Dear Sir

I should like to strongly endorse the article by Andy Pickett,1 published in the September issue of ATLA, in which he criticises the lack of published information relating to a new in vitro method developed by Allergan Inc., which has received regulatory approval as a replacement for the LD50 mouse bioassay for the potency testing of a botulinum neurotoxin (BoNT) series of products.

Clearly, there is much interest in developing alternatives to the mouse LD50 potency test for BoNT, which is understandable, in view of the highly severe endpoint and the large numbers of animals involved.2–11 For example, cell-based assays have been devised that use either continuous cell lines, such as neuro-2a, PC12, and SK-N-SH cells, or primary neurons derived from chicken, mouse and rat spinal cord cells.12 However, none of these methods have been fully developed, validated and accepted by regulatory authorities.

It was therefore with great interest that I learned that Allergan Inc. (which currently produces the vast majority of the pharmaceutically-used BoNT products, as BOTOX® [onabotulinumtoxin A]) had developed a replacement potency test, which has received regulatory approval.13 Until June 2011, the mouse LD50 potency assay was the only method approved by regulatory authorities, anywhere in the world. Allergan announced that the FDA had approved a fully in vitro, cell-based assay for the potency testing of its products (BOTOX® [onabotulinumtoxin A] and BOTOX® Cosmetic) for stability and potency.

The company stated that the new assay is specifically applicable to its botulinum toxin type A product, and that the newly-approved test was to be implemented immediately for the release of product for sale in the US. This approval does not extend to botulinum toxins made by other manufacturers. It is estimated that use of the new assay will eventually reduce Allergan’s animal-based assay testing by up to 95% over the next three years.

It was also reported that the UK’s Medicines and Healthcare Products Regulatory Agency (MHRA) has approved the assay for BOTOX® vials sold in the UK. In addition, it was noted that the first positive opinion, from Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS), relating to VISTABEL®, paves the way for approval in 29 countries in the European Union. The second positive opinion was granted by the Irish Medicines Board (IMB) for BOTOX®, and covers 14 European Union countries involved in the Mutual Recognition Process. Since then, Hong Kong and Switzerland have also approved use of the new assay, and, apparently, registrations are ongoing in several other countries worldwide.

On the face of it, this would seem to be very good news, and the development and acceptance of the assay has been welcomed by several scientific and animal welfare bodies. The method has apparently been well-documented in regulatory submissions, and has been subjected to a validation study by Allergan. However, as Spielmann noted,14 it is essential for the scientific basis of the new cell-based potency assay and the validation data to be put into the public domain, in order for the method to be available for use by other toxin producers.

It is also important for all of this information to be published, to permit independent scrutiny and assessment of the validation data obtained, and for other scientists to be able to see if the method is reliable and relevant when performed in their laboratories, as is standard practice with the validation of other novel replacement safety testing methods.15,16 In this context, it should be noted that Allergan is discussing how to license the technology “to other parties that share its commitment to implementing non-animal alternatives to animal-based assays in the manufacture of their medical products”.13

So, what do we know about the new assay from the paucity of details publicly available? Two US patents, filed by the company in June 2012, contain potentially relevant information on the development of methodology to screen for cell lines responsive to BoNT/A intoxication. One of these17 discloses details of methods for making and detecting the binding of antibodies against the epitope of the carboxyl-terminus at the P1 residue of the alpha-SNAP-25 BoNT/A protein cleavage product. The other patent18 describes work that appears to have paved the way for the development of an in vitro cytotoxicity luminescent gene reporter assay involving genetically-engineered neuronal cell lines (e.g. H1), highly sensitive to very low amounts of BoNT/A activity. This is coupled with immunological detection of the SNAP-25 cleavage product, presumably by using the specific antibodies described in the other patent. Toxin potency in the assay is expressed as a ratio of BoNT/A activity to cell viability.
The detection of neurotoxin activity by using SNAP-25 is based on the fact that the L-chain of BoNT-A has endopeptidase activity, enabling it to specifically degrade the SNAP-25 protein required for the release of neurotransmitters from nerve axon endings.19 One such method, based on the above mechanism, has been validated at the National Institute for Biological Standards and Control (NIBSC) in the UK, for internal use.20 The method is precise and reproducible, and has provided potency estimates which are highly comparable to those obtained by manufacturers using the LD50 assay. A version of such an assay has been in use at NIBSC to verify manufacturers’ in vivo data for many years, during independent batch evaluation.21 However, the method has never been accepted for product quality testing and release by any regulatory agencies worldwide, since it only measures one property of botulinum toxin (the toxin L-chain functional domain activity), so the activities of the toxin’s other functional domains are not detected.

