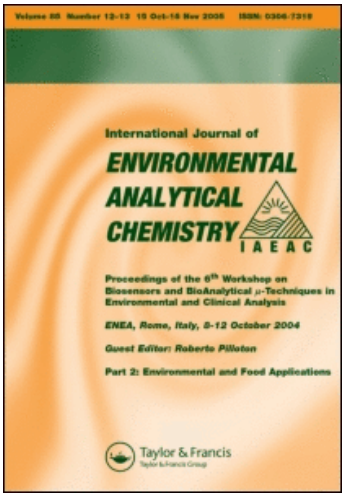

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Micellar electrokinetic chromatography and laser induced fluorescence detection of botulinum neurotoxin type A activity using a dual-labelled substrate

Hongping Wei[a]; Yan Kang[b]; Zhi-ping Zhang[a]; Zong-qiang Cui[a]; Yafeng Zhou[a]; Xian-En Zhang[a]
[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China [b] College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China

To cite this Article Wei, Hongping , Kang, Yan , Zhang, Zhi-ping , Cui, Zong-qiang , Zhou, Yafeng and Zhang, Xian-En(2008) 'Micellar electrokinetic chromatography and laser induced fluorescence detection of botulinum neurotoxin type A activity using a dual-labelled substrate', International Journal of Environmental Analytical Chemistry, 88: 13, 947 — 956
To link to this Article: DOI: 10.1080/03067310802065208
URL: http://dx.doi.org/10.1080/03067310802065208

Intern. J. Environ. Anal. Chem.
Vol. 88, No. 13, 15 November 2008, 947–956

Taylor & Francis
Taylor & Francis Group

Micellar electrokinetic chromatography and laser induced fluorescence detection of botulinum neurotoxin type A activity using a dual-labelled substrate

Hongping Wei[a], Yan Kang[b], Zhi-ping Zhang[a], Zong-qiang Cui[a], Yafeng Zhou[a] and Xian-En Zhang[a]*

[a]*State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China;* [b]*College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China*

(*Received 5 November 2007; final version received 18 March 2008*)

The activity of Botulinum neurotoxin type A (BoNT A) can be measured by monitoring the toxin's endopeptidase reaction with its peptide substrate. In this report, a sensitive and simple capillary electrophoresis (CE) method for analysing BoNT A activity was developed using a peptide substrate labelled with Fluorescein isothiocynate (FITC) at the N-terminal and biotin at the C-terminal. This dual labelling enables not only highly sensitive laser induced fluorescence (LIF) detection of the reaction product, but also good analytical separation of the product from the peptide substrate by Micellar Electrokinetic Chromatography (MEKC). The separation between the product peak and the substrate peak was approximately 5 min using the dual-labelled substrate, while just about 1 min using the FITC-labelled substrate without biotinylation. Using the current assay method, BoNT A with concentration as low as 0.1 ng ml^{-1} (3.6 U mL^{-1} in mouse LD_{50}) in water was detected with a S:N ratio of 3 (RSD <19%) and a linear range of four orders of magnitude. With CE's advantages of very small sample volume needed, this method may find particular applications as in assays of BoNT A activity in water samples and kinetic analyses of toxin activity.

Keywords: botulinum neurotoxin A activity; MEKC; LIF; dual labelling

1. Introduction

Prevalent in soil and marine sediments worldwide, botulism-causing *Clostridium botulinum* bacteria and their spores are also often found on the surfaces of fruits and vegetables, and in seafood. The bacteria and spores themselves are harmless. But the botulinum neurotoxins (BoNT; types A to G) produced by the bacteria are the most lethal biotoxins known, and thus pose a bioterrorism threat [1–4]. BoNTs, especially BoNT A, are also important medical drugs to treat muscle dysfunction, inflammation, and chronic pain [4–7]. These toxins selectively target cholinergic nerve endings, where they act as zinc-dependent endoproteases, cleaving proteins involved in the release of acetylcholine [8].

*Corresponding author. Email: zhangxe@most.cn

ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310802065208
http://www.informaworld.com

Like most other proteins, BoNTs are subject to losing activity upon exposure to unfavourable conditions, such as heating, surfactants and extreme pH [9]. Therefore, these conditions must be avoided in any analytical method for BoNTs. It is important to monitor the activity of BoNTs for assessing BoNT bioterrorism threats in the environment and foods, diagnosis of botulism, and quality control of the therapeutic effects of BoNT medicines.

