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Analytical Biochemistry 353 (2006) 248–256
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Rapid detection of *Clostridium botulinum* toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection

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Received 2 February 2006
Available online 20 March 2006

Abstract

Sensitive and specific electrochemiluminescence (ECL) assays were used to detect *Clostridium botulinum* neurotoxins serotypes A, B, E, and F in undiluted human serum, undiluted human urine, assay buffer, and selected food matrices (whole milk, apple juice, ground beef, pastry, and raw eggs). These novel assays used paramagnetic bead-based electrochemiluminescent technology in which biotinylated serotype-specific antibodies were bound to streptavidin-coated paramagnetic beads. The beads acted as the solid support and captured analyte from solution. Electrochemiluminescent detection relied on the use of ruthenium chelate-labeled anti-serotype antibodies and analysis with a BioVeris M-Series M1R analyzer. The sensitivities of the assays in clinically relevant matrices were 50 pg/ml for serotypes A and E, 100 pg/ml for serotype B, and 400 pg/ml for serotype F. The detection limits in selected food matrices ranged from 50 pg/ml for serotype A to 50 to 100 pg/ml for serotypes B, E, and F. The antibodies used for capture and detection exhibited no cross-reactivity when tested with the other serotypes. When purified native toxin was compared with toxins complexed to neurotoxin-associated proteins, no significant differences in assay response were noted for serotypes A, B, and F. Interestingly, the native form of serotype E exhibited reduced signal and limit of detection compared with the complexed form of the protein. We suspect that this difference may be due to trypsin activation of this particular serotype. The assays described in this article demonstrate limits of detection similar in range to the gold standard mouse bioassay, but with greatly reduced time to data. These rapid sensitive assays may have potential use in clinical settings, research studies, and screening of food products for botulinum toxins.
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Keywords: Botulinum toxin; Toxin detection; Clinical samples; Food; Paramagnetic beads; Immunodetection; Electrochemiluminescence assay; *Clostridium botulinum*

Of the seven *Clostridium botulinum* neurotoxin (BoNT)[1] serotypes, only A, B, E, and F are associated with human intoxications [1]. These toxins selectively target cholinergic nerve endings, where they act as zinc-dependent endoproteases, cleaving proteins involved in the release of acetylcholine and, in severe cases, resulting in paralysis or death [2]. BoNTs have high substrate specificity with respect to amino acid length and composition [3]. Their enzymatic activities and substrate specificities have been the focus of intense research designed to discover inhibitors capable of reversing the neurotoxin-induced morbidity. Even with progress in this area, difficulties in the treatment and diagnosis of botulism remain. The exceedingly small quantity of toxins needed for intoxication (<1 μg/kg) requires rapid and sensitive assay detection for these proteins, often in complex matrices. The mouse lethality assay remains the most sensitive and most commonly used test

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[1] *Abbreviations used:* BoNT, *Clostridium botulinum* neurotoxin; PCR, polymerase chain reaction; nt., purified; comp., complexed; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CRDA, cooperative research and development agreement; Ru, ruthenium; S/B ratio; signal/background ratio.

0003-2697/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ab.2006.02.030

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
20 MAR 2006	**N/A**	**-**

4. TITLE AND SUBTITLE
Rapid detection of selected botulinum toxins in serum, urine, and assay buffer using paramagnetic bead technology, Analytical Biochemistry 353:248 - 256

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Rivera, VR Gamez, FA Rossio, J White, J Poli, MA

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases,Fort Detrick

8. PERFORMING ORGANIZATION REPORT NUMBER
RPP-04-450

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
Sensitive and specific electrochemiluminescence (ECL) assays were used to detect Clostridium botulinum neurotoxins serotypes A, B, E, and F in undiluted human serum, undiluted human urine, assay buffer, and selected food matrices (whole milk, apple juice, ground beef, pastry, and raw eggs). These novel assays used paramagnetic bead-based electrochemiluminescent technology in which biotinylated serotype-specific antibodies were bound to streptavidin-coated paramagnetic beads. The beads acted as the solid support and captured analyte from solution. Electrochemiluminescent detection relied on the use of ruthenium chelate-labeled anti-serotype antibodies and analysis with a BioVeris M-Series M1R analyzer. The sensitivities of the assays in clinically relevant matrices were 50pg/ml for serotypes A and E, 100pg/ml for serotype B, and 400pg/ml for serotype F. The detection limits in selected food matrices ranged from 50pg/ml for serotype A to 50 to 100pg/ml for serotypes B, E, and F. The antibodies used for capture and detection exhibited no cross-reactivity when tested with the other serotypes. When purified native toxin was compared with toxins complexed to neurotoxin-associated proteins, no significant differences in assay response were noted for serotypes A, B, and F. Interestingly, the native form of serotype E exhibited reduced signal and limit of detection compared with the complexed form of the protein. We suspect that this difference may be due to trypsin activation of this particular serotype. The assays described in this article demonstrate limits of detection similar in range to the gold standard mouse bioassay, but with greatly reduced time to data. These rapid sensitive assays may have potential use in clinical settings, research studies, and screening of food products for botulinum toxins.

