Towards Cruelty-free Botulinum Toxin Testing: Current Situation and Roadblocks

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Plain Language Summary

Botulinum neurotoxin (BoNT), more commonly known as Botox, is a biological toxin produced by bacteria and is characterized by its paralytic effect on nerve cells. BoNT is used for medical applications such as muscle spasms but is also known for its use in the cosmetic treatment of facial wrinkles. Since BoNT is a biological product, the characteristics and potency of the toxin can differ between production batches. For this reason, potency tests need to be performed on each batch of BoNT product to ensure its efficacy and safety. For these potency tests, European guidelines require the use of a mouse LD50 test. In this mouse model, a dilution series of BoNT product is injected into the abdomen of the mice, and the concentration at which 50% of injected mice die is used to determine the potency of this particular batch of BoNT. Over the course of the test, mice will become unable to walk and eventually suffocate due to the failure of respiratory muscles. However, during the test mice often die of starvation or dehydration because they are unable to move to their food bowls and water taps because of muscle paralysis, making the test unreliable. Both outcomes inflict great suffering on the mice. It has been estimated that over 400,000 mice have died annually across the EU for BoNT potency testing during manufacturing. In 2011 Allergan was the first major BoNT manufacturer to develop and receive approval for a cell-based model for BoNT potency testing. Unlike other available alternatives to the mouse model, this cell-based model incorporates all crucial steps of the neurotoxin's mechanism of toxicity. Different BoNT products use different ingredients which can cause problems if the assay is not adapted for these specific products, resulting in a time-consuming validation process. This contributed to the other two major BoNT manufacturers, Merz and Ipsen, taking years to validate a cell-based assay for their own products. Even though all three major BoNT manufacturers in Europe now use a cell-based assay to replace some amount of their animal testing, the number of mice used for BoNT potency testing had actually increased in the years leading up to 2019. This is likely the result of new manufacturers bringing their BoNT products to the EU market, EU regulatory guidelines stating that some quality control tests still need to be performed using the mouse model, and the total amount of produced BoNT increasing to keep up with rising consumer demand. Animal rights organizations are disappointed with this development, especially considering BoNT is commonly used for cosmetic treatments. Even though animal testing for cosmetics has been banned in the EU since 2013, BoNT products avoid this ban due to having genuine medical applications and are thus classified as medicines. However, in both the Netherlands and the rest of the EU, off-label prescription of BoNT for vanity reasons is common. Even though LD50 testing is prohibited in the Netherlands, BoNT products which were tested on mice are still marketed and sold there for cosmetic purposes. Since 2019, the EU has done little to help finalize the replacement of LD50 testing for BoNT. EU regulatory guidelines need to be updated to fully remove LD50 testing as part of the quality control process and until all BoNT products are 'cruelty-free', off-label prescription of these products for vanity reasons should be prohibited.

Abstract

Botulinum toxin type-A has been used in various medical applications as well as for the cosmetic removal of facial wrinkles for the past thirty years. Use of the cruel mouse bioassay for routine potency testing of botulinum toxin during pharmaceutical manufacturing has led to scientists and animal rights organizations to call for its replacement with an animal free alternative. Even though the three major manufacturers of botulinum toxin products have developed and validated a cell-based assay for potency testing, reports indicate that the number of animals used for potency testing had in fact increased in the years leading up to 2019. This review seeks to investigate the current situation regarding the use of animals for botulinum toxin batch potency testing as well as identify the scientific, economic, and legal roadblocks that stand in the way of full replacement of the cruel mouse model. No manufacturer has been able to achieve full replacement due to the guidelines in the European Pharmacopoeia requiring the use of a reference standard calibrated in LD50 units if manufacturers want to use an alternative test. Use of the alternative tests has not been legally enforced, leading to continued use of the mouse model by other manufacturers. Despite common off-label use as vanity treatments, botulinum toxin products are still allowed to be tested on animals, circumventing the EU ban on animal testing for cosmetics. Until regulators and manufacturers come together to fully replace the mouse model, off-label prescription of these products for cosmetic reasons should not be permitted.

Introduction

Botulinum neurotoxins (BoNT) are biological products of the anaerobic bacterium *Clostridium botulinum* (Luvisetto, 2021). BoNTs exist in seven structurally unique serotypes (BoNT type A-G) (Montecucco & Rasotto, 2015). Their neurotoxic effect consists of blockage of acetylcholine neurotransmitter release at neuro-muscular nerve endings (Luvisetto, 2021; Nepal & Jeong, 2020). Acute intoxication with BoNT leads to muscular paralysis among other adverse effects and can result in death via asphyxiation due to respiratory muscle failure (Johnson & Montecucco, 2008). This neuroparalytic condition is known as botulism and can occur in both humans and animals. The most common cause of botulism is the consumption of food that has been contaminated with *Clostridium botulinum* bacteria (Johnson & Montecucco, 2008). BoNTs exert their neuroparalytic effects even at minute concentrations and are thus considered to be among the most toxic substances in existence. (Pellett, 2012)

The mechanism of toxicity of all BoNT subtypes relates to the release of the neurotransmitter acetylcholine (ACh) at neuro-muscular nerve endings (Luvisetto, 2021). ACh is an excitatory neurotransmitter and responsible for activating muscle cells, leading to muscle contraction, which is crucial for bodily functions such as breathing and movement (Lauder & Schambra, 1999; Luvisetto, 2021). ACh release consists of several important steps (See figure 1a). First, synaptic vesicles containing the neurotransmitter bind to the inner cell membrane (Pirazzini et al., 2017). This process is mediated by the formation of a complex of three different N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins: Synaptobrevin, which is attached to the synaptic vesicle, and SNAP-25 and Syntaxin, which are attached to the cell membrane. After the formation of this protein complex, the synaptic vesicle is fused with the cell membrane, leading to the release of ACh into the synaptic cleft. Subsequent binding of ACh to receptors on the post-synaptic neuron or muscle cell leads to transmission of the signal or muscle cell contraction respectively (Nepal & Jeong, 2020).