Characterising BoNT activity traditionally relies on functional assays, such as toxic effects to mice, and immunoassays [9,10]. The mouse lethality assay remains the most sensitive and is the most commonly used test for identifying BoNT activity [10,11]. However, the mouse bioassay requires up to four days for preliminary screening, followed by toxin neutralisation using specific antibodies for serotype identification and confirmation. Efforts to address this time-consuming problem have resulted in a variety of activity assay methods for BoNTs, which take advantage of BoNTs' endopeptidase activity and peptide substrate specificity [12–18]. In these methods, pioneered by Shone's group [18], a peptide substrate is incubated with a specific type of BoNT for a fixed time. After reaction, the cleaved products are assayed either by fluorescence [12,16,17,19], immunoassay [18] or mass spectrometry (MS) [14,20,21]. The amount of the cleaved product(s) is proportional to the activity of the BoNT. Compared with the mouse bioassay, these methods are much more convenient, faster and of higher throughput. Among current assays reported, peptide substrates labelled with fluorescent tags are widely used in a 96 well plate format for screening therapeutic compounds [12,16,17]. Although it is very useful, this format is not suitable for kinetic analysis of toxin activity since the substrates are immobilised on the plate surface. It is also not suitable for analysing BoNT in environmental samples because non-related proteases could cleave the fluorescent substrate and generate false positives. For analysing BoNT A activity in real environmental samples, methods based on expensive mass spectrometers have been proven to be good methods after suitable sample pretreatment [14,20,21].

Capillary electrophoresis (CE) has been emerging as another promising way for analysing BoNT's activity because of the extremely small sample volume needed per analysis and high throughput potential [19,22]. During the assay, CE is used to separate the product from the peptide substrate after reaction with BoNTs. Since the toxin activity is assayed in solution phase, the CE format allows kinetic analyses of toxin activity [22]. However, due to the small light-path length in CE, it is necessary to use sensitive detectors to detect the low concentrations of BoNT. So far in CE, the most sensitive detection is laser induced fluorescence (LIF). To use LIF, the peptide substrates must be labelled with fluorescence dyes. In research for screening a library of peptide that inhibit BoNT A activity [19], fluorescent CBQCA dye was used for labelling the reaction mixtures of BoNT A and its peptide substrates. Because CBQCA dye will label all proteins and peptides existing in the mixtures, separation conditions had to be adjusted for different samples [19]. To solve this problem, we report here a more sensitive and simpler CE method for analysing BoNT A's activity that uses a peptide substrate labelled dually with FITC at the N-terminal and biotin at the C-terminal. The FITC labelling enables highly sensitive LIF detection, while the biotin labelling facilitates good separation of the cleaved product (containing no biotin) from the peptide substrate by Micellar Electrokinetic Chromatography (MEKC) using a sodium dodecyl sulphate (SDS) separation buffer.

2. Experimental

2.1 *Chemicals*

Purified BoNT A was purchased at $1\,mg\,mL^{-1}$ in 50 mM sodium citrate buffer, pH 5.5 from Metabiologics (Madison, WI, USA). The toxin activities in mouse LD_{50} or units (U) of specific toxicity were $3.6 \times 10^7\ U\,mg^{-1}$ according to the provider. Stock solutions ($100\,\mu g\,mL^{-1}$) were kept at 4°C in sterile 50 mM sodium acetate buffer (pH 4.2), 2% gelatin, and 3% bovine serum albumin (BSA). Working dilutions in various matrices were prepared immediately before use. The peptide substrate used for the activity assay has a sequence of FITC-GGGSNRTRIDEANQRATRMLGGK-Biotin. The substrate was synthesised and labelled with biotin at the C-terminal and FITC at the N-terminal by GL Biochem Ltd (Shanghai, China). The peptide's sequence was confirmed by mass spectrometry and the purity was analysed by HPLC as 95% before shipping. The lyophilised peptide powder was dissolved into water to make a stock solution with a concentration of $0.5\,\mu mol\,L^{-1}$.

2.1.1 *Assay procedures for endopeptidase activity of BoNT A*

Assays were conducted by mixing the stock BoNT A solution or water samples with an assay buffer consisting of 50 mM N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) (pH 7.3), 25 mM dithiothreitol (DTT), 0.25 mM $ZnCl_2$, $1\,mg\,mL^{-1}$ BSA and $1\,nmol\,L^{-1}$ peptide substrate. Two kinds of the peptide substrate were used, i.e., peptide substrate labelled just with FITC at the N terminal (single labelled substrate) and peptide substrate labelled with FITC at the N terminal and biotin at the C terminal (dual-labelled substrate). Various amounts of the purified BoNT A were added into the assay buffer to prepare the reaction mixtures with the final BoNT A concentrations indicated. For studying the effects of BSA to the activity assay, assay buffers with different concentrations of BSA (all other components were the same as the above assay buffer) were used. When analysing tap water samples spiked with BoNT A, the reaction mixtures were prepared by mixing 10 μL of the water sample with 40 μL of the assay buffer. All the reaction mixtures prepared above were incubated at 37°C in dark for 6 h unless otherwise indicated and then 0.1 M guanidine HCl was added in to stop the reaction. Finally the reaction mixtures were introduced into the capillary for CE.