15. SUBJECT TERMS
Clostridium botulinuim, neurotoxin, electrochemiluminescence assay, ECL, paramagnetic bead technology, detection, methods

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**9**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

for identifying BoNT intoxication. The bioassay is very time-consuming because it requires time for preliminary screening, followed by toxin titration and finally toxin neutralization by specific antibodies for serotype identification and confirmation. In addition, increasing concern about the use of laboratory animals underscores the need for replacement assays that are equally sensitive and reliable. Attempts to address this problem have resulted in the development of a variety of assays for BoNTs. Activity assays take advantage of the endopeptidase activity and substrate specificity and have been developed for serotypes A, B, and F [3,4]. One problem with such assays is the susceptibility of the assay substrates to nonrelated proteases and the consequent risk of false positives. A variety of antibody-based assays that are both sensitive and serotype specific have been proposed [5–8]. A drawback with all immunoassays is their inability to distinguish biologically active toxin or toxin fragments from biologically inactive ones. The antibodies used to capture and detect the toxins do not differentiate between "native" toxin and denatured toxin or toxin fragments that are not biologically active but contain recognizable antibody binding epitopes. The same statement applies to polymerase chain reaction (PCR) tests that can detect the relevant BoNT gene fragment but are unable to determine the presence or toxicity of the expressed toxin.

The use of paramagnetic bead-based electrochemiluminescent detection offers significant advantages over the use of traditional assays. The signal is generated after capture of complexes containing paramagnetic beads complexed with capture and detection antibodies and the antigen of interest by a magnet on the instrument electrode. Most potential interfering substances in the matrix are washed away during an online bead wash step; thus, matrix effects are minimized or eliminated. The near solution phase reaction is rapid and does not require prebinding of the capture antibody to the plate, antigen coating, or any manual blocking or wash steps. It is a one-step assay involving the mixing of all reagents in a microtiter plate, a brief incubation step, and an automated plate read on the bead-based electrochemiluminescent detector.

In today's political environment, biological warfare/bioterrorism possibilities associated with the *C. botulinum* toxins are of potential concern. Therefore, rapid and sensitive detection, confirmation of biological activity, and an understanding of the in vivo pharmacokinetics of these toxins are of high priority. This article describes the use of paramagnetic bead-based electrochemiluminescent technology for the rapid and sensitive detection of BoNTs A, B, E, and F in undiluted human serum, undiluted human urine, assay buffer, and selected food matrices. These tests originally were developed to monitor the toxins in food samples [8]. Work described herein expands on that application and provides evidence suggesting that the sensitivity of these assays could permit studies of the pharmacokinetics of biologically active *C. botulinum* toxins in animal models without the need for radioactive toxin analogs.

Materials and methods

Toxins

Purified (nt.) and complexed (comp.) forms of BoNTs A (nt. $= 1.6 \times 10^7$ mouse units [MU]/100 μg; comp. $= 4.5 \times 10^6$ MU/100 μg), B (nt. $= 7.0 \times 10^7$ MU/100 μg; comp. $= 1.4 \times 10^6$ MU/100 μg), E (nt. $= 4.5 \times 10^6$ MU/100 μg; comp. $= 2.5 \times 10^5$ MU/100 μg), and F (nt. $= 4.4 \times 10^5$ MU/100 μg; comp. $= 2 \times 10^5$ MU/100 μg) were purchased from Metabiologics (Madison, WI, USA). Stock solutions (10 μg/ml) were kept at 4 °C in sterile 50 mM sodium acetate buffer (pH 4.2), 2% gelatin, and 3% bovine serum albumin (BSA) as per the manufacturer's instructions. Working dilutions in the various matrices were prepared immediately before use. The biological activity (mouse bioassay) and purity (gel electrophoresis) of the native and complexed neurotoxins were confirmed by the manufacturer before shipment.

Reagents

Assay reagents

Assay buffer consisted of 60 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 and 0.2% BSA. Normal human urine was purchased from Sigma Chemical (St Louis, MO, USA) as a lyophilized powder and reconstituted in distilled deionized water. Normal human serum was purchased from Cambrex Bio Science (Walkersville, MD, USA).

Paramagnetic bead-based electrochemiluminescence detection procedure

Test kits for BoNTs A and B (part 145006) and BoNTs E and F (part 145010) were provided by BioVeris International (Gaithersburg, MD, USA) as part of a cooperative research and development agreement (CRDA). The sandwich immunoassay-based test kits (Fig. 1) used a biotinylated rabbit anti-serotype antibody and streptavidin-labeled paramagnetic beads that serve to capture toxin on the bead surface. The toxins were detected using a second rabbit antibody labeled with a ruthenium (Ru) chelate. When toxin was present in the sample, an immunocomplex in which both capture and detection antibodies were bound to the antigen was formed, resulting in a Ru-labeled paramagnetic bead. Within the flow cells of the M-Series M1R analyzer (BioVeris), a magnet collected the microbeads onto the surface of an electrode within the instrument. In the presence of toxin, the Ru-containing complexes on the surface of the bead led to an electrochemiluminescent signal that is detected and quantitated by the instrument. Specifically, 100 μl of each spiked sample matrix was added to a 96-deep-well microtiter plate (0.5 ml/well), followed by 150 μl of assay diluent. Then 100 μl each of capture antibodies (prebound to streptavidin-coated microbeads) and detection reagent (Ru-labeled anti-serotype antibodies) were added to all wells. The microplate was incubated at