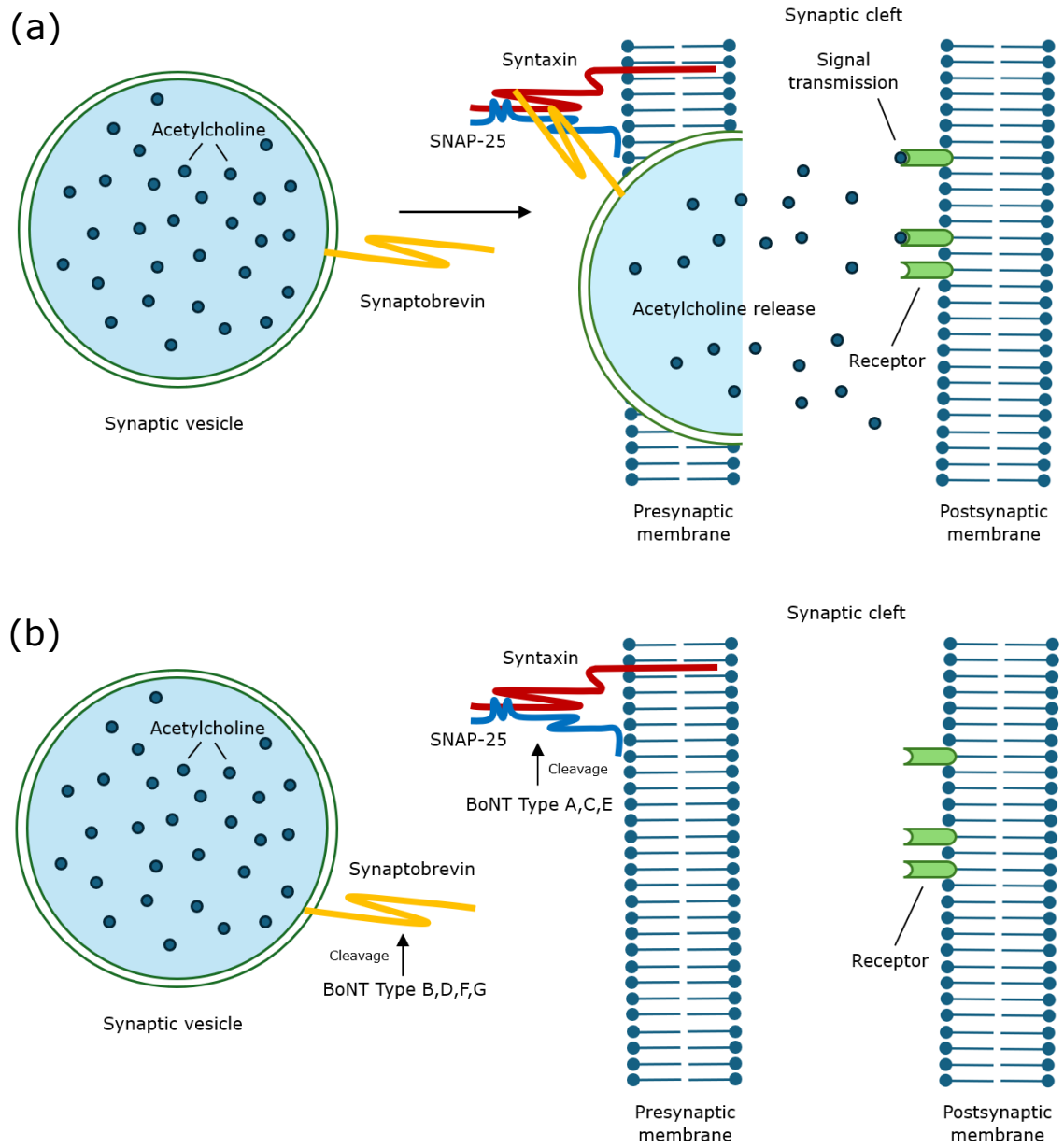

Figure 1 *– Schematic overview of the mechanism of acetylcholine neurotransmitter release and signal transmission. (a) normal neuron function (b) BoNT presence preventing acetylcholine release. Image adapted from Nepal et al. 2020.*

The structure of every serotype of BoNT consists of a 150 kDa single-chain protein, which can be cleaved by proteases to yield a 100 kDa heavy chain and a 50 kDa light chain. Internalization of the BoNT is mediated by the heavy chain, its C-terminus able to bind to certain ganglioside receptors and proteins on the surface of nerve cells (Pirazzini et al., 2017). After receptor binding, the neurotoxin is taken up via endocytosis. Protonation of the BoNT inside the endosome leads to its release into the nerve cell's cytosol (Nepal & Jeong, 2020). Inside the cytosol, the disulfide bond between the heavy chain and the light chain is broken, which leads to the release of the proteolytic light chain. The light chain is capable of cleaving one of the three SNARE proteins involved in acetylcholine release, though the exact cleavage site differs per serotype of BoNT (Pirazzini et al., 2017). BoNT-A, which is the serotype most commonly used for medical and cosmetic purposes,

cleaves the SNAP-25 protein (See figure 1b). Cleavage of any of the three SNARE proteins prevents membrane fusion of synaptic vesicles and the subsequent release of acetylcholine into the synaptic cleft (Nepal & Jeong, 2020). This absence of excitatory ACh results in unresponsive motor neurons and muscle cells (muscle paralysis).

For over thirty years, local injections containing minute dosages of BoNT type A or B have been used to treat various conditions, such as muscle spasticity, excessive sweating, and chronic migraines (Chen, 2012; Luvisetto, 2021). Additionally, an ever-increasing number of BoNT injections have been used since around the year 2000 as a cosmetic treatment for the temporary removal of facial wrinkles (Satriyasa, 2019). A group of doctors from the Erasmus Medical Centre in Rotterdam estimated that around 250,000 cosmetic BoNT treatments were administered in 2019 in the Netherlands alone. They extrapolated this number for an estimate of nearly eleven million cosmetic BoNT treatments Europe-wide in 2019 (Decates et al., 2021). These cosmetic treatments consist of intramuscular BoNT injections in various facial areas that lead to the relaxing of muscles responsible for facial wrinkles, resulting in the purely cosmetic effect of the skin appearing smoother (Satriyasa, 2019; Taylor, 2019). The paralytic effect of the BoNT injections fades over the course of a three-month period, so additional injections are required to maintain the cosmetic effect (Satriyasa, 2019).

As BoNT is one of the most dangerous neurotoxins on earth, each batch of BoNT product needs to have its potency tested to ensure its safe use in patients (Adler et al., 2010; Pellett, 2012). During manufacturing, BoNT is obtained from a purified liquid taken from a broth-culture of *Clostridium botulinum* bacteria (Taylor, 2019). Since BoNT is a biological product and consists of a complex of proteins, the composition and biological activity of each batch can vary due to structural differences (Taylor, 2019). Furthermore, cases of iatrogenic botulism, where BoNT spreads to undesired targets leading to potentially lethal adverse effects, can rarely occur after both therapeutic and cosmetic treatments (Floresta et al., 2020). This highlights the importance of the so-called ‘batch potency tests’ which are performed on all BoNT products to ensure patient safety.

