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FAST PROTEIN LIQUID CHROMATOGRAPHY OF BOTULINUM NEUROTOXIN TYPES A, B AND E

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SUMMARY

Three antigenically different botulinum neurotoxins (NTs, relative molecular mass ~150 000), classically distinguished only by specific antisera, were for the first time chromatographically resolved. Mixed NTs eluted from a Mono-Q column in order of types E, A and B, and from Mono-S as B, E and A. Type A and B NTs were successfully chromatographed on the cation-exchange Mono-S column above their isoelectric points. Purification of type A and B NTs by automated liquid chromatography was also accomplished for the first time. Type A, B and E NTs were purified by application on an anion-exchange Mono-Q column, followed by use of a cation-exchange Mono-S column.

INTRODUCTION

The sole cause of the neuroparalytic disease botulism is a protein of relative molecular mass (M_r) of approximately 150 000. The neurotoxin (NT) blocks release of the neurotransmitter acetylcholine from the presynapses at the neuromuscular junctions. Seven serotypes of the NT, called types A, B, C_1, D, E, F and G, are known that are antigenically distinct but are pharmacologically similar. The protein produced by *Clostridium botulinum* is found in the bacterial cultures as tightly bound complexes of the NT and non-neurotoxic proteins which often have hemagglutinin activity [1,2]. Isolation of an NT serotype generally involves purification of this stable complex (hereafter referred to as complex) through several steps. In the final step these complexes are dissociated at moderately alkaline pH (7.5–8.0), in the presence of ca. 0.15 *M* NaCl, and the NTs are chromatographically separated from the non-neurotoxic protein. The well known crystalline botulinum toxin [3] is such a complex containing only ca. 20% (w/w) of the type A NT [4].

Current procedures for purification of NT types A, B and E are based on conventional chromatography developed between 1966 and 1970 (see below) and

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routinely yield >95% pure NT. These procedures have been adequate so far to work with the intact NT. Modern high-performance liquid chromatographic techniques that provide rapid highly reproducible chromatograms by minimizing the variables in particle size of resin (gel), flow-rate, elution gradient slope, etc., have yet to be exploited for botulinum NTs. Only type E NT has been purified by high-performance ion-exchange chromatography [5].

Various fragments of the NTs, to be derived from controlled proteolytic cleavages, will be needed for further studies on structure (e.g. sequence) and structure–function relationship (e.g. polypeptide regions critical for neurotoxicity and antigenic determinants). Isolation of pure preparations of the NT fragments will require (i) ability to examine small amounts of proteolytic digests of NT at analytical level with rapid turn around time and (ii) the option to work with scaled up sample size without a need to reestablish the chromatographic parameters. Examination of the effect of fragmentation on biological activities (neurotoxicity, antigenicity) will require separation of the fragments from the remaining undigested (intact) parent molecule and prior knowledge of chromatographic behavior of the intact NT in the new system.

To derive the benefits of an automated modern system we employed the fast protein liquid chromatographic (FPLC) system (Pharmacia) to (i) examine whether isolation of an NT from its complex can be made more efficient (operation time, yield and purity of NT) and (ii) establish the chromatographic parameters of NT. We now present the results of first such study of three NT serotypes following our preliminary report [6].

Purification of a botulinum NT was first accomplished by isolating type A NT (M_r 150 000) from the crystalline botulinum type A toxin (M_r ~900 000) initially using DEAE-Sephadex A-50 (Pharmacia) and then DEAE-cellulose (cellex-D, Bio-Rad) columns [4,7,8]. Pure type B NT (M_r 165 000) was isolated next by fractionating the type B complex (M_r ~500 000) using DEAE-cellulose [9]. Other investigators exploited similar approaches to purify type B NT (M_r 167 000) using DEAE-cellulose [10] and isolate pure type E NT (M_r 150 000) from the type E complex (M_r ~350 000) using DEAE-Sephadex A-50 [11].