According to the press release,13 Allergan’s new assay was allegedly validated against a picomolar-sensitive immuno-based method for BoNT/A activity, by using a toxin standard. It is not known if it was also validated against the LD50 mouse test. Allergan has reported substantial progress in the optimisation of the assay, resulting in increased sensitivity and improvements in assay read-out. Apart from the patents filed in June 2012, the only other available information released by Allergan is that the performance criteria, against which the new cell-based potency assay needed to be validated, included the ability to assess all primary modes of action of the neurotoxin.13 In addition, the new assay format had to be suitable for use in an intensely quality-controlled environment, and at the high capacity needed to support commercial production.

Like Pickett,1 I have attempted to obtain further information on the assay, from the relevant regulatory agencies and from Allergan. To date, the only reply I have received indicated that, as the details are confidential and owned by Allergan, I need to apply via US Freedom of Information (FOI) rules to see them.

I believe that this is unacceptable, and that it is wrong for a regulatory body to accept a test method for regulatory use, on the basis of the submission of confidential information. This situation differs from that in which regulatory decisions are made on the basis of the submission of data that have been obtained by using a test method. This is the norm with regulatory toxicity testing, when, understandably, the results are kept confidential to maintain a competitive advantage with the development of novel products. However, in regulatory toxicity testing, the methods used are conducted according to published protocols. Moreover, in the case of new in vitro toxicity tests, these methods must be validated before regulatory use for specific purposes in several different laboratories, the results of which are then independently reviewed and endorsed. This is necessary to ensure that a method that is going to be used to test many samples and products for safety is fit for purpose.

The above situation should apply to Allergan’s new test, irrespective of the fact that it is, for the moment at least, intended to be solely used by the company and only for its own products. With test methodology, precedence should be given to scientific disclosure, at the expense of any competitive disadvantage that a company might experience by not being the sole user of the test. In any case, it would seem that Allergan has some protection afforded through the patents it has filed. This is to avoid the possibility that an inappropriate test would generate spurious safety data for many batches of product.

What are the prospects for developing a method for use by other laboratories for testing all BoNT products that could replace the LD50 test? The use of human stem cells shows great promise, due to their high sensitivity, and it is believed that such an approach will ultimately provide the most useful alternative models.21 Indeed, Whitemarsh et al.22 have recently shown that neurons derived from human induced pluripotent stem cells (hiPSCs) provide a highly sensitive platform for BoNT potency determination, for neutralising antibody detection, and for mechanistic studies. Their neuronal preparation consisted predominantly of gamma amino-isobutyric acid (GABA)ergic and glutamatergic neurons. These cells expressed all of the necessary receptors and substrates for BoNT intoxication by all BoNT serotypes, the latter being an advantage over Allergan’s method. Neuronal toxicity was assessed by analysing cell lysates by Western blot for SNAP-25 or VAMP2 cleavage (vesicle-associated membrane protein 2, another substrate for botulinum toxin23).

Whitemarsh et al.22 concluded that their assay has the potential to largely replace the LD50 bioassay for BoNT potency determination, once it has been standardised, optimised, and validated by using an appropriate toxin standard. Comparative testing of four different lots of hiPSC-derived neurons indicated good lot-to-lot consistency. The authors emphasised the fact that, as these neurons are human cells derived from stem cells and not animal cell lines, they provide a more-relevant model system for testing BoNT preparations.

In conclusion, I consider it erroneous, and also injudicious, for regulatory authorities to accept any new test method, such as that developed by Allergan, on the basis of information that is not in the public domain. One possible argument for regulators to act as they did is that the test is designed for use by one organisation for one prod-
uct. However, any method should be demonstrated to be reliable and relevant for its stated purpose in more than one laboratory, before results generated by it are accepted as being definitive, whatever restrictions there are to be on its intended usage.