2.1.2 *Safety precautions*

Because of the extreme toxicity of BoNT A, all operations such as preparation of BoNT A solutions and mixing samples should be performed in a sealed glove box within a lab with biosafety at least at Level II. Protection clothes and masks have to be used and measures should be taken to avoid forming aerosol during the experiments.

2.2 *Capillary electrophoresis*

A Prince 560 CE instrument (Prince Technologies, Netherlands) with a LIF detector (Picometrics, France) was used for CE analyses. The apparatus employed a 488 nm line of an argon ion laser for fluorescence excitation. An uncoated fused-silica capillary (70 cm length × 75 μm i.d. × 360 μm o.d.) was used throughout the experiments. The length from the injection end to the detection window was 40 cm. The capillary was

maintained at 25 ± 0.1°C. Electrophoresis was performed in a running buffer (SDS separation buffer, pH 9.2) consisting of 56 mM boric acid, 24 mM Na_2CO_3 and 40 mM SDS by 20 kV with positive polarity at the injection end. The samples were injected into the capillary using a pressure pulse of 50 mbar × 0.3 min. Minimum sample volume needed in the sample vial was 10 μL. The capillary was washed with the running buffer for 2 min prior to each run. At the end of each run, the capillary was rinsed with 0.1 M NaOH for 1 min, followed by a rinse with deionised water for 1 min.

Mouse bioassays as described previously (Bacteriological Analytical Manual Online, Chapter 17, http://www.cfsan.fda.gov/~ebam/bam-17.html) were performed to determine the BoNT A toxicity after mixing with the SDS separation buffer. Briefly 1 mL of the SDS buffer was passed through a 0.45 μm sterile membrane filter and then the purified BoNT A was added to prepare a 1 μg mL^{-1} BoNT A solution. At the same time, another 1 μg mL^{-1} BoNT A solution was prepared with the SDS-free running buffer. After incubation at 37°C for 10 min, the two BoNT A solutions were diluted 50 times with a sterile gelatin-phosphate buffer (2.0 g gelatin and 4.0 g Na_2HPO_4 in 1.0 L water, pH 6.2). Then, for each of the solutions, 0.5 mL of the diluted BoNT A solutions was injected intraperitoneally (i.p.) into two mice, respectively, for toxicity test. As negative controls, parallel mouse bioassays were also performed with another two mice after heating the diltuted BoNT A SDS-free solution at 100°C for 10 min.

3. Results and discussions

3.1 *Assay format considerations*

The endopeptidase activity of BoNT A was analysed based on its ability to cleave the peptide substrate (Figure 1). Based on previous studies, the sequence of the substrate was taken from the residues 187–203 of mouse brain protein SNAP-25 [14–16,20,21]. The peptide was modified with biotin at the C-terminal residue and FITC at the N-terminal residue during synthesis. Upon reaction with BoNT A, the peptide is cleaved between the residues Q and R. Therefore, the activity of BoNT A could be determined by measuring the fluorescent product after separating it from the substrate by CE.

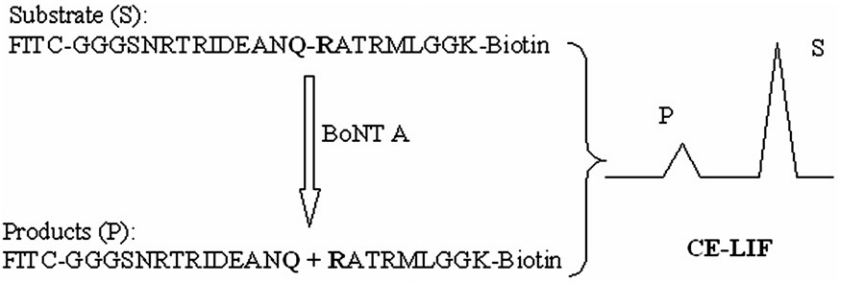

Figure 1. The assay format of BoNT A activity.
Notes: BoNT A cleaves the dual-labelled peptide substrate between Q and R to produce two cleaved products: a fluorescent product (P: FITC-GGGSNRTRIDEANQ) and a non-fluorescent biotinated product. Using a LIF detector, only the fluorescent product (P) needs separating from the peptide substrate (S: FITC-GGGSNRTRIDEANQRATRMLGGK-Biotin) by CE.