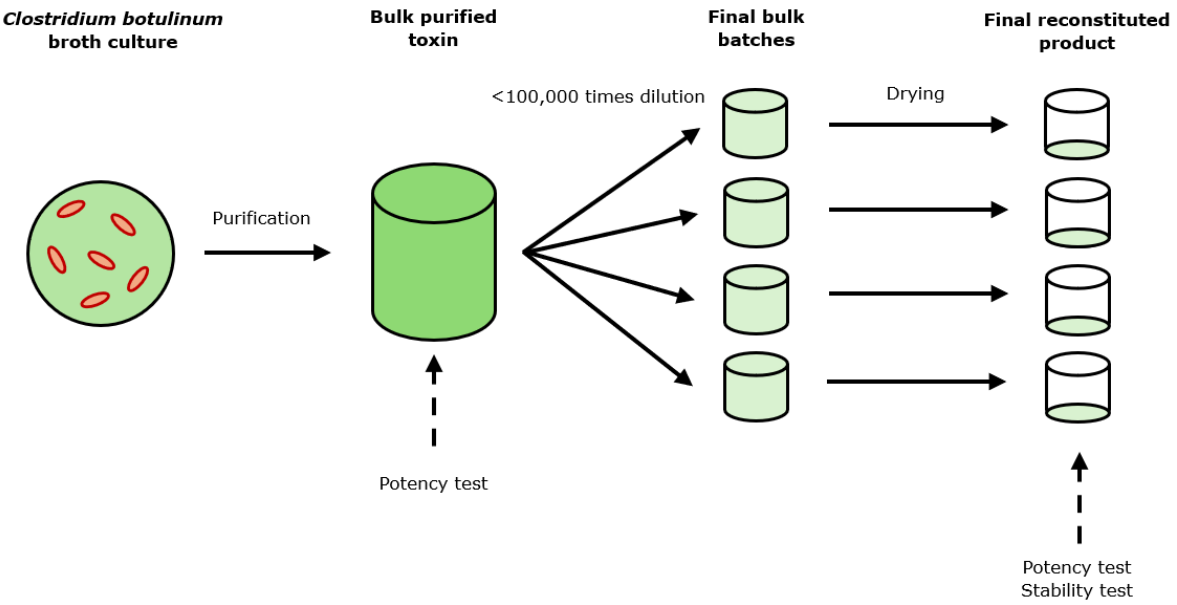

Figure 2 *– Schematic overview of the BoNT manufacturing process, indicating where potency and stability tests need to be performed according to the Ph. Eur. Figure adapted from Adler et al. 2010.*

The 'golden standard' of these batch potency tests has been the mouse bioassay (MBA), also referred to as the mouse lethality assay (MLA) (Bitz, 2010). The MBA is a median lethal dose (LD50) test, where a range of BoNT dosages are injected into different groups of mice before assessing the lethality in each group. The LD50 value is defined as the concentration of toxin at which 50% of the injected mice die (Bitz, 2010). During the test, mice are expected to die from asphyxiation, a slow and painful process, and thus the MBA is widely considered to inflict 'severe suffering' on the animals (Bitz, 2010; Taylor, 2019). Using the LD50 value from the test, the potency of the neurotoxin can be determined and the safe, intended dose for use in human treatments calculated. BoNT potency is assessed for purified bulk BoNT, but also for batches of the product in their final formulation prior to distribution as well as for product stability (See figure 2) (Adler et al., 2010). This means that without the use of animal-free alternatives, the manufacturing process for BoNT products results in testing which inflict great suffering on mice (Bitz, 2010; Taylor, 2019).

The official and legal and scientific guidelines for these batch potency tests are contained in the European Pharmacopoeia (Ph. Eur.), which is maintained by the European Directorate for the Quality of Medicines and Healthcare (EDQM).[1] Monograph 2113 of the Ph. Eur. addresses the manufacturing and quality control guidelines for BoNT-A products. It states that BoNT batch potency testing must be performed using the MBA, or an alternative test with the use of a reference standard of BoNT that was calibrated with the MBA (Taylor, 2019). This means that the alternative, animal-free test must still use a control sample of BoNT that was confirmed to have a certain potency by determining its LD50 value using the mouse model.

Use of the MBA for BoNT testing is allowed because these products circumvent the 2013 EU ban on all animal testing for cosmetic products.[2] This is because all BoNT products are administered as injections and are therefore considered to be medicines, even if the application for which they are used is merely cosmetic (Taylor, 2019).[2] BoNT injections against skin wrinkles are therefore often referred to as an 'aesthetic' treatment to highlight the fact that these are legally not considered a cosmetic treatment, such as for example the use of face creams (Taylor, 2019). The use of mice in routine testing for products which are commonly used for vanity purposes has resulted in backlash from both scientists and animal rights organizations. Over the past twenty years, BoNT manufacturers have made progress in the adoption of animal-free alternative tests, yet to this day the mouse model remains in use for BoNT potency testing. The goal of this review is to identify the steps that need to be taken to fully replace the MBA for BoNT potency testing in Europe. An overview of available animal-free alternatives is provided and their advantages and disadvantages for routine potency testing are discussed. Additionally, the history of the adoption of animal-free tests by BoNT manufacturers is reviewed to determine which obstacles these companies face when replacing the mouse model. Lastly, the current legal requirements of BoNT production and distribution in the Netherlands and Europe are listed to identify which legislative changes could be made to ensure that 'cruelty-free' BoNT products can be attained.

Limitations and Concerns of the MBA

The MBA is a LD50 assay where groups of mice are injected in their peritoneal cavity (abdomen) with dosages of BoNT product that differ per group (Bitz, 2010). The number of dead mice per dosage group are used to determine the LD50 value of the BoNT batch (Adler et al., 2010). Generally, over 90% of mice die in the highest dosage group and 10% in the lowest dosage group, though all surviving mice are still terminated at the end of the test (Adler et al., 2010). As death is the endpoint of the MBA, it is considered by many regulatory authorities to be in the 'severe suffering' category of animal tests. Additionally, the mice experience great distress and suffering during the test and no form of pain relief is given to the mice (Hartung, 2008; Taylor, 2019). In an attempt to reduce suffering, testing laboratories employ 'humane killing' when it becomes clear a mouse likely to die before the next observation period (Bitz, 2010). This is indicated by mice showing 'severe symptoms of paralysis, including difficulty breathing and cyanosis'.[3] However, reports indicated that merely 10-30% of mice were killed by the humane endpoint at various laboratories. (Adler et al., 2010; Taylor, 2019) The reasons for this are likely a combination of a reluctance to kill the mice too early as well as an insufficient frequency of observation points during the period of severe toxicity.[3] Furthermore, the British Union for the Abolition of Vivisection (BUAV, now called Cruelty Free International) performed an undercover investigation at Wickham laboratories, who were performing MBAs for BoNT manufacturer Ipsen.[3] They found that the observation points were indeed not frequent enough to euthanize the mice by the humane endpoint and that improper killing methods were employed.[3] The mice were euthanized either via 'cervical dislocation using a ball point pen or by carbon dioxide poisoning', the former being extremely painful if not properly performed and the latter being a slow death (Bitz, 2010).[3]

These findings highlight that the addition of a humane endpoint to the MBA has not been successful in reducing the amount of suffering the mice experience.

This severe suffering is experienced by a high number of mice for each individual MBA, further raising ethical concerns. The Ph. Eur. does not recommend a sample size for the MBA. This has resulted in each manufacturer using different numbers of mice per test, the exact number being kept confidential (Bottrill, 2003; Pickett, 2011). The BUAV's investigation into Wickham laboratories revealed that hundreds of mice are used per MBA, though the exact number was kept confidential.[3] MBA testing in Europe is known to have been performed in control testing laboratories in Ireland, the United Kingdom and Germany, upon request by BoNT manufacturers (Taylor, 2019). In 2019, it was estimated by the European Coalition to End Animal Experiments (ECEAE) that over 400,000 mice were used yearly for BoNT batch potency testing (Taylor, 2019). Based on EU reports on animal testing, Cruelty Free International estimated in 2020 that 273,955 mice were used in tests for BoNT potency assessment.[4] The EU is currently the only market region where figures on animal usage in testing are published.[4] The global number of mice subjected to MBA testing for BoNT can only be estimated by extrapolating European testing based on sales but is likely to surpass 600,000 mice annually (Bitz, 2010). The exact number of mice used in MBAs for BoNT potency testing is difficult to determine as BoNT manufacturers have not been transparent about their MBA usage. Nearly all information on this topic has been obtained from specific manufacturer press statements and questioning by animal rights organizations (Taylor, 2019). The combination of the severe suffering the mice face and the high number of mice that are killed or sacrificed each year creates an urgent need to replace the MBA with an animal-free alternative.