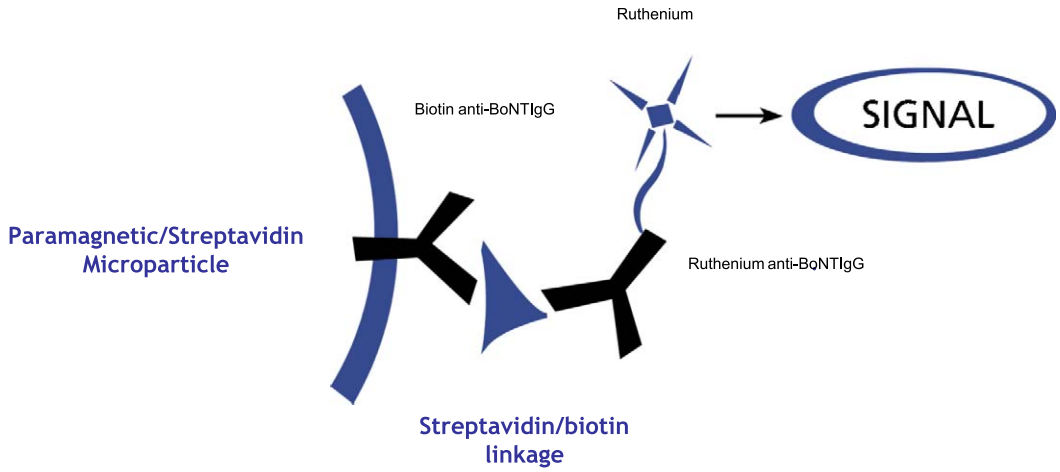

Fig. 1. Assay format. The toxin tests used in this study employed a sandwich immunoassay format. An antibody specific to the toxin binds the antigen to a paramagnetic microparticle through a streptavidin/biotin linkage. The toxin is detected using a second antibody that is labeled with BV-TAG label. When the toxin is present in the sample, both antibody components of the assay bind to the antigen, resulting in a BV-TAG-labeled microparticle. A magnet is used to collect the microparticle on the surface of an electrode within the instrument system. If the toxin is not present in the sample, no BV-TAG label is bound to the microparticles and no signal is observed.

room temperature for 60 min with shaking and was read on the M-Series M1R analyzer. The entire assay, from sample preparation to final results, required only approximately 2 h.

Cross-reactivity studies with other serotypes

Cross reactivity studies for serotype A toxin were performed in the presence of homologous antibodies to serotypes B, E, and F following the paramagnetic bead-based electrochemiluminescence detection procedure described above. The procedure was repeated for serotype B, E, and F toxins versus the respective test antibodies.

Sample preparation

Serum and urine were used undiluted and without sample filtration or other preparation. The various foods were homogenized with equal volumes of PBS (Sigma Chemical) with no pH adjustment after extraction. The homogenates were then centrifuged at 13,000*g* for 15 min at 4 °C. Whole milk, ground beef, apple juice, pastry, and raw eggs were purchased from a local supermarket. Clinical samples and food extract supernatants were processed as described above and spiked with the various concentrations of BoNTs.

Data analysis

Assays were evaluated for slope and coefficient of regression of standard curves based on average of quadruplicate determinations. Assay sensitivity was defined as the minimal concentration of antigen that provided a signal of two times the background signal (minus antigen). Each data point represented the average and standard deviation of four replicate wells (data points). The assays for each serotype were developed and optimized for incubation time and reagent (and/or sample) volumes to achieve the desired sensitivity in an incubation time of 1–2 h.

Results

Standard curves in serum, urine, and assay buffer

In this study, standard curves for BoNTs A, B, E, and F were constructed from data derived by spiking serum, urine, and buffer (Figs. 2–5). The threshold for a positive reading was determined by calculating a signal/background (S/B) ratio. An S/B ratio of 2 (for serotypes A, B, and E) or higher (for serotype F) was considered to be positive. Standard curves for serotypes A, B, E, and F in undiluted human serum are shown in Figs. 2A, 3A, 4A, and 5A, respectively. The backgrounds were low for all serotypes in this particular matrix, ranging from 220 to 331 relative luminescence units. S/B ratios for serotypes A, B, E, and F were 2.1, 1.5, 3.1, and 1.1, respectively, at a toxin concentration of 50 pg/ml. Quenching of the signal was observed in the presence of serum. This effect was present regardless of the serotype but did not interfere with the limits of detection of the assays. The assays effectively measured 50 pg/ml (22.5 pg/well) for serotypes A and E, 100 pg/ml (45 pg/well) for serotype B, and 400 pg/ml (180 pg/well) for serotype F.

Figs. 2B, 3B, 4B, and 5B show standard curves of data for toxin detection in undiluted human urine for serotypes A, B, E, and F, respectively. Unlike serum, no signal suppression was noticed for this particular matrix. Our assays again showed low backgrounds for BoNTs A (344), B (471), E (411), and F (403), with S/B ratios of 4.7, 2.4, 7.4, and 2.3, respectively, indicating a sensitivity of

