 two meetings, one held by the European Directorate for the Quality of Medicines (EDQM) and one by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the US National Toxicology Program (NTP) and the European Centre for the Validation of Alternative Methods (ECVAM) were held to promote alternative methods to the LD_{50}. The second workshop was initiated by a nomination submitted by the Humane Society of the United States (HSUS) in November 2005, asking for a review of the validation status of available *in vitro*, *ex vivo*, and non-lethal *in vivo* test methods as potential replacements for the classical LD_{50} and requesting that ICCVAM work with partners and stakeholders to validate one of the available alternative methods (ICCVAM/NICEATM/ECVAM, 2008).

In 2009 an international workshop with experts consisting of animal welfare, authorities, science and industry organised by the German Federal Institute of Risk Assessment took place in Berlin, Germany to discuss the outcome of the previous meetings and the *status quo* of alternatives to BoNT testing with respect to the question how the classical LD_{50} test can be replaced. As an advanced result a BoNT Expert Working Group, commissioned by ZEBET, was established to regularly evaluate the status of alternative methods to the LD_{50} and to define criteria to further their regulatory acceptance (Federal Institute of Risk Assessment, 2009).

5 Legal considerations

BoNT products are generally subjected to the regulation valid for pharmaceuticals despite their widespread use for cosmetic purposes. This results in a legal dilemma, as the EU Cosmetic

Directive outlaws animal experiments for cosmetic products and ingredients. However, this dilemma is bypassed by the fact that cosmetic products are defined as products applied to the skin. BoNT products, in contrast, are injected and thus the testing requirements for pharmaceuticals are applied. In practice this means that according to the requirements of the *European Pharmacopoeia* the LD_{50} assay in mice for the determination of the potency of products containing botulinum toxin must be carried out for each batch. Additionally, stability tests have to be carried out over a period of five years after registration when modifications in the manufacturing are made or a new product is registered.

Although the three methods described above are included as alternatives in the *European Pharmacopoeia* subject to their validation and are considered preferable with respect to animal welfare aspects (European Pharmacopoeia, 2006), their use is not enforced. Furthermore, although reduced or refined LD_{50} assays (using fewer animals or humane endpoints) are accepted for the testing of chemicals, in the LD_{50} test for BoNT products suffering and death are still used as endpoints.

6 Conclusions

The legal loophole that allows testing of BoNT for cosmetic purposes using the LD_{50} test must be closed. Three alternative methods to the LD_{50} for BoNT have been developed and are accepted by the *European Pharmacopoeia* subject to validation. Their implementation would save more than 600,000 mice per year from severe distress and pain, the non-animal method being most preferable from an animal welfare point of view. Responsible authorities in Europe must delete the LD_{50} test for BoNT from the *European Pharmacopoeia*, since this would force BoNT producing companies to validate the recommended alternative methods and use them. A shift towards animal-free methods for BoNT potency testing would benefit animal welfare, consumer protection and the economy.

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