(12)

EDGEWOOD
RESEARCH,
DEVELOPMENT &
ENGINEERING
CENTER

ERDEC-CR-002

DETECTION OF PICOGRAM QUANTITIES OF BOTULINUS TOXIN-B USING THE LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR

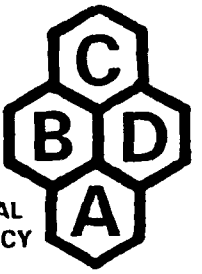
James P. Chambers

UNIVERSITY OF TEXAS AT SAN ANTONIO
San Antonio, TX 78285

James J. Valdes

RESEARCH AND TECHNOLOGY DIRECTORATE

December 1992



U.S. ARMY
CHEMICAL
AND BIOLOGICAL
DEFENSE AGENCY

Aberdeen Proving Ground, Maryland 21010-5423

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1992 December	Final, 91 Sep - 92 Jan

4. TITLE AND SUBTITLE
Detection of Picogram Quantities of Botulinus Toxin-B Using the Light Addressable Potentiometric Sensor

5. FUNDING NUMBERS
C-DAAA15-C-0008

6. AUTHOR(S)
Chambers, James P. (University of Texas at San Antonio), and Valdes, James J. (ERDEC)

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas at San Antonio, San Antonio, TX 78285

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
DIR, ERDEC,* ATTN: SCBRD-RT, APG, MD 21010-5423

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
ERDEC-CR-002

11. SUPPLEMENTARY NOTES
*At the time this work was completed, ERDEC was known as CRDEC.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Detection of picogram quantities of Botulinus toxin-B (BoTX), using Protein A purified biotin- and fluorescein-labelled antibodies, is described in this report. All assays were performed on the Light Addressable Potentiometric Sensor. Results indicate a near linear dose response curve for increasing the amounts of BoTX (10-1,000 pg). The presence of 1-30 ng of toxin resulted in a dose response curve that yielded high output signals (>4,000 μV/s) at saturation (approximately 25 ng). When challenged with nine different venoms, the biosensor indicated very low output signals (15% control), which indicated minimal biosensor nonspecificity.

14. SUBJECT TERMS
Receptors Toxins
Biosensors

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Blank

PREFACE

The work described in this report was authorized under Contract No. DAAA15-C-0008. This work was started in September 1991 and completed in January 1992.

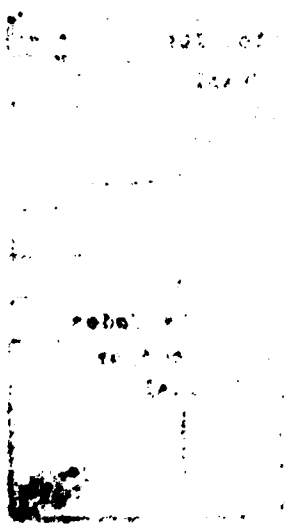
The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for release to the public.

Acknowledgments

The authors thank Dr. Eppie Rael (University of Texas at El Paso, El Paso, TX) for supplying the immunoreagents; James Colston and Dr. Pramod Kumas (University of Texas at San Antonio, San Antonio, TX) for technical support; and Marcy Goforth (Research and Technology Directorate, ERDEC) for preparing the final manuscript.

Blank



CONTENTS

Blank

DETECTION OF PICOGRAM QUANTITIES OF BOTULINUS TOXIN-B USING THE LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR

1. INTRODUCTION

The extremely potent exotoxins produce by Clostridium botulinum [also known as botulinus toxin (BoTX)] cause fatal neurotoxicity in both animals and humans. Seven serologically distinct BoTXs were isolated from this organism and each share similar structural characteristics. A 145,000-150,000 M_r protoxin is enzymatically cleaved into the active toxin, which consists of two peptides of approximately 50,000 and 100,000 M_r linked by a disulfide bridge.[1] The active toxin binds specifically to gangliosides and prevents presynaptic release of acetylcholine by an unknown mechanism.[2]