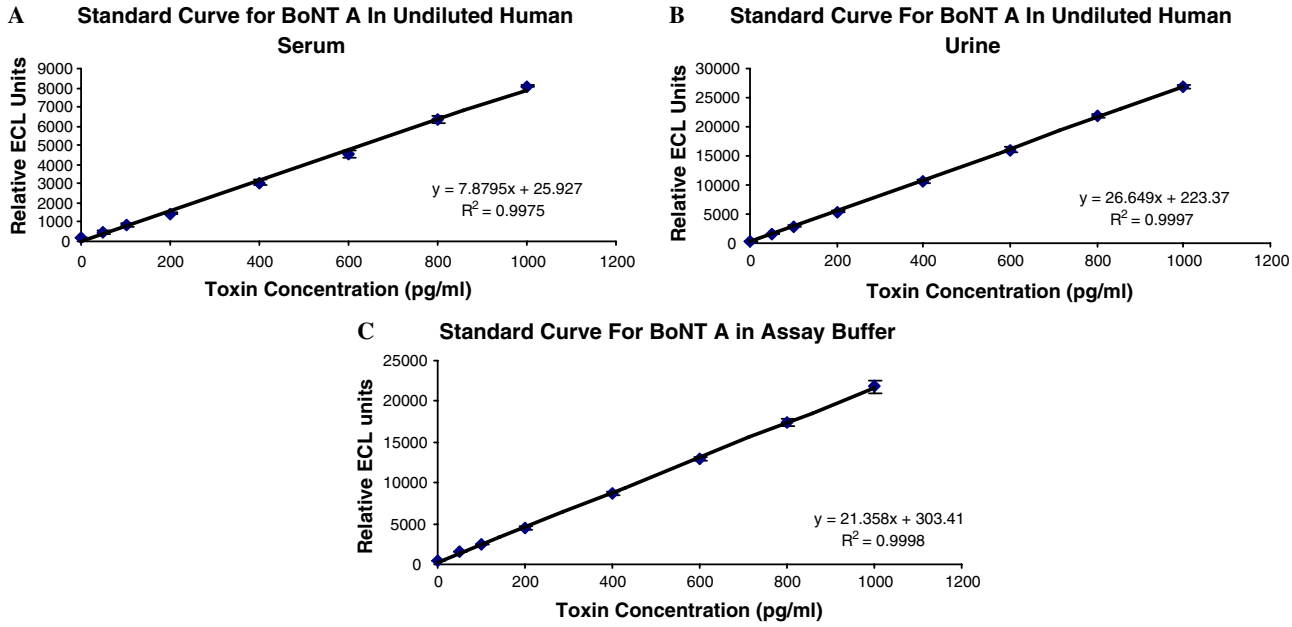

Fig. 2. Standard curves for BoNT A in undiluted human serum (A), urine (B), and assay buffer (C). The concentrations described reflect the actual spiked values per milliliter of matrix.

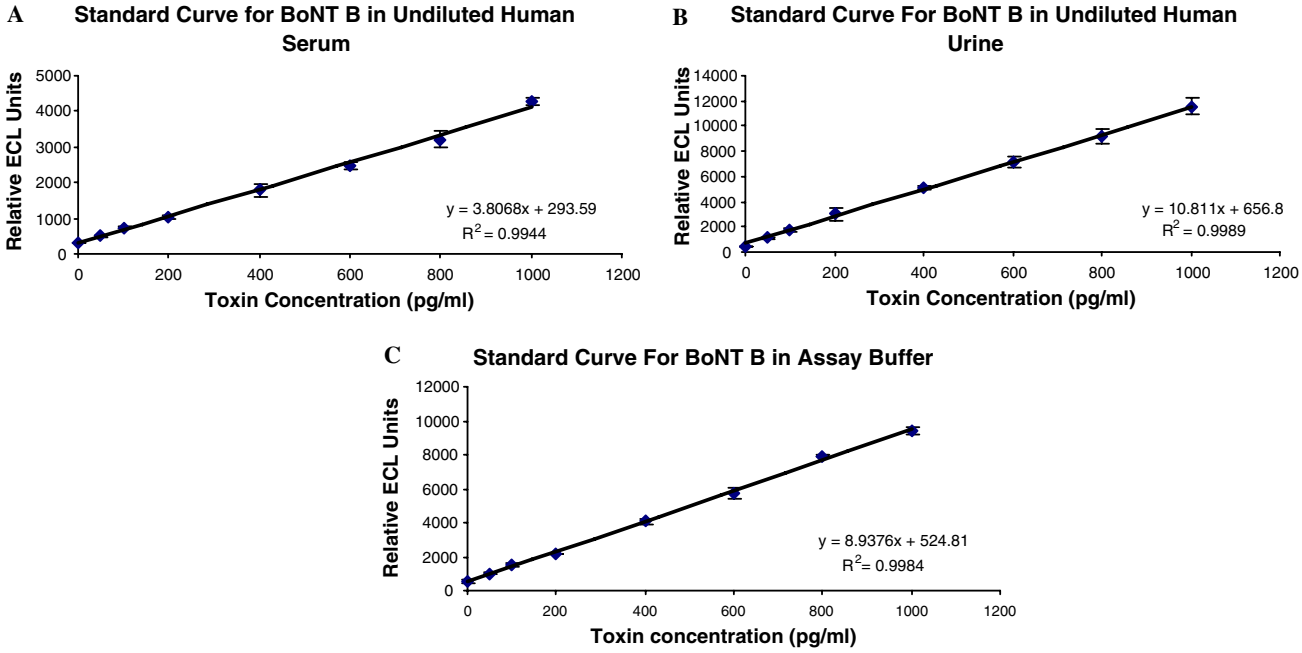

Fig. 3. Standard curves for BoNT B in undiluted human serum (A), urine (B), and assay buffer (C). The concentrations described reflect the actual spiked values per milliliter of matrix.

50 pg/ml (22.5 pg/well) or less for all of the serotypes in this particular matrix.

The assays also performed very well in assay buffer for all of the serotypes (Figs. 2C, 3C, 4C, and 5C for serotypes A, B, E, and F, respectively). The detection limits for serotypes A, B, and E were $\geqslant$50 pg/ml (22.5 pg/well), and the detection limit for serotype F was approximately 400 pg/ml (180 pg/well).