It is to be hoped that all relevant information on Allergan’s new methodology, together with the results of the validation study, will be published as soon as possible, in the interests of scientific transparency. In the meantime, efforts should continue to find other, less specific in vitro tests, based on the methods discussed above.

Yours faithfully

Robert D. Combes
Consultant
122 Cavendish Court
Recorder Road
Norwich NR1 1HX
UK
E-mail: robert.d.combes@gmail.com

References


Note added in proof:
Allergan has at last published details of its new cell-based botulinum toxin potency assay in an open-access journal.24 The basis of the assay is essentially that which was described earlier in the two patents discussed above, but includes a great deal of supporting information. However, the details and results of the validation study remain to be published — all we are told is that it took place in a quality-controlled laboratory.

24 Fernández-Salas, E., Wang, J., Molina, Y., Nelson, J.B.
Dear Sir

As a former campaigner who targeted Allergan for using the mouse LD50 Test to assess the potency of its flagship product, BOTOX®,1,2 I was prepared to embrace Andy Pickett’s critique of the company.3 Allergan announced that it had developed a non-animal alternative to this test nearly a year and a half ago, yet the company only recently published details of its assay (after publication of Pickett’s critique).4 Moreover, there has been no indication that Allergan has begun to license or otherwise share its assay with other manufacturers of botulinum neurotoxin (BoNT)-based products, such as Ipsen and Merz, a possibility mentioned in Allergan’s press release announcing its new assay.5 Allergan’s silence on these issues at relevant international meetings has been frustrating.

Yet Pickett’s commentary is as much a strident defence of the continued use of the LD50 Test in the rest of this niche industry as it is a broadside against Allergan. He takes jabs, not just at Allergan, but at nearly all the stakeholders — critics of the industry’s use of the LD50, independent scientists developing alternatives, and the press. And nowhere in the commentary does Pickett explicitly reveal that he was the point-person on the LD50 issue for Allergan’s competitor, Ipsen.

Pickett does not seem to grasp why many people are concerned that some companies are still using the LD50 Test — industry standard or not for BoNT products — and why this use is all the more disturbing in the context of a prominent application of these products, namely, the temporary smoothing of facial wrinkles. Yes, there are therapeutic applications, and yes, some people who get the “aesthetic” treatment may benefit psychologically from their improved appearance, but we’re talking about a test that has been used in the BoNT arena since 19196 (if not earlier) and that induces paralysis and/or suffocation in perhaps hundreds of thousands of animals per year. Where is the scientific ingenuity in the rest of this industry?

Pickett’s commentary comes across to this observer as a cranky defence of Allergan’s competitors, rather than a principled and much needed prod to induce Allergan to move the issue forward.

Although progress is being made,7–9 much more work remains to be done to replace the mouse LD50 assay in all its BoNT applications, including the detection of this deadly toxin in environmental samples, as well as in the assessment of potency and quality for pharmaceuticals that use BoNT in clinical and aesthetic applications. This is especially so in the USA, where testing practices have not kept pace with scientific developments, owing to the issue’s low profile. It has been six years since the federal Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) held a workshop on alternatives to the mouse LD50 assay.10 In the prevailing post-September 11th (2001) atmosphere, perhaps the push to develop measures for detecting and countering exposures to BoNT and other potential bioterrorism agents will drive the BoNT field forward.

Yours faithfully

Martin L. Stephens
Center for Alternatives to Animal Testing
Johns Hopkins University
615 N. Wolfe Street, W7026/W7032
Baltimore, MD 21205
USA
E-mail: mstephen@jhsph.edu

References

**Re: Pickett, The Botulinum Toxin LD50 Potency Assay — Another Chapter, Another Mystery**
Dear Sir

I am grateful to Bob Combes and Marty Stephens for their responses to my commentary on recent events surrounding the mouse lethality assay (MLA) used for commercial botulinum toxin (BoNT) products. ¹ Perhaps we all get a little “cranky” as we get older, but my intention was not to reflect that ageing process in my comments, as Marty suggests!

