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A chip-based assay for botulinum neurotoxin A activity in pharmaceutical preparations

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Abstract The production of botulinum neurotoxin A (BoNT/A) for therapeutic and cosmetic applications requires precise determination of batch potency, and the enzymatic activity of BoNT/A light chain is a crucial index that can be measured in vitro. We previously established a SNAP-25 chip-based assay using surface plasmon resonance (SPR) that is more sensitive than the standard mouse bioassay for the quantification of BoNT/A activity. We have now adapted this procedure for pharmaceutical preparations. The optimized SPR assay allowed multiple measurements on a single chip, including the kinetics of substrate cleavage. The activity of five different batches of a pharmaceutical BoNT/A preparation was determined in a blind study by SPR and found to be in agreement with data from the in vivo mouse lethality assay. Biosensor detection of specific proteolytic products has the potential to accurately monitor the activity of pharmaceutical BoNT/A preparations, and a single chip can be used to assay more than 100 samples.

Keywords Botulinum neurotoxin · Surface plasmon resonance · Toxin sensor · On-chip enzyme assay

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Introduction

Clostridium botulinum neurotoxins (BoNTs) are composed of a heavy (100 kDa) and a light chain (50 kDa), linked by a disulfide bridge (Dhaked et al. 2010; Rossetto et al. 2014). The heavy chain binds to receptors located on nerve terminals and delivers the light chain into the neuronal cytoplasm. The latter step requires disulfide reduction, which severs the link with the heavy chain, and also turns on the metalloprotease activity intrinsic to the light chain. BoNT light chains are endoproteases that specifically attack synaptic SNARE proteins in the cytosol, thus inhibiting vesicular fusion and the calcium-dependent exocytosis of neurotransmitters. While eight distinct BoNT serotypes (BoNT/A–H) have been described (Popoff 2014), only BoNT/A and also to a minor extent BoNT/B are currently used therapeutically in humans.

BoNT/A has been formulated in different drug products that are currently used in a wide range of therapeutic and cosmetic applications (Chen 2012; Dressler 2012, 2013) to inhibit transmitter release from nerve terminals and suppress muscular hypercontraction (e.g., dystonia, strabism, blepharospasm, facial wrinkles) or glandular hypersecretion (e.g., hyperhidrosis). Quality control of pharmaceutical batches during production relies on toxicity tests, based on an in vivo mouse assay. BoNT concentration is quantified from median lethality in a group of mice and expressed as median lethal dose (LD_{50}) Units per milliliter. However, animal testing is controversial from an ethical point of view, and current legislation aims at phasing it out. Thus, there is a pressing need for automated in vitro technology to replace the mouse assay (Adler et al. 2010).

The endoprotease activity of BoNT/A light chain specifically cleaves a peptide bond (Q_{197}–R_{198}) located in the C-terminal domain of synaptosomal-associated protein of 25 kDa (SNAP-25), a component of the synaptic SNARE complex. Measurement of enzymatic activity can constitute

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a reliable means to monitor BoNT/A potency for quality control and can be included in a battery of in vitro assays to characterize therapeutic preparations.

Microfluidic devices based on chip technology are well suited to probe biomolecular processes in terms of miniaturization and integrated and automated operations (Kohler and Henkel 2005). Biosensor apparatuses based on optical label-free detection methods such as surface plasmon resonance (SPR) are mainly used to measure protein interactions and are particularly adapted to automated product-testing in biotechnology and the pharmaceutical industry (Helmerhorst et al. 2012). One of the proteins is immobilized on a sensor chip surface, whereas the binding partner is injected into buffer flowing over this surface, via an integrated microfluidic flow system. SPR responses are measured in resonance units (RU) and are proportional to the number of molecules bound to the surface. The goal of this report is to compare a novel in vitro biosensor assay we have developed (Lévêque et al. 2013) with the standard mouse bioassay to measure the activity of BoNT/A in a commercial BoNT/A formulation (Dysport). Quantification of batches activities was consistent with parallel results from the mouse bioassay, corroborating the potential contribution of SPR biosensors to replacing animal testing.