Standard curves in food extracts

Standard curves of spiked BoNTs in selected food matrices are shown in Fig. 6. The assay could detect BoNT A (Fig. 6A) in all selected matrices with a detection limit of $\leqslant$50 pg/ml with S/B ratios of 3.6 for milk, 4.5 for meat, 4.4 for both eggs and pastry, and 2.9 for apple juice. The signal was most intense for apple juice, followed by pastry, meat,

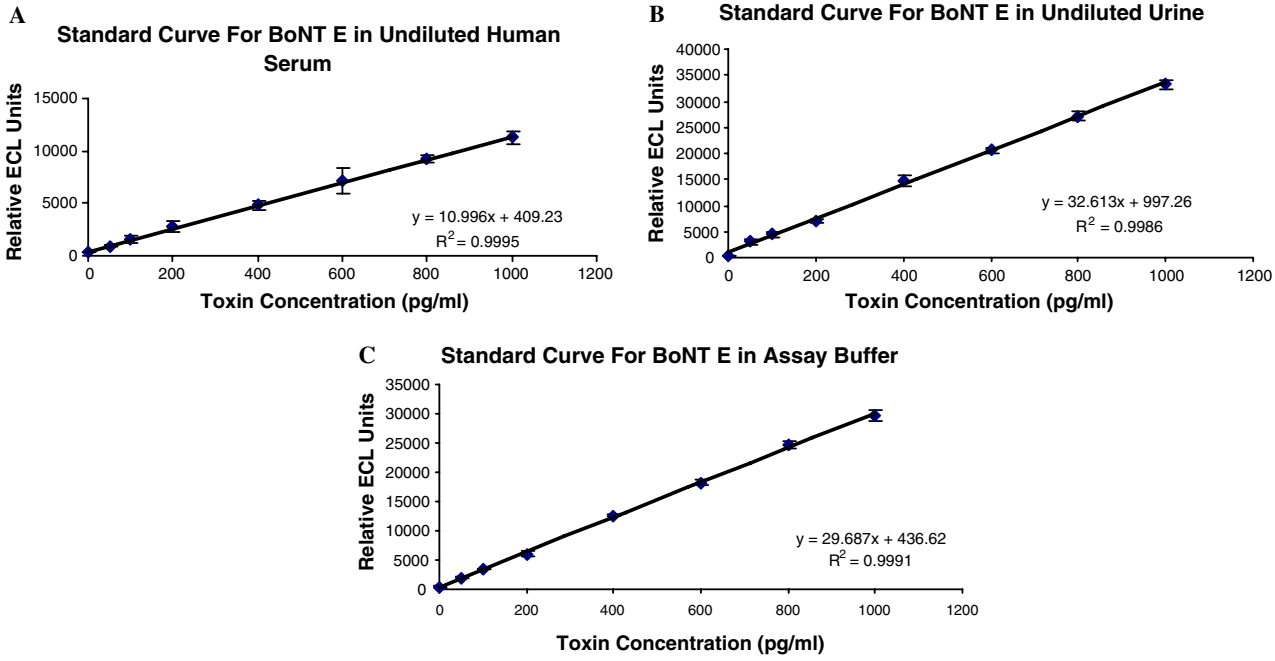

Fig. 4. Standard curves for BoNT E in undiluted human serum (A), urine (B), and assay buffer (C). The concentrations described reflect the actual spiked values per milliliter of matrix.