3.2 *CE separations with single labelled substrate*

In order to test the effects of biotin labelling on CE separation, the assay buffer containing the single labelled substrate reacted with BoNT A first. Figure 2 shows electropherograms of the assay buffers after reaction with different concentrations of BoNT A for 4 h at 37°C, respectively. We could see that a major substrate peak appeared around 8 min, which was just about one minute earlier than the product peak. In order to separate the substrate peak well from the product peak, a lower substrate concentration (0.2 nmol L^{-1}) and smaller injection time (50 mbar × 0.2 min) have to be used. Because of these, it is not easy to identify BoNT A with concentrations lower than 10 ng mL^{-1}.

3.3 *Activity assay of BoNT A with dual-labelled substrate*

In order to test the feasibility of the double labelled substrate, we first analysed the activity of BoNT A with a concentration of 10 μg mL^{-1}. Figure 3 shows electropherograms of the assay buffers after reaction with BoNT A for 2, 4 and 6 h at 37°C, respectively. We can see that a major substrate peak at about 17 min as well as some minor peaks were detected in the blank solution without adding BoNT A (Figure 3A). Because the dual-labelled peptide substrate was about 95% pure, the minor peaks were clearly due to the impurities in the peptide substrate. In the solutions after reaction with BoNT A, a new product peak was detected at about 12 min and the product peak became higher with increase in the incubation time (Figures 3B, 3C and 3D). These results show that BoNT A can cleave the dual-labelled substrate and the assay format using dual-labelled substrate is well suitable to measure BoNT A endopeptidase activity. Compared with the result using the

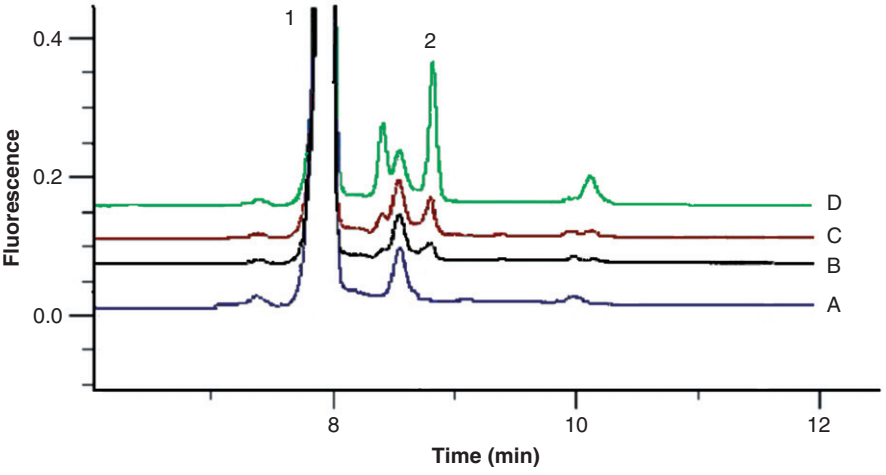

Figure 2. Electropherograms of the reaction mixtures after reaction of the single labelled substrate with different concentrations of BoNT for 6 h.
Notes: (A) Blank solution without adding BoNT A; (B) 10 ng mL^{-1}; (C) 100 ng mL^{-1} (D) 2 μg mL^{-1}. Peak identification: 1. The peptide substrate; 2. The cleaved fluorescent product. Assay buffer: 50 mM Hepes (pH 7.3), 25 mM DTT, 0.25 mM $ZnCl_2$, 1 mg mL^{-1} BSA and 0.2 nmol L^{-1} peptide substrate; Incubation temperature: 37 °C. Separation buffer: 56 mM boric acid, 24 mM Na_2CO_3 and 40 mM SDS, pH 9.2. Injection: 50 mbar × 0.2 min. Separation voltage: 20 kV. The Y scale plotted applies to the electropherogram A only. For other conditions see 'Experimental' sections.

