HPLC analysis of recombinant Botulinum neurotoxins: expanding the analytical toolbox

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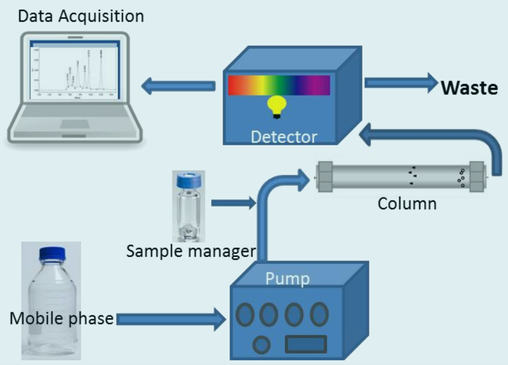
Introduction and Objectives

- High Performance Liquid Chromatography (HPLC) is a powerful tool widely used in the pharmaceutical industry to assess process and product-related impurities within different molecules. (1)
- Methods used to monitor different purity attributes of molecules such as size, aggregation, shape, truncation, hydrophobicity etc.
- Chromatographic separation of toxins carries multiple technical challenges to equipment set-up and consideration of assay design.
- Having found solutions to overcome the safety challenges, here we present Reverse Phase (RP) HPLC and Size Exclusion (SE) HPLC methods developed for various serotypes of recombinant Botulinum neurotoxins (BoNTs) and novel re-targeted BoNTs (Targeted Secretion Inhibitors- [TSI]).

Figure 1. HPLC operational schematic



Methods

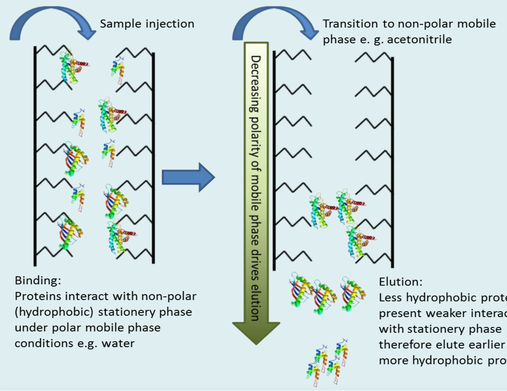
Safety considerations

Table 1. Safety considerations prior to HPLC analysis of BoNTs

Challenge	Solution
User safety during HPLC analysis of BoNTs	The whole HPLC system enclosed in the safety cabinet
Best practise - HPLC mobile phase stored in glass containers → risk of injury from sharps	Solvent resistant plastic sourced and proved to be fit for purpose
Solvent containing BoNT waste decontamination	Segregated waste stream established for off-site destruction
Decontamination of the HPLC system	Safe and effective decontamination procedure in place

Reverse phase HPLC

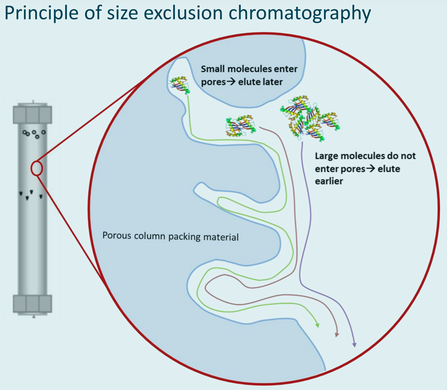
Figure 2. Principle of reverse phase chromatography



- Purity, impurity and activation assay.
- Separation based on polarity of molecules. Molecules with similar polarity tend to be attracted to each other whilst those with dissimilar polarity do not exhibit any attraction, and may even repel one another.
- The following parameters were optimised:
  - column screening (industry standards)
  - column regeneration
  - gradient
  - column temperature
  - sample concentration and preparation
- Assay's performance in terms of precision and linearity was assessed to establish assay acceptance criteria and gain confidence in the assay performance, ensuring suitability for qualification.