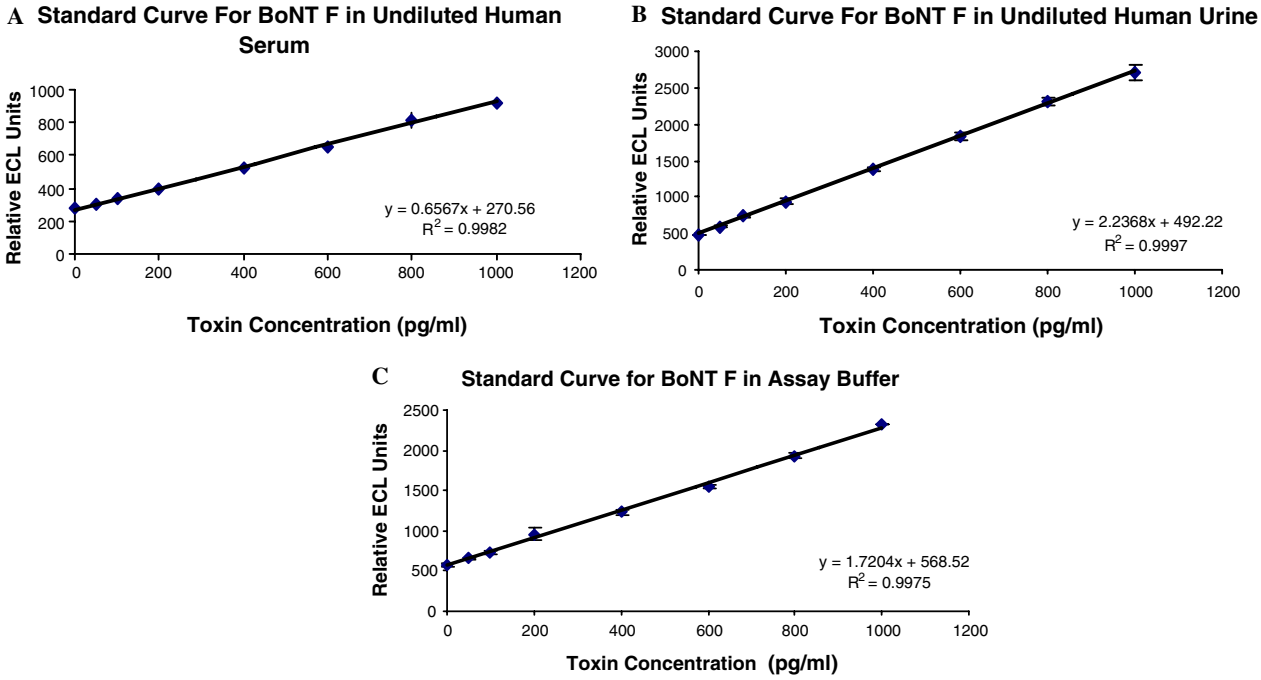

Fig. 5. Standard curves for BoNT F in undiluted human serum (A), urine (B), and assay buffer (C). The concentrations described reflect the actual spiked values per milliliter of matrix.

and eggs, with milk consistently being the lowest for all serotypes. Longer incubation times—approximately $1\frac{1}{2}$ to 2 h—will result in a more robust assay and increased sensitivity. Because it was our goal to perform all assays in 2 h or less from sample preparation to results, we settled on a 1-h incubation.

Similar results were observed for BoNT B (Fig. 6B). The detection limits were in the range of 100 pg/ml for milk, meat, and eggs, with S/B ratios of 2.3, 2.1, and 2.5, respectively. Pastry and apple juice showed limits of detection of approximately 200 pg/ml, with S/B ratios of 2.0 for these two matrices.

Matrices spiked with BoNT E (Fig. 6C) had detection limits of approximately 100 pg/ml, showing S/B ratios ranging from 2.0 to 2.6. The least sensitive assay tested was that for BoNT F (Fig. 6D). The lower limit of detec-

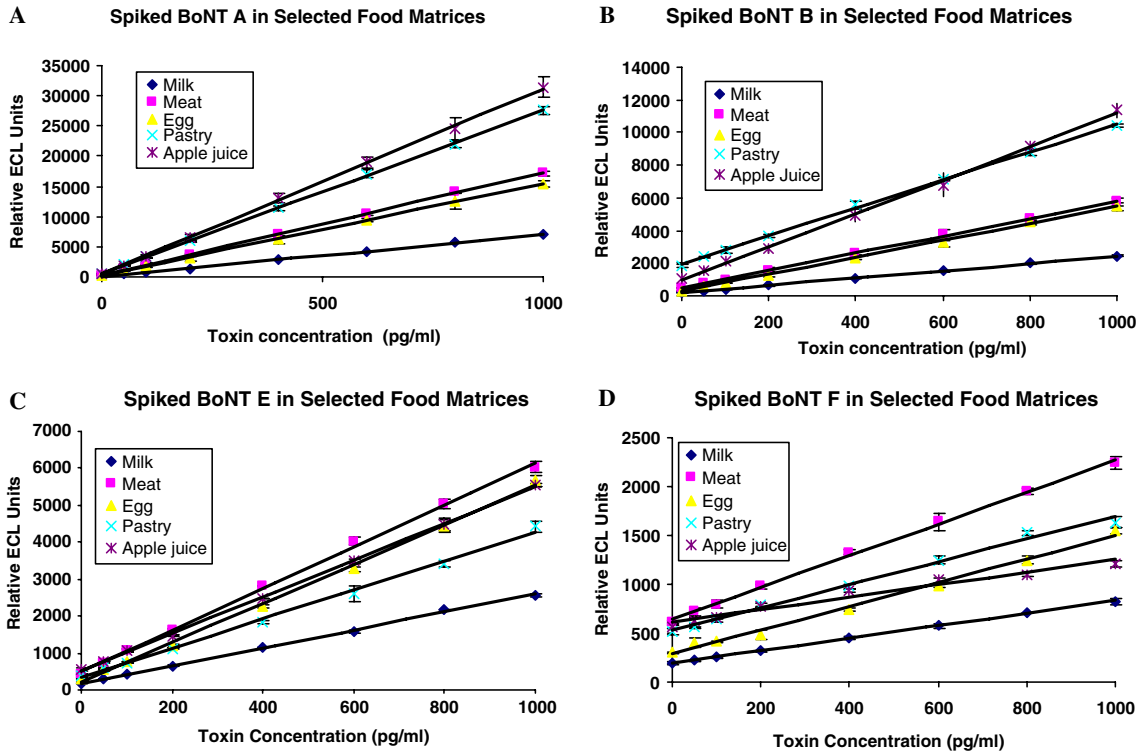

Fig. 6. Standard curves of BoNTs A (A), B (B), E (C), and F (D) in selected food matrices. The concentrations described reflect the actual spiked values per milliliter of matrix.

tion for this particular serotype was in the range of 400 pg/ml for milk, meat, and eggs, with S/B ratios of 2.3. 2.2, and 2.4, respectively. Pastry had a limit of detection of approximately 600 pg/ml, with an S/B ratio of 2.4, and apple juice had a limit of detection of approximately 800 pg/ml.

