Chapter 2
Botulinum Toxin as a Clinical Product: Manufacture and Pharmacology

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Abstract The use of botulinum neurotoxins as clinical products has required the development of suitable manufacturing processes meeting the highest standards of safety and quality for pharmaceutical products. The exceptional potency of the molecule has imposed safety requirements not just for the products but also for the staff and working environments during manufacture. There are now a number of clinical products available, produced using different manufacturing processes and with different final formulations. These differences and their implications are reviewed in this chapter. Future developments in the manufacture of clinical neurotoxin products are also considered.

Keywords Botulinum neurotoxin · cGMP · Drug substance · Drug product · Formulation · Containment · Pharmacology

2.1 Introduction

Botulinum neurotoxin (BoNT) has become widely accepted as a major, first-line treatment for a number of debilitating conditions that previously received no adequate therapy [1]–[3]. From the initial pioneering work of Alan Scott in ophthalmology [4], the use was expanded and extended into neurological conditions where muscles were in spasm [5], [6]. In the 20 years since the first licensed products became available—Oculinum® in the USA and Dysport® in the UK—BoNT has found established uses not just in neurology but in rehabilitation, urology, pain, headache, hyperhidrosis and aesthetic treatments [7]–[14], together with a wide range of new, experimental applications (see, for example, [15], [16] and Chaps. 3–6 of this book). In the majority of these indications, the products have provided long-term, stable results for patients [17]–[19].

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Key to this predictable, long-term use, in many cases for the full duration of product availability over the decades, the provision of consistent, high-quality, reproducibly potent products has been essential [20]–[22]. Without this quality of product, clinicians could not have confidence that patients would have repeat, long-term therapy. Yet surprisingly, little has been published about how the BoNT products are manufactured, and data to show their consistency are scarce.

There are currently several manufacturers of type A BoNT for clinical use. Allergan, based in Irvine, CA, and Westport, Ireland, are the main manufacturers of the leading Botox®/Botox® Cosmetic/Vistabel®/Vistabex®/Vista® range. Ipsen, based in France and Wrexham, UK, are the second largest with the brands Dysport® and Azzalure® (an early brand name Reloxin® intended for aesthetic use in the USA [23] was finally not allowed by the US Food and Drug Administration, FDA). Merz, from Germany, have the Xeomin® and Bocouture® brands. Three other manufacturers are based in Asia. Medy-Tox, in Korea, have the Meditoxin®/Neuronox®/Siax® brand of type A BoNT which started in Korea and is now becoming available in other countries, for example, in Latin America. Hugel Inc., also based in Korea, have a further type A BoNT called Botulax (also Zentox or Regenox in other countries). Lanzhou Institute for Biological Products (LIBP), in Lanzhou, China, has produced a licensed type A BoNT available since 1997 called BTXA™ which is distributed by Hugh Source, in Hong Kong, and by other local/regional companies under different names such as Lantox in Russia; Lanzox in Indonesia; Prosigne in Brazil; Liftox in Ecuador; and Redux in Peru—in total, available in over 30 countries.

One serotype B BoNT has been available for a decade, called Myobloc®/Neurobloc®. The product was originally developed by Athena Neurosciences in the USA, subsequently acquired by Elan, then Solstice NeuroSciences LLC, now acquired in August 2010 by US WorldMeds based in Kentucky, USA. In Europe, the product is marketed by Eisai Europe Limited. Initially, the product was targeted to patients who had developed an antibody response to a type A product and so could be treated with type B BoNT since the two serotypes are immunologically distinct [24]. Follow-up treatment with immunoresistant patients has limited success in the long term [25]. Clinical data exist for licensed use of the product in cervical dystonia [26]–[28], but other clinical uses have been examined [25], [29]. However, large doses of the product are required, perhaps 50 times that for a type A product due to a lower potency per unit of toxin protein. Patients initially responded to this alternative but soon developed further antibodies to the type B molecules and became refractive to further therapy. The treatments were short-lived. Attempts have been made to revive the product as, for example, an aesthetic treatment [30]–[32] but the product is never used in routine aesthetic practice, despite these data. Of particular relevance to use of this serotype, recent data on receptor binding have shown that human synaptotagmin II, as used by type B BoNT, does not have a high affinity for the molecule [33]. This phenomenon is specific to humans and chimpanzees and potentially explains why high doses of type B BoNT are required to achieve therapeutic effects.

The present chapter describes how BoNT products are made for clinical use and the data available on those products, focusing on the three main BoNT-A families since these are the most prominent products in the marketplace today. The processes

and their constraints are considered and how these link together is discussed. The pharmacology of the products is also discussed, especially with reference to actual clinical uses.

The mechanism of action of BoNT is described in detail elsewhere in this book (see Chaps. 2 and 6–8 of the companion volume to this book, KA Foster (ed) *Molecular Aspects of Botulinum Neurotoxin*, Springer, New York), and so is not included here. Also, clinical data are not considered in this section, unless applicable to the discussion.

2.2 The Two Stages of Manufacture

The manufacture of clinical BoNT is carried out in two, clearly distinct stages. All stages and all testing are performed in accordance with current good manufacturing practice (cGMP), which is a similar but not identical set of standards and requirements laid down by the regulatory authorities in each country and/or each region of the world. These standards will not be reviewed here, they are too extensive; the reader is referred to the freely available information of the guides and regulations on the web sites of the FDA and the European Medicines Evaluation Agency (EMEA) as typical examples of the stringency of manufacture and testing that are required.

2.2.1 Bulk Active Toxin: Drug Substance

The first stage is the production of the active BoNT in what are often described as 'bulk quantities'. In reality, exceptionally large amounts of highly potent active BoNT can be made in small, almost laboratory-scale equipment, unlike the type of quantities required for other active biomolecules. This involves cultivation of the production strain, initial separation of the crude BoNT, purification of the BoNT as either a complex or purified neurotoxin and final storage. Quality control (QC) testing of this material against a fixed and regulatory-approved specification is then carried out; in-process QC testing is also a stringent requirement. The resultant concentrated BoNT is called bulk toxin, bulk active substance, or, in the current modern terminology of the regulatory agencies, the active pharmaceutical ingredient (API) or the drug substance (DS). This multiplicity of terms is not so helpful; DS will be used here.

2.2.2 Finished Product Vials: Drug Product

After testing is satisfactorily completed and release of the DS for onward processing is approved by the quality functions in the company, very small volumes are used for dilution, formulation with excipients, dispensing into vials and then preservation by either freeze drying or vacuum drying or just as a liquid, dependent on the product family. The dilution factor may be 10,000 times or greater [34]. The output is called either finished product or, in current terms, drug product (DP).

2.2.3 Quality Testing and Release to Market

In certain cases, the regulatory authority of a country where a product is approved may require separate testing and release of the DS to be carried out, either by their own laboratories or by a designated and approved contract testing house, before any DP is manufactured. This can even be on an individual batch basis. In turn, each batch of DP may be individually released by the licensing authority of a country before that batch can be commercialised within the country. Within Europe, one control laboratory, designated an Official Medicines Control Laboratory (OMCL), will carry out formal testing and release on behalf of all European countries in accordance with the Official Control Authority Batch Release (OCABR) guidelines [35]. A fee is levied, currently about 2,300 € (US $ 3,200), against the manufacturer for the testing and release of each batch carried out together with the subsequent issue of a release certificate; but for the fee, time constraints are placed on the OMCL for release purposes. The OCABR for Human Biologicals is, in fact, a specific network within the OMCL network [36]. The FDA carry out similar testing for the products destined for the USA, in compliance with the Code of Federal Regulations (which define the cGMP requirements) 21CFR610 2(b), but no fee is levied. For other specific countries where products may be approved for sale, certification of testing and release from the home country of the manufacturer is required before the product batch is considered for commercialisation in the second country. For certain countries, repeat testing of any batch of DP submitted for commercial sale will be carried out by the respective authorities in that country, as part of the marketing authorisation when granted.

In most cases, the release authorities in a country carry out testing in accordance with the licensed testing procedures. This may go to extremes where, for example, even specific strains of mice are imported for the potency release testing of BoNT products. However, there are specific examples where a release authority may perform their own tests or even experimental tests as part of their release procedure. Those authorities maintain a rigid right to independent testing, to the extent that even the format and results from such tests are not revealed to the manufacturer. This has often been considered as unreasonable by manufacturers (and even other release authorities) to the extent that debate has often occurred on the reasonableness of such an approach. Changes have not, however, been brought about.

2.3 The Production Organism

2.3.1 Key Aspects

Clostridium botulinum is an obligate, spore-forming anaerobe, a gram-positive bacillus that is ubiquitous in nature. The nature of the bacterium leads to three distinct aspects that must be dealt with for the production of clinical material to occur successfully.

Firstly, the anaerobic requirements mean that oxygen must be excluded from the first stages of the production system, when the bacteria are grown in a fermenter or a glass vessel. A fermenter is the preferred usual choice in these cases, since this may be steam sterilised under pressure and fully validated in accordance with modern regulatory requirements and cGMP. The culture media employed generally include a reducing agent to aid this anaerobic requirement, and additional nitrogen may be used as an overlay. During growth, the bacteria produce several volatile acids that help to maintain an anaerobic environment and give the characteristic odour of a fermenting culture!

Secondly, the production of toxin progresses from the first stages of growth [37], only recently reported for a strain used in a clinical product process [38]. Suitable precautions to ensure that the culture fluids are appropriately contained and handled correctly, to prevent contamination of the environment or the operators, are essential. Gases evolving from the growth stages will build up pressure in the growth vessel and therefore must be filtered before release to prevent bacterial aerosols escaping.

