Development of separation methods for recombinant Botulinum neurotoxins, using a statistical modelling-based approach

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In the Biopharmaceutical field, the development of separation methods is essential for the release, stability testing and characterization of therapeutic proteins to ensure purity, efficacy and safety of the clinical product. Size Exclusion (SEC) and Ion Exchange chromatography (IEC) are common separation techniques used for aggregate, fragment and charge isoform characterization, being robust qualitative and quantitative methods. SEC separates protein based on size and is used for monitoring aggregate and fragments. IEC uses the protein surface charge to indicate the charge heterogeneity of the molecule. The goal of this thesis is to establish and optimise the Size Exclusion and the Ion Exchange chromatography methods for different endonegative (inactive) serotypes of recombinant Botulinum neurotoxins. To develop these methods, computational Design of Experiments (DoE) were performed to evaluate different mobile phases, pH, temperature, flow rate, columns and run times, to maximize the peak resolution and recovery and, also, to minimize peak tailing.

The work described in this thesis demonstrates the presence of arginine as a mobile phase additive is crucial for SEC performance, and that the same optimised conditions can be used for different toxin serotypes. However, IEC development is more product specific, thus different conditions must be developed for the different serotypes. For the serotype studied, pH and temperature were shown to be critical factors.

Keywords: Botulinum neurotoxins, SEC, IEC, separation methods, DoE

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1. INTRODUCTION

In neurosciences, Botulinum neurotoxin as a biotherapeutic is used to treat several diseases, such as spasticity, cervical dystonia, hemifacial spasm and blepharospasm [1].

Botulinum neurotoxins (BoNT) are produced by neurotoxigenic strains of anaerobic and spore forming bacteria of the genus Clostridium (Clostridium botulinum, Clostridium butyricum, Clostridium barati, and Clostridium argentinensis) [2]. This neurotoxin is the most lethal acute toxin known and when ingested in contaminated food can interfere with muscle function causing paralysis or death [3]. However, it is a successful therapeutic agent used for clinical and cosmetic applications [1].

The development of BoNT as a biopharmaceuticals is complex and highly regulated by regulatory bodies, such as the FDA and EMA, require a full understanding of the drug. So, size exclusion (SEC) and ion exchange chromatography (IEC) are important analytical tool [4]. SEC is required to identify and measure aggregates and fragments. IEC characterize and identify charge isoforms of the protein therapeutic [5].

Size Exclusion Chromatography (SEC) separates molecules based on differences in size and shape. The biomolecules pass through a spherical porous particle stationary phase with a specific pore size and diffuse...
depending on the molecular size using a mobile phase. High molecular weight species, for example aggregated biomolecules, cannot penetrate the pores of the stationary phase and therefore elute first from the column. Low molecular weight species, for example protein fragments, partially or completely enter the porous particles, slowing their progression and therefore eluting later [6][4].

To develop a SEC method with good resolution, many factors must be considered such as column (particle size and uniformity, bed height, diameter and length), flow rate, sample volume and viscosity. The mobile phase composition, pH and ionic strength are also important but are not related directly with the resolution. However, these last factors influence the conformation or biological activity [4][6][7]. Also, an important consideration for a good SEC method establish is the possible resin-protein interaction: electrostatic and hydrophobic interactions. To avoid that a good choice of the resin and, specially, the mobile phase should be performed. To suppress electrostatic interactions the salt or the ionic strength of the buffer can be optimized. It is important to consider the salt concentration because, at low salt concentration, electrostatic interaction dominates, and, at high salt concentration, the hydrophobic interaction dominates. The reduction of hydrophobic interactions can also be achieved by addition of organic solvents, such as acetonitrile and alcohol [4][6][8]. Arginine is a common additive used to suppress non-specific interactions between protein-resin and prevent additional aggregation, giving better resolution and higher aggregation and protein recovery [9][10][11].

Ion Exchange Chromatography (IEC) is a separation method based on net surface charge of proteins. The net surface charge depends on the protein charged groups, each with different pKa values (acid ionization constants). Ion exchange separation occurs by a reversible interaction between a charged surface protein and an oppositely charged chromatography medium [12][13].

The choice of the IEC stationary phase and mobile phase gradient depends on the isoelectric point of the protein. If the solution pH is higher than the pI, the protein will be negatively charged and therefore bind to a positively charged anion exchanger. If the solution pH is lower than the pI, the protein will be positively charged and therefore bind to a negatively charged cation exchanger. The anion exchanger has positively charged functional groups linked to the matrix, so the more negatively charged variants elute later than the less negatively charged variants. On the other hand, the cation exchanger has negatively charged functional groups linked to the matrix, so the more positively charged variants elute later than the less positively charged variants. The analyte retention depends on mobile phase pH, ionic strength, and counter ion additives. The interactions between stationary phase and the protein varies along the mobile phase gradient that alters the charged state and solubility of the analyte or and ionization state of stationary phase [13][14][15].