A common counterargument against the full replacement of the MBA is the fact that *in vivo* assays are currently the only way to assess the effect of pharmacokinetics on BoNT potency (Bitz, 2010; Nepal & Jeong, 2020). However, it can be argued that if the pharmacokinetics do not resemble the situation of the products eventual use case, the value of their inclusion into the model may be overestimated. For example, the end use of cosmetic BoNT products is a highly diluted injection into facial muscles, with the effect being precise localized paralysis (Luvisetto, 2021). In the MBA however, the BoNT is injected straight into the peritoneum (abdomen), from which the toxin's paralytic effects spread across the entire body of the mice (Bitz, 2010). Other medical applications for BoNT also employ minute concentrations at specific locales, which is again not the situation in the MBA. Furthermore, biological differences between mice and humans naturally lead to different pharmacokinetic behavior of drug compounds (Nau, 1986). Species-specific differences are not limited to kinetics, as the BoNT activity of specific serotypes differs between mice and humans (Dressler & Benecke, 2007). Lastly, as the only outcome of the MBA is death, which is considered to be an unreliable outcome, it is questionable to which extent pharmacokinetics can be accurately assessed (Bitz, 2010). Nevertheless, the pharmacokinetic behavior of BoNT is relevant for potency assessment, and thus the ongoing development of novel 3D *in vitro* models of neuromuscular junctions is of great interest for the future of animal-free BoNT potency testing (Natarajan et al., 2019).

Aesthetic use of BoNT

These MBA potency tests are performed for all BoNT products, including the ones that end up being used for cosmetic treatments. BoNT manufacturers have generally not been transparent about the number of sales between medical and cosmetic BoNT products, but over the years some indications have come up. Irish manufacturer Allergan reported in 2017 that their cosmetic BoNT product Vistabel® made up 43% ($1,369.2 million) of their global BoNT product sales (Taylor, 2019). However, considering that their BoNT product for general medical applications (Botox®) is commonly prescribed off-label for cosmetic treatments as well, it can be assumed that over 50% of Allergan's sold BoNT products are used for cosmetic purposes (Taylor, 2019). Since a large portion of manufactured BoNT product is merely being used for vanity, use of LD50 testing for BoNT products especially has received major criticisms from animal rights organizations, which have pressured the manufacturers to adopt alternative tests for the past twenty years (Bottrill, 2003).[5,6]

Available Alternatives

The previously mentioned ethical concerns, alongside the high monetary costs associated with performing the MBA have resulted in scientists and BoNT manufacturers developing alternative tests for BoNT potency assessment. An overview of the BoNT assays that were developed in the last thirty years is given in Table 1 and the details individual assays are described in subsequent sections.

Assay	Type	Advantages	Limitations	Extent of adoption for BoNT manufacturing
SNAP-25 assay	*In chemico*	-Simple and inexpensive design -Animal-free	-Only one step of BoNT toxicity is assessed (cell binding, internalization, intracellular activity, ACh release and muscle paralysis are **not** modelled)	Included in the Ph. Eu. in 2005, limited adoption confirmed by Ipsen.
Non-lethal mouse flaccid paralysis assay	*In vivo*	-Less severe outcome compared to MBA -Uses less mice compared to MBA -Assesses all steps of BoNT toxicity (from cell binding to muscle paralysis)	-Not animal-free -Outcome is assessed with a scoring system and therefore less robust	Included in the Ph. Eu. in 2005, validation unsuccessfully attempted by Wickham laboratories.
Mouse phrenic nerve hemidiaphragm test	*Ex vivo*	-Assesses all steps of BoNT toxicity (from cell binding to muscle paralysis)	-Complicated procedure -Difficult to scale up for routine testing -Not animal-free	Included in the Ph. Eu. in 2005, validation unsuccessfully attempted by Merz.
Cell-based assay	*In vitro*	-Assesses most steps of BoNT toxicity (from cell binding to ACh release) -Possibilities for human cells eliminate species-specific differences -Animal-free depending on the used cell type	-BoNT product excipients can affect the assay, complicating the validation process.	Included in Ph. Eu. in 2012, adopted by Allergan (2011), Merz (2015) and Ipsen (2018) for the replacement of the MBA in batch potency testing.

Table 1 *– Overview of each alternative test to the mouse bioassay that is accepted by the official guidelines for BoNT-A potency testing in the European Pharmacopeia. Advantages and disadvantages of each alternative test are listed, alongside the extent to which they have been adopted by manufacturers.*

SNAP-25 Assay

The SNAP-25 assay, also known as the endopeptidase method, is an *in chemico* method originally developed in 1997 by the British National Institute for Biological Standards and Control (NIBSC) as a replacement for the MBA (Ekong et al., 1997). Since then, the NIBSC has used this alternative instead of the MBA to check the accuracy of the manufacturer's batch potency test results of BoNT-A products distributed in the UK.[7] During the SNAP-25 assay, BoNT is added to a substrate containing SNAP-25 protein derived from mouse stem cells. Cleavage

of the protein can then be detected using various techniques, all involving the fluorescent labelling of the cleavage products (Ekong et al., 1997). Though this assay is highly sensitive to BoNT, it does not include multiple steps crucial to the potency of BoNT. These include cell binding, internalization, intracellular activity, and potential structural changes to the BoNT protein (Ekong et al., 1997). Manufacturers did not fully adopt this assay as a replacement for the MBA because it does not incorporate these crucial steps. Ipsen has stated that using the assay alongside of the MBA did result in 25% less animals being required (Taylor, 2019). Despite more recent improvements to the sensitivity of the assay, its *in chemico* design cannot assess all factors of BoNT potency and thus it cannot fully replace the MBA in BoNT potency testing (Nepal & Jeong, 2020).

Non-lethal Mouse Flaccid Paralysis Assay

The non-lethal mouse flaccid paralysis assay (NFPA) is an *in vivo* local paralysis assay originally developed in 1996 by the NIBSC as an alternative to the MBA.[7] Compared to the MBA, it is considered to be more economical and less severe while employing five times fewer mice (Nepal & Jeong, 2020). The NFPA uses local paralysis as a more humane endpoint, circumventing the systemic toxicity which leads to the death of mice in the MBA. During the test, mice receive a subcutaneous, non-lethal injection of BoNT-A in the groin.[7] Relaxation of the abdominal muscles would occur over the course of the next 48 hours, leading to abdominal ptosis (swelling). The severity of the swelling would be assessed using a ranking system, a score on a five-point scale being assigned to the mice based on the intensity of abdominal swelling.[7] Though the NFPA inflicted less suffering and uses significantly fewer mice, it was never fully adopted by manufacturers, even though it is listed as an acceptable alternative in the Ph. Eur. (Adler et al., 2010; Taylor, 2019). Skepticism towards the scoring system, as well as manufacturers being required to validate the assay themselves may be responsible for the assay not being adopted as a replacement for the MBA. Additionally, the NFPA is not ideal from an animal rights perspective, as it still requires the use and sacrifice of at least a couple dozen mice (Nepal & Jeong, 2020).

