RESEARCH NOTE

Rapid Method Using High Performance Liquid Chromatography for the Purification of Tetanus Toxoid

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The tetanus toxin is produced by the *Clostridium tetani* and appears into the medium after lysis of the bacteria (B Bizzini et al. 1969 *Ann Inst Pasteur 116:* 686-711, R Veronesi 1981 Prophylaxis p. 238-263. In R Veronesi *Tetanus important new concepts*, Excerpta Medica, Amsterdam). As this powerful toxin is fatal after contact with the organism, its neutralization by formaline before purification by ammonium sulfate precipitation is a preferable aproach to industrial methods of purification. In the other hand, this procedure results in the production of alergenic contaminants present in the purified anatoxin. So, different methods have been described to purify the toxoid (L Levine, JL Stone 1951 *J Immunol 67*: 235-241, WC Latham et al. 1965 *J Immunol 95:* 487-493, 1967 *Appl Microbiol 15*: 616-621, K Ozutsumi et al. 1985 *Appl Environ Microbiol 49*: 939-943, SMA Prado et al. 1992 *Bol Biotecnol 3*: 11-16, 1993 *Bol Biotecnol 4*: 3-8) and two important points were recomended in the control of purified toxoids. These are purity and absence of specific toxicity (R Donikian 1991 p. 26-31. In CR Manclark *Department of Health and Human Services, United State Public Health Service*. Publication No. (FDA) 91-1174, Bethesda, Maryland, USA). In the course of numerous high-performance liquid chromatography (HPLC) determinations of the tetanus toxoid content of industrial preparations, it was observed that the antigenic compound could always be obtained as a peak distinct from those of most other components. This suggested the possibility of developing a simple procedure for the isolation of tetanus toxoid by direct gel permeation.

The samples of tetanus toxoid employed in this study were generously provided by Butantan Institute (BI) (Lot 92/23; Lf 490/ml; 1.53 µg/µl), São Paulo and Vital Brazil Institute (VBI) (Lot 11/89; Lf 1750/ml; 6.4 µg/µl), Rio de Janeiro, Brasil. Briefly, the toxin was obtained from broth medium [Mueller medium modified by WC Latham et al. (1965 *J Immunol 95*: 487-493)] of *C. tetani*, Harvard-Caracas strain, concentrated 6-10 times and sterilized by ultrafiltration and inactivated during 30 days at 37°C using 10% (v/v) formaline. The toxoid was water washed (1:2, v:v) and concentrated using an ultrafiltration (Pellicon, Millipore) system.

For the analysis and purification of the active toxoid an automatic high performance liquid chromatography system (Model 6A, Shimadzu, Kyoto, Japan) was employed using a Shim-pack Diol 150 gel filtration column (5 µm; 50 cm x 7.9 mm; Shimadzu, Kyoto, Japan) at a variable flow rate (0.6-1.2 ml/min). The peaks were collected automatically and the specific tetanus toxoid detected by double imunodifusion (O Ouchterlony 1962 p. 30-154. In P Kallas, BH Wasman *Progress in Allergy*, Basel, Karger, Swizerland). The plates were incubated overnight at room temperature with 10 µl of standard horse sera anti-crude tetanus toxin, supplied by WHO (Stateus Seruminstitut, Copenhagen, Denamark), and aproximately 0.2 µg of each peak protein. Then washed two days in phosphate-buffered saline containing 0.2% NaN_3 at room temperature and stained with 0.25% Coomassie brilhant Blue R in ethanol/acetic acid/water (5:1:5) for 5 min. Plates were destained in 35% (v/v) ethanol/10% (v/v) acetic acid and photographed.

Figure 1 shows the elution profile on a Diol-150 gel filtration HPLC column of two different industrial preparation of tetanus toxoid. Only 26 min was required for this HPLC step at a flow rate of 1 ml/min using 50 mM phosphate buffer pH 7.0. Flow rates below (0.6 or 0.8 ml/min) or above (1.2 ml/min) this value were not useful for separate the peak 1 and 2 (data not shown). The elution profile showed at least six peaks. Ouchterlony analysis with polyclonal antibodies showed that the