Cation-exchange chromatography of botulinum NT, accomplished first with pure type A NT at pH 5.55 on Amberlite resin IRC-50 (Rohm & Haas) [12,13], has been used only with highly purified preparations of type A NT at pH 6.3 on SE-Sephadex-C-50 [14] and at pH 7.0 on SP-Sephadex C-50 [15], type B NT at pH 5.9 on SE-Sephadex C-50 [16] and type E NT at pH 6.5 on SP-Sephadex C-50 (unpublished results). Cation-exchange chromatography has not been useful to isolate NT from the complex because the operational pH is not conducive for the dissociation of the complex. In our laboratory chromatography on SP-Sephadex C-50 (SE-Sephadex is no longer available) columns is routinely done for type A at pH 7.0 [15], type B at pH 5.9 [16] and type E NT at pH 6.5, 0.02 *M* Na_2HPO_4–NaH_2PO_4 buffer (unpublished results) following their isolation from the respective complexes at alkaline pH.

Gel permeation on Sephadex G-200 first attempted with type A [4] and then with type B [9,10] has not been very effective in isolating NT from the complex because NT and non-neurotoxic protein peaks overlap.

EXPERIMENTAL

Description of FPLC system

The FPLC system consisted of two P-500 pumps controlled by a GP-250 gradient programmer, a GM-1 gradient mixer, a V7 valve for sample application and two V7 valves for selection of attached Pharmacia (Uppsala, Sweden) Mono-Q HR 5/5 and Mono-S HR 5/5 (anion and cation exchangers, respectively) columns. Samples were applied with either the 10- or 50-ml Superloop or the 500-μl loop. Effluent was monitored for protein content at 280 nm with a UV-1 single-path monitor and collected with a FRAC-100 fraction collector equipped with a PSV-100 valve to divert flow to fractions or a waste vessel. A REC-482 two-channel recorder was used to simultaneously record effluent absorbance and gradient.

Buffers

Phosphate buffers were prepared by titrating 0.02 *M* Na_2HPO_4 with 0.02 *M* NaH_2PO_4 until the desired pH was reached. Tris–HCl buffers were prepared by titrating solutions of TRIZMA base (Tris, tris(hydroxylmethyl)aminomethane, Sigma, St. Louis, MO, U.S.A.) with concentrated hydrochloric acid until the desired pH was reached and addition of water to make the TRIZMA concentration 0.05 *M*. Portions of phosphate and Tris buffers were enriched with 1.0 *M* NaCl for generation of gradients. All reagents used were of at least analytical grade. Deionized water was further purified by passage through a Millipore Milli-Q reagent water system. All buffers were filtered through 0.2-μm pore membranes (Acrodisc, Gelman Sciences, Ann Arbor, MI, U.S.A.) and degassed before use on the FPLC system.

Neurotoxins

Preparation of toxic complexes and isolation of pure type A, B and E NTs from their complexes were described [15,17,18]. The protein preparations were dialyzed against a suitable buffer (buffer A), filtered through 0.2-μm membranes and degassed prior to chromatography.

General method of chromatography

The column, equilibrated with buffer A, was loaded with protein, washed with two to five times the gel bed volume (1 ml) with buffer A and then eluted with an increasing concentration of NaCl. NaCl gradients were generated by mixing increasing proportion of buffer B (buffer A plus 1.0 *M* NaCl) to buffer A. Generally, at the end of the gradient, the column was washed with 100% buffer B to remove any tightly bound material and then re-equilibrated with buffer A. The concentration of NaCl at which an NT eluted (apex of the protein peak) was determined from the chart recording of absorbance at 280 nm and gradient, and from the data stored in the FRAC-100. All columns were run at 1.0 ml/min at room temperature (23–25°C). Details of chromatographic parameters are included with presented chromatograms. Protein concentrations of type A, B and E NTs were based on A_{278} (1 mg/ml) of 1.63, 1.85 and 1.4, respectively. Purity of NT was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis

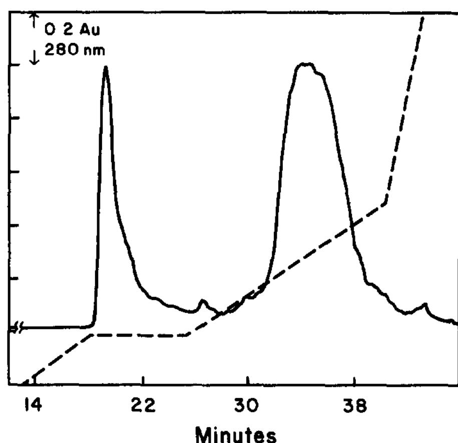

Fig. 1. Isolation of type A NT from the complex on the Mono-Q column eluted with 0.02 *M* phosphate buffer pH 8.2 at 1.0 ml/min and room temperature. Column load: 11.4 mg of type A complex in 10 ml, equilibrated in pH 8.2 buffer. Type A NT and non-toxic proteins were sequentially eluted with a 20-min gradient from 1 to 350 m*M* NaCl with a hold at 94 m*M* NaCl. The yield of A NT, eluted at ~94 m*M* NaCl, was ~20% of the applied complex.