Mouse Phrenic Nerve Hemidiaphragm Test

The mouse phrenic nerve hemidiaphragm (MPN) test is an *ex vivo* test utilizing hemidiaphragm muscle tissue containing the phrenic nerve harvested from euthanized mice (Bigalke & Rummel, 2015). The test aims to imitate the endpoint of the MBA, respiratory paralysis. For the assay, slices of murine muscle tissue are placed in an organ batch which is constantly electrocuted at a frequency of 1 Hz using two electrodes (Bigalke & Rummel, 2015). This causes the phrenic nerve within this tissue to be electro-stimulated, causing the muscle tissue to continuously contract. The amplitude of the isometric contraction, in other words, how strongly the muscle tissue is able to contract, is then measured (Bigalke & Rummel, 2015). The incubation solution is then replaced with a BoNT-containing solution, and the time until the assay endpoint of a 50% decrease in contraction amplitude is measured (Bigalke & Rummel, 2015). The effect of BoNT on contraction amplitude is dose-dependent and the assay shows the same sensitivity as the MBA (Nepal & Jeong, 2020). Even though it is listed

in the Ph. Eur. as an acceptable alternative to the MBA, several factors make this test unsuitable for BoNT batch potency testing (Taylor, 2019). The assay requires skilled personnel in order to be performed, as the dissection process to obtain the phrenic nerve muscle tissue is quite delicate (Nepal & Jeong, 2020). Furthermore, only a limited number of samples can be assessed during a single assay, and it is therefore difficult to scale the assay up to accommodate for the large number of batches that require potency testing during manufacturing (Nepal & Jeong, 2020). Furthermore, the sacrifice of mice is still required to obtain the muscle tissue, and thus ethical concerns are not fully eliminated.

Cell-based Assays

Cell-based assays (CBAs) are an *in vitro* model for BoNT detection and have been used to determine the mechanism and potency of BoNT for over forty years at this point (Pellett, 2012). Generally, nerve cells are exposed to BoNT, and its potency can be assessed by the detection of the products of SNAP-25 cleavage and neurotransmitter release assays (Pellett, 2012). Nerve cells from a variety of sources can be used for this purpose, an overview of which is provided in Table 2. CBAs were originally used to study the characteristics and mechanism of toxicity of BoNT, as unlike other assays, complex mechanisms like cell entry and inhibition of acetylcholine release can be investigated using the CBA (Pellett, 2012). Primary neurons from various animals, often mice, were cultured for these earlier tests (Pellett et al., 2011). These neurons would be obtained by sacrificing a pregnant mouse, extracting the pups from the womb, and dissecting out the spinal cord from the pups (Pellett et al., 2010, 2011). Nowadays, the spinal cords can also simply be purchased. It was not until about fifteen years ago that cell culture technology advanced to the point that primary cell-based assays surpassed the MBA in sensitivity for BoNT potency testing (Pellett, 2012). Primary cell-based assays proved to yield reliable and reproducible results for BoNT potency testing. However, since the primary neurons need to be obtained from mice, species-specific differences between the relevant SNARE-proteins remain a major limitation (Pellett, 2012). After all, the activity of different BoNT serotypes differs between mice and humans. Most notably, BoNT-B activity is roughly forty times lower than than BoNT-A in humans, whereas in mice these two serotypes show similar potency (Dressler & Benecke, 2007). Furthermore, the primary-cell methods still require the sacrifice of mice to obtain the nerve cells for each separate assay, meaning that for routine testing in manufacturing, a large number of mice would still need to be sacrificed.

Cell type	Advantages	Disadvantages
Murine primary neurons	-Sensitive to BoNT and yields reliable results -Commercially available	-Murine origin leads to species-specific differences -Sacrifice of mice is required to obtain the cells for each individual assay
Human neuroblastoma cells	-Human origin eliminates species-specific differences -Simple and inexpensive to maintain cell line compared to stem cells	-Cancerous origin can lead to poorly defined cell characteristic and differences in BoNT sensitivity
Murine embryotic stem cell-derived neurons	-Sensitive to BoNT and yields reliable results	-Murine origin leads to species-specific differences -Differentiation and culturing of embryotic stem cells require specialized equipment and trained staff -Procurement of the cells is more expensive than maintaining a continuous neuroblastoma cell line.
Human-induced pluripotent stem cell-derived neurons	-Sensitive to BoNT and yields reliable results -Human origin eliminates species-specific differences -Commercially available	-Procurement of the cells is more expensive than maintaining a continuous neuroblastoma cell line.

Table 2 – *Overview of the different types of cell(-line) types that have been utilized to culture nerve cells for the purposes of BoNT potency testing. The advantages and disadvantages of each cell type are listed.*

Continuous cell-lines are indefinitely maintained cell lines usually obtained from cancerous cells (Yowler et al., 2002). Originally, continuous cell-line derived nerve cells did not provide the needed sensitivity for BoNT potency testing, but advancements in cell culturing technology resulted in equal sensitivity compared to the MBA, improved cell line stability, and allowed for the use of human neuroblastoma cells (Fernández-Salas et al., 2012; Yowler et al., 2002). Continuous cell lines are relatively simple and inexpensive to maintain compared to other cell lines, but nerve cells derived from continuous cell lines can show poorly defined cell characteristics and differences in BoNT sensitivity due to their cancerous nature (Pellett, 2012).

Lastly, stem cell-derived neurons can be utilized for CBAs (Pellett, 2012). Embryotic stem (ES) cells can be differentiated into neurons with the addition of retinoic acid, a process that takes two weeks (Zhang, 2006). CBAs employing neurons derived from murine ES cells showed BoNT sensitivities in line with the MBA and primary neuron CBA (Pellett et al., 2011). However, the differentiation process of ES cells is time consuming and requires experienced staff in a laboratory specialized in stem cell work (Pellett, 2012). These factors, combined with the fact that these murine neurons show species-specific differences with humans, can explain why murine ES-derived neurons remain unused in CBAs for commercial BoNT product testing. Human ES cells could be employed to eliminate these differences, but the ethical concerns regarding the way these cells are obtained are likely the reason no CBA for BoNT potency was never developed using

human ES cells (Pellett, 2012). This ethical problem was nullified when advancements in stem cell technology made it possible to convert human adult somatic cells into stem cells. Similarly to ES cells, these human-induced pluripotent stem (hiPS) cells can be differentiated into nerve cells, allowing for the ethical procurement of human neurons for testing purposes (Takahashi et al., 2008). Cryopreservation of well-defined differentiated hiPS-derived neurons allows for them to be sold commercially and utilized in the pharmaceutical industry for CBAs without the need for specialized stem cell culturing-experience and equipment (Pellett, 2012). In 2012, a group of researchers from the University of Wisconsin a highly sensitive CBA was developed for BoNT detection using hiPS-derived neurons (Whitemarsh et al., 2012). Use of these neurons in BoNT potency testing has two major advantages compared to other types of neuronal cell lines. Not only are the cells of human origin, eliminating species-specific differences, but hiPS cells also resemble well-defined regular somatic nerve cells, unlike the cancerous continuous cell-line derived neurons (Whitemarsh et al., 2012).