Materials and methods

Chemicals and reagents

Amine coupling kits were from GE Healthcare. $ZnCl_2$, Tween 20, dithiothreitol (DTT), and fatty acid-free bovine serum albumin were from Sigma. His_6-SNAP-25 and mAb 10 F12 were prepared as described (Lévêque et al. 2013). Dysport samples containing lyophilized *C. botulinum* type A toxin-hemagglutinin complex, human serum albumin, and lactose were generously supplied by IPSEN Biopharm (Wrexham, UK). Reconstitution of Dysport was carried out using high-pressure liquid chromatography (HPLC)-grade water obtained from Merck. All experiments were performed in accordance with French and European guidelines for handling botulinum neurotoxin.

Sample preparation

Dysport batches Botulinum toxin preparations were calibrated in LD_{50} units by a mouse LD_{50} assay, and expressed in mouse LD_{50} Units/ml. Vials from a reference batch were reconstituted with distilled water at 1000 LD_{50} Units/ml. The reference batch was diluted in cleavage buffer (50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES)-NaOH pH 7.4, 0.1 % bovine serum albumin, 10 mM DTT, 1 % Tween 20, 100 μM $ZnCl_2$) to establish a standard curve with 9 data points between 10 and 90 LD_{50} Units/ml, aliquoted and stored frozen at −20 °C for subsequent analysis. In a blind study, vials from five Dysport batches with unknown concentrations and a reference vial were reconstituted with 0.5 ml of distilled water each, diluted 25-fold in cleavage buffer and incubated for 20 min at 37 °C before analysis. Samples were either directly placed in the rack of the SPR apparatus at 4 °C or aliquoted and frozen for subsequent analysis. Frozen samples were thawed less than 2 h before injection to avoid potential loss of enzymatic activity.

Sensor chip preparation

All experiments were performed at 34 °C on a Biacore 3000 system using HEPES-buffered saline (HBS: 10 mM HEPES-NaOH, 150 mM NaCl, pH 7.4) as running buffer. SNAP-25 was amine coupled at pH 4.5 to CM5 sensor chips (GE Healthcare) as described in (Lévêque et al. 2013), using automated programs. After SNAP-25 coupling, the chip was primed by injection of cleavage buffer, followed by background determination consisting in five injections of mAb 10 F12 diluted in HBS at 10 μg/ml. The mAb binding was systematically regenerated with a regeneration buffer (glycine-HCl 10 mM pH 1.7).

SPR assay for Dysport batches

A cycle of sample analysis was as follows: cleavage buffer was injected into flowcell 1 (Fc 1, reference flowcell) and then samples were injected for 2 min separately through unconnected flowcells (Fc 2, 3, and 4). Standard reference samples were introduced in a random manner throughout the analysis. Then the four flowcells were connected, flushed with regeneration buffer, and mAb10F12 was injected for 2 min before regeneration Measurement of reaction kinetics was carried out discontinuously by repeating sequential injections of enzyme and mAb. As kinetics were linear, in most experiments, only two injections were used to determine the slope. SPR chips can be reused for at least 40 assay-regeneration cycles without significant loss of binding capacity. Chips were discarded when more than 5 % of the substrate was cleaved.

All SPR measurements were established after subtracting nonspecific background obtained on the reference flowcell with coupled SNAP-25 and treated with cleavage buffer.

In vivo assay

The mouse bioassay of Dysport batches was previously performed by IPSEN Biopharm using published methodology (Straughan 2006).

Results

Optimization of the on-chip assay for pharmaceutical batches

We used a Biacore 3000 apparatus as a biosensor because of its performance, in terms of signal to noise ratio and precision. This biosensor has four flowcells that are serially connected, and samples can be introduced into individual or connected flowcells. Briefly, the biosensor method comprises two steps: (i) substrate cleavage by injection of BoNT/A over recombinant SNAP-25 immobilized on an SPR chip; (ii) detection of cleavage by monitoring binding of an antibody (mAb10F12) to the SNAP-25 cleavage product. MAb10F12 recognizes a neo-epitope produced when the protease activity of BoNT/A light chain cleaves the Q_{197}–R_{198} peptide bond releasing a nine amino acid peptide from the C-terminus of SNAP-25. However, mAb10F12 does not recognize intact SNAP-25.