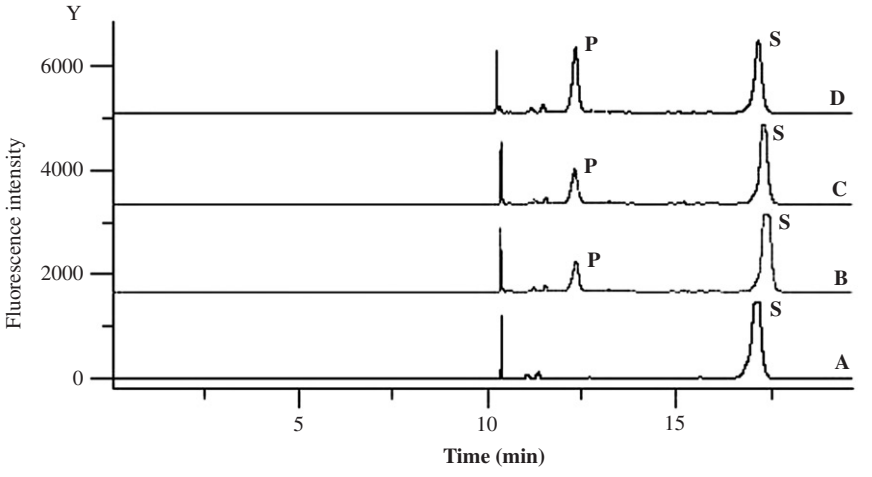

Figure 3. Electropherograms of the reaction mixtures after reaction of the dual-labelled substrate with 10 μg mL^{-1} BoNT A and various incubation times.
Notes: (A) Blank solution without adding BoNT A; (B) 2 h incubation; (C) 4 h incubation; (D) 6 h incubation. Peak identification: P. the cleaved fluorescent product; S. the peptide substrate; Assay buffer: 50 mM Hepes (pH 7.3), 25 mM DTT, 0.25 mM $ZnCl_2$, 1 mg mL^{-1} BSA and 1 nmol L^{-1} peptide substrate; Incubation temperature: 37°C. Separation buffer: 56 mM boric acid, 24 mM Na_2CO_3 and 40 mM SDS, pH 9.2. Injection: 50 mbar × 0.3 min. Separation voltage: 20 kV. The Y scale plotted applies to electropherogram A only. Other conditions see 'Experimental' sections.

substrate without biotinylation (Figure 2), the separation between the product peak and the substrate was increased from about 1 minute to approximately 5 minutes even when a higher concentration of the substrate was used (1 nmol L^{-1}), which justified biotinylation of the peptide substrate.

3.4 *Effect of BSA on activity of BoNT A*

In some studies BSA was included in the assay buffers [18,20]. In other studies BSA-free buffers were used [12,16]. Therefore, in our experiments, we compared the activity assay results using assay buffers with and without BSA. Figure 4 shows that the assay buffer containing 1 mg mL^{-1} BSA gave about five-fold higher amount (on average) of the cleaved product than the buffer without BSA after the same reaction time. This result demonstrated that BSA could improve the activity of BoNT A in aqueous solutions. Further increasing BSA concentration to 2 mg mL^{-1} showed similar results to that of 1 mg mL^{-1} BSA. Actually in previous reports [23,24], human serum albumin and BSA were found to help stabilise the toxicity during lyophilisation of BoNT A and could activate BoNT A also through a direct effect on the kinetic constants of the reaction [24]. Although the actual mechanism behind using BSA to improve BoNT A activity is still unclear, it is beneficiary to include BSA in the assay buffer for increasing sensitivity. Therefore, 1 mg mL^{-1} BSA was included in the assay buffer throughout the following experiments.

Also by comparing the electropherograms shown in Figures 4B and 4C, we can see that there were no additional peaks detected after adding BSA into the assay buffer. This result