Finally, sporulation of the bacteria can occur at low levels during the growth stages, but particularly in abundance as the bacterial life cycle ends and the bacteria die. A high spore count in the final stages of the fermentation is inevitable [39]–[41]. Non-sporulating mutants of type A or B strains have not been reported but new mutants with reduced sporulation have recently been generated; these also have reduced BoNT production [42], indicating the two processes may be linked genetically. The absence of spores has been reported for one production strain, but this may simply be due to the fermentation conditions, including the medium, employed [43]. Stages therefore need to be added to the manufacturing process to ensure that no spores are present in the final active BoNT DS used for clinical product, usually by filtration. The spores are highly resistant to heat and radiation and the use of these techniques is not possible.

The nutritional growth requirements of *C. botulinum* are not known in detail. Perhaps one of the most comprehensive reviews of growth and toxin production was described in a series of papers in the late 1950s and early 1960s by Bonventre and Kemp [37], [44]–[46]. Complex growth media are therefore used. As these can originate from plant and/or animal sources, the provenance must be established before use. Only certified sources of animal materials that are free from adventitious agents must be used. Even plant material derivatives can be processed by the suppliers using enzymes of animal origin and so careful investigation of the sources and the processes used for the materials is essential before purchase. One BoNT manufacturing process described for clinical material, essentially the original Botox® process, has used no materials of animal origin [47]. Inevitably, BoNT product manufacturers closely guard the finer details of their processes, especially production conditions used, as their intellectual property and know-how. There is certainly evidence to suggest that the process described by Schantz and Johnson [47] has now changed [48].

2.3.2 Strains Used

The type A BoNT production strains are all from the original collections of Professor Ivan Clifford Hall, a distinguished and enigmatic anaerobe specialist and epidemiologist of botulism food poisoning, amongst several of his specialities. Hall worked in the first half of the 1900s and collected many strains of *C. botulinum* from all over the USA. For an unknown reason(s), many BoNT type A production strains became known as 'the Hall strain', which is inaccurate [49]. Hall was known to have several type A strains in his collection, even in the earliest days of his work [50]. Despite this, one manufacturer has recently stated that type A products are from the same strain [51], which is again inaccurate [20]. In fact, recent genetic analysis of type A strains, including clinical product strains, has shown them to be clearly distinct from each other [52].

Detailed genetic analysis of the neurotoxin gene within the Allergan production strain has been published [53], [54] and complete genome sequencing of another strain, designated ATCC 3502 and as used by Merz [51], has been available for several years [55]. The exact strains used by Ipsen and MedyTox have not been published, other than being described as 'a Hall strain' [34], [56]. The neurotoxin sequence of the strain used for the production of BTXA by Lanzhou Institute for Biological Products has also been published [57].

The BoNT type B production strain used is designated the Bean strain. The production method was originally described in detail by Siegel and Metzger [58], which was updated for the clinical product by Setler 20 years later [59].

To date, only BoNT serotypes A and B have been made available for clinical uses. Other serotypes have been tested in man [60]–[64], but none have yet become commercial products and have progressed no further than proof-of-principle tests in man.

2.4 The Critical Mixture of Safety and Good Manufacturing Practice

There are two main challenges that override the manufacturing processes for clinical BoNT production.

The keyword to drive the processes is 'containment'. Due to the high potency of BoNT and the sporulation abilities of the production organism, a detailed but risk-based approach to safe operation must be taken to protect both the operators and the environment. The production environments will normally be high-grade rooms with special air-handling systems and these must not become contaminated or lengthy decontamination procedures will need to be followed that ensure (and are validated to ensure) the elimination of any spores, bacteria and BoNT—all of which must be considered separately. Only limited data are available on, for example, the persistence of BoNT after decontamination of surfaces [65]. Information on typical methods of decontamination by formaldehyde or other fumigants is also available and useful [66], [67].

Coupled with the safety aspects, manufacture must proceed in accordance with the cGMP requirements of the national or regional licensing authorities from whom marketing authorisations are sought for the products. These are laid down in detail and few, if any, exceptions are granted by the authorities because of the nature of either the product or the organism. This is especially the case since the current manufacturers have been compliant with these standards for many years, firmly setting the quality goals that any new manufacturer must meet for market entry.

The safety and containment requirements for facilities handling human pathogens such as *C. botulinum* are defined in three documents which are widely available as the basis for the standards. These are from the World Health Organisation [68], the United States Health and Human Services (HHS; Center for Disease Control and Prevention, CDC) [69] and the United Kingdom Health and Safety Executive Advisory Committee on Dangerous Pathogens [70]. Each of these contains descriptions of the standards to be applied. Within each country, different organisations will also be involved in the legislative and standards process—for example, CDC is the branch of the HHS responsible for both *C. botulinum* and, separately, for work with BoNT, including handling, storage and shipping. Amounts of BoNT up to 0.5 mg are not regulated under current US legislation (see Code of Federal Regulations 42CFR73.3 ((d)(3))).

The *C. botulinum* organism is currently considered in somewhat different ways by different countries. In the USA, BoNT (the toxin and not the producing organism) is one of six Category A Bioterrorism Agents defined by CDC. The definition of such an agent is as follows [71]:

High-priority agents include organisms that pose a risk to national security because they

- *Can be easily disseminated or transmitted from person to person;*
- *Result in high mortality rates and have the potential for major public health impact;*
- *Might cause public panic and social disruption and*
- *Require special action for public health preparedness.*

Both BoNT and the bacterium are however included on the select agent list in the USA [72] and in 42CFR 73.3. A detailed publication on what is required for the security of such agents has been prepared by both CDC and the US Department of Agriculture [73] and describes all aspects of a facility security policy and procedure, from personnel access to chain of custody requirements. These are additional to the other biosecurity measures that have to be taken for safe handling and processing, as described earlier.

In the UK, *C. botulinum* (*not* BoNT) is defined as a hazard group 2 biological agent whereas, in contrast, *Bacillus anthracis* is in hazard group 3 [74]. The list also indicates that a vaccine is available, but vaccination of individuals involved with the organism also has potential risks associated with the vaccine itself. In fact, the only commonly available vaccine has been withdrawn from use mainly due to efficacy issues [75]. New generation BoNT vaccines are being actively developed [76], [77]. Detailed risk assessments on vaccination are therefore called for before

its adoption as a standard procedure in the production environment. Availability of a suitable vaccine remains a key issue if any company risk assessments indicate that vaccination is a vital part of their production strategies.

To meet these dual requirements, manufacturers generally can adopt two approaches. Firstly, mainly due to the significant quantities of BoNT being handled and the high concentrations of the production organisms, which are quite unlike those found in a normal pathology or microbiology environment such as a diagnostic laboratory, manufacturers can uprate the pathogen-handling categorisation for their facilities to the next level or higher. This enables a high degree of re-assurance that handling of the organism and toxin is under the strictest containment procedures. Secondly, an increase in the categorisation means that the work should be performed in class III microbiological safety cabinets, which are fully contained, negative pressure units with glove-port access only (Fig. 2.1). The exhaust air from these cabinets is then high-efficiency particulate arrestant (HEPA) filtered through monitored and tested specific grades of filter specifically for environmental protection. Occasionally, pictures of these production cabinets from manufacturers are shown at presentations in symposia or in their product literature, but, apart from these, nothing else is published about the technology involved.

2.5 The Production Processes

There are many references to procedures and processes for the manufacture of BoNT, covering several decades (see, for example, [47], [78]–[81]), but until the 1990s, these of course did not focus on (or even envisage) any clinical uses and hence clinical quality of product. Nevertheless, these original processes formed the basis for clinical product manufacture that subsequently occurred.

2.5.1 Botox® Family

The only detailed publication that describes manufacture of clinical products is that of Schantz and Johnson, in the early 1990s [47]. As the originators of Oculinum® (later to become Botox®), they published in detail the exact process used for the manufacture of DS at that time (Fig. 2.2), which, as described, was based on the original method of Duff and colleagues [82], [83]. In particular, the method described was that used for DS batch 79-11, made in November 1979. This batch was critical in the clinical trials for the product and, subsequently, for the launch of commercial product. The batch lasted for many years after manufacture and was distributed as Botox® by Allergan until 1997 [84] (approval by FDA of alternative DS) or later in other markets outside the USA. This was despite the fact that batch 79-11 was reported as losing potency over the nearly 20 years since manufacture [47]! The process was ‘classical’, involving the original first stage of BoNT precipitation with an acidification step, followed by several stages with salt solution, ethanol precipitation