The activation of the BoNT/A light chain protease requires reduction of the disulfide bond which links the heavy and light chains. Initial experiments demonstrated that mAb10F12 displayed binding to a SNAP-25 chip following injection of a Dysport sample in the presence of DTT. However, no binding was detected when the Dysport sample was diluted in cleavage buffer in the absence of the reducing agent (Fig. 1).

The optimization of the SNAP-25 on-chip cleavage assay followed several axis: (i) using a single chip for a direct comparison of different Dysport batches; (ii) decreasing analysis time; (iii) diminishing sample consumption; (iv) using the same flowcell for cumulated cleavage rounds; (v) randomizing flowcell usage to avoid flowcell differences bias; and (vi) increasing analysis precision using slope (RU/min) rather than single end-point (RU) measurements. As SPR analysis of mAb binding was very precise and the chips could be completely regenerated (Lévêque et al. 2013), the same flowcell was used for several rounds of cleavage. This led to a cumulative increase in the mAb binding signal which correlated with the number of BoNT/A injections. The number of assays per flowcell depends on the concentration and cleavage time of injected BoNT/A. We calculated that measuring in a minimal cleavage time small increments in mAb SPR signal (10–20 RU), allows about 100 assays on the same flowcell of a Biacore 3000 apparatus, without significantly affecting substrate cleavability (data not shown). To increase assay precision, Dysport samples were injected several times in the same flowcell and mAb10F12 binding measured after each injection. Figure 2 illustrates measurement of cumulative amounts of cleaved SNAP-25 produced by three Dysport concentrations, each injected three times into a single flowcell. Injections induced a regular increment in the signal generated by mAb10F12 binding, which is related to BoNT/A concentration (Fig. 2a). Plotting the cumulated bound mAb in Fig. 2a yielded three linear segments (Fig. 2b). The slope of each segment was proportional to the amount of reaction product formed in a given period of time and kinetics of sample activity can be expressed in RU per minute (Fig. 5a).

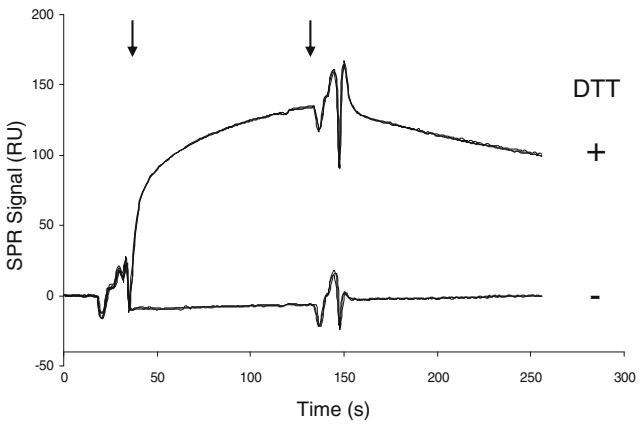

Fig. 1 SPR detection of Dysport activity. SNAP-25 was coated on a CM5 chip of a Biacore 3000 apparatus. One flowcell was exposed for 5 min to 60 LD_{50} Units/ml of a diluted Dysport sample in cleavage buffer containing the reducing agent DTT. The chip was then probed with mAb10F12 for 2 min. The lower traces are the result of an identical experiment performed in cleavage buffer in the absence of DTT. *Arrows* indicate the start and end of mAb injections (triplicate)

Flowcell-to-flowcell reproducibility

To determine the reproducibility of measurements between different flowcells, SNAP-25 was coupled to four flowcells. Cleavage buffer alone was injected in flowcell 1, while a Dysport sample was injected independently in flowcells 2, 3, and 4. The four flowcells were then connected in series, and mAb10F12 was injected to measure SNAP-25 cleavage. As shown in Fig. 3, reproducibility between flowcells was high with a calculated CV of 2 % between flowcells.

Assay protocol for Dysport samples

We determined that the minimum injection time that yielded reproducible measurements was 2 min (data not shown). In these conditions, background was close to zero (−2 to −4 RU), the detection threshold was 1 RU (mean of background+3× standard deviation) and the threshold for quantification was 4 RU (mean of background+10×standard deviation).