TABLE I

ISOLATION OF NT FROM COMPLEX USING THE ANIONIC AND CATIONIC COLUMNS

Chromatography parameter	Type A	Type B	Type E
Mono-Q column buffer	0.02 *M* phosphate	0.02 *M* phosphate	0.02 *M* phosphate
pH	8.2	8.2	7.5
NaCl[a] (m*M*)	94[b]	118[c]	64[d]
Recovery range[e] (%)	13-20	40-45	28-38
Average recovery[f] (%)	19	42	33
Mono-S column buffer	0.02 *M* phosphate	0.02 *M* phosphate	0.02 *M* phosphate
pH	7.0	5.8	6.5
NaCl[a] (m*M*)	188[g]	185[h]	171[i]
Recovery range[j] (%)	13-19	29-49	24-32
Recovery average[k] (%)	16	39	28

[a]Concentration of NaCl required to elute NT in a 20-min gradient.
[b]Gradient from 1 to 350 m*M* NaCl with a hold at 94 m*M* NaCl.
[c]Gradient from 1 to 300 m*M* NaCl with a hold at 118 m*M* NaCl.
[d]Gradient from 1 to 350 m*M* NaCl with a hold at 64 m*M* NaCl.
[e]Recoveries of NT from the total amount of complex applied to Mono-Q column.
[f]Recovery of NT from complex on Mono-Q averaged from similar methods.
[g]Gradient from 1 to 500 m*M* NaCl; average of six chromatograms.
[h]Gradient from 1 to 300 m*M* NaCl; average of nine chromatograms.
[i]Gradient from 1 to 300 m*M* NaCl; average of three chromatograms.
[j]Recoveries of NT from Mono-S column from the total amount of complex applied to preceding Mono-Q column.
[k]Average recovery calculated in *j*.

(SDS-PAGE). Known molecular weights of single-chain NTs, L and H chains, very well established by SDS-PAGE [1,19] were used, hence calibration curves are not shown here. Preparation of samples for SDS-PAGE and the conditions used for electrophoretic run have been reported (see refs. 3 and 18 in ref. 20).

RESULTS

Isolation of NTs from complexes and chromatographic behavior of the NTs

The type A, B and E complexes were dissociated on a Mono-Q column (0.02 *M* sodium phosphate buffer, pH 7.5 or 8.2). In each case two major peaks emerged: the first peak under the gradient of increasing NaCl was NT and the second peak was the non-neurotoxic protein (hemagglutinin in cases of types A and B, see Introduction). Fig. 1 for type A is a representative chromatogram. The concentration of NaCl in the gradient that eluted NT types A, B and E and their recoveries are summarized in Table I. Examination of the isolated NT by SDS-PAGE occasionally revealed presence of a small amount of impurity (Coomassie blue-stained bands running ahead of the main NT band). Each of the NT types isolated from their respective complex when chromatographed on Mono-Q (Tris-HCl or phosphate buffer) appeared as a single peak (Fig. 2). Chromatography of NT on Mono-S (Fig. 3), following its isolation from the complex (on the Mono-