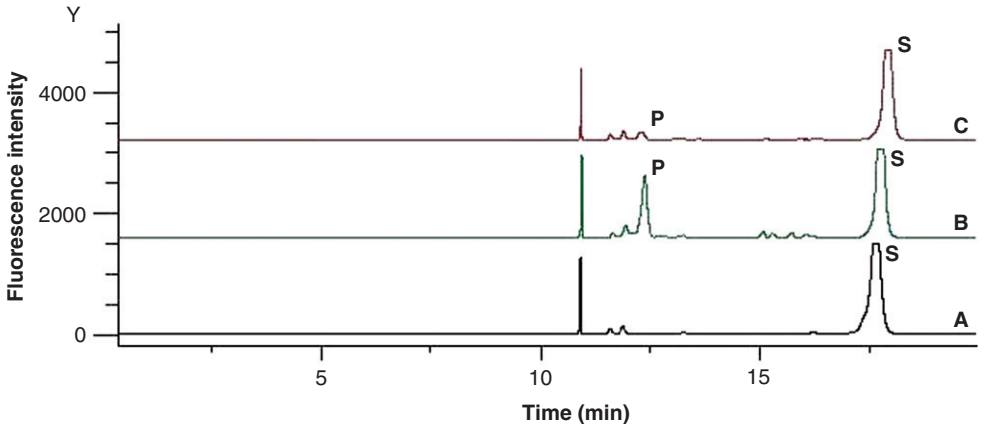

Figure 4. Effects of BSA on activity of BoNT A.
Notes: (A) Blank solution without adding BoNT A; (B) 1 mg mL^{-1} BSA in the assay buffer; (C) No BSA in the assay buffer; Incubation time: 6 h. The Y scale plotted applies to electropherogram A only. Other conditions are same as in Figure 3.

demonstrates that the current assay does not have the problem of co-migration and a uniform separation buffer could be used, compared with the previous post-reaction libelling [19].

3.5 *Roles of SDS in the separation buffer*

Because of the high toxicity of BoNT A, safety during experiments and disposal of the toxin-contaminated waste CE buffer are very important issues. It has been reported that SDS solutions above the micelle concentration could interact with BoNT and cause irreversible changes of the toxin's structures [25]. The toxicity of BoNT A should be deactivated during the MEKC separation since 40 mM SDS in the separation buffer is well above its micelle concentration. To test if 40 mM SDS could deactivate BoNT, mouse assays were performed to detect the toxicity of the solution. No mouse was found dead within 4 days of the assay for the BoNT A solution prepared with the SDS separation buffer. However, another two mice were dead within two days after injecting the BoNT A SDS-free solution. These results showed that the toxicity of BoNT A in the CE waste buffer vial should be quite low after the MEKC separations. The results also demonstrated that besides forming micelles to separate the biotinylated substrate from the cleaved product during the MEKC separations, SDS could be used as an additive to minimise risks of the CE separation buffer contaminated with BoNT A and therefore, provided safer BoNT analysis.

3.6 *Sensitivity of the assay*

Finally we tested the sensitivity of the assay method for activity assay of BoNT A at a fixed incubation time of 6 h. BoNT A with concentration as low as 0.1 ng mL^{-1} could be analysed at a Signal:Noise ratio 3 with relative standard deviation (RSD) <19% (three parallel tests) with a linear range of four orders of magnitude (Figure 5).

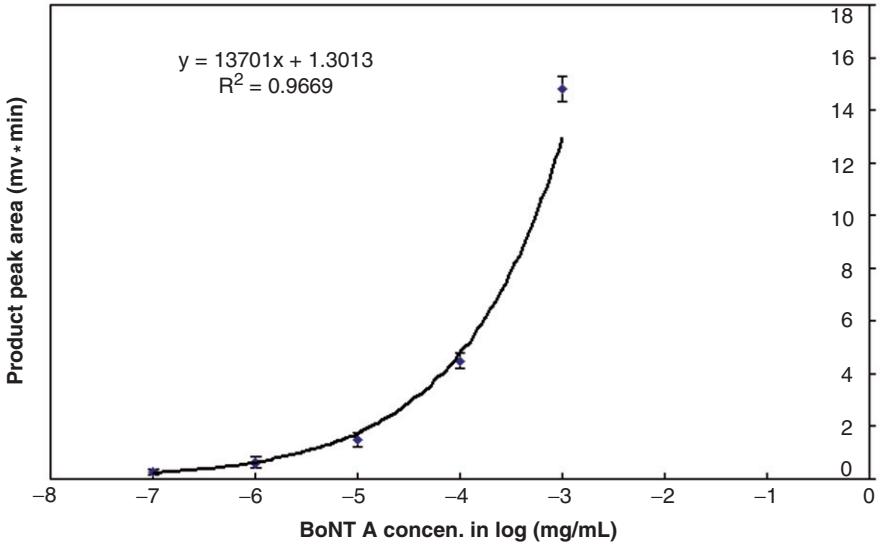

Figure 5. Linear range of the assay by plotting the peak area of the cleaved fluorescent product *vs.* the concentration of BoNT A in log.
Notes: Incubation time: 6 h. Other conditions are same as in Figure 3.

Because the linear range is quite wide, the calibration curve is plotted as peak area of the cleaved product *vs.* BoNT A concentration in log. The current assay demonstrated a detection limit (0.1 ng mL^{-1} or 3.6 U mL^{-1}) close to that of the standard mouse bioassay (1 U mL^{-1}) [26] and the MALDI-TOF-MS for BoNT A (1.25 U mL^{-1}, 4 hours reaction) [20]. The reproducibility of the assay was found with RSD $< 7\%$ for 1 ng mL^{-1} BoNT A calculated by three repeating assays on the same day and RSD $<10\%$ between days.