A diagram of typical rounds of injection and detection is shown in Fig. 4. Cleavage buffer was injected independently into each flowcell, in the presence or absence of Dysport. Dysport batches were successively injected for 2 min, in flowcells 2, 3, and 4 and cleavage buffer alone in flowcell 1. At the end of the injection period, the four flowcells were connected in series and probed with mAb10F12. As illustrated in Fig. 4, samples 1, 2, and 3 displayed an increase in antibody binding with respect to the control trace (Fc1). At the end of the detection step and after regeneration, a new cycle is started, each cycle lasting about 25 min.

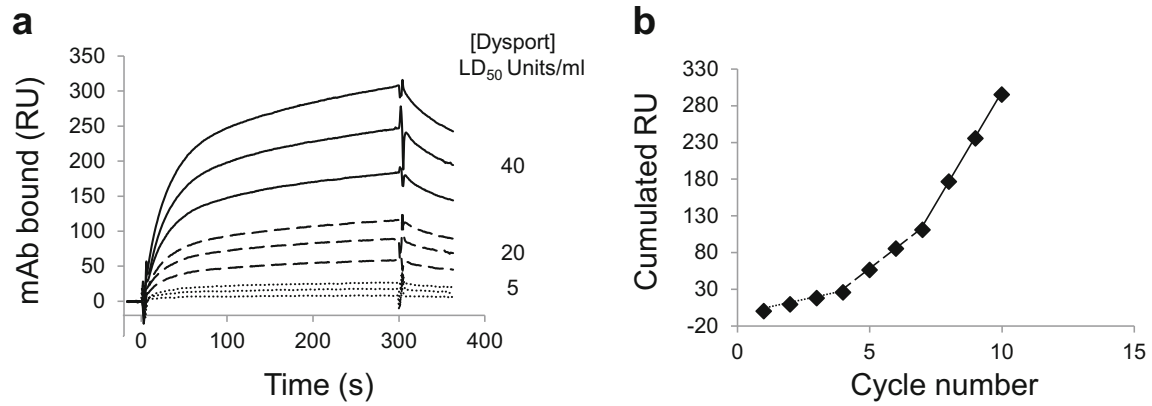

Fig. 2 Kinetics of Dysport endoprotease activity. **a** Dysport samples diluted to 5, 20, or 40 LD_{50}Units/ml were each injected three times for 5 min over a single flowcell. After each round of injection, mAb10F12 was injected and the amount of SNAP-25 cleavage product quantified at the plateau of mAb binding. **b** Cumulated RU obtained from **a**. Each segment corresponds to a different Dysport concentration: 5 LD_{50}Units/ml (….), 10 LD_{50}Units/ml (–), and 40 LD_{50}Units/ml (—)

Measurement of the endopeptidase activity of Dysport batches

Vials from five different Dysport batches (1 to 5) and a reference batch as internal control (ref.) were assayed and quantitative data obtained by interpolation in a standard curve. A range of dilutions of a Dysport standard, calibrated in LD_{50} Units/ml was used to establish the reference curve. These standards were introduced into series of samples to be assayed, using a random positioning protocol. The kinetics of cleavage were then measured by repeating sequential injections of samples and mAb10F12. We found that the plot of the slopes obtained for standard BoNT/A concentrations between 10 and 90 LD_{50}Units/ml yielded a linear calibration curve that was reproducible between experiments (Fig. 5a). Interpolation was then used to measure the activity of the five unknown samples. Results expressed in total contents per vial, showed that batches 1 (612 Units), 2 (565 Units), and 3 (559 Units) were not significantly different from the reference (577 Units, whereas batches 4 (339 Units) and 5 (164 Units) had lower contents (Fig. 5b). The SPR data indicated that the Dysport content of batch 4 was 59 % of the reference batch, whereas batch 5 had 28 % of the content of the reference. These results are in good agreement with the mouse assay: reference= 516 Units, batch 4=285 Units, 55 % of reference, batch 5= 199 Units, 39 % of reference. The coefficient of variation measured for three batches with six different vials per batch (n=18) was between 9 and 11 %. This value includes potential vial to vial content fluctuation and methodological reproducibility.