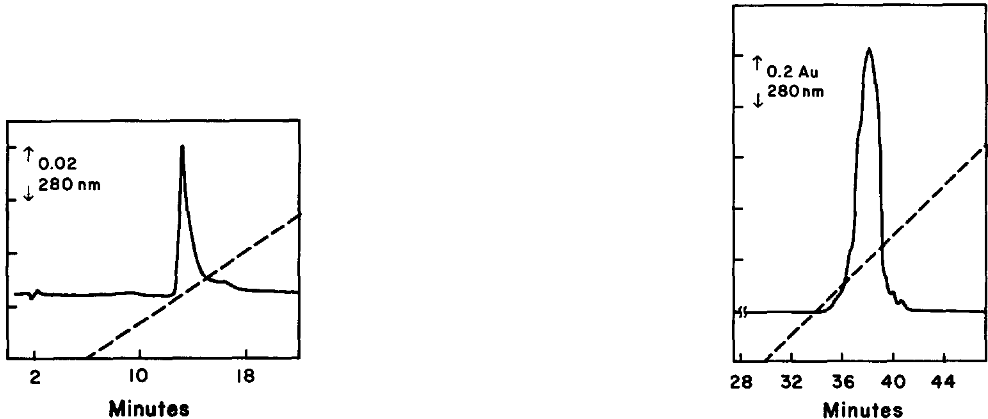

Fig. 2. Chromatography of type A NT on the Mono-Q column eluted with 0.05 *M* Tris-HCl buffer pH 8.0 at 1.0 ml/min and room temperature. The column was loaded with 0.08 mg of A NT (in 0.5 ml), equilibrated in pH 8.0 buffer and eluted with a 20-min gradient from 1 to 350 m*M* NaCl. Recovery of A NT, eluted at 118 m*M* NaCl, from the Mono-Q usually was greater than 90%.

Fig. 3. Chromatography of type A NT on the Mono-S column eluted in 0.02 *M* phosphate buffer pH 7.0 at 1.0 ml/min and room temperature. Column equilibrated in pH 7.0 buffer was loaded with 4.7 mg of NT in 24 ml. Recovery of A NT, eluted at 170 m*M* NaCl with a 20-min gradient from 1 to 500 m*M* NaCl, was ~98% of the applied material. The concentration of NaCl required to elute the NT has ranged from 170 to 203 m*M* with an average of 188 m*M* from six runs of same gradient slope. Recoveries ranged from 72 to 98%. Low recoveries are due to material that fails to bind the column and was not accounted for in determining amounts of recovered protein. The recovery of A NT from the complex was 19% of the material applied to the Mono-Q.