Comparison of pure BoNTs with complex forms

All testing described above was done with toxin standards composed of highly purified toxins devoid of associated proteins. Under natural conditions, BoNTs are synthesized and released from the bacteria with hemagglutinins or other "noncovalently associated" proteins [9]. Although these proteins are not implicated in toxicity, they are known to protect the toxins from the acidic environment of the stomach and from possible degradation in the intestinal tract. It is important to evaluate any assay against the complexed forms as well because these are most likely to be found in real intoxication situations. It is difficult to accurately compare both forms on a mass unit basis because the native pure form will be more toxic per unit of protein than will the complex form [6,7]. The relationship of toxicity to protein toxin concentration was determined by the manufacturer and noted in the certificate of analysis provided with each serotype as described in Materials and methods. MUs were used as the basis for our comparison between complexed and pure neurotoxins. Based on this, we determined that our antibodies recognized the complex and the neurotoxin approximately equally for serotypes A, B, and F (Figs. 7C, D, and A, respectively). These data agree with previous findings by Poli and coworkers [6] and Szilagyi and coworkers [7]. We also observed that this recognition was different for serotype E (Fig. 7B). The production of mature serotype E protein involves a trypsin activation step. We suspect that the trypsin activation step used in the process of this particular serotype may have altered the recognition epitopes that are necessary for binding by the antibodies used in this assay.

Cross-reactivity between serotypes

The reagent pairs for the various serotypes were tested for cross-reactivity (Fig. 8) against heterologous toxin serotypes at the range of the standard curve used for the assay. There was no significant cross-reactivity for any of the serotypes tested.

Discussion

Bead-based electrochemiluminescence detection assays have been developed for BoNTs A, B, E, and F in undiluted human serum, undiluted human urine, assay buffer, and selected food matrices. The assays performed well in all matrices. Variation among four replicate well data points was less than 5% for all assays. Linearities, as measured by the correlation coefficients (r^2) of the regressed lines, ranged from 0.9944 to 0.9997 for all assays. Detection limits for all serotypes, as defined by an S/B ratio of 2.0, were

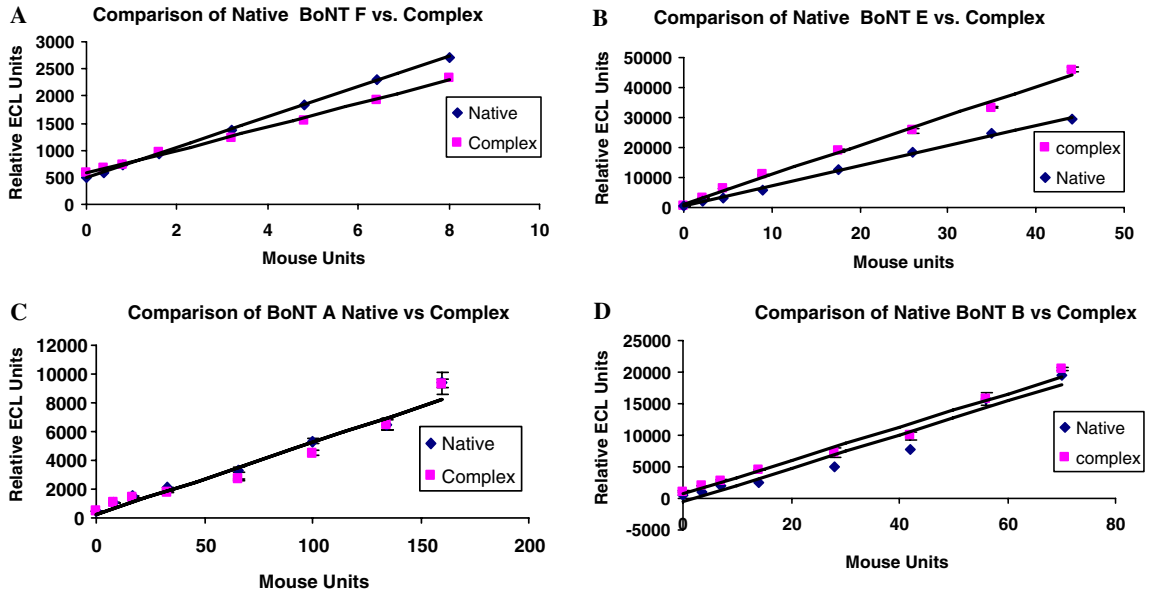

Fig. 7. Recognition of toxins with associated nontoxic proteins. (A–D) The detection of native toxin versus toxin complexed with associated nontoxic protein. Although there is no significant difference in the detection of serotypes A, B, and F (C, D, and A, respectively), there is a difference in the recognition by antibody to serotype E (B).