Mass spectrometry can be used to identify charge modifications due to the increase or decrease in mass of a basic or acidic isoform relative to the unmodified protein. There are three ways to analyses therapeutic data: intact mass; reduced mass (Middle) and digestion for peptide mapping (Bottom). The intact mass give information of the overall heterogeneity of the protein, but not provide the type of modification or location. The reduced mass provides information of the light chain and heavy chain or anthers fragments confirming the distribution gave by intact mass and detecting where is the sequence modification (LC or HC). The peptide mapping identifies small modifications or no mass changes in the intact protein, giving a better understanding of the modifications on the protein [16].

2. DEVELOPMENT METHODOLOGY

The main strategy of this project was to use the power of Design of Experiments (DoE) to define the main method factors and interactions that influence resolution, peak tailing and recovery and predict optimized conditions for improved resolution. The DoE approach can evaluate multiple factors at the same time with reduced runs than the conventional ‘one factor at a time’ approach. The application of DoE in chromatography method development provides a detailed mathematical analysis of the system and resolution of the problem with minimal, well-planned experiments that avoid time-consuming trial and error methodology [19][20]. For the DoE and respective analysis two statistical software packages were used: JMP and Minitab. The flow diagram for SEC and IEC method development and optimization is on fig. 1.

Fig. 1. Simplify SEC and IEC development methodology.
3. EXPERIMENTAL

A. SEC

The SEC experimental procedure included: mobile phase preparation, sample preparation, ULPC run and data analysis.

Different mobile phases were prepared with L-Arginine monohydrochloride, sodium chloride, sodium phosphate monobasic anhydrous, Sodium phosphate monobasic anhydrous and ultra-pure water. Always, adjusting the pH and filtering through a 0.22µm filter.

Sample preparation was the dilution of the type BoNT in PBS if required. The blank was mobile phase.

The sample was introduced in Water Acquity UPLC system to be separated with the column Waters Acquity BEH SEC 200 1.7µm, 4.6 x 150 mm.

The Empower 3 was the software used to preform the separation and process the data.

B. IEC

The IEC experimental procedure include: mobile phase preparation, sample preparation, ULPC run and data analysis.

For IEC, it was required a buffer A and buffer B (for the gradient). Different buffer A where prepared with MES monohydrated, MES sodium salt, Trisodium Citrate, Tris base, Tris HCl, Sodium phosphate dibasic anhydrous, Sodium phosphate monobasic anhydrous and ultra-pure water. The buffer B was the buffer A preparation plus sodium chloride. The adjust of the pH and filter through a 0.22µm filter was always performed.

Sample preparation was the dilution of the type BoNT in PBS if required. The blank was buffer A.

The sample was introduced in Water Acquity H-Class UPLC system to be separated with the columns: Propac WCX-10 4 X 250 mm; Protein-Pak Hi-Res Q 4.6 x100mm 5µm; Sepax Proteomix SEX 4.6 x 100mm 5µm.

The Empower 3 was the software used to preform the separation and process the data. For the last study of the influence of temperature and pH on IEC profile, it was used Autobled Plus.

C. IEC fractionation and MS

For the IEC fractionation, the method was described on fig. 2. To buffer exchange the fractionated samples, 100 mM Tris pH 7.6 was used. The samples where collected from IEC column as represented on fig. 9.

For mass spectrometry, buffer A was a solution of formic acid and buffer B was formic acid with acetonitrile solution. The equipments were: Synapt G2Si (QTOF) from Waters, UPLC Acquity H-class and column Protein BEH C4 300A 1.7μm 2.1 x 50 mm. The data was analyzed by MassLynx.

4. RESULTS & DISCUSSION

A. SEC

A full factorial design with a centre point in Minitab (20 runs) was chosen to evaluate 4 factors:

- Sodium phosphate concentration: 20-200 mM;
- Sodium chloride concentration: 0-300 mM;
- Arginine concentration: 0-300 mM;
- Serotype: 2 and 3.

The main response evaluated was USP tailing. The analysed data of the DoE are on fig. 3 and 4.