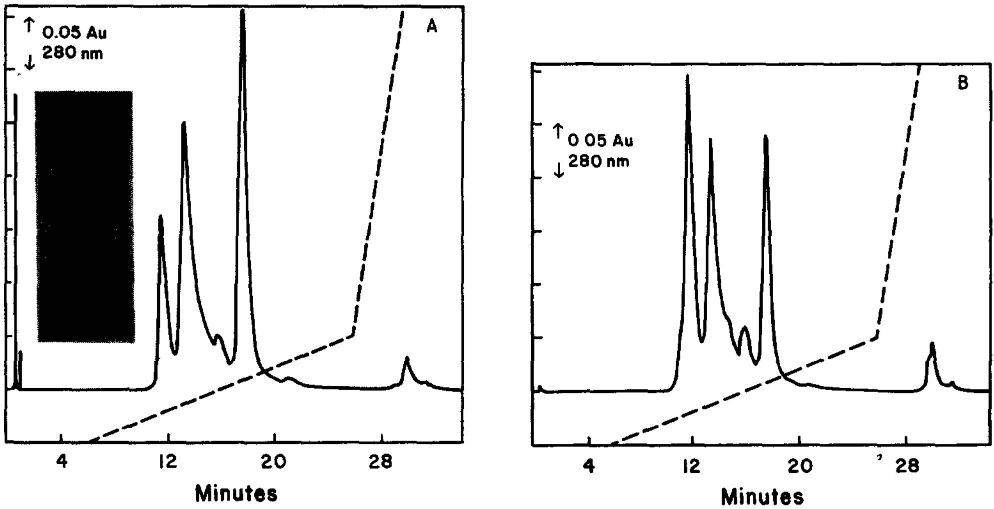

Fig. 4. (A) Resolution of a mixture of type A, B and E NTs on the Mono-Q column eluted with 0.02 M phosphate buffer pH 7.0 at 1.0 ml/min and room temperature; 0.3 mg A NT, 0.21 mg B NT and 0.13 mg E NT in 1.4 ml equilibrated in pH 7.0 buffer were applied in 1.4 ml and eluted with a 20-min gradient from 1 to 200 mM NaCl. The order of elution (left to right) is E, A and B NT, at 51, 68 and 111 mM NaCl. The shoulder or small peak following A NT is also A NT. SDS-PAGE (gels of 7.5% cross-link were run 5.5 h at 9 mA/gel, migration from top to bottom) of the chromatographically resolved NTs following reduction with mercaptoethanol is also shown. From left to right: gel 1, single-chain type E NT (M_r 147 000); gel 2, bands at 10, 19 and 35 mm are single-chain type A (M_r 145 000), H (M_r 97 000) and L (M_r 53 000) chains of dichain type A NT, respectively; gel 3, bands at 11, 19 and 36 (very faint) are single-chain type B (M_r 152 000), H (M_r 104 000) and L (M_r 51 000) chains of dichain type B NT. These M_r values determined by SDS-PAGE are very well established [1,20]. (B) Resolution of a mixture of type A, B and E NTs on the Mono-Q column eluted with 0.02 M phosphate buffer pH 7.5 at 1.0 ml/min and room temperature. A mixture of 0.28 mg A NT, 0.14 mg B NT and 0.27 mg E NT in 1.7 ml, equilibrated in pH 7.5 buffer, was applied and eluted with a 20-min gradient from 1 to 200 mM NaCl. The order of elution (left to right) was E, A, and B NT at 51, 69, and 110 mM NaCl. The shoulder or small peak following A NT is also A NT.

Q), also improved its purity (comparable to the electrophoretic results shown in Fig. 4A). The parameters of chromatography of the NT types A, B and E on Mono-Q and Mono-S columns are summarized in Table II.

Type A complex (at pH7.0–7.5) stored at 4°C as precipitate in 55% saturated ammonium sulfate over several weeks has produced a small peak immediately behind the main NT peak. Some batches of type B complex yielded NTs that were found to retain traces of hemagglutinin (e.g., see Fig. 4A, gel 3, faster of the two bands at 19 mm). When this happened no FPLC method yet tried could completely remove the hemagglutinin.

Chromatography of mixtures of pure type A, B and E NTs and their resolution

Types A, B and E NTs (following their isolation from complexes on Mono-Q and further purification by Mono-S column) were dialyzed against buffer A separately. They were mixed in known proportions and then chromatographed on Mono-Q (Fig. 4) as well as Mono-S (Fig. 5) columns. The eluted NTs were ini-

TABLE II

BEHAVIOR OF NEUROTOXINS ON ANIONIC AND CATIONIC COLUMNS

Numbers in parentheses indicate number of replicate experiments for averaging.

Column	Buffer	pH	mM NaCl at which NT eluted*		
			Type A	Type B	Type E
Mono-Q	0.05 *M* Tris-HCl	8.0	119 (2)	140 (2)	118 (3)
Mono-Q	0.02 *M* phosphate	7.5	106 (3)	137 (4)	70 (4)
Mono-Q	0.02 *M* phosphate	7.0	93 (4)	134 (4)	71 (3)
Mono-S**	0.02 *M* phosphate	7.0	184 (1)	0 (3)	163 (2)
Mono-S	0.02 *M* phosphate	6.5	212 (4)	98 (4)	196 (4)
Mono-S	0.02 *M* phosphate	6.0	240 (3)	175 (3)	227 (4)

*The average concentrations of NaCl required to elute serotypes A, B, and E NTs as a function of buffer pH and the ion-exchange column used (injected singly or in combination with other NTs). Elution of an NT was not appreciably affected by the presence of the other NT. A 20-min linear gradient from 1 m*M* to 350 m*M* NaCl was used for all injections. A decrease in gradient slope generally resulted in lower concentrations of NaCl to elute NT, e.g., see Fig. 4A; at pH 7.5 when the gradient was 1-200 m*M* NaCl the NTs A, B and E were eluted at 69, 110 and 51 m*M* NaCl, respectively.
**Type B NT did not bind at pH 7.0.