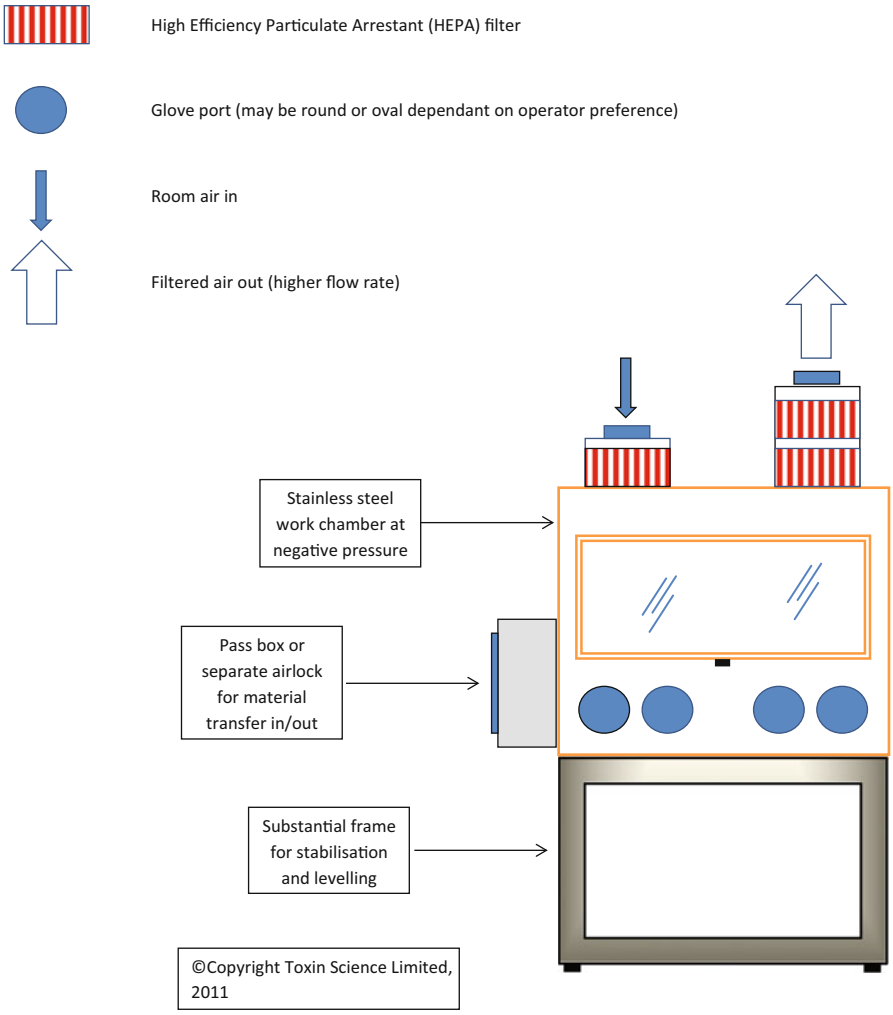


Fig. 2.1 Schematic drawing of class III microbiological safety cabinet used for manipulation of high-potency/volume BoNT-containing materials

and crystallisation in the presence of ammonium sulphate [47]. A clear specification for the DS was established (Table 2.1), as they described [47]. Interestingly, the specification included a measurement of the amount of residual nucleic acids present, as a proportion of the protein obtained (expressed as an A_{260}:A_{280} ratio), which indicated that these were considered contaminants at that time. The DP was stabilised with human serum albumin (HSA) in a formulation that was intended to be physiological in properties, with sodium chloride as the second component [47].

Some years later, in 2008, a further paper—this time published by Allergan—claimed that the method was still used to manufacture the Botox® DS [85], [86]. However, in between times, an inspection report of an Allergan-contracted

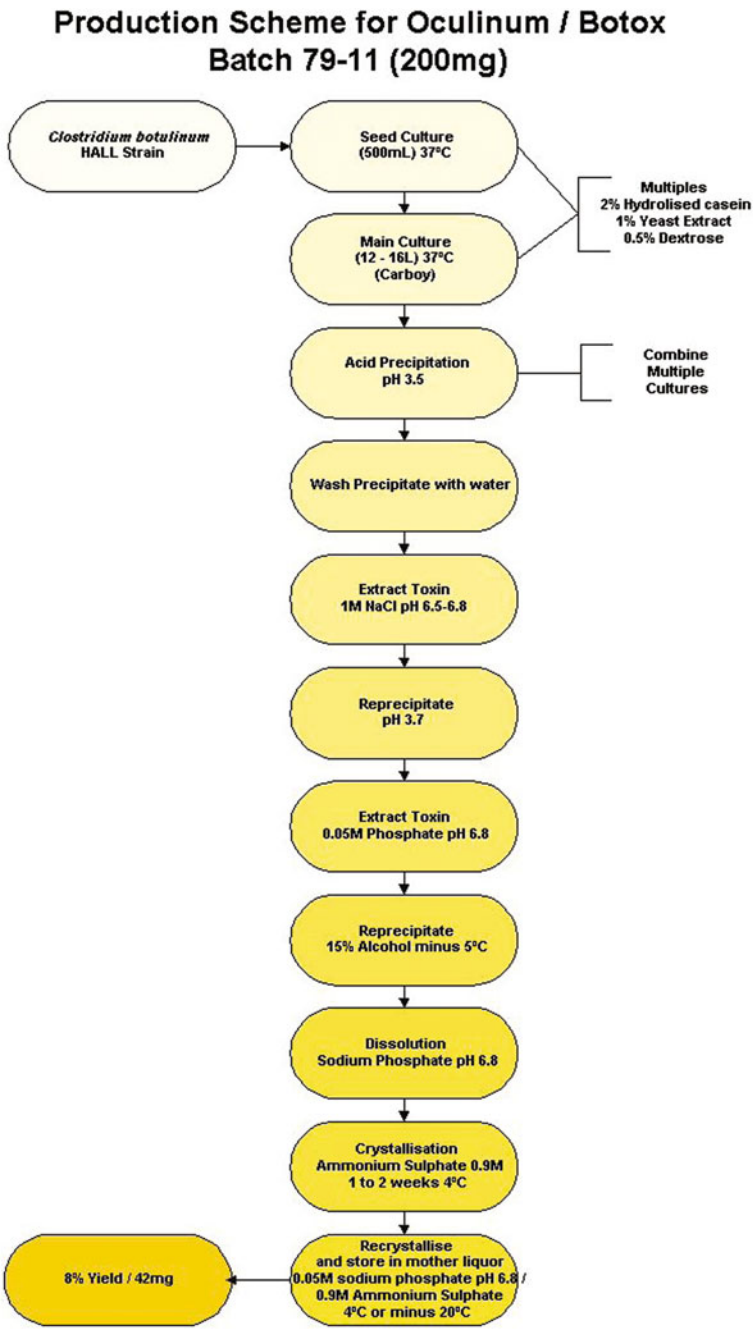


Fig. 2.2 Production scheme employed for manufacture of Oculinum®/Botox® batch 79–11, the original product first licensed in the USA in 1989. (Scheme based on details in reference [44])

Table 2.1 Drug substance (bulk active BoNT) specification for original Oculinum®/Botox® produced by Edward Schantz for initial clinical trials and subsequent product licensure. (Based on reference [47])

Product parameter	Specification
Strain	Hall
Absorbance (in 0.05 M sodium phosphate buffer pH 6.8)	Maximum at 278 nm
A260/A278 nm ratio	0.6 or less
Specific toxicity (mouse potency)	$3 \times 10^7 \pm 20$ % mouse LD_{50} per mg
Extinction coefficient (absorbance) 1 mg per ml toxin in 1 cm light path	1.65

establishment [87] indicated that the process had changed in various aspects from the original Schantz and Johnson [47] method and this was raised in the follow-up correspondence [48]. No direct response was received to these questions and the subject has not been addressed since. Although Allergan produced DS at this contractor for a time, the current production of DS is believed to take place in Irvine, Orange County, CA with finished DP originating from the Allergan Westport facility, in Ireland—as noted on the labelling in most countries for the product family.

2.5.2 Dysport® Family

Two papers have summarised how the Dysport® family of DP are manufactured [34], [88]. The process is described as 'precipitation, column chromatography and dialysis'. The use of column chromatography in the Dysport® DS process was in fact criticised by Schantz and Johnson in their main paper [47] as something that should be avoided, but the Dysport® experience has shown and demonstrated the validity of using such methods of production.

Many attempts have been made by authors in the past to cite a specific reference [80] as describing how the Dysport® family of products is manufactured. However, these attempts have been repeatedly criticised by the manufacturer (see, for example, [89]). The publication cited was nearly 10 years before Dysport® was licensed as a therapeutic product at the end of 1990. The errors in such claims have been detailed [89]. A second publication [81] has recently been cited as again describing the production process [51], but there are very few details therein, which only mention an eight-stage process with 46 in-process and quality control tests using a 30-litre stainless steel fermenter. There is no literature or other information to support if these data are accurate for the Dysport® process today.

These incorrect citations have, in turn, led to errors in published product-specific data, especially product characteristics, which has not been helpful to clinicians [89]. These errors have also included data on DP formulations [21]. Consequently, Pickett and Perrow published in 2010 [90] a definitive description of the current BoNT formulations available in the market, with DS characteristics included (Table 2.2). Regrettably, reviews are still being published, at the time of writing the present chapter, that include erroneous product data [21], [91] and which do not even cite the source of the data they used.

Table 2.2 Characteristics of current major BoNT products. (©2012 Toxin Science Limited. Reproduced with permission)

Product	Production strain	Process	U/vial [Product specific]	Excipients (in vial)
Dysport®	Hall	Fermentation Dialysis Chromatography	300/500 sU	HSA 125 ug Lactose 2.5mg
Azzalure®	Hall	Fermentation Dialysis Chromatography	125 sU	HSA 125 ug Lactose 2.5mg
Botox®	Allergan "hyper"	Fermentation Precipitation "Crystallisation"	100/200 B	HSA 500 ug NaCl 0.9mg
Vistabel® & Vistabex®	Allergan "hyper"	Fermentation Precipitation "Crystallisation"	50 V	HSA 500ug NaCl 0.9mg
Xeomin®	Hall	[Unpublished]	100/200 X	HSA 1mg Sucrose 5mg
Bocouture®	Hall	[Unpublished]	50 B	HSA 1mg Sucrose 5mg

2.5.3 Xeomin® Family

No publication exists to describe the manufacture of the Xeomin® product family. Even a recent publication, claiming in the title to consider manufacturing of the product, does not [92]. As for the facilities and equipment used, limited data have been presented in congresses on the manufacturing process and this seems to follow other processes used previously for purified BoNT. In addition, data included in patent applications by the scientists who developed the product have contributed towards the process data available [93]. This source is, however, somewhat restrictive and not mainstream for clinicians seeking further information on this product family. Repeated requests have been made for such data to be published and hence made available to the clinical community, but this has not yet happened.