Replacement of the MBA

In 1997, the NIBSC was the first institution to replace their use of the MBA with an alternative, namely the SNAP-25 assay (Ekong et al., 1997). However, BoNT product manufacturers were not allowed to use any alternative tests for the EU market until 2005, when the Ph. Eur. monograph 2113 was updated to state the following:

After validation with respect to the LD50 assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare, including 1 of the following:

1. Endopeptidase assay in vitro (SNAP-25 assay)

2. Ex vivo assay using the mouse phrenic nerve diaphragm

3. Mouse bioassay using paralysis as the endpoint

For these other methods, the potency is calculated with respect to a suitable reference preparation calibrated in mouse LD50 units.

In Article 13(1) on the Directive 2010/63 on the protection of animals used for scientific purposes, the European Commission states that alternative tests listed in the Ph. Eur. are recognized under EU legislation and must be used instead of the animal tests they replace (Taylor, 2019). However, the manufacturers themselves are required to validate an alternative method for each BoNT product in respect to the MBA, meaning they need to show that the dose-dependent potency of BoNT is the same in both models. The Ph. Eur. does not provide any detailed information on the acceptable alternative methods and there has not been any support for the validation process on a European level (Taylor, 2019). The manufacturers themselves must provide updated market authorizations to every country-specific European medicine regulatory body, which will then decide if their validation of the alternative method is acceptable. Even though use of the alternative methods is supposedly a legal requirement, their adoption by manufacturers remained limited in the years after the Ph. Eur. was updated in

2005 (Adler et al., 2010). Manufacturer Ipsen claimed that the endopeptidase-assay was not suitable as a complete replacement for the MBA, as merely the proteolytic property of BoNT is measured (Taylor, 2019). This sentiment was shared by European regulators, who only approved the use of the endopeptidase assay for every second yearly stability test on BoNT product batches (Adler et al., 2010). Around this time there were also attempts to validate the NFPA and MPN tests by Wickham labs and manufacturer Merz respectively, but both attempts are presumed to have been unsuccessful (Adler et al., 2010; Taylor, 2019).[7]

The first major step for the replacement of the MBA in BoNT potency testing occurred in 2011, when manufacturer Allergan finished the development of a cell-based assay with the aim to completely replace the MBA for BoNT batch potency testing (Fernández-Salas et al., 2012; Taylor, 2019). Their CBA utilizes a human neuroblastoma cell line and BoNT-A activity is measured using a sandwich ELISA for SNAP-25 cleavage product (Fernández-Salas et al., 2012). According to Allergan, the development of their CBA costed $65 million and was a decade long process (Taylor, 2019). After the assay was validated for their own BoNT products, Allergan received approval for this updated method for the United States market in 2011 and for the European market in 2012. In the same year, the Ph. Eur. monograph 2113 was updated to state the following:

"The LD50 is associated with severe suffering of animals and manufacturers are strongly encouraged to develop and validate assays that will reduce the number of animals used or refine or replace the test procedure with the goal of promoting animal welfare".

"After validation with respect to the LD50 assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare, for example mouse bioassays using paralysis as the endpoint, ex vivo assays using mouse phrenic nerve diaphragm, endopeptidase assays in vitro and cell-based assays. For alternative replacement methods the potency is calculated with respect to a suitable reference preparation calibrated in mouse LD50 units".

This change added a statement on the severe suffering associated with the MBA, alongside the addition of cell-based assays as an accepted alternative. Even though the EDQM claims here that development and validation of alternative assays is "strongly encouraged", there still has not been any European-level support to the manufacturers nor have there been any legal repercussions for continuing the use of the MBA (Taylor, 2019).[5] Allergan published a paper on the design and validation of their CBA in 2012 (Fernández-Salas et al., 2012). However, other companies were unable to copy the design from this article due to omitted details, while Allergan was not willing to assist them in the adoption of the CBA (Pickett, 2012). Considering the amount of time and money Allergan had spent on the development process however, it is not surprising that they were unwilling to provide aid to their competitors. The other two major BoNT manufacturers in Europe, Ipsen and Merz, started a collaboration in 2011 to develop a CBA together, but separated about a year later to validate the assay for their own products (Taylor, 2019). In 2015, Merz received European approval for their use of the CBA for testing their products while Ipsen did not receive approval until 2018 (Taylor, 2019). Considering it took Merz roughly three years to validate

the CBA for their products, and Ipsen took about six years, this process does not appear to have progressed smoothly.

Scientific Roadblocks

The reason why the validation process was so time-consuming for both Ipsen and Merz is not clear but may have been a combination of genuine scientific issues, strict demands of European regulators and these manufacturers not being willing to spend large amounts of funds on the validation process (Taylor, 2019). An overview of existing roadblocks and possible solutions is given in Table 3. It is known that adapting the CBA for different products is complicated due to the excipients present in the final formulation of BoNT products (Pellett, 2012). Excipients such as the diluent and stabilizing protein not only influence assay sensitivity, but also on BoNT activity in general. This is the reason why BoNT units are not interchangeable between different products, different LD50 values being assigned to each individual product (Pellett, 2012). As for the effect of excipients on the CBA, the commonly used stabilizing protein human serum albumin has shown to increase the sensitivity of the assay, while saline, which is commonly used to resuspend dried BoNT prior to administration, can affect the cells during the assay due to its salt contents (Pellett, 2012).

Roadblock	Type	Possible solutions
The development and validation of alternative tests is expensive and complicated, leading to manufacturers being unwilling to work towards the replacement of the mouse model.	Economical / Scientific	-Regulators and manufacturers sharing knowledge on how the alternative tests should be performed -Enforcing the legal requirement of using alternative tests to the mouse bioassay
Some regulators do not consider the current cell-based models adequate.	Regulatory / Scientific	-Co-operation between scientists and manufacturers to work towards a novel 3D *in vitro* model for BoNT potency testing -Country specific regulatory bodies could ask for other alternative tests to confirm findings in cell-based model
Botulinum toxin is considered a medicine and therefore not subjected to the 2013 EU ban on animal testing on cosmetics.	Regulatory	-Prohibiting off-label prescription of botulinum toxin for cosmetic purposes, only allowing use of the product for genuine medical applications.
Use of an MBA-calibrated reference standard is required by the European Pharmacopeia.	Regulatory	-Updating the European Pharmacopeia to longer require the use of this reference standard. -Supporting standardization between laboratories so that use of a cruelty-free reference standard of botulinum toxin can be facilitated.

Table 3 – Overview of the different roadblocks that prevent full replacement of the mouse bioassay for BoNT potency testing. Possible solutions for each roadblock are suggested.

The validation process can also be complicated by the innate variability of the MBA. The CBAs need to be validated against the current 'golden standard' MBA, even though the mouse model is known to show different LD50 values for the same BoNT product at different laboratories (Bitz, 2010). This can be attributed to a major limitation of the MBA and LD50 testing in general, which is the fact that death the is only outcome of the assay (Bitz, 2010). Even though it is assumed all deaths are caused by asphyxiation, mice often die of dehydration or starvation during the MBA because they are unable to move and feed themselves due to muscle paralysis (Pickett, 2011). The validation of the alternative CBA test is troubled if the results between the models do not align because the observed lethality in the MBA does not reflect the actual potency of the BoNT.