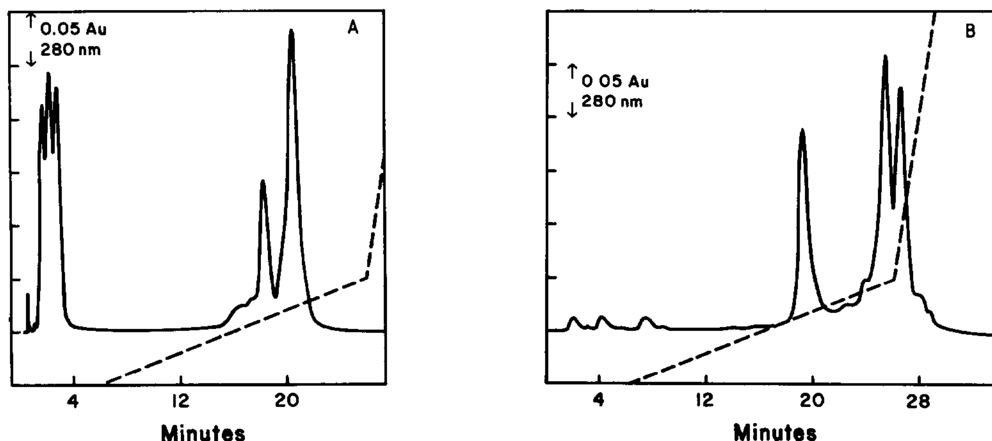

Fig. 5. (A) Resolution of a mixture of type A, B and E NTs on the Mono-S column eluted with 0.02 *M* phosphate buffer pH 7.0 at 1.0 ml/min and room temperature. A mixture of 0.3 mg A NT, 0.21 mg B NT and 0.13 mg E NT was applied in 1.4 ml, equilibrated in pH 7.0 buffer and eluted with a 20-min gradient from 1 to 200 m*M* NaCl. The order of elution (left to right) was B, E and A NT. B NT did not bind; E and A NTs were eluted at 119 and 141 m*M* NaCl. Because the sample mixture was injected in three 500-μl portions, B NT was split into three peaks. (B) Resolution of a mixture of type A, B and E NTs on the Mono-S column eluted with 0.02 *M* phosphate buffer pH 6.0 at 1.0 ml/min and room temperature. A mixture of 0.27 mg A NT, 0.17 mg B NT and 0.23 mg E NT in 1.5 ml, equilibrated in pH 6.0 was applied and eluted with a 20-min gradient from 1 to 200 m*M* NaCl. The order of elution (left to right) was B, E and A NT at 126, 188 and 200 m*M* NaCl.

tially identified by their retention characteristics. The resolved peaks were examined by SDS-PAGE and further identified from their well known electrophoretic mobility pattern (Fig. 4A, see Discussion section for further information). The order of elution from the Mono-Q column (Fig. 4) was types E, A and then B. The small peak behind type A NT peak (Fig. 4) was identified as type A NT on the basis of SDS-PAGE. Chromatography of mixtures of NT types E, A and B in ratios (w/w) 2:2:1 at pH 7.5 (Fig. 4B) or 1:2.3:1.6 at pH 7.0 (Fig. 4A) did not show significant change in resolution.

Similar mixtures were chromatographed on Mono-S columns. At pH 7.0, the type B NT did not bind to the column, while types E and A were retained. Increasing Na^+ gradient eluted the E NT first and then the A NT (Fig. 5A). At pH 6.0 all three NTs were retained, and they were eluted in the order B, E and A (Fig. 5B). The eluted protein peaks were identified by SDS-PAGE as described above (gels not shown here). Separation between E and A at pH 7.0 was better than at pH 6.0 at an identical gradient slope. Parameters of chromatography are summarized in Table II.

DISCUSSION

The experimental results lead to four discussion topics: dissociation of the complexes to isolate the three NT types and their purity, chromatographic behavior of the three NT types on anionic and cationic resins, ionic interaction between the column resins and these large proteins that are pharmacologically similar but antigenically different, and potential beneficial use of the FPLC system.

Isolation of an NT serotype from the complex was patterned after conventional methods mentioned in the Introduction. A complex was first dissociated on an anionic Mono-Q column. Buffer composition, pH and gradients were altered to enhance yield and purity of NT. Isolated NT was chromatographed on either Mono-Q or Mono-S column. The purity and the yield of the three NTs from the FPLC method is as good as that from conventional methods (see Introduction). The same conclusion was reached in another laboratory when type E NT was purified by high-performance ion-exchange chromatography [5], but there are some advantages of FPLC over the conventional columns: (1) Compared to conventional column methods that take four or five days (primarily in packing and equilibrating) the NT can be isolated and purified in two days by use of the presented FPLC methods. (2) The consistent performances of the Mono-Q and Mono-S columns have made it possible to obtain NT of high purity from old batches of complexes or the occasional batch of a complex that were difficult to dissociate on conventional columns. (3) Elution profiles of NTs and non-neurotoxic proteins following dissociation of their complexes are much more uniform than conventional columns. Gradients formed by the P-500 pumps are more reproducible, hence the concentration of NaCl necessary to elute the NT from the Mono-Q and Mono-S columns are highly predictable and can be more accurately determined.