3.7 *Detection of BoNT A in tap water*

Water sources and drinking water supply systems may be targets for bioterrorism or sabotage. Because BoNT A is one of the most potent biotoxins known, tasteless and odourless, it would generate great concern if drinking and water supply systems were intentionally contaminated by BoNT A. A tap water spiked with 1.0 ng mL^{-1} BoNT A was analysed to test if the method would be applicable for tap water samples. It can be estimated from the toxicological data [27] that the oral lethal dose of this spiked water for a 70 kg human being is about 70 mL. From Figure 6, we can see that a product peak could be well detected after reaction of the water sample with the dual-labelled peptide substrate for 6 h. The result showed that the current method could work for analysing water samples spiked with BoNT A.

To conclude, a sensitive and simple CE-LIF method for detecting activity of BoNT A was developed by labelling its peptide substrate dually with biotin and FITC. A number of improvements compared with previous CE activity assay methods were demonstrated. First, a much simpler CE analysis can be used because just two fluorescent compounds in the reaction mixtures, the cleaved fluorescent product and

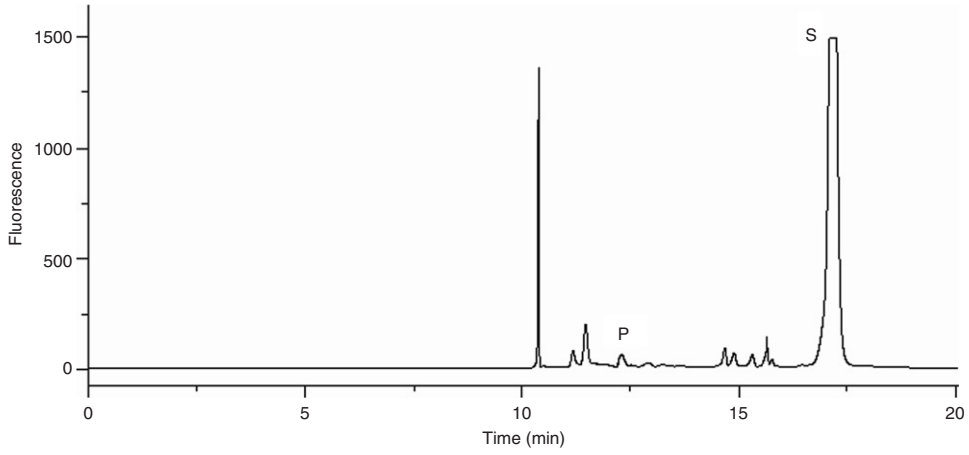

Figure 6. Electropherogram of the reaction mixture after reaction with a tap water sample spiked with 1.0 ng mL^{-1} BoNT A.
Notes: Peak identification: P. the cleaved fluorescent product; S. the peptide substrate; Assay buffer: 50 mM Hepes (pH 7.3), 25 mM DTT, 0.25 mM $ZnCl_2$, 1 mg mL^{-1} BSA and 1 nmol L^{-1} peptide substrate; Incubation temperature: 37°C; Incubation time: 6 h; Other conditions are same as in Figure 3.

the substrate itself, need separating during CE-LIF analysis. Other proteins and peptides in the reaction mixtures, such as the peptide product labelled with biotin and BSA added in the assay buffer, do not need to be considered because they are non-fluorescent. Therefore, uniform separation conditions and highly sensitive LIF detection can be used compared with the previous CE methods using UV detection and post-reaction labelling [19,22]. Second, the biotinylated substrate facilitates good quantitative determination of the cleaved product in the presence of large amounts of the peptide substrate. The BoNT reaction is actually an enzyme reaction. According to enzyme kinetics, higher concentration of substrate will achieve wider assay range for BoNT A. If the substrate is labelled with FITC only, the substrate and the cleaved fluorescent product migrate at only slightly different rates during CE (see Figure 2) because of their similar charge:mass ratio. The major peak of the substrate could affect accurate analysis of the nearby product peak, or even hide the product peak if they are not well separated from each other. Finally, since SDS can interact with BoNT and cause irreversible changes of the toxin's structure [25], the SDS buffer used in the study has an additional advantage of providing a safer way to work with BoNT A. With CE's advantages of small sample volume and high throughput potential, this method may find particular applications as in activity assay of BoNT A in water samples and kinetic analysis of BoNT A activity.