Sensitive and rapid detection of BoTX is invaluable for testing contamination of foodstuffs. In this report, we describe an immunoligand assay that used a Light Addressable Potentiometric Sensor (LAPS), purchased from Molecular Devices Corporation (Menlo Park, CA), to detect picogram (pg) quantities of BoTX. The LAPS is a recently developed, sensitive and reliable technology utilizing nitrocellulose filters onto which fluorescein-labelled immunocomplexes are immobilized and labelled with an antifluorescein urease conjugated antibody.[3,4,5] Quantitation of toxin is accomplished by inserting immobilized, urease-labelled immunocomplex into a reader containing a solution of urea. Enzymatic breakdown of urea produces a potentiometric shift proportional to the amount of urease, hence, toxin, which is detected rapidly with high sensitivity and reproducibility. Biotin-and fluorescein-labelled anti-BoTX polyclonal antibodies were used in this study to detect picogram quantities of toxin on the LAPS.

2. MATERIALS AND METHODS

2.1 Reagents.

Goat polyclonal antiserum against BoTX-B was obtained from Biodesign International (Kenne Bunkport, ME). Antibodies were biotinylated and fluoresceinated using a labelling kit that contained the N-hydroxysuccinimide esters of DNP-biotin and carboxyfluorescein. Labelling reagents were reconstituted with dimethylformamide. Samples were concentrated using Centricon-30 Microconcentrators (Amicon Division, W.R. Grace and Company, Beverly, MA). All general laboratory reagents were of the highest quality. Venoms from the Heloderma horridum, Naja nigricollis, Agkistrodon acutus, Apis mellifera, Leiurus quinquestriatus, Bitis nasicornus, Naja atra, Crotalus, viridis helleri, and Bufo americus were purchased from Sigma Chemical Company (St. Louis, MO).

2.2 Instrumentation.

All assays were performed on the LAPS, and the data were collected and stored digitally on a microcomputer.

2.3 Purification and Labelling of Immunoglobulin (IgG).

IgG was purified from serum by passage over a Protein A column, following the procedures described by the manufacturer (Promab, JRH Biosciences, Lenexa, KS). Antibodies were concentrated using a Centricon-30 microconcentrator (20,000 x g for 20 min) and washed two times with phosphate buffered saline (PBS) until a volume of 1 mL remained. The final concentration of purified IgG was 1.36 mg/mL as determined by the Bradford method[6] using bovine serum albumin as a standard.

Antibody preparations were separated into two aliquots. One aliquot was biotinylated [DNP biotin labelling reagent for 2 hr at room temperature using a molar coupling ration (MCR) of 30:1]. The remaining aliquot was fluoresceinated (fluorescein labelling reagent for 2 hr at room temperature using an MCR of 30:1). We determined (data not shown) that a n MCR of 30:1 provides adequate and efficient labelling of IgG antibodies. Unreacted haptens were removed by dialysis following six, 1-L changes of PBS. Dialyzed samples were stored at 4 °C.

2.4 Assay Procedure.

For each test, BoTX and venoms were dissolved in a 100-μL assay buffer (Molecular Devices Corporation). Labelled antibodies were then diluted in the assay buffer to a final concentration of 2 ng/μL. Prior to assaying, biotinylated and fluoresceinated antibodies were combined in a 1:1 ratio in that each 100 μL contained 100 ng of each labelled antibody. We determined this ratio and concentration of antibodies to be optimal for sensitive detection of BoTX (data not shown).