![Fig. 2. IEX fractionation conditions.](image)

![Fig. 3. Main Effects plot for USP tailing factor.](image)

The arginine concentration is the factor that has more impact on the USP tailing factor response. The increasing arginine causes a clear reduction of the USP tailing. In the presence of high arginine concentration, the variance in sodium phosphate or NaCl concentration had a low impact on the response. However, without arginine, the highest concentrations of sodium phosphate and NaCl improved the peak tailing. The serotype is the factor with the least effect on tailing, confirming the possibility of using the same HPSEC method for the different serotypes. This was also confirmed by visualization of the chromatograms.

By the response surface for USP tailing and recovery, it was concluded that the sodium phosphate concentration should not be too low or too high, so to have reduced...
tailing and better recovery the sodium phosphate concentration range should be around 100-150 mM. The tailing is always low and the total area is higher when the arginine concentration is high.

To a better understanding of the mobile phase impact, it was performed size exclusion of 3 weeks degraded BoNT 3 (25°C). The mobile phases assessed were 115Mm sodium phosphate, 300mM NaCl and 300Mm L-Arginine (Minitab optimal composition); 115Mm sodium phosphate and 300mM NaCl (to confirm the importance of L-Arginine in the mobile phase) and 50Mm sodium phosphate, 200mM NaCl and 200Mm L-Arginine (used in previous Ipsen work). The results are showed on fig. 5.

These results confirm the presence of arginine is essential to achieve better resolution, less tailing and baseline drift and reduce the retention time of BoNT 3. Also, there is an improvement in the results when all the compounds are present at higher concentrations. It should be noted with the increased of arginine is observed an increase in aggregate peak area. The same event was described by Arakawa, demonstrating that arginine improves the reliable quantification of aggregates [19]. The data also demonstrates that the arginine not promote aggregation, preventing the protein binding to the column but cannot dissociate irreversible bound protein. Consequently, it can be concluded the same approach because the amount of monomer eluting is not decreased by the arginine (around 97 percent). This can be justified because the arginine has a preferential binding of unbound protein, which is in equilibrium with the surface bound protein. Consequently, arginine shifts the equilibrium binding toward the dissociated state [11][19].

Once the optimal mobile phase conditions were confirmed (115mM sodium phosphate, 300mM NaCl and 300mM L-Arginine), the 25°C heat stressed material of BoNT 2 and 3 during one, two and three weeks were analyzed. BoNT 2 is stable at 25°C, because no aggregation increasing was observed. However, BoNT 3 aggregation increase during the time, demonstrating degradation over the time at these conditions. So, the optimised HPSEC method is able to analyse degraded samples and is therefore suitable as a stability indicating assay for BoNT 2 and 3.

To conclude the HPSEC development, the method was qualified to demonstrate it is robust, linear and precise. Prior to starting the qualification, the NaCl concentration was reduced to prevent reduction of the column life time. Arginine at 400mM was also evaluated to demonstrate method robustness around the 300mM arginine limit with BoNT 3. The reduction of NaCl concentration to 200mM generated a USP tailing of 1.336 and a USP resolution between the main peak and following peak of 7.213. These results supported the use of reduced NaCl concentration. No difference was observed between 300mM and 400mM arginine, which demonstrated robustness around the arginine concentration. A linear regression analysis (R2) of total peak area vs. concentration was calculated to be 0.9992 over the range of 0.05-0.5mg/mL of sample. The precision (repeatability) of the assay was calculated as a Percent Relative Standard Deviation of six replicate injections of sample. The Percent Relative Standard Deviation for total peak area and monomer peak area, monomer percent peak area and monomer retention time were lower than five percent. Overall the results show the HPSEC method is suitably precise. The final SEC methods is showed on fig. 6.

B. IEC

To develop an IEC method for BoNT 2 a statistical modelling approach using Design of Experiments was used to save time and material. Before starting with DoE, it was required to define the conditions and factor ranges.

The first approach was the use of weak cation exchange. Several factors were changed like buffer, pH, sample buffer exchange. Unfortunately, none of the solutions showed good separation due to the instability of BoNT 2 at those conditions. Consequently, a new strategy was implemented which started the IEC development from the beginning.

It is known that BoNT 2 is stable at pH 6.5 at 100Mm
salt concentration and the pI is 6.3 therefore a basic pH between 7 and 8.5 for the mobile phase was chosen. It was also considered that the toxin has to be stable at the column loading pH which is slightly more basic due to the Donan effect. To minimize this effect, the buffer concentration shouldn’t exceed 50mM and the chosen buffer should have a pKa equal to the desired pH ± 0.5 to achieve a sufficient buffer capacity. Based on this, Tris was selected as the buffer due to its pKa (25 °C) of 8.07. Consequently, Strong Anion Exchange was chosen because the buffer pH is more basic than the toxin pI, so the biomolecule net charge is negative. The term strong means that the charge density on resin surface is maintained over a broad pH range, and therefore these resins retain their selectivity and capacity over a wide pH range [14][15]. The first AEX column chosen was Protein-Pak Hi-Res Q 4.6x100mm 5 µm that has a quaternary ammonium group with a pKa of 10.5. The other factors and choices are described more in the deep on the thesis.