Acknowledgements

This work was supported financially by the National Science Foundation of China (grant: 20545001) and the Chinese Academy of Sciences (grant: GFCX-YJ-23).

References

[1] S.S. Arnon, R. Schechter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A.D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, and K. Tonat, Jama **285**, 1059 (2001).
[2] K.I. Berns, R.M. Atlas, G. Cassell, and J. Shoemaker, Crit. Rev. Microbiol. **24**, 273 (1998).
[3] R.M. Atlas, Crit. Rev. Microbiol. **24**, 157 (1998).
[4] C. Shone, J. Ferreira, A. Boyer, N. Cirino, C. Egan, E. Evans, J. Kools, and S. Sharma, Neurotox. Res. **9**, 205 (2006).
[5] M. Barnes, J. Rehabil. Med. **41** (Suppl.), 56 (2003).
[6] N. Mahant, P.D. Clouston, and I.T. Lorentz, J. Clin. Neurosci. **7**, 389 (2000).
[7] K.A. Foster, E.J. Adams, L. Durose, C.J. Cruttwell, E. Marks, C.C. Shone, J.A. Chaddock, C.L. Cox, C. Heaton, J.M. Sutton, J. Wayne, F.C. Alexander, and D.F. Rogers, Neurotox. Res. **9**, 101 (2006).
[8] G. Schiavo, M. Matteoli, and C. Montecucco, Physiol. Rev. **80**, 717 (2000).
[9] M. Lindstrom and H. Korkeala, Clin. Microbiol. Rev. **19**, 298 (2006).
[10] M. Wictome and C.C. Shone, Symp. Ser. Soc. Appl. Microbiol. **27**, 87S (1998).
[11] H. Kondo, T. Shimizu, M. Kubonoya, N. Izumi, M. Takahashi, and G. Sakaguchi, Japan J. Med. Sci. Biol. **37**, 131 (1984).
[12] J.J. Schmidt and R.G. Stafford, Appl. Environ. Microbiol. **69**, 297 (2003).
[13] S.R. Kalb, M.C. Goodnough, C.J. Malizio, J.L. Pirkle, and J.R. Barr, Anal. Chem. **77**, 6140 (2005).
[14] J.R. Barr, H. Moura, A.E. Boyer, A.R. Woolfitt, S.R. Kalb, A. Pavlopoulos, L.G. McWilliams, J.G. Schmidt, R.A. Martinez, and D.L. Ashley, Emerg. Infect. Dis. **11**, 1578 (2005).
[15] J.J. Schmidt and K.A. Bostian, J. Protein Chem. **14**, 703 (1995).
[16] J.J. Schmidt, R.G. Stafford, and C.B. Millard, Anal. Biochem. **296**, 130 (2001).
[17] C. Anne, F. Cornille, C. Lenoir, and B.P. Roques, Anal. Biochem. **291**, 253 (2001).
[18] B. Hallis, B.A. James, and C.C. Shone, J. Clin. Microbiol. **34**, 1934 (1996).
[19] T.D. Laing, A.J. Marenco, D.M. Moore, G.J. Moore, D.C. Mah, and W.E. Lee, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **843**, 240 (2006).
[20] A.E. Boyer, H. Moura, A.R. Woolfitt, S.R. Kalb, L.G. McWilliams, A. Pavlopoulos, J.G. Schmidt, D.L. Ashley, and J.R. Barr, Anal. Chem. **77**, 3916 (2005).
[21] S.R. Kalb, H. Moura, A.E. Boyer, L.G. McWilliams, J.L. Pirkle, and J.R. Barr, Anal. Biochem. **351**, 84 (2006).
[22] M. Adler, H.F. Shafer, H.A. Manley, B.E. Hackley Jr, J.D. Nicholson, J.E. Keller, and M.C. Goodnough, J. Protein Chem. **22**, 441 (2003).
[23] M.C. Goodnough and E.A. Johnson, Appl. Environ. Microbiol. **58**, 3426 (1992).
[24] J.J. Schmidt and K.A. Bostian, J. Protein Chem. **16**, 19 (1997).
[25] B.R. Singh, M.P. Fuller, and B.R. DasGupta, J. Protein Chem. **10**, 637 (1991).
[26] M. Szilagyi, V.R. Rivera, D. Neal, G.A. Merrill, and M.A. Poli, Toxicon **38**, 381 (2000).
[27] D.M. Gill, Microbiol. Rev. **46**, 86 (1982).