Size exclusion HPLC

Figure 3. Principle of size exclusion chromatography



Size exclusion HPLC

- Aggregation assay.
- Separation based on size of molecules. Smaller particles can enter the beads of the resin and subsequently elute later than larger particles which are excluded from more of the column volume.
- The following parameters were optimised:
  - column selection (industry standards)
  - mobile phase components and concentrations
  - sample preparation
- BoNTs are prone to secondary interactions with the surface of stationary phase, mainly due to electrostatic and hydrophobic interactions which results in peak shape changes, such as tailing. A common approach to mitigate this involves an increase of the ionic strength of the mobile phase and addition of modifiers, such as organic solvents or arginine. (2)
- Assay's performance in terms of precision and linearity was assessed.

Results

Reverse phase HPLC

Figure 4. Separation of BoNT by RP HPLC under reducing and non-reducing conditions

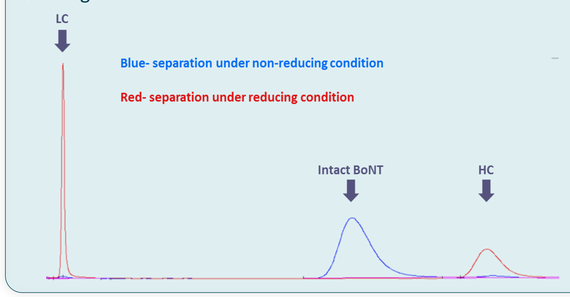
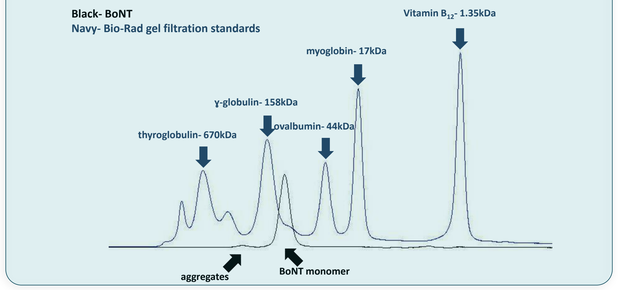


Table 2. Results for the RP HPLC assay

Method attribute	Method parameter varied	Result
Carryover (retention of material on the column)	Column selection, temperature, washing procedure	Carryover decreased from 60% to less than 1%
Peak shape	Column selection, temperature	Sharper peak and better symmetry at higher temperature
Peak resolution	Acetonitrile gradient	Multiple step gradient- good separation without a significant increase in run time
Reduction of disulphide bond	Reducing agents screen	Full activation achieved without protein precipitation

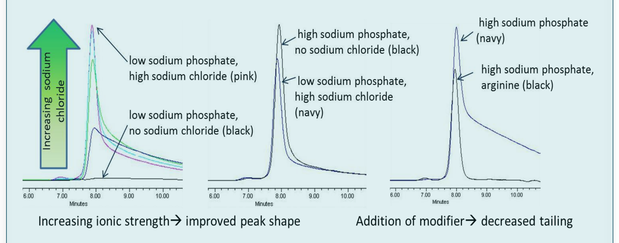
Size exclusion HPLC

Figure 5. Separation of BoNT and standards on the SE HPLC



- Column selection based on manufacturer's claimed molecular weight range was confirmed using protein standards (Bio-Rad).
- In the initial testing of sodium chloride concentration progressively increasing peak height with less tailing was observed with the increasing salt. Further increase of ionic strength of the mobile phase did not bring any improvement of tailing.
- Addition of arginine was found to sufficiently mitigate unspecific interaction with the column resulting in good peak shape.

Figure 6. Improvement of the peak shape in SE HPLC



Conclusions

- Prior to introducing any of the new chromatographic separation methods, it was necessary to perform rigorous safety checks and process review.
- Solutions were found to overcome technical issues allowing chromatographic analysis of BoNTs.
- A suite of analytical chromatography methods has been developed allowing assessment of purity and impurity profiles of rBoNTs and TSIs.
- Certain elements of assay development are transferable across multiple serotypes but individual optimisation is required.
- Precision and linearity of developed assays were assessed and assays proved to be suitable for qualification.

References

1 'HPLC of peptides and proteins methods and protocols' Marie-Isabel Aguilar from 'Methods in molecular biology, vol251'

2 'A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates' P. Hong et al, 2012

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