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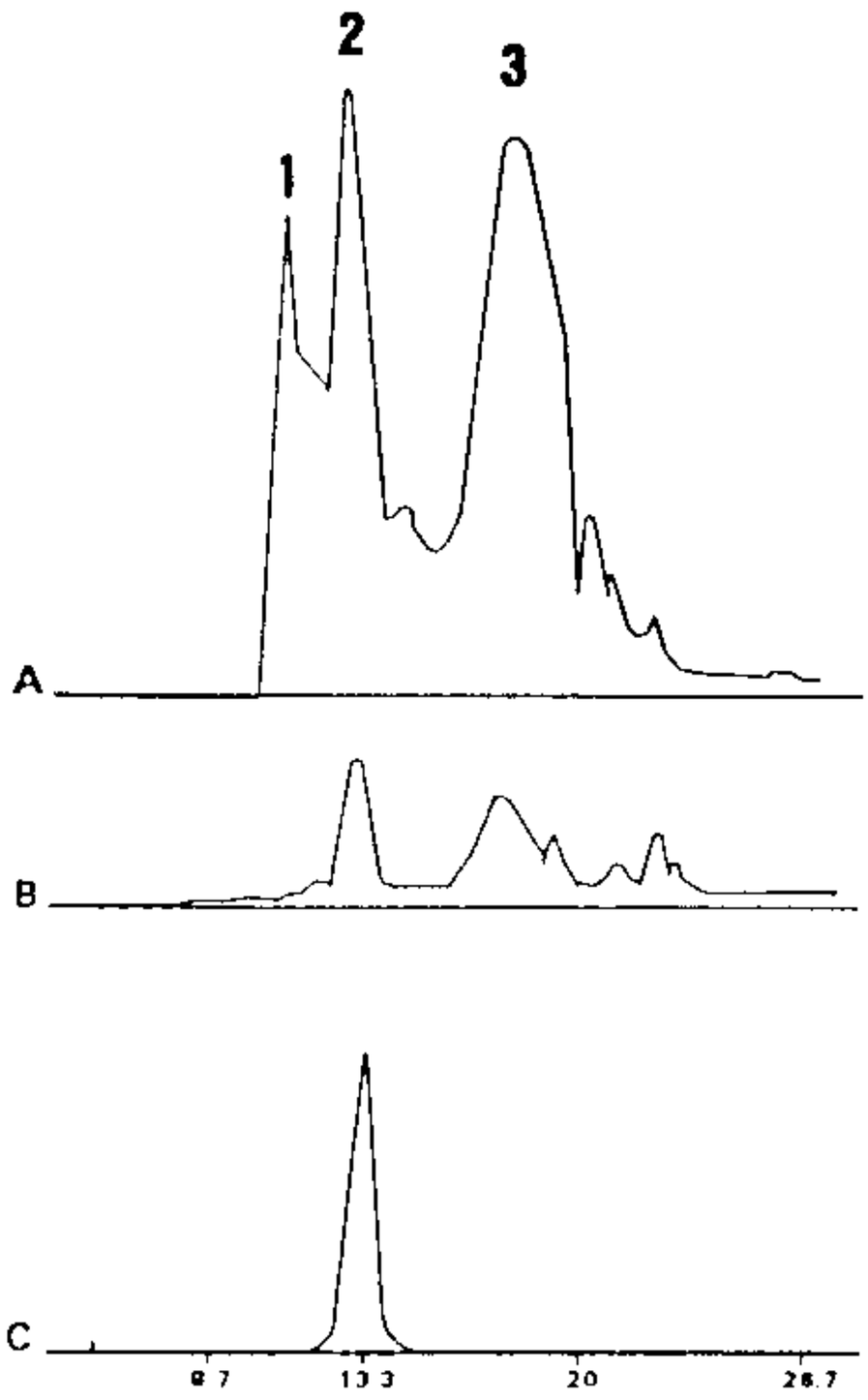

Fig. 1: HPLC analysis of different toxoid preparation using a gel permeation column (50 cm x 7.9 mm ID). Flow rate were 1ml/min and the absorbance measured at 280 nm using AUF= 0.02. A: Vital Brazil Institute preparation (120 µg); B: Butantan Institute preparation (60 µg); C: recromatography of the VB purified peak 2 (60 µg).

major antigenic protein is present in the peak centred at 13 min (Figs 1,2). Collection of the toxoid-containing fraction was realized automatically using a collector fraction (Advantec, Toyo Kaisha Ltd, Japan) coupled to the HPLC system and was initiated when two thirds of the peak height had been reached, and was terminated when the peak descended.

In addition, the immunological analysis showed that this material did not share major antigenic determinants with the peaks 3 and 4 which could represent major contaminants. Some reactivity with the peak 1, was observed with the Vital Brazil Institut toxoid preparation (data not shown). However, its presence in the VBI preparation could be due to different metodologies employed by the manufacturing in the first steps of toxin neutralization. The Butantan Institute preparation did not presented this component (Fig. 1B).

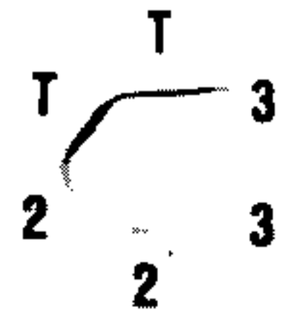

Fig. 2: double immunodifusion analysis of HPLC peaks using horse anti-crude toxin serum (100 Lf/ml). Portion (about 0.2 µg) of peaks 2 (2) and 3 (3), total toxoid (T) preparations (about 3 µg) and 10 µl of anti-tetanus toxin antibodies (central circle) were incubated in agarose plates overnight at room temperature, washed for two days in phosphate-buffered saline containing 0.02% NaN_3, stained with Coomassie Blue for 5 min and destained as described in the text. The double numbers 2 and 3 represent the analysis in duplicate of the two different (VB and BI) toxoid preparations.

Similar elution profiles were obtained by increasing the amount of sample to 150 µl (1 mg) of protein. At rechromatography on HPLC, both collected fractions from the major peaks of the purified toxoid preparations gave only a single peak (Fig. 1C). Restabilization of the column with buffer for another 10 min at a flow rate of 1.2 ml/min renders it ready for the next run.

The final recovery of toxoid as a percentage of the total material aplied onto the HPLC column was 23% (VBI) and 33% (BI). This difference is probably due to different industrial procedures used for purification of the toxoid. The reprodutibility of the method was demonstrated since the variation in the retention time of the active antigenic was less than 2% in more than 30 analysis.

In conclusion, using a gel permeation column of I.D. 7.9 mm, the optimum batch size was 1 mg of the formalized residue. Using the procedure described, each run could be completed in about 26 min, yielding 330 µg of tetanus toxoid and several runs could be done in succession. Using larger preparative columns (2.5 by 50 cm and above), which are commercially available, the batch size can be increased to 1g. Moreover, as the column is compatible with ammonium sulphate the fractionation will be realized without previous dialysis, saving time for industrial toxoid production. Thus, the present method of preparing highly purified tetanus toxoid will be useful not only for experimental but also for pratical purposes. In addition using the power of the HPLC method coupled with the limit of floculation /protein nitrogen (Lf/PN) determination an accurated quality control for tetanus toxoid production can be obtained.