Primary immunocomplexes were formed by adding 100 μl antibody solution to the reaction mixture containing toxin. After gently vortexing, assay mixtures were incubated at 37 °C for 45 min. After incubation, 1 mL of capture reagent (Molecular Devices Corporation) containing streptavidin was added to each reaction mixture followed by a brief mixing. Each assay mixture was then transferred immediately to separate test wells, and vacuum filtered through a biotinylated nitrocellulose filter mounted on a test stick (Molecular Devices Corporation). Immunocomplexes were captured (immobilized) by formation of a nitrocellulose biotin-streptavidin-biotin conjugate. Unbound material was removed by adding a 2-mL wash buffer (Molecular Devices Corporation) to each test well and followed by vacuum filtration.

Urease conjugated, antifluorescein, antibody solution (Molecular Devices Corporation) was added to each test well and filtered. A final wash with a 2-mL wash buffer was performed, and the nitrocellulose test sticks were removed and then placed in a beaker containing wash buffer until read. Each nitrocellulose stick was inserted into the LAPS reader, and the respective immunoconjugates were monitored via hydrolysis of urea.

3. RESULTS

The effect of increasing amounts of BoTX on output signal is shown in Figure 1. A linear response ($r = 0.98$) over the lowest range of toxin from 100 to 1,000 pg was observed (shown in A, Figure 1). A lower detection limit of 250 pg (78.7 ± 1.5 μV/s) is attainable under these conditions. A dose response curve of increasing amounts of BoTX (1-30 ng) that indicates saturation to occur at approximately 25 ng with a corresponding mean output signal of 4219.4 ± 301.4 μV/s is shown in B, Figure 1. Further, the effect of increasing BoTX over an extended concentration range (10^{-10} - 10^{-6} g) is shown in C, Figure 1. Figure 2 shows the effect of various venoms on the output signal, and the results indicate very low, assay nonspecificity (<15% of control).

4. DISCUSSION

Active BoTX-B consists of heavy and light chains (M_r approximately 100,00 and 50,000, respectively) linked by a disulfide that are bridged together with several Botulinum-associated protein that yield a complex a with a molecular weight of nearly 500,000 datlons.[7] Toxins produced from nonproteolytic cultures require exogenous protease treatment for protoxin activation.[8] However, in this study, BoTX-B was not treated with exogenous protease. Previous studies in our laboratory indicated the toxin complex to be extensively nicked (5 pieces), which upon Western blotting, revealed approximately 70% of the immunoreactivity to reside in 2 peptides (M_r = 37,480 and 50,470).[9]

Detection of picogram quantities of BoTX was achieved using labelled polyclonal IgG antibodies. Data in Figure 1 indicate the lowest range of detection under these assay conditions to be between 100 and 250 pg. This amount of toxin approaches the minimal lethal dose in the mouse.[10,11] Although the output signals observed when detecting picogram quantities of BoTX are rather low (<200 μV/s), they are very reproducible, which are indicated by the fact that the standard error bars are not visibly distinct from the data points shown in A, Figure 1.

Output signal produced by toxin in the 1-30 ng range (B, Figure 1) indicated saturation to occur in approximately 25 ng toxin. BoTX in excess of 30 ng results in a sharp decrease in the signal shown in C, Figure 1, is most likely due to dilution of antibody by excess toxin. It is essential that antibody concentration be in relatively large excess when compared to that of toxin for reliable detection. Detection of toxin in excess of 30 ng is possible, but larger amounts of labelled antibody is required, which results in a reduction of sensitivity.

It was important to determine the extent of biosensor nonspecificity. To accomplish this, the effect of nine different challenge venoms on the signal output was determined. No attempt was made to isolate and purify individual proteins from the venoms for the purpose of exposing the assay to the maximum amount of toxic venom component(s). When compared to an