Regulatory Roadblocks

On a regulatory level, there are additional roadblocks which further complicate the validation process of alternative tests. The Ph. Eur. does not contain any detailed information on how to conduct the accepted alternative tests, and manufacturers have essentially been told to 'figure it out' on their own (Taylor, 2019). The US FDA however, presented a roadmap in 2023 in which BoNT testing is directly addressed. Their plans include the technical characterization and validation of a 3D-neuromuscular junction chip model for BoNT potency testing, complete replacement of animal testing in the FDAs network for testing foodborne cases of Botulism and supporting the development of regulatory guidelines in the biopharmaceutical industry to accelerate the use of alternative tests in industry.[8] The EDQM has not presented similar plans to support the replacement of animal testing for BoNT potency testing in Europe.[9] Additionally, though BoNT manufacturers are supposedly 'strongly encouraged' by the EU to adopt these alternative tests, there have been no repercussions for continued use of the mouse model since the Ph. Eur. was last updated in 2012 (Taylor, 2019). Lastly, skepticism for some regulators towards the alternative CBA has confounded the validation process, especially considering the need to receive market authorizations in each separate European country (Taylor, 2019).

Efforts to push regulators into limiting the use of the MBA for cosmetic BoNT products have seen little success due to the legal grey area between medicine and cosmetic that these products occupy (Taylor, 2019). This legal loophole is largely contributed to by off-label prescription of BoNT products. In the United Kingdom, animal rights organization Cruelty Free International had started two Judicial Review proceedings against the UK government for their authorization of the MBA. In the license agreement for MBA-testing in contract testing facility Wickham Labs, it was stated that the MBA may only be performed "for medicinal products". Cruelty Free International argued that according to this license, the products tested with the MBA may not be used for cosmetic purposes, yet the BoNT products still ended up in cosmetic clinics (Taylor, 2019). The UK government admitted it was merely checking if the products tested using the mouse model were authorized for medical use and did not confirm that the products were being used for vanity reasons (Taylor, 2019). Like many European countries, the UK considers facial wrinkles that have 'an important psychological impact', to be a medical skin disorder (Taylor, 2019).[2] Since the manufacturer has no impact on how cosmetic doctors

prescribe the BoNT treatment however, they can simply assume that the BoNT product is used according to medical guidelines to remedy the 'important psychological impact', rather than for off-label vanity reasons. The judge ruled that the MBA should indeed not be permitted to be used for BoNT batches that end up being sold as cosmetic treatments but determined that the UK government was doing as much as they could to prevent this (Taylor, 2019).

Continued Use of the MBA

Even though the use of CBAs has been approved for batch potency for the three major BoNT manufacturers, none of them have been able to achieve full replacement of the MBA. The mouse model is still used for bulk BoNT potency testing, stability tests and for tests requested by country-specific regulatory agencies (Taylor, 2019). The Ph. Eur. states:

"*For alternative replacement methods the potency is calculated with respect to a suitable reference preparation calibrated in mouse LD50 units".*

Therefore, use of the MBA to obtain reference standards using previously tested products is still required. Since each company is testing products with different potencies using different adaptations of the CBA, there is currently no universal reference standard (Adler et al., 2010; Taylor, 2019). BoNT bulk potency tests and stability tests, for which the MBA is still used, require the use of this MBA-calibrated reference standard as well (Taylor, 2019).

In 2019, even though all three major European BoNT manufacturers had received approval for their product-specific validated CBAs, the ECEAE found that use of mice for BoNT testing had actually increased during that decade (Taylor, 2019). The possible explanations they gave included problems with the validation process and the requirement for the MBA-calibrated reference standard (Taylor, 2019). Furthermore, growth of the BoNT production throughput and new manufacturers entering the European market could also contribute to this increase in animal use. In 2018, Japanese manufacturer Eisai sold the development and marketing rights of their BoNT-B product Neurobloc® to the American company Sloan Pharma.[10] Even though Eisai appeared to have been in the process of validating their own CBA, Sloan Pharma opted to continue using the MBA in 2019, receiving a license to perform the LD50 test on 46,800 mice in Germany (Taylor, 2019).[5]

It is not clear to which extent the number of mice used in MBAs for BoNT has decreased in the last few years since the report of the ECEAE in 2019. Manufacturers are still as secretive as always about their animal use, and annual animal testing reports from the EU and specific countries often bundle data on BoNT batch potency tests with other potency tests, such as ones for veterinarian and human vaccines (Taylor, 2019).[11] Furthermore, batch release requirements for BoNT products are not publicly disclosed by country-specific regulatory bodies, meaning it is difficult to determine to what extent the MBA has been replaced for specific products. In February of 2024 however, Animal right organization 'Focus on severe suffering' found that there had been an 89% decrease in the number of severe animal tests performed in Ireland between 2013 and 2022.[12] Allergan is known to have performed their MBA tests in Ireland, and the large volume of that

companies BoNT product sales contributed massively to the total number of animal tests in Ireland (Taylor, 2019). Indeed, the European Coalition to End Animal Experiments estimated that Allergan's BoNT potency testing may have singlehandedly been responsible for close to 74% of all animal tests performed in Ireland (Taylor, 2019). It can be assumed that this large drop in severe animal tests in Ireland is the result of Allergan slowly phasing out their use of the MBA over the course of the last ten years.[12] The ECEAE however, stated that in 2021, 82,000 mice were killed in Ireland for BoNT testing, while BoNT tests on another 22,420 mice were approved in Germany.[9] Their statement also highlighted that the EDQM has not revealed any new plans to support the further replacement of the MBA in BoNT potency testing. All in all, a lack of transparency on LD50 testing from manufacturers, regulatory bodies and governments alike has obfuscated the progress on MBA replacement for BoNT potency testing. Instead, patients and consumers are reliant on animal rights organizations to directly question and pressure the manufacturers and regulatory bodies to provide information on their efforts in replacing the mouse model (Taylor, 2019).

BoNT Treatments in the Netherlands

BoNT-A injections, both for therapeutic and cosmetic end-goals, are classified in the Netherlands as medicines.[2] This is in line with European legislation, as only products that are applied to the skin like make-up, deodorant, shampoo, skin creams and toothpaste are classified as cosmetics.[2,13] Animal tests for the development or testing of cosmetics have been prohibited in the Netherlands since 1997. In the same year, the Dutch government prohibited the general use of LD50 tests.[14] Only if there are no available alternative tests can an exception be requested, which has led to use of LD50 tests being limited to studies such as environmental toxicity studies using fish.[15] As the Ph. Eur. has stated multiple alternatives since 2005, it is highly likely no BoNT potency testing using the MBA has been performed in the Netherlands in the past two decades. Though the Dutch government releases a public document containing figures on animal tests performed in the country each year, only the total number of performed quality control tests is shown, with no specific statistics for BoNT potency testing.[11] However, in 2014 the Dutch State Secretary for Economic Affairs did state that no LD50 tests are performed on mice in the Netherlands for BoNT.[15] This does not entail that all BoNT products that are authorized and distributed in the Netherlands are 'cruelty-free', as the products are tested in other European countries where LD50 testing is allowed (Taylor, 2019).