2.5.4 Myobloc®/Neurobloc®

There are publications available which describe how the type B BoNT product is manufactured [59], [94], [95]. The process applied is similar to that for BoNT type A except that an additional stage of BoNT activation is required for full activity to be obtained. [59], [95]–[97]

2.6 Consistency and Quality of Products

For clinicians to be able to judge the merits of the different products available, clear and unequivocal product data need to be made readily available [21], [91]. Aspects such as consistency of products manufactured over time, stability, biochemical characteristics and potency are key to assist in the comparisons. Such data are, however, very limited for reasons that are not entirely clear.

Because of the product licensing procedures and the subsequent DP quality release procedures by the licensing authorities, consistency of final products should be inherent, or the products would not be available for marketing. These are facts. However, data should be available to the clinical community in order to judge product consistency for themselves.

2.6.1 The Initial Oculinum®/Botox® Issues

When Oculinum® was first licensed by the US FDA, all the product originated from one batch of bulk toxin [47]. This batch was labelled as 79-11 being made in November 1979. The batch lasted for many years of commercial production and sales, but was found to have significant issues about potency [47], [98]. Approximately 90 % of each finished product vial was found to be inactive BoNT, material that had been inactivated by the final stages of the manufacturing process for the vials [83], [98]. For patients, this meant that they were being given some ten times more BoNT-related protein than needed to achieve the therapeutic effect. As a consequence, many patients developed neutralising antibodies to BoNT protein, especially when being treated with the higher doses used for conditions such as cervical dystonia or stroke-related paralyses. This was recognised in the early 1990s [99]–[101]. In addition, the treatment regimens at that time often used so-called booster injections, where patients would be retreated after a short time interval (usually weeks) if their initial response was not felt to be sufficient [102]. Up to 17 % of patients with cervical dystonia were reported with these antibodies [103] and hence became refractory to BoNT therapy. These data have been revised downwards more recently, based upon long-term experience with the current Botox® product [104], [105]. Interestingly, this issue did not seem to occur as much with patients outside the USA [83] as another batch of Botox® DS was apparently used to supply those markets.

These issues relating to the product were reported by the originators of the BoNT DS [47], [83], but not by Allergan. The originators also identified ways in which the inactivation in the DP manufacturing process could be eliminated and proposed new formulations that might be considered which retained almost the entire potency [98].

In the event, Allergan chose to replace the DS batch with a new designated BCB-2024 [84]. New preclinical testing was carried out [84], followed by clinical studies in cervical dystonia. Initial comments were that the new DS batch was of significantly higher potency and caused side effects in patients, but later reports recorded equivalence in efficacy and safety profiles with the original [106]–[108]. FDA finally approved the new DS in 1997 [84]. There was no change in the formulation composition of the DP, but no details have ever been made available as to whether the actual DP process was changed or not, in line with the recommendations from the originators of the product [83], [98].

These initial issues with Botox®, effectively a significant inconsistency of the product to yield consistent clinical results over time, have not occurred again. Allergan have only published limited analytical data on the later and current DS materials used [48], [85], [86], but have offered no reassurances to the clinical community that such issues will not occur again. Allergan does, however, appear to continue to focus on the science related to neutralising antibody formation [109]–[111] and has recently published a meta-analysis on the use of Botox® in various clinical indications, which demonstrates an overall low level of antibody response to the product when used in various clinical applications [105]. Today, the number of patients developing neutralising antibodies to the product is no greater than for any of the other type A BoNT products [112].

2.6.2 Consistency of the Dysport® Product Family: The History of Protein Load

In 2003, Ipsen made available the first data on the consistency of Dysport® over time [113]. These data were published due to an argument that had developed between the manufacturers as to how much toxin-related protein (both neurotoxin and complex-related proteins) was there in a vial of each product. At that time, the Merz products were not available and the debate was between Dysport® and Botox®.

Because of the number of patients who had developed antibodies to early BoNT therapies, the subject of ‘protein load’ had emerged. Protein load was defined as the amount of all toxin-related protein per vial of product—both neurotoxin and complex-related proteins (but excluding the HSA excipient) and was represented in nanograms. Giving a patient the least possible amount of toxin-related protein was considered one way of minimising the potential for that patient to develop antibodies and hence become ‘resistant’ to treatment [114].

One early reference had cited a predecessor product to Dysport® as containing 12.5 nanograms protein load per vial [115]. Conversely, the data available for Botox® had indicated that this only contained 5 nanograms per vial [84], [108], [116]. The argument was established.

On review, the Stell reference [115] was found not to describe Dysport® and this was documented several times by Ipsen scientists [34], [117], [118]. In evidence, they produced in total four publications [34], [113], [117], [118], the last of which in 2008 [34] demonstrated a 15-year consistency of product data and only 4.35 nanograms of toxin-related protein per vial. On a per treatment basis (taking into account the potency unit differences), this meant that patients treated with Dysport® only received 40–50 % of the toxin-related protein that patients treated with Botox® were given. The 2008 data also demonstrated DS consistency over the life of the product in detail and reported biochemical properties including binding and enzymatic activity. Overall, the product was shown to be of high consistency and quality, with a number of DS batches being used throughout the product life cycle.

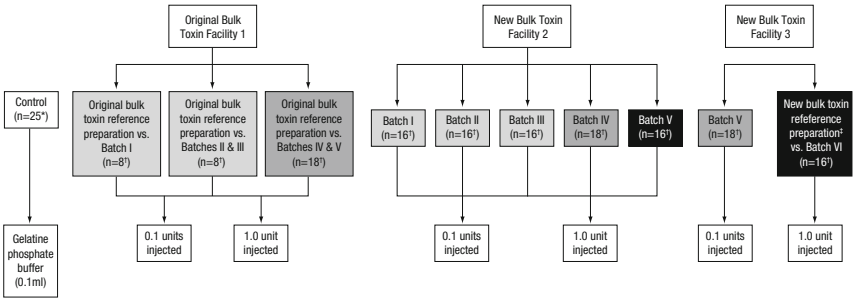
The consistency of Dysport® DP has also been demonstrated in a non-clinical model of muscle force in the rat (the rat muscle force (RMF) model) [119]–[121]. The model, based on one reported earlier [122] and subsequently developed further by Ipsen in conjunction with the University of Florida, is clinically relevant since muscle force is temporarily weakened in a dose-dependent manner using doses that are similar to those used in humans (on a per kilo basis). The data provided were reproducible and useful as measures of consistency (Fig. 2.3). The model was used to show consistency of Dysport® DS made in three different facilities. Unlike other non-clinical animal models that have been used for BoNT products, such as the digital abduction scoring (DAS) method [84], [123], the RMF method used a quantitative, non-subjective assessment of the effect of BoNT, in comparison with a placebo.

One further source of consistency data exists for the Dysport® family in the publically available review reports from the FDA which have been made available subsequent to product licence approval in the USA. In the clinical sections of these reports, data are provided of a trial in humans with cervical dystonia which used DS sourced from two different manufacturing sites (trial reference Y-97-52120-096 [124]). These are highly relevant consistency data since they originate from man. The data show clearly that no differences in clinical response were found using material from either source. Essentially, this demonstrates the robustness of the DS manufacturing process, since transfer between manufacturing sites can be a severe test of the reproducibility of any biological manufacturing process. An earlier description of biological product manufacture used to be ‘the process defines the product’, a short way of saying how process-dependent biological products used to be.

As Botox® was licensed in the USA many years prior to Dysport®, no such publically available data exist from FDA records. One publication has described the licensing process for Botox® [125] and the somewhat abbreviated dossier that was originally filed and approved.

2.6.3 The Xeomin® Family

No data exist in the public domain on the consistency of the Xeomin® product family.

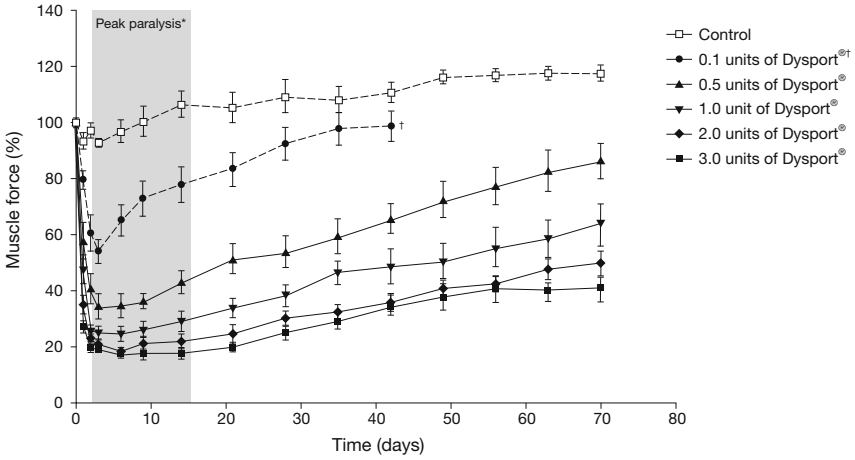


*Of the 25 rats given control material, eight rats were from Study 1, nine were from Study 2 and eight were from Study 3.

†50% of rats given each batch were injected with 0.1 units, the other 50% were injected with 1.0 unit.