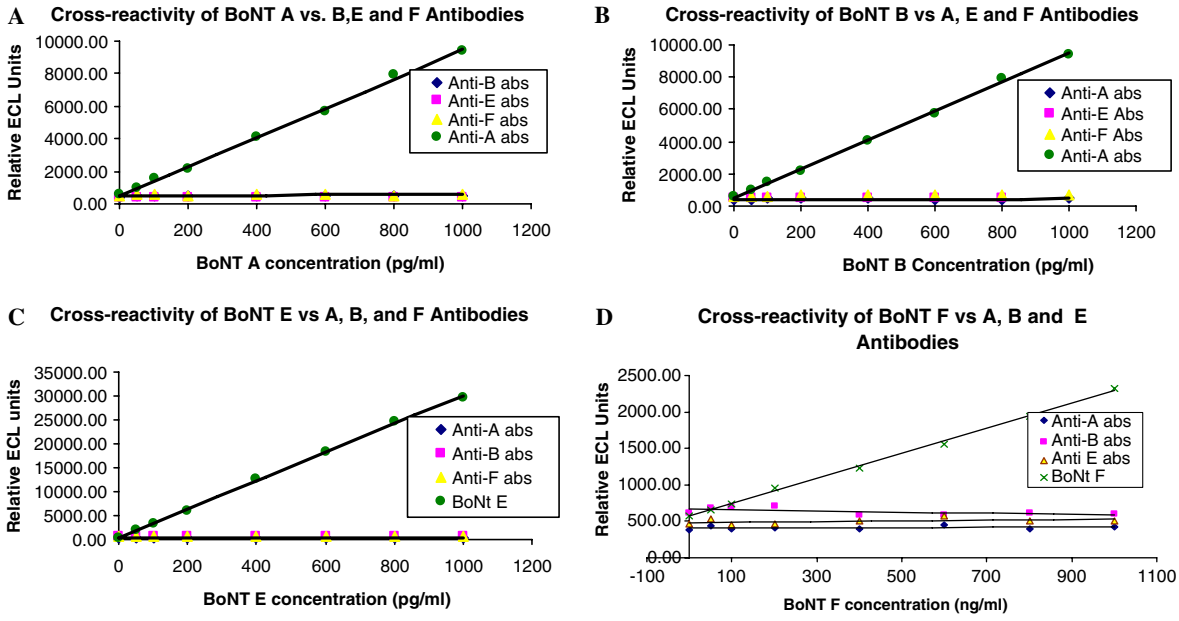

Fig. 8. Cross-reactivity among serotypes A (A), B (B), E (C), and F (D). The reagent pairs to the various serotypes were tested for cross-reactivity against heterologous toxin serotypes at the range of the standard curve used in the study. There was no significant cross-reactivity for any of the antibodies, indicating the serotype specificity of the antibodies.

in the low picogram/milliliter range. These new assays offer a promising alternative for detecting botulinum toxins in a rapid, sensitive, and reliable manner. Importantly, these assays offer sensitivity comparable to that of the gold standard mouse bioassay without the time involved and the use of animals. It is interesting that even with 30–60% homology among serotypes, many of the polyclonal antibodies against the various serotypes exhibited little to no cross-reactivity. This opens the possibility of a "multiplex" type of assay in which a variety of detection antibodies could be used in one single platform for detecting these toxins rapidly and reliably. This is important in the management of an outbreak of the illness in which serotype identification is important for the effective administration of immunotherapy. The use of "near real time" rapid diagnostics that are accurate, sensitive, and reliable is critical to the identification and administration of the correct treatment.

BoNT A has become widely used as a therapeutic agent for treating a variety of muscular conditions [10] and is also popular in cosmetic applications. The therapeutic reagent

is evaluated using the mouse bioassay to monitor its biological activity and determine the effective dose. The use of functional assays to monitor the biological activity has been proposed [11,12]. Even with the successful implementation of functional assays, it is important to determine the actual amount of toxin/protein present in each preparation. One of the problems in many recipients of the therapeutic toxin products is the eventual development of immune-induced resistance that reduces the therapeutic benefit [13,14]. By accurately measuring the actual toxin concentration (by immunoassays) and the active fraction (by functional assay), one can maximize effectiveness while minimizing side effects. Our assay is capable of detecting concentrations similar to the mouse bioassay in approximately 2 h, including assay preparation time, and should be ideally suited for this type of application.

Sensitive and specific detection of these toxins is needed not only for therapeutic applications but also for investigations of suspected outbreaks of botulism-based food poisoning. Few assays described in the literature [15–18] approach the required sensitivity for low-level detection and are generally not commercially available. One exception is the assay developed by Doellgast and coworkers [19], but this test has limited application because it is very expensive and involves extensive manipulations and a complex amplification system.

The most prominent disadvantage of immunoassays is the inability to differentiate between active toxins and inactive toxins. This problem could be overcome by the use of a platform that combines activity/functional-based assays with immunodetection. Advances in this field [10,11] and our unpublished work suggest that these will be available in the near future. The bead-based electrochemiluminescence assays are rapid because they avoid the plate preparation, offline washes, and extended incubation times required with similar immunoassay protocols. Although matrix-induced signal quenching occurred for normal human serum, it did not reduce the limits of detection in this particular matrix. There was negligible cross-reactivity of antibodies against the other toxin serotypes. Together, these data suggest that sensitivities afforded by these new assays could be used to support pharmacokinetic studies of these toxins in animal models using unlabeled toxin, thereby avoiding the complications associated with the use and disposal of radiolabeled toxin analogs.

The sensitivity of these newly described assays, like that of any other immuno-based assay, depends on the use of high-quality reagents. The use of high-affinity capture antibodies combined with affinity purification, signal amplification, and low background qualifies the bead-based electrochemiluminescence detection as one of the most sensitive and versatile systems available. Recent advances in the field of bioengineering have resulted in a variety of methods for engineering antibodies with increased antigen affinity and specificity [20]. Elegant work by Nowakowski and coworkers [21] demonstrated that it is possible to generate recombinant antibodies of high affinity that are capable of toxin neutralization for therapeutic purposes. This potentially offers an unlimited supply of high-affinity antibodies that could be customized and used in assay development.

Rapid reliable diagnostic tests are a required tool in the management of botulinum intoxication. The BioVeris electrochemiluminescence test kits provide fast and accurate results (1–2 h) compared with other immunoassays. The tested ability of these assays to recognize both complexed and pure forms of the serotype A, B, E, and F neurotoxins provides an important screening tool in the management of *C. botulinum* intoxication.

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