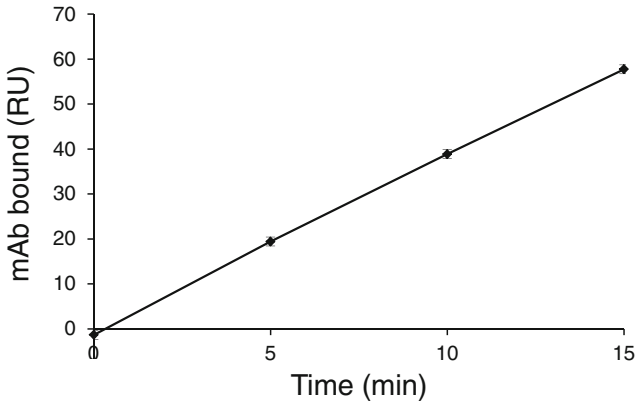

Fig. 3 Estimation of flowcell-to-flowcell reproducibility. The same concentration of Dysport (20 LD_{50}Units/ml) was injected three times independently into flowcells 2, 3, and 4 for 5 min. After each round of injection, the amount of cleaved SNAP-25 was determined by measuring mAb10F12 binding to all flowcells connected in series. *Error bars* represent the SD of the mean signal from the three flowcells

Discussion

We have adapted an SPR-based chip method (Lévêque et al. 2013) to provide a straightforward assay for the enzymatic activity of BoNT/A. The assay involves (i) BoNT/A injection over a SNAP-25 substrate chip and (ii) detection of proteolyzed SNAP-25 by injecting a mAb that specifically binds to the cleavage product generated by BoNT/A. We had previously optimized this method for maximum sensitivity, which is crucial for applications at the limit of detection, notably in surveillance and diagnostics. In this format, using a prolonged (5 h) substrate cleavage step and amplified detection with a secondary antibody, we can detect as little as 0.01 LD_{50} BoNT/A (Lévêque et al. 2014).

Several enzymatic assays have been reported (Capek and Dickerson 2010), but they can be subject to interference from the excipient contained in the formulation (Hunt et al. 2010; Pickett and Perrow 2010). ELISA- or HPLC-based method to detect the cleavage product, after elimination of interfering agents, appeared particularly appropriate to measure BoNT/A activity in pharmaceutical preparations (Ekong et al. 1997; Hunt et al. 2010).

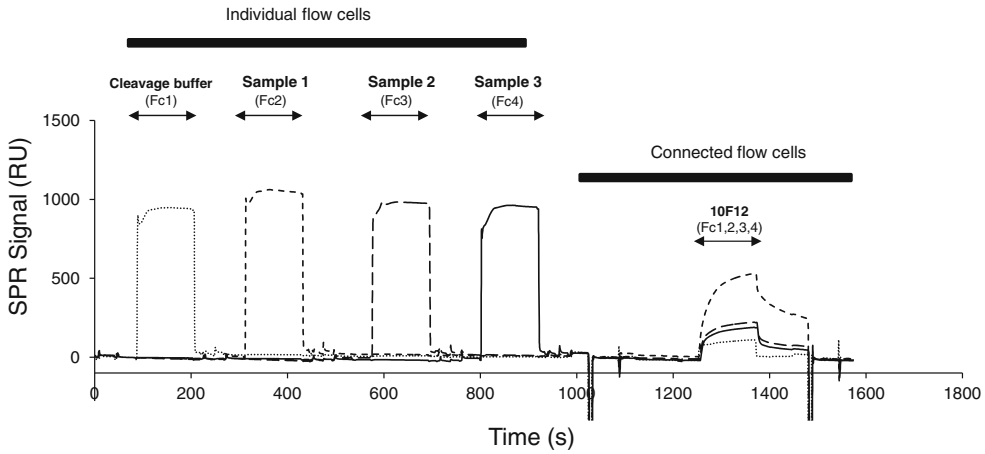

Fig. 4 Assay principle. The enzymatic activity of Dysport batches was measured using the four flowcells (Fc1–4) of a CM5 chip with coupled SNAP-25. Cleavage buffer (flowcell 1) and three Dysport samples were injected sequentially for 2 min into distinct flowcells (flowcell 2, 3, or 4). The four flowcells were connected in series, and then mAb10F12 was injected for 2 min to quantify cleavage. A rapid pulse of pH 1.7 was performed before and after (see off scale downward deflections of SPR signal) injection of mAb10F12 to regenerate the sensor surface