‡The new reference preparation is the same as Batch V from Study 2, which was licensed for commercial use prior to commencement of Study 3.

a



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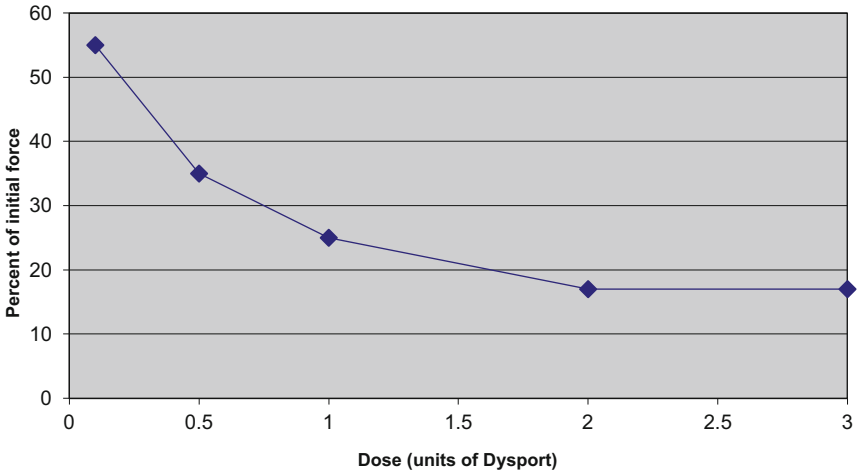

c

Fig. 2.3 Use of the rat muscle force (RMF) model for the assessment, characterisation and comparison of clinical batches of Dysport® botulinum toxin type A. **a** Detailed schematic diagram of how the product batches from different manufacturing sources were tested using the method.

A useful publication, providing detailed non-clinical data and characteristics, was made available early on in the product's life (when the product was still known by the Merz compound number NT201) [126]. This did not, however, provide DS or DP consistency data.

Unusually, one clinical paper exists which discusses what might be an early *inconsistency* of Xeomin® in clinical use [127], which was further tested by the reporters in additional clinical studies of glabellar line treatment. No continuing problems were identified and the original issues identified were not repeated.

2.7 Stability of BoNT Products

The subject of stability of the BoNT products has evolved since the products were first licensed. Table 2.3 shows the licensed storage conditions that are granted now, for the three main product families.

2.7.1 Shelf-Life Storage

Originally, Botox® had to be stored below − 5 °C whereas Dysport has always been maintained at refrigerator temperatures of 2–8 °C. Botox® now has a mixed set of storage conditions, either below − 5 °C or at 2–8 °C. Xeomin storage is below 25 °C and this has not changed since the product was first licensed. For biological products, the duration of shelf-life storage for Botox® and Dysport® has increased until the maximum of 3 years and 2 years, respectively. Of 67 biologics examined recently for length of shelf life, licensed over an 18-year period, none had a shelf life longer than 3 years [128].

Since first arriving on the market, Merz has made much of the elevated storage temperature of Xeomin® and has published storage stability data several times [129], [130]. In different publications (see, for example, [130]), storage for short periods at temperatures up to 60 °C has been reported. The stability of the product is excellent under such conditions. Why? The Xeomin® family has the highest concentration of HSA excipient per vial (Table 2.2), eight times that found in Dysport® and twice that of Botox®. HSA is an exceptionally stable human protein. The HSA-manufacturing process has always included a step of heating at 60 °C for 10 hours as one of the viral

b RMF generation results from a study to identify suitable doses for future studies in the model. The period of peak paralysis is represented by the band of shading. In this case, peak paralysis is defined according to specified acceptance criteria (days 2–15). The 0.1 unit dose was used to assess duration of effect. When muscle force generation returned to 100 %, no further measurements were made. **c** Dose–response relationship for Dysport® product batches tested in the RMF model. Peak paralysis response values highlighted in (b) were used. (Reprinted from Pickett A, O'Keeffe R, Judge A, Dodd S (2008) The in vivo rat muscle force model is a reliable and clinically relevant test of consistency among botulinum toxin preparations. Toxicon 52(3):455–464, Copyright (2008), with permission from Elsevier)

Table 2.3 Current storage conditions licensed for the different BoNT product families in major regions of the world. (Data taken directly from the official information provided by each regulatory authority web sites or official product web sites in each region)

Product[a]	EU	USA	Japan	Australia
Dysport® (300 and 500 Speywood units)	2 years at 2–8 °C	12 months at 2–8 °C; protect from light	N/A	2 years at 2–8 °C
Azzalure® (125 Speywood units)	2 years at 2–8 °C	N/A	N/A	N/A
Botox® (50, 100 and 200 units)	3 years at 2–8 °C or at/below −5 °C	3 years at 2–8 °C (100 units) or 2 years at 2–8 °C (200 units)	Below 5 °C (stability data for 50 and 100 unit sizes is provided for 2 years)	2 years at 2–8 °C (200 units) or 3 years (100 units)
Botox® Cosmetic (50 and 100 units)/Vistabel®/ Vistabex® (50 units)/Botox Vista® (50 units)	3 years at 2–8 °C (Vistabel®/ Vistabex®)	3 years at 2–8 °C (Botox® Cosmetic)	Below 5 °C (Botox Vista®)	N/A
Xeomin® (50 and 100 units)	4 years at 25 °C or below (100 units) 3 years at 25 °C or below (50 units)	Up to 3 years at room temperature 20–25 °C (68–77 °F), in a refrigerator at 2–8 °C (36–46 °F), or a freezer at − 20 to − 10°C (− 4 to 14 °F).	N/A	N/A
Bocouture®	3 years at less than or equal to 25 °C	N/A	N/A	N/A
Myobloc®/ Neurobloc®	3 years at 2–8 °C; protect from light	3 years at 2–8 °C; protect from light	N/A	N/A

N/A product not available in that country
[a]Not every product unit size is available in every country

inactivation stages used to provide assurance of freedom from human adventitious agents [131]. The higher HSA concentration in Xeomin® may, therefore, confer high temperature stability for the product, probably acting as a protecting protein or similar. No explanations have been forwarded by Merz for this effect. The standard storage conditions of the Xeomin® family, at less than 25 °C (perhaps less than 30 °C in some countries, such as Brazil), cover many shipping conditions, but in higher temperature climates and summer conditions this will be exceeded, necessitating shipping under controlled temperature conditions, as for the other BoNT products. The higher storage temperature for Xeomin® does mean that refrigeration is not required by most pharmacies for the product.

Certain of the newer low-unit formulations, such as Azzalure® or Vistabel®, have the shelf life of their parent. As each product has been submitted for licensing, individual stability data also have to be submitted to the regulatory authorities for their review and approval. In cases where the shelf life is shorter than the parent product, this is likely due to the new product only having stability data to support the initial shelf life at the time of licensing. This does not mean the new product is 'less stable' or of 'lower quality' than the parent and no such conclusions should be drawn. In due course, these shelf lives will be extended.

2.7.2 Reconstitution Stability

The stability of the BoNT products after reconstitution, using the recommended diluent of sterile 0.9 % sodium chloride, has also been an area of commercial competition. The post-reconstitution time is variable depending on the requirements of the individual licensing authorities. Details of several licensed conditions are shown in Table 2.4.

As more data have been generated by the manufacturers, they have been able to gain extensions to post-reconstitution storage conditions until now when the maximum post-reconstitution storage time has been reached in the various markets.

Clinicians regularly ask the manufacturers to supply information on reconstitution stability. In many cases, especially in the aesthetic world, a long reconstitution time is sought, beyond that stated in the standard supplied product information. The manufacturers have only been able to supply limited information, basically re-stating what conditions have been approved by the licensing authorities occasionally supplemented by small additional studies that they have performed to provide data if, for example, excursions in storage condition occur. Consequently, clinicians have performed numerous studies of their own to look at, for example, extended reconstitution times or even alternative diluents. These studies have provided valuable data for the clinical community and should be taken into account in any discussions relating to the individual products. A summary of such studies is provided in Tables 2.5 and 2.6.

These studies have, importantly, looked at maintenance of clinical effects after long-term reconstitution, together with the microbial content (that is, sterility) of the reconstituted product—one of the main risks, since the products are essentially a protein-rich medium (with their content of HSA) for microbial growth after reconstitution. Typically, the studies by Hexsel and co-workers on Dysport® [132] and Yang and colleagues on Botox® [133] have been significant contributors to data on reconstituted products.

Table 2.4 Current reconstitution storage conditions licensed for the different BoNT product families available in different regions of the world

Product	EU	USA	Japan	Australia
Dysport®	Up to 8 h at 2–8 °C following reconstitution	Up to 4 h at 2–8 °C following reconstitution; protect from light	N/A	Up to 8 h at 2–8 °C following reconstitution
Azzalure®	Up to 4 h at 2–8 °C following reconstitution	N/A	N/A	N/A
Botox®	Up to 24 h at 2–8 °C following reconstitution	Up to 24 h at 2–8 °C following reconstitution	Use immediately	Up to 24 h at 2–8 °C following reconstitution
Botox® Cosmetic/ Vistabel®/ Vistabex®/Botox Vista®	Up to 4 h at 2–8 °C following reconstitution (Vistabel®/ Vistabex®)	Up to 24 h at 2–8 °C following reconstitution (Botox® Cosmetic)	Use immediately (Botox Vista®)	N/A
Xeomin®	Up to 24 h at 2–8 °C following reconstitution	Up to 24 h at 2–8 °C following reconstitution	N/A	N/A
Bocouture®	Up to 24 h at 2–8 °C following reconstitution	N/A	N/A	N/A
Myobloc®/ Neurobloc®	If diluted, use immediately	If diluted, up to 4 h at 2–8 °C; protect from light	N/A	N/A

Reconstitution conditions often state that from a microbiological point of view, the product should be used immediately after reconstitution
N/A Product not available in that country

2.8 New Product Formulations

The current BoNT product formulations have existed, for the main products, for more than 20 years. In that time, they have changed in no respect. They have shown themselves to be stable (see above), convenient for use and excellent for patient treatments. The number of reports of patients developing adverse responses immediately or shortly after injection is limited, taking into account the usage of the products across the world now [134]. Only recently, with the advent of aesthetic uses, have alternative versions become available, but these have only reduced the number of BoNT units per vial and not altered the formulation in other ways. Higher potency preparations of Botox®, at 200 units/vial, have also been made available recently, but no similar increase for Dysport® has been marketed.