It was performed a salt gradient 0-100 percent in 40 min to understand where the protein eluted in the mobile phase gradient. It was observed one peak between 12-14 percent of salt with poor separation and baseline. With this information and, after gradient slope optimisation, the gradient chosen was 0-25 percent of salt in 40 min. The definitive screening DOE was generated in JMP to understand what are the main factors that affect resolution, recovery and peak number (18 runs). The factors were:
- Flow rate: 0.3-0.5 ml/min;
- pH: 7.5-8;
- Temperature: 25-30 degree Celsius. Previous study showed that the BoNT 2 is not stable at temperature higher than 30 degree Celsius.
- Tris concentration: 20-30 mM;
- Sodium chloride concentration: 0.5-1 M.

The results show that temperature and pH are the most relevant factors as on fig. 7.

The custom design was used to further understand temperature and pH as factors and build a response surface (12 runs). Also, Auto Blend Plus was used to prepare the different buffers automatically. the new ranges were:
- pH: 7.5-8.5;
- Temperature: 15-25 degree Celsius.

By fig. 8, for resolution, the critical factor is pH of the mobile phase. From the response surface, pH around 8 give a good resolution. The predictive response for resolution is a good model that described well the variance of the data and it is not over-fitted. However, for the total area is difficult to find a good model to fit on the data, so it is less reliable. This model, show the combination of low temperature has the highest recovery. However, for the middle pH, 25 °C give highest area. This results were confirmed by visually analysis of the chromatograms.

The final IEX method is described on fig. 2, after, used for IEX fractionation.

**C. IEC fractionation and MS**

IEC fractionation coupled with mass spectrometry was used to characterize and potentially identify the BoNT 2 isoforms. The IEX profile for BoNT 2 is described on fig. 9 and it was collected the five fractions.

It was identified two main isoforms of BoNT 2. The initial hypothesis for the two isoforms was due to the presence or absence of residues Gly-Ile-Arg from the LC which the first peak is the isoform with GIR and the second without GIR. The reason for that hypothesis is because, during the activation step of BoNT, trypsin cleaves the inactive single polypeptide BoNT chain between Arg 421 – Lys 422 to generate the di-chain made of a 50kDa light chain and a 100kDa heavy chain linked by a disulfide bond. The light chain is then cleaved again between Lys 418 - Gly...
Fig. 9. IEC fractionation for BoNT 2 - zoom chromatogram

419 which removes GIR [3][20]. This phenomenon is well described by the fig. 10.

Fig. 10. BoNT 2 activation by Trypsin

The Intact mass results discard this hypothesis because the two isoforms have a similar MW that both correspond to the form without GIR as shown in fig. 11. So, some future work includes peptide mapping of the fractions to understand the small modifications responsible for generating the two different isoforms.

Fig. 11. Theoretical and measured MW for BoNT 2 (-)

5. CONCLUSION

In the biopharmaceutical field, development of an analytical tool box to fully characterize the drug substance and control the quality and purity of the product, batch to batch, is mandatory. To build the tool box several methods are required including size exclusion chromatography and ion exchange chromatography. These methods were already established for BoNT 1, nevertheless, the development for BoNT 2 and 3 were, also, essential.

The aim of this thesis was, therefore, developing HPSEC and IEC methods for BoNT 2 and 3. HPSEC is used to measure aggregation and IEX measures charge isoforms. With this purpose, a statistical based approach, including Design of Experiments, was firstly used in Wrexham site, which allowed faster method development and a key understanding of the main factors and how their interactions impacted outputs, such as peak tailing, resolution and recovery.

HPSEC development was performed with full factorial design. This confirmed the importance of arginine in the mobile phase, needed to suppress the hydrophobic and electrostatic interactions between the neurotoxins and the resin. Furthermore, the DoE demonstrated that HPSEC conditions can be used for both BoNT 2 and 3.

The separation of degraded BoNT 2 and 3 (incubation at 25 °C) by the final HPSEC method revealed that the technique is appropriate as a stability test, able to detect aggregates and fragments. Likewise, it was also shown with degraded BoNT 3 that arginine improved aggregate recovery.

The HPSEC was validated by linearity and repeatability tests, confirming that the HPSEC assay development was successful and is suitable