The problem with purification of type B NT

Type B complex is prepared in two molecular forms, L (large) and M (medium), by gel permeation [2,21]. Separation of the type B NT from the non-toxic protein by conventional anion-exchange chromatography is less if the source material is L complex rather than M complex [21]. The occasional difficulty we have experienced in purifying type B is probably due to the presence of some L in the M complex which was our source material.

The criteria of purity and identification of type A, B and E NT based on SDS-PAGE

The NT is synthesized as a single-chain protein ($M_r \sim 150\,000$). It is then cleaved (nicked) at one third the distance from the N- to C-terminal into dichain (nicked) NT by a protease endogenous to the bacteria. In the absence of this protease the single-chain protein remains unnicked. The two chains of the dichain NT are held together by weak non-covalent bonds and at least one –S–S– bond. The heavy chain ($M_r \sim 100\,000$) is twice the size of the light chain. When isolated from bacterial culture the type A NT is > 95% in the nicked (i.e. dichain) form, with the rest in the unnicked (single-chain) form; the type B is primarily in the unnicked form with a small amount in the nicked form; the type E is the single-chain form. These NTs, following their isolation from complexes, migrate in SDS–PAGE as single band ($M_r \sim 150\,000$). Reduction of the –S–S– between the heavy ($M_r \sim 100\,000$) and light chains ($M_r \sim 50\,000$) brings about their characteristic electrophoretic pattern. Type A yields strong heavy and light chain bands and a thin unnicked NT band. Type B yields strong bands of unnicked NT and traces of heavy and light chain bands. Type E, the unnicked protein, runs as a single band [19]. The band patterns in Fig. 4A are consistent with this insight.

The order of elution of the NTs proteins from Mono-Q column (first type E, then A and lastly B) was not entirely inverted when Mono-S column was used: they emerged as types B, E and lastly A. The type B NT did not bind to the cation exchanger at pH 7.0, whereas the other two did. The surface charges on the type B NT (available to form electrostatic bonds with the anionic and cationic groups on the ion-exchange resins) appear to be more different than type A and E. Note that types A and B NTs bind to the cation-exchange columns at pH 7.0 and 6.5 (Table II) that are above their isoelectric points, pH 6.1 for type A and pH 5.25 for type B [19].

The total number of lysine, histidine and arginine residues in type B (118+7+39, respectively; sum=164), type E (104+14+35, respectively; sum=153) and type A (100+14+43, respectively; sum=157), known from their amino acid compositions [20], does not provide ready explanation for the elution order of type B from the cation-exchange Mono-S column. However, fewer histidines in type B (7 versus 14 in types A and E) and the low pK value of imidazole group are consistent with the observed pattern.

Fragments of any of these NT serotypes to be derived enzymatically or chemically are likely to differ more in their cationic (particularly in histidine content) than anionic amino acid contents because sum of Asx and Glx (i.e. aspartic acid, asparagine, glutamic acid and glutamine) for types A, B and E are 314, 342 and

358, respectively [20]. Hence separation of fragments is more likely on the Mono-S because of the probable greater differences in the cationic charges.

The three antigenically distinct NT serotypes were for the first time chromatographically distinguished. Evidently the Mono-Q and Mono-S columns in the FPLC system can distinguish differences in the ionic properties of the three large proteins. Chromatographic separation between them were wide enough, thus it is possible that other serotypes, e.g. C_1, D, F and G, would also be separable from a mixture, whether in the presence or absence of types A, B and E. This is not a mere hypothetical application of FPLC system. It is a real issue because certain strains of *C. botulinum* prduce both type A and B, or A and F, or C and D NTs (ref. 22 and references therein) as exceptions to the "general rule" that only one NT serotype is produced by one strain. The NTs in these binary mixtures were identified by serological reactions using type-specific antisera. The individual NTs in these mixtures have not been isolated, purified and biochemically characterized (e.g. N-terminal amino acid sequence) presumably because of limitations in the resolution of the conventional chromatography. The FPLC presents the feasibility of resolving two NT serotypes, e.g. types A and B produced by a *C. botulinum* strain [22].

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