Table 2.5 Reconstitution stability studies (performed with non-clinical assessments) under various conditions for the different BoNT product families show a range of conditions and diluents tested over the past 20 years

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Gartlan, M. G. Hoffman, H. T.	1993	Crystalline preparation of botulinum toxin type A (Botox): degradation in potency with storage	Botox®	Otolaryngol. Head Neck Surg. 1993; 108 (2), 135–140	Normal saline	Measurement of mouse potency of product reconstituted, immediately frozen and stored for 2 weeks	. . . a 69.8 % loss in potency was found when Botox was reconstituted, immediately frozen, and then assayed 2 weeks later ($p < 0.0001$). Statistically significant degradation in potency was seen after refrigerator storage for 12 hours ($p = 0.007$), but not for 6 h ($p = 0.16$)
Jabor, M. A. Kaushik, R. Shayani, P. Ruiz-Razura, A. Smith, B. K. Morimoto, K. W. Cohen, B. E.	2003	Efficacy of reconstituted and stored botulinum toxin type A: an electrophysio-logic and visual study in the auricular muscle of the rabbit	Botox®	Plast. Reconstr. Surg. 2003; 111 (7): 2419–2426	Normal saline	Assessment of product either freshly reconstituted or stored frozen after reconstitution up to 12 weeks for potency, duration of action (by duration of nerve conduction effects) and microbial contamination	In conclusion, using the rabbit model, it seems that reconstituted and stored botulinum toxin type A has the same initial potency but the duration of action is affected sometime after 2 weeks of storage. No bacterial contamination was associated with storing unpreserved reconstituted botulinum toxin type A for up to 12 weeks

Table 2.5 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Hexsel, D. M. Alencar de Castro, I. Zechmeister, D. Almeida do Amaral, A.	2004	Re: Hexsel, et al. Multicenter, double-blind study of the efficacy of injections with botulinum toxin type A reconstituted up to six consecutive weeks before application	Botox®	Dermatol. Surg. 2004; 30 (5): 823	Normal saline	Brief report on microbiological analysis of vials stored for 14 & 15 weeks including after fully opening. No contamination detected in any vial	Nevertheless, it is recommended that all the precautions in diluting and storage of botulinum toxin type A be observed, especially after opening the vials
Alam, M. Yoo, S. S. Wrone, D. A. White, L. E. Kim, J. Y.	2006	Sterility assessment of multiple use botulinum A exotoxin vials: a prospective simulation	Botox®	J Am Acad Derm 2006; 55 (2): 272–275	Bacterio-static saline	Reconstitution of vials and then repeat use for up to 7 weeks. Total of 127 vials used. End vials tested microbiologically; no evidence of contamination	Routine refrigerator storage of medication vials containing reconstituted botulinum toxin does not result in microbial contamination of the contents even after serial re-extraction of solution from these vials, and after handling of such vials by multiple personnel. Storage and subsequent reuse of botulinum toxin appears safe for at least 7 weeks after reconstitution

Table 2.5 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Elmas, C. Ayhan, S. Tuncer, S. Erdogan, D. Calguner, E. Basterzi, Y. Gozil, R. Bahcelioglu, M.	2007	Effect of fresh and stored botulinum toxin A on muscle and nerve ultrastructure: an electron microscopic study	Botox®	Ann. Plast. Surg. 2007; 59 (3): 316–322	Normal saline	Study on rabbits injected with either saline, freshly reconstituted product or product reconstituted and stored at 4 °C for 2 weeks into anterior auricular muscles. Muscle and motor nerve examined 5 days or 12 weeks after injection	Alterations in muscle and nerve structures after botulinum toxin injection revealed that there is no significant difference between freshly reconstituted and stored toxin for 2 weeks, at the onset of effect. However, when stored toxin was used atrophic changes in the muscles has started to return earlier or it is less severe than the fresh toxin. . . . However, there is no significant difference between the effects of fresh and stored toxin on nerve ultrastructure at 3 months
Menon, J. Murray, A.	2007	Microbial growth in vials of botulinum toxin following use in clinic	Dysport®	Eye (Lond) 2007; 21 (7): 995–997	Unspecified (probably normal saline)	Microbiological assessment of vials reconstitutes and stored at room temperature for 4 h and after refrigeration for 5 days at 3–5 °C. No microbial contamination detected	This pilot study suggests that if aseptic precautions are followed during the use of botulinum toxin, the contents of the bottle remain sterile despite being exposed to room temperatures for up to 4 h

Normal saline is often referred to as preservative-free saline and bacteriostatic saline as preserved/preservative-containing saline (contains 0.9 % benzyl alcohol)

[a] Conclusions presented as verbatim quotations from publication

Table 2.6 Reconstitution stability studies (including clinical assessments), performed under various conditions for the different BoNT product families, show a range of conditions and diluents tested over the past 15 years

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Sloop, R. R. Cole, B. A. Escutin, R. O.	1997	Reconstituted botulinum toxin type A does not lose potency in humans if it is refrozen or refrigerated for 2 weeks before use	Botox®	Neurology. 1997; 48(1):249–53	Normal saline	Measure of the decline in extensor digitorum brevis (EDB) M-wave amplitude (percent paralysis) following injection of freshly reconstituted BTX compared with the effect of BTX that was refrozen (− 20°C) or refrigerated (+ 4°C) for 2 weeks after reconstitution	Essentially no difference in the muscle paralysis resulting from fresh BTX compared with refrozen or refrigerated BTX, and no statistical difference between groups was noted
Alam, M. Dover, J. S. Arndt, K. A	2002	Pain associated with injection of botulinum A exotoxin reconstituted using isotonic sodium chloride with and without preservative: a double-blind, randomized controlled trial	Botox®	Arch. Dermatol. 2002; 138 (4): 510–514	Bacterio-static saline	Prospective (blinded) and retrospective clinical assessments on pain of injection when using either 0.9 % saline (recommended) or 0.9 % saline with benzyl alcohol preservative. Split-face study	Reconstitution of botulinum toxin with preservative-containing saline can markedly decrease patient discomfort at the time of injection. The difference is statistically and clinically significant

Table 2.6 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Hexsel, D. M. De Almeida, A. T. Rutowitsch, M. De Castro, I. A. Silveira, V. L. Gobatto, D. O. Zechmeister, M. Mazzuco, R. Zechmeister, D.	2003	Multicenter, double-blind study of the efficacy of injections with botulinum toxin type A reconstituted up to six consecutive weeks before application	Botox®	Dermatol. Surg. 2003; 29 (5): 523–529	Normal saline	Double-blind study of 88 volunteers treated for glabellar lines with product reconstituted over 6 weeks	BTX-A may be applied up to 6 weeks after reconstitution without losing its effectiveness. Other factors, which are probably individual, may influence the response to BTX-A injections
Trindade De Almeida, A. R. Kadunc, B. V. Di, Chiacchio N. Neto, D. R.	2003	Foam during reconstitution does not affect the potency of botulinum toxin type A	Botox®	Dermatol. Surg. 2003; 29 (5): 530–531	Normal saline	Clinical assessment of vigorous shaking on product efficacy	Our article demonstrates that when reconstituting Botox, the presence of foam does not affect the potency of the product
van Laborde, S. Dover, J. S. Moore, M. Stewart, B. Arndt, K. A. Alam, M.	2003	Reduction in injection pain with botulinum toxin type B further diluted using saline with preservative: a double-blind, randomized controlled trial		J. Am. Acad. Derm 2003; 48 (6): 875–877	Normal saline and bacterio-static saline	Double-blind, randomized study of 15 volunteers in split-face treatment of upper face dynamic lines to determine if diluent affected pain on injection	Use of preservative-containing saline to further dilute botulinum toxin type B can significantly decrease patient discomfort on injection

Table 2.6 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Kwiat, D. M. Bersani, T. A. Bersani, A.	2004	Increased patient comfort utilizing botulinum toxin type a reconstituted with preserved versus non-preserved saline	Botox®	Opthal. Plas. Recon. Surg. 2004; 20 (3): 186–189	Normal saline and bacterio-static saline	Double-blind, split-face clinical study on 20 volunteers for pain on injection using either diluent	Injection of botulinum toxin reconstituted with preserved saline is less painful than non-preserved saline preparations
Sarifakioglu, N. Sarifakioglu, E.	2005	Evaluating effects of preservative-containing saline solution on pain perception during botulinum toxin type-A injections at different locations: a prospective, single-blinded, randomized controlled trial		Aesth. Plas. Surg. 2005; 29 (2): 113–115	Normal saline and bacterio-static saline	Single-blind randomized clinical study of pain in 93 patients injected in face, neck or axilla using either diluent	The authors conclude that the preservative-containing saline solution significantly decreased pain perception during BTX-A injections ($p = 0.000$)
Thomas, J. P. Siupsinskiene, N.	2006	Frozen versus fresh reconstituted botox for laryngeal dystonia	Botox®	Otolaryngol. Head Neck Surg. 2006; 135 (2), 204–208	(Unspecified)	Open label crossover study of 43 patients with adductor spasmodic dysphonia randomly treated with either freshly reconstituted or previously frozen product (2 occasions for up to 8 weeks)	BTX-A may be safely used after being reconstituted and frozen or refrozen without a significant loss of effectiveness or additional side effects. In our experience, the period of freezing was on 2 occasions for up to 8 weeks