I am also very pleased to clearly confirm that, yes, I was part of the manufacturer Ipsen’s efforts to find alternative methods to the MLA for many years, and I was very pleased to be a part of trying to change the landscape for the better. I was also part of the group that set up the European EWG after that first experts meeting in April 2009,² with the intention of finding a positive way forward on many aspects. But, during those years, we did not have the technology that many people thought existed to replace the MLA (mainly through comments and articles that were not correctly researched or informed). I discussed this in my recent commentary and the situation has not changed.¹ The publication of the original 2005 European Pharmacopoeia monograph on BoNT, with the comments about alternative methods, was also unfortunate, in that no accompanying commentary was included to state those same facts. Therefore, one of my roles was to try and better inform the commentators about the situation, which was not readily accepted then and is the case even today.

Another chapter opened in this long-running story shortly after my commentary was published. Allergan’s new cell-based method was published on 21 November 2012.³ The timing after my article was a coincidence: my article certainly played no role in this coming forward, since we can see that the manuscript had been many months in the pipeline before being accepted. But, finally, we could see what Allergan had achieved and what the results were. Or could we?

Allergan’s published work is comprehensive and commendable.³ The basis of their new assay is as expected — a selected cell line combined with a sensitive detection method for SNAP-25 target cleavage. But, like any commercially-based publication such as this, the manuscript is perhaps more interesting for what is not there than what is actually included. Allergan scientists have also now presented their work at a recent international meeting, and confirmed several aspects.⁴ The following points are worth noting:

— As expected and discussed by Bob Combes and myself, the method is covered by a US patent.⁵ This is a very detailed legal document, and is more comprehensive than the publication (although many pages contain DNA and protein sequence data only).

— The published method is not the one used for their product batch release and testing. A variant is used for that purpose, as is also mentioned in the publication.

— The method published is used by their research scientists in research settings, but not by their Quality Control group.

— The method is not specific to their own products, since other BoNT-A products have been assessed, as well as a commercial product. Obviously, further adaption of the method would be needed for other commercial products, but, on the basis of what has been described, this could be rather minor, since the other BoNT-A products are rather similar in formulation.

Re: The Mouse Lethality Assay for Botulinum Toxin Products

— No cross-validation data with the MLA are included — there are no MLA data anywhere in the publication (except as nominal stated potencies for a commercial product), so performance comparisons are not possible.

— An interesting form of statistics has been used. A test product batch has been compared to a reference batch, and a “relative potency” of 0.82 was obtained with confidence intervals 0.7–1.1. This is not equivalence. The new statistics involve a statement that these results indicate “the potency of the two lots is indistinguishable, since the confidence interval included the number one”. But there are many other numbers in that interval as well!

Perhaps, though, the most interesting aspect of their work is that there is a “critical reagent” used in the method, which requires a “constant and reliable supply”. This is a monoclonal antibody raised in mice and purified from ascites. So we now have the new concept of using mice to develop a reagent to use in an assay to reduce the use of mice! No further details on how many mice are used to produce this reagent are provided.

My intention here is not to criticise, but to inform. Allergan’s work is important, but clarity on the commercial situation behind such a new assay is needed, and their results, as published, must as ever be interpreted for commentators, other scientists and the public with interests in and concerns about this subject.

Since my first involvement with BoNT 25 years ago, I have been acutely aware of the issues surrounding the MLA. Marty’s comments, that I do not grasp people’s concerns about the use of such a method, are simply wrong. Nor do I defend the use of the MLA, as suggested. However, what I am equally sensitive to is the great and genuine benefits that BoNT has brought to many patients, young and old, also in the aesthetic world, as well as in the therapeutic world. To continue to demean the product with the term “cosmetic” is now entirely inappropriate. Commentators and the public should be correctly informed in the correct ways and with the correct information.

Yes, yet another chapter and yet another mystery!

Yours faithfully

Andy Pickett
Toxin Science Limited
The Sycamores
Wrexham
LL12 8DU
UK

Adjunct Professor
Botulinum Research Center
University of Massachusetts Dartmouth
North Dartmouth
MA 02747
USA

Head of Development
Q-Med, a Galderma Division
Seminargaten
Uppsala
Sweden
E-mail: andy@toxinscience.com

References