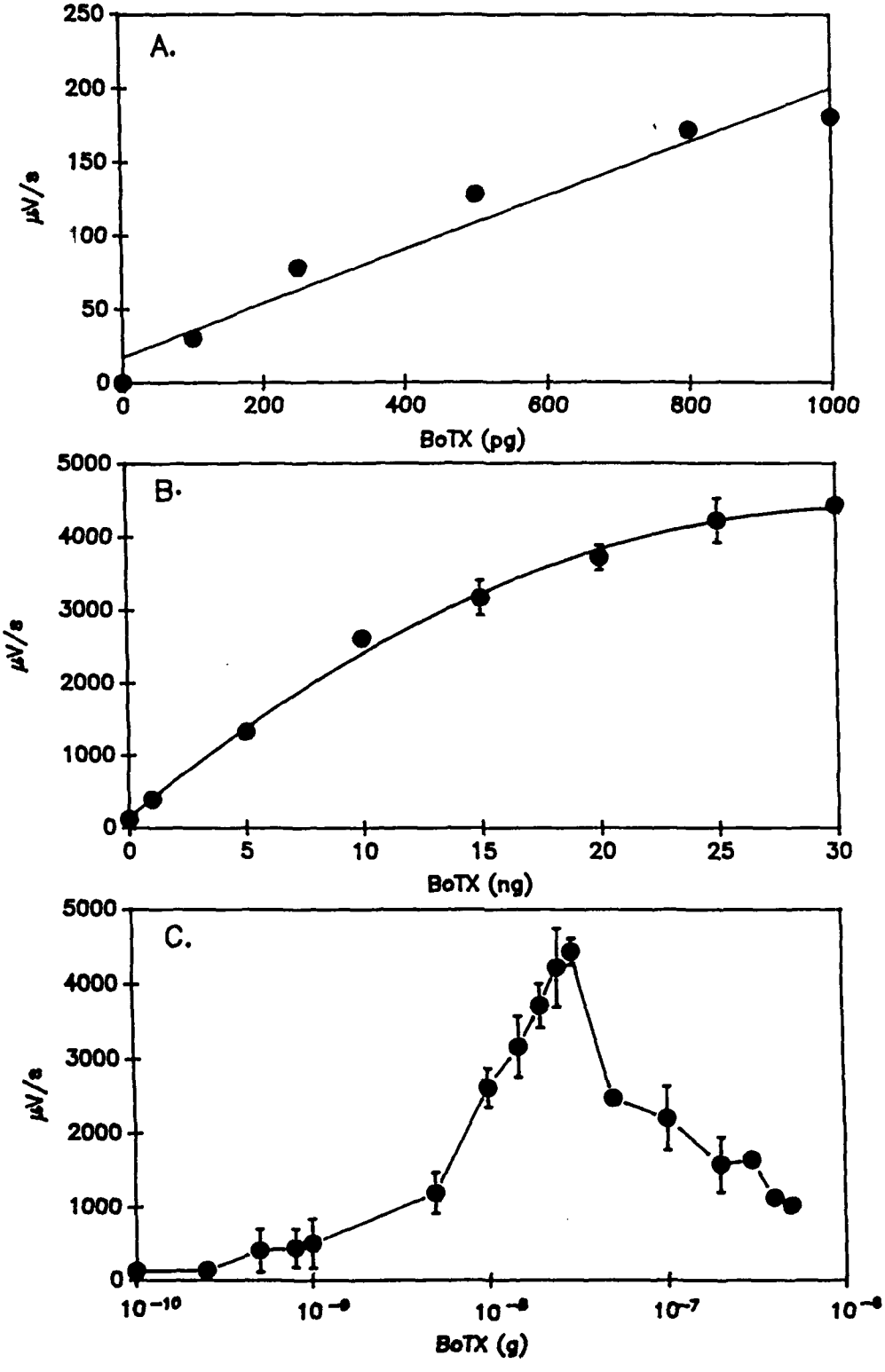


Figure 1. Detection of BoTX

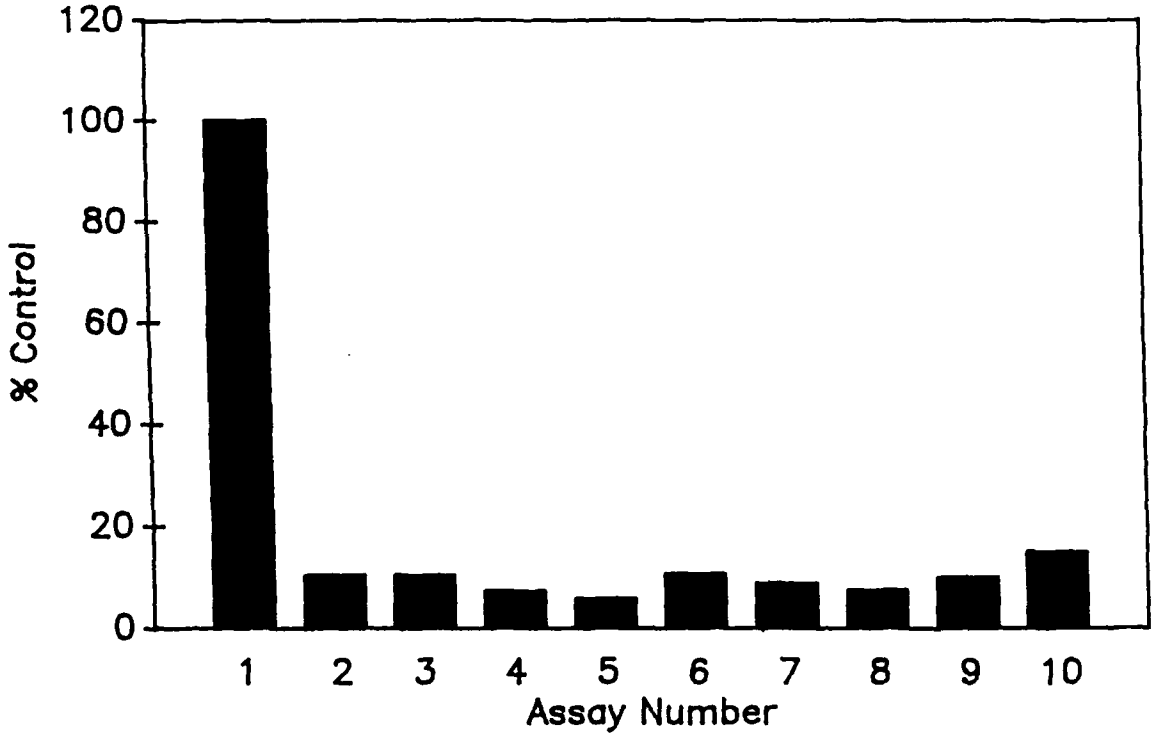


Figure 2. Nonspecific Output Signal Produced by Nine Difference Venoms

LITERATURE CITED

1. DasGupta, B.R., and Sugiyama, H., Biochem. Biophys. Res. Commun. Vol. 48, p 108 (1972).

2. Burgen, A.S.V., Dickens, F., and Eatman, L.J., J. Physiol. London Vol. 109, p 10 (1949).

3. Briggs, J., Nature, Vol. 329, p 565, 1987.

4. Hafeman, D., Parce, J., and McConnell, H., Science, Vol. 240, p 1182, 1988.

5. Parce, J., et al., Science, Vol. 240, p 1182, 1988.

6. Bradford, M.A., Anal. Biochem. Vol. 72, p 248 (1976).

7. Wagman, J., and Bateman, J.B., Arch. Biochem. Biophys. Vol. 31, p 424 (1951).

8. DasGupta, B.R., J. J. Physiol. Paris Vol. 84, p 220 (1990).

9. Kumar, P., et al., "Detection of Botulinus Toxin Using an Evanescent Wave Immunosenor," Recent Advances in Toxicology Research, Vol. III, pp 361-374, 1992.

10. Hanig, J.P., and Lamanna, C., J. Theor. Biol. Vol. 77, p 107 (1979).

11. Schantz, E.J., and Johnson, E.A., Microbiol. Vol. 56, p 80 (1992).