Table 2.6 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Hui, J. I. Lee, W. W.	2007	Efficacy of fresh versus refrigerated botulinum toxin in the treatment of lateral periorbital rhytids	Botox®	Ophthal. Plast. Reconstr. Surg. 2007; 23 (6): 433–438	Bacterio-static saline	Double-blind randomized study on 45 volunteers split-face design using product freshly reconstituted or product stored for 2 weeks at 4 °C after reconstitution	This study demonstrates that 2 weeks of refrigeration does not appear to significantly affect the time of onset or efficacy of botulinum toxin in the treatment of lateral periorbital rhytids
Lizarralde, M. Gutierrez, S. H. Venegas, A.	2007	Clinical efficacy of botulinum toxin type A reconstituted and refrigerated 1 week before its application in external canthus dynamic lines	Botox®	Dermatol. Surg. 2007; 33 (11): 1328–1333	Normal saline	Double-blind, randomized clinical study of 30 volunteers treated in canthal lines with product either freshly reconstituted or stored after reconstitution at 4 °C for 1 week. Assessments by photographs or nerve conduction	BTX-A, reconstituted and refrigerated 1 week before its application, has similar clinical efficacy in treating external canthus dynamic lines as does fresh BTX-A
Parsa, A. A. Lye, K. D. Parsa, F. D.	2007	Reconstituted botulinum type A neurotoxin: clinical efficacy after long-term freezing before use	Botox® Cos-metic	Aesthetic Plast. Surg. 2007; 31(2):188–91: discussion 192–193	Normal saline	Clinical assessment of freezing Botox® in syringes after reconstitution for up to 6 months compared to unfrozen product used within 4 hours	Reconstituted BoNT/A may be frozen, thawed, and injected without losing its potency up to 6 months, with efficacy equivalent to freshly prepared BoNT/A

Table 2.6 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Hexsel, D. Rutowitsch, M. Castro, L. Zechmeister-do-Prado, D.	2008	Multicenter double-blind study of the efficacy of injections with a commercial preparation of botulinum toxin type A reconstituted in different weeks	Dysport®	J. Am. Acad. Dermatol. 2008; 58 (2) suppl. Presented as a poster at AAD meeting, 2008	Normal saline	Clinical assessment of 105 volunteers (3 groups) treated for glabellar lines with product reconstituted for 8 h, 8 days and 15 days before use. No microbial contamination detected	This study showed that the time of dilution up to 15 days before applications had no influence on the efficacy and safety of the 500 U BT-A
Kazim, N. A. Black, E. H.	2008	Botox: shaken, not stirred	Botox®	Ophthal. Plast. Reconstr. Surg. 2008; 24 (1): 10–12	Normal saline	Double-blinded, randomized 6-month trial of seven volunteers injected in frontalis with product reconstituted as manufacturer's instructions or vigorously shaken for 30 s	The effect of botulinum toxin type A is maintained and has the same duration when it is reconstituted vigorously compared with when it is reconstituted gently
Yang, G. C. Chiu, R. J. Gillman, G. S.	2008	Questioning the need to use Botox within 4 hours of reconstitution: a study of fresh vs 2-week-old Botox	Botox®	Arch. Facial. Plast. Surg. 2008; 10 (4): 273–279	Normal saline	Double-blind, randomized trial of 40 volunteers treated for forehead rhytides using product freshly reconstituted or stored for 2 weeks at 4 °C or − 20 °C	No measurable difference was found in the potency or duration of efficacy of Botox in the treatment of forehead rhytides after 2 weeks of refrigeration or freezing compared with fresh reconstituted Botox

Table 2.6 (continued)

Authors	Year	Title	Product	Reference	Diluent	Summary	Conclusions[a]
Hexsel, D. Rutowitsch, M. S. De Castro, L. C. Do Prado, D. Z. Lima, M. M.	2009	Blind multicenter study of the efficacy and safety of injections of a commercial preparation of botulinum toxin type A reconstituted up to 15 days before injection	Dysport®	Dermatol. Surg. 2009; 35: 933–940	Normal saline	(Full publication of 2008 poster)	The results confirm the possibility of injecting 500U of BT-A up to 15 days after its reconstitution safely and without loss of efficacy
Shome, D. Nair, A. G. Kapoor, R. Jain, V.	2010	Botulinum toxin A: is it really that fragile a molecule?	Botox®	Dermatol. Surg. 2010; 36 (suppl. 4): 2106–2110	Normal saline	Potency study in mice examining reconstituted product inverted 30 times a minute for up to 6 weeks at 4 °C	OnabotulinumtoxinA is an extremely stable molecule, and vigorous agitation does not impair its potency, even after 6 weeks
Allen, S. B. Goldenberg, N. A.	2012	Pain difference associated with injection of abobotulinumtoxinA reconstituted with preserved saline and preservative-free saline: a prospective, randomized, side-by-side, double-blind study	Dysport®	Derm. Surg. 2012; 38 (6): 867–870	Normal saline and bacterio-static saline	Double-blind, randomized study of 20 volunteers over 2 weeks in split-face treatment of glabellar lines and crow's feet to determine if diluent affected pain on injection	Reconstitution of abobotulinumtoxinA with preserved saline results in significantly less pain on injection than with preservative-free saline. Preserved saline may be the reconstituent of choice for reconstitution of abobotulinumtoxinA

Normal saline is often referred to as preservative-free saline and bacteriostatic saline as preserved/preservative-containing saline (contains 0.9 % benzyl alcohol)
[a]Conclusions presented as verbatim quotations from publication

So where to now with the formulations? What new approaches are being considered or studied?

Pickett and Perrow [90] have discussed new formulations, in particular replacement of human plasma-derived HSA with a fully recombinant version; but this change has not appeared in commercial products to date. The replacement of other components to 'modernise' the formulations was also discussed in their work [90].

Pickett [135] has considered whether a liquid formulation might appear in the future, perhaps in pre-filled syringes or similar administration devices, effectively removing the need for reconstitution.

The concept of a liquid formulation for BoNT products has been in existence for many years. BoNT type B product has already been provided as a liquid in a vial since license approval in December 2000. This product may be painful when injected, likely to be due to the acidic pH of the solution [136]. One key to a successful future liquid product is, therefore, a formulation that is not painful to the patient and which has a physiological solution for injection. Shelf-life stability will also be essential to compete with the existing products, as discussed above.

The patent literature contains numerous references to liquid formulations, filed by Allergan, Ipsen, Elan/Solstice and MedyTox, together with other individuals (Table 2.7). These formulations all have different stabilisers and components, including the omission of HSA. As such, they should be considered next-generation formulations and will be made available as soon as stability and presentation issues are determined by the manufacturers. Also, these new formulations will have to show that they work equivalently in the clinical applications when compared to the current marketed products!

Devices are now starting to appear that are designed to make BoNT injections easier. An example is the botulinum toxin automatic injector called Talent™ BT, recently presented by the Swiss company Primequal [137]. This is effectively a repeat pen injector, containing the reconstituted product in a syringe which 'clicks' each time a trigger is pressed. Does this offer any real advantage? Probably not to the experienced clinician. The repeat 'click' could also be somewhat disconcerting to the patients. More importantly, the cost of the devices is high (20 € each). Is there a need for such high precision of injection volumes? This has not been raised to date as an issue by the clinical community. Nevertheless, the device has recently gained innovation awards.

2.9 Pharmacology of Clinical BoNT Products

In accordance with accepted definitions, pharmacology is the study of what drugs are, how they work and what they do (see, for example, [138]). The pharmacology of products for human therapy must be determined. Licensing of the products without these data is not possible. The study of drug pharmacology is therefore a wide subject, with several subsets such as clinical pharmacology, neuropharmacology, pharmacogenetics, pharmacoepidemiology and toxicology. However, in the case of the BoNT products, the major aspect of their pharmacology is the fact that they

Table 2.7 BoNT liquid formulation patents and patent applications 2008 to present (United States Patent and Trademark Office only)

Patent/Application number	Date granted/published	Title	Abstract	Assigned	Comments
7,211,261	May 1, 2007	Stable liquid formulations of botulinum toxin	Liquid formulations of botulinum toxin stable to storage in liquid form at standard refrigerator temperatures for at least 1–2 years and to storage at higher temperatures for at least 6 months	Moyer, E.; Hirtzer, P. Solstice Neurosciences LLC	Succinate-buffered saline pH range 5.4–5.8. With HSA
8,173,138	May 8, 2012	Stable liquid formulations of botulinum toxin	Liquid formulations of botulinum toxin stable to storage in liquid form at standard refrigerator temperatures for at least 1–2 years and to storage at higher temperatures for at least 6 months	Moyer, E.; Hirtzer, P. Solstice Neurosciences, Inc.	Phosphate, phosphate-citrate and succinate buffers pH 5–6. With HSA. Continuation of US 7,211,261
Patent application	*Date published*				
20080050352	February 28, 2008	Pharmaceutical composition comprising botulinum, a non-ionic surfactant, sodium chloride and sucrose	Botulinum toxin complex or purified neurotoxin in liquid (or solid) with crystalline agent	Webb, P.; White, M.; Partington, J. Ipsen	Includes buffer, sodium chloride, non-ionic surfactant and disaccharide
20080102090	May 1, 2008	Pharmaceutical composition containing BoNT A2	First application involving type A2 in a liquid with range of benefits compared top type A1	Panjwani, N.; Webb, P.; Pickett, A. Ipsen	Buffer containing surfactant, disaccharide and sodium chloride

Table 2.7 (continued)

Patent/Application number	Date granted/published	Title	Abstract	Assigned	Comments
20090291062	November 26, 2009	Protein formulations and methods of making same	Protein and water formulations of low conductivity and/or low osmolality	Fraunhofer, W.; Bartl; A.; Krause; H-J.; Tschoepe, M.; Kaleta, K.	Includes non-ionic surfactants (polysorbate), non-ionizable excipients (sugar alcohol or polyol) and a method of preparation
20100168023	July 1, 2010	Injectable botulinum toxin formulations	Advantages including reduced antigenicity, reduced tendency for localized diffusion, increased duration of efficacy, faster onset of clinical efficacy, and/or improved stability	Ruegg, C.L.; Stone, H.F.; Waugh, J.M. Revance Therapeutics, Inc.	Positively charged carrier with positively charged backbone including polyamino acid
20100291136	November 18, 2010	Pharmaceutical liquid composition of botulinum toxin with improved stability	A liquid pharmaceutical composition of botulinum toxin which is improved in stability. It comprises botulinum toxin, polysorbate 20, and methionine and optionally isoleucine	Jung, H.H.; Yang, G.H.; Kim, H.W.; Woo, H.D.; Rhee, C.H. Medy-Tox Inc.	Specified ranges of concentrations and pH 5.5–7.0
20100330123	December 30, 2010	Albumin-free botulinum toxin formulations	Botulinum toxin formulations that are stabilized without the use of any proteinaceous excipients	Thompson, S.A.; Ruegg, C.L.; Waugh, J.M.	Non-reducing di/tri-saccharide, non-ionic surfactant, physiologically compatible buffer, different salt compositions

Table 2.7 (continued)

Patent/Application number	Date granted/published	Title	Abstract	Assigned	Comments
20110268765	November 3, 2011	Injectable botulinum toxin formulations	Advantages including reduced antigenicity, a reduced tendency to undergo unwanted localized diffusion following injection, increased duration of clinical efficacy or enhanced potency relative, faster onset of clinical efficacy, and/or improved stability	Ruegg, C.L.; Stone, H.F.; Waugh, J.M. Revance Therapeutics, Inc.	Use of a carrier and a suitable formulation to stabilise against diffusion, reduce antigenicity and reduce diffusion
20120107361	May 3, 2012	Albumin-free botulinum toxin formulations	Botulinum toxin formulations that are stabilized without the use of any proteinaceous excipients	Thompson, S.A.; Ruegg, C.L.; Waugh, J.M.	Non-reducing di/tri-saccharide, non-ionic surfactant, physiologically compatible buffer, different salt compositions

Most patents and patent applications also have equivalent European and World registrations, occasionally with different titles

are, quite simply, lethal in minute quantities! Classical pharmacokinetic studies on adsorption, distribution, metabolism and excretion (ADME studies), for BoNT products used clinically, have not been reported, indeed not performed for obvious reasons mostly related to limitations on detection. A recent discussion of the subject by Simpson [139] only dealt with BoNT that had caused intoxication through classical routes and not through clinical application. The entire pharmacology of the BoNT products is therefore skewed as to what can actually be achieved in practice and what data can be obtained using standard techniques that is of value in the assessment of their suitability.

There are several reviews of what has been titled the pharmacology of BoNT, but in reality, these are subsets such as clinical pharmacology or the mode of action in animals [95], [109], [140]–[143].

Coupled to this, of course, is the fact that until now every batch of every product released commercially has been potency tested in animals, a unique situation in the pharmaceutical world. Added to this are the independent release procedures enforced by the regulatory authorities, as described earlier. The safety elements are, therefore, highly enforced and the likelihood of a product being released to the market that is either super- or subpotent is remote.

The pharmacological aspects are also significantly biased by the fact that animals are known to have a different sensitivity to the BoNT products than humans (see, for example, [142], [144]). Mice and rats have different sensitivities to each other [142] and are significantly less sensitive than human adults. These essential facts have been overlooked by many scientists studying the effects of BoNT in animals, most especially when they subsequently make extrapolations to human clinical uses [145]. Studies have been carried out where, if extrapolated to adults, a dose of 5,000 Botox® units or greater would have been administered (see, for example, [146]–[148]). Explanations for these differences in BoNT sensitivities between animals and humans are only now finding explanations, probably in relation to the reduced sensitivity of human BoNT receptors compared to those of other species [33].

No detailed review of the various animal studies and the related pharmacological data obtained is possible in the context of the present work since this would warrant a further chapter. But two aspects are discussed below which are representative of past approaches and future directions in this area, as illustrations of the subject.

2.9.1 Attempts to Relate Animal Data to Safety in Humans

One of the biggest uses of animal studies with BoNT, relating to clinical use, started in the late 1990s, when the first work emerged that attempted to relate the results of animal studies with safety of use in humans. A model was adapted from other uses that claimed to demonstrate useful results when BoNT was included [123]. The original model, called the tail suspension test, was adapted shortly after this initial work to be called the digit abduction scoring (DAS) assay. Basically, mice were injected in one hind limb with different doses of BoNT and then suspended by

the tail. Trained observers could then 'measure' the angle that the foot digits were extended to in what is normally described as 'the startle response' of the animal [84]. However, the scale of effect is arbitrary and the result qualitative. Doses as high as 30 Botox® units/kg were employed and, as commented by the workers themselves, 'do not reflect clinical doses' [123]. In addition, the species difference between rodents and man was also acknowledged by the investigators.

Nevertheless, this work was evolved to the point at which claims of safety differences between the BoNT products were being made, based upon these animal pharmacological studies [149]. The investigators revised their earlier comments into using the animal data as representative of human doses ('translational medicine').

When these studies were looked at in detail, the results were clearly not as presented. Exceptionally high comparative doses of products were being used (up to 100 units/kg) [149] and the statistical analysis was flawed [150]. The kinetic analyses, which purported to show different product characteristics, were shown subsequently to be identical, when appropriate differences in the potency units of the products under study were taken into account [150]. There was actually no demonstrable difference in the dose–response of the products tested [150].

2.9.2 Modern Approaches to Pharmacological Studies

Since the studies of the early 2000s, several groups have regrettably repeated the errors when trying to understand the mechanisms of clinical action, the pharmacology, of the BoNT products, as discussed above. But new approaches are slowly emerging that could be of significant value in the future.

The recent work of Hale and colleagues [151] has used the technique of near-infrared whole body analysis to examine the pharmacokinetics of a detoxified BoNT molecule in mice. The images clearly show the kinetics of BoNT diffusion throughout the animals (Fig. 2.4). Intramuscular injections in the tail remained localised over the duration of the study (24 H), but intravenous, intraperitoneal and oral administration showed dissemination throughout the animal. The mutated BoNT molecule had previously been shown to bind to neuronal cells and has been well characterised [152], [153].

This modern approach may herald a new era in ADME studies of BoNT. Future data and publications are awaited with great enthusiasm by the BoNT scientific and clinical communities.

2.10 Conclusions

The manufacture and quality aspects of the various main families of clinical BoNT products are defined and established. Although more than 25 years old, the major products are still finding new clinical applications today and, as always has happened, clinicians are leading the way in developing those new uses.

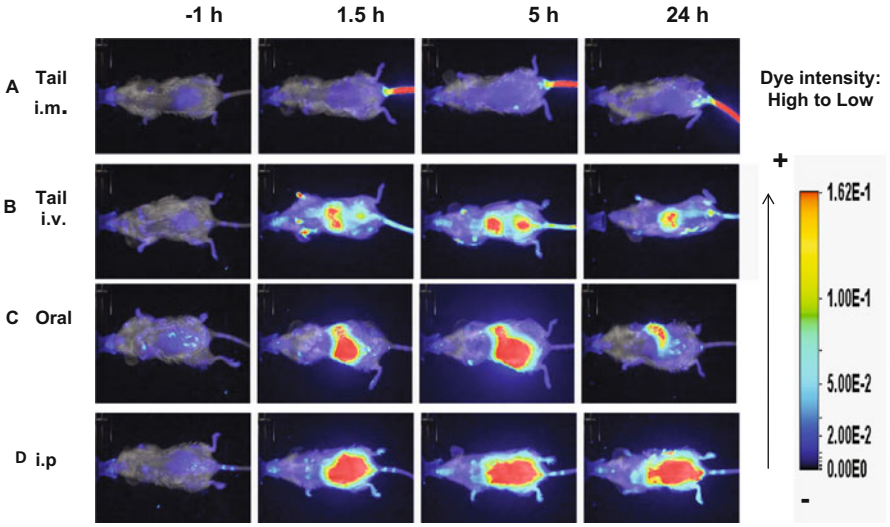

Fig. 2.4 Use of near-infrared technology for whole body in vivo monitoring of metabolism of a detoxified recombinant botulinum toxin labelled with the dye 800 CW. Images for each scan were captured at white light, 700 and 800 nm and fluorescent signals were analysed using the Pearl Cam software supplied with the Odyssey Imaging System (LiCor Inc). (Reprinted from Hale M, Riding S, Sing Bal Ram (2010) Near-infrared imaging of Balb/c mice injected with a detoxified botulinum neurotoxin A. Botulinum J 1(4):431–441, Copyright (2010), with permission)

As the decades pass, new technology on formulations and pharmacological aspects are finding their way into use, to advance even further our understanding of these essential, potent and fascinating products. The coming 25 years are awaited with great anticipation.

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