Similar to ELISA or HPLC method, this SPR-based assay allows detection of cleavage product in a controlled buffer avoiding potential interference during the cleavage step. It has been successfully used for determination of BoNT/A enzymatic activity from purified and non-purified BoNT/A from culture supernatants or sera from patients with type A botulism. We now report optimization of this method to assay Dysport, a pharmaceutical BoNT/A preparation. In this case, for the purposes of quality control, we accorded a priority to rapidity and precision rather than sensitivity. The method was adapted for multiple measurements on a single flowcell of a Biacore biosensor, which yields high precision with minimal injection volumes. As the concentration of BoNT/A in therapeutic batches is significantly higher than in clinical specimens, sensitivity was not a requirement. Thus, the substrate cleavage step was reduced to 2 min and amplification of SPR signals with a secondary antibody was not necessary. With a 2-min contact with the substrate chip, the threshold for quantification was 10 LD_{50} Units/ml Dysport, which allows 50 assay points to be determined with a single 500-Unit vial. The procedure is rapid and straightforward and only requires two specific biological reagents: recombinant SNAP-25 and a mAb to detect cleavage products. The enzymatic activity of six unknown samples and six calibration points can be measured in 4 h.

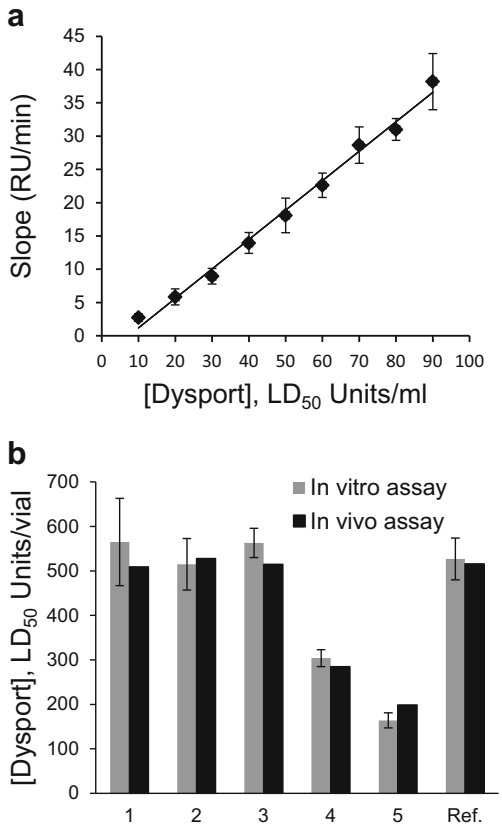

Fig. 5 Assay of Dysport samples. **a** Standard curve for Dysport, (n=4 independent experiments). **b** Comparison between on-chip and mouse assays. SPR analysis of three different vials from five different Dysport batches was performed for each batch. *Error bars* correspond to three independent experiments

The procedure involves four steps: sensor chip functionalization, sample preparation, sample analysis, and data analysis, which can all potentially be automated. Chip functionalization is completely automatic and uses certified coupling reagents. This process can be monitored via the SPR readout to check normalization of lanes and the amount of SNAP-25 coupled. Although we have developed the method with recombinant SNAP-25 as a substrate, it may be advantageous to use a synthetic peptide substrate for routine use in an industrial environment. Sample preparation was carried out manually in the present study, but this step could also be achieved using the automated sampling and dilution functions incorporated in the Biacore 3000 apparatus. The vial contents would be solubilized in cleavage buffer, incubated to reduce

disulfide bonds, and then diluted at a range of concentrations before injection onto the chip. Sample analysis includes measurement of the background signal produced by nonspecific binding of the mAb used to detect cleavage products, sequential injection of the toxin, and the mAb and chip-regeneration, which are all automated.

The mouse bioassay is still the standard method because it evaluates all three functions necessary for toxicity (binding, translocation, endoprotease activity). An intrinsic limitation of endoprotease assays for BoNT/A is that they only report on light chain activity and deleterious alterations to the heavy chain may go undetected. Thus, enzyme activity may not always correlate precisely with LD_{50} data, as binding and translocation, which are also essential for toxicity, are conferred by specific domains on the heavy chain. However, the chip-based assay we present here for measurement of endoprotease activity may be used in association with other in vitro procedures to evaluate heavy chain properties and contribute to reducing the number of mice used in quality control of commercial BoNT/A batches.

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