There are seven different BoNT-A formulations that have been approved in the Netherlands for therapeutic and cosmetic treatment (See Table 4).[16] These all originate from the three largest BoNT product manufacturers in Europe: Ipsen, Allergan and Merz, which have all adopted a CBA for their batch potency testing. It is not clear if adoption of the cell-based assay for batch potency testing is the reason why only products from these three manufacturers received market authorizations. It is important to note that Alluzience®, Azzalure®, Bocouture® and Vistabel® are exclusively approved for cosmetic applications.[16] Additionally, the way these are marketed by the manufacturers heavily implies they are intended

for vanity use. Terms such as 'confidence' and 'grace' are commonly used in promotional material for these cosmetic BoNT formulations.[17]

Product	NL Approved Uses	Manufacturer	MBA usage
Alluzience®	Moderate to severe glabellar lines while frowning	Ipsen (France)	CBA approved in 2018, MBA still used for bulk and stability testing
Azzulure®	Moderate to severe glabellar lines while frowning, crow's feet* while smiling	Developed by Ipsen, manufactured, and distributed by Galderma Benelux (Subdivision of Galderma, Switzerland)	Same as Alluzience®
Bocouture®	Moderate to severe lateral frown lines,	Merz (Germany)	CBA approved in 2015, MBA still used for bulk and stability testing
Botox®	Blepharospasms, hemifacial spasms, cervical dystonia, focal spasticity, chronic migraine, overactive bladder, excessive sweating	Abbvie (United States), originally Allergan (Ireland) before acquisition by Abbvie.	CBA approved in 2011, MBA still used for bulk and stability testing
Dysport®	Blepharospasms, hemifacial spasms, cervical dystonia, focal spasticity, excessive sweating	Ipsen (France) Dysport was originally developed by Speywood (United Kingdom) before acquisition by Ipsen.	Same as Alluzience®
Vistabel®	Moderate to severe glabellar lines while frowning, crow's feet* while smiling, lateral frown lines	Abbvie (United States), originally Allergan (Ireland) before acquisition by Abbvie.	Same as Botox®
Xeomin®	Blepharospasms, hemifacial spasms, cervical dystonia, focal spasticity, chronic sialorrhea	Merz (Germany)	Same as Bocouture®

Table 4 *– Overview of each market approved BoNT-A formulation in the Netherlands and the applications for which they were approved. *Crow's feet are laugh lines that appear around the outer part of the eye sockets while smiling or laughing.*

In the Netherlands, BoNT injections for cosmetic end goals may only be performed by registered medical doctors that can properly administer BoNT.[2] These are often plastic surgeons or doctors at cosmetic clinics. Additionally, cosmetic BoNT treatments are only authorized for skin wrinkles if they have an 'important psychological impact' on the patient and only if the patient is between the ages of 18 and 65.[16] In this specific case, the facial wrinkles are considered to be a medical 'skin-related disorder'. In reality, the doctor prescribing the treatment will often be the owner of a cosmetic clinic. Considering none of the cosmetic BoNT products are covered by healthcare insurance, the patient will be paying out of pocket and money will go directly to the cosmetic clinic.[18] This results in BoNT products commonly being prescribed 'off-label', allowing them to be utilized for treatments they were not authorized for. Simply by signing a consent form about the potential risks of using BoNT injections to treat facial wrinkles, anyone who wishes to undergo cosmetic treatment for any superficial reason is able to do so.[19] The ease

of receiving these treatments may be responsible for the drastic increase in the number of cosmetic clinics providing BoNT injections in the Netherlands in the past fifteen years.[20] This growth has put a significant strain on healthcare regulators, resulting in cases where incompetent doctors perform the BoNT injections and unauthorized BoNT products from outside the European market are used.[20] Considering the fact that the quality control of these products may still be entirely performed using the MBA, off-label prescription of these foreign BoNT products also poses further ethical concerns. Though the competency of personnel performing cosmetic BoNT treatments has been more strictly regulated since 2021, off-label prescription of BoNT for vanity reasons remains a widespread occurrence.[21]

Possibilities for Legislative Change

To further prevent the use of animal-tested BoNT products in the Netherlands, it is important to consider the impact previous bans on animal testing had. The Dutch ban on LD50 tests simply led to companies performing these tests in other European countries where these tests are allowed, such as the United Kingdom, Belgium, and Germany (Taylor, 2019). BoNT products still end up being sold in the Netherlands, even though the cruel potency tests that were performed for them would not be allowed there.[15] It is not public knowledge if Dutch drug regulators ask these companies for LD50 data when providing market authorization for their products. It is known however that all market authorized BoNT products in the Netherlands are manufactured by companies who have at least partially replaced the MBA in the past fifteen years.[16]

Another relevant ban is the 2013 EU ban on all animal tests for the development and safety testing for cosmetics and the restriction of all trade in cosmetic products that were tested on animals.[13] This strict, wide approach was successful in preventing about 50,000 animal tests annually, as the trade ban meant cosmetic manufacturers could not simply relocate their animal testing to another country.[22] The ban was not a perfect solution, as individual ingredients could still be tested on animals if they were also used in other non-cosmetic products.[22] Nevertheless, this ban can be considered a great step towards the replacement of animal tests for consumer products.[22]

The effects of the Dutch ban on LD50 testing and the European ban on animal testing for cosmetics highlight the need for EU-level legislation on animal testing for cosmetic BoNT products. Products like Alluzience®, Azzulure®, Bocouture® and Vistabel® are clearly marketed for 'aesthetic' purposes yet are still given the green light for animal testing due to fringe medical applications.[16,17,23] An update to the Ph. Eur. removing the requirement for MBA-calibrated reference standards is necessary to finalize the replacement of the cruel mouse model. As long as animal tests for BoNT products remain, the off-label prescription of these should not be permitted. After all, the MBA is only allowed for potency tests because of the genuine medical applications of BoNT.[2,16] In reality however, over half of BoNT sales are likely due to cosmetic treatments and it is highly doubtful all these treatments are to remedy an 'important psychological impact' on the customer (Taylor, 2019). To ensure that only patients with genuine psychological problems receive cosmetic BoNT treatments, doctors or physicians with no affiliation with

the cosmetic clinics performing the procedures should be responsible for prescribing these treatments.

Conclusion

The MBA should not be regarded as the ‘golden standard’ for potency testing anymore. The tests inflict great suffering on the mice while being notoriously unreliable. High variability of the MBA has complicated the validation process of alternative models, which have proven themselves to be just as sensitive and even more reliable than the mouse model when properly adapted and validated for specific BoNT products. The Cell-based models now incorporate all crucial steps of BoNT toxicity: cell entry, release of the proteolytic light chain, cleavage of the SNAP-25 protein and the inhibition of acetylcholine neurotransmitter release. The three major BoNT manufacturers have made significant progress to fully replace the mouse model, but a lack of transparency has made it difficult to determine how many mice are being saved. To achieve full replacement of the MBA, the Ph. Eur. should no longer state that the reference standard for BoNT potency tests should be calibrated using an LD50 mouse test. Furthermore, the regulatory guidelines need to be stricter across the EU and co-operation between regulatory bodies and BoNT manufacturers is required to ensure those who have not adopted a cell-based assay yet do so in a reasonable time. As long as the manufacturing process of all BoNT products, both for medical and cosmetic treatments, involves any amount of animal testing, off-label prescription for vanity reasons should not be permitted if the harm:benefit assessment for animal tests is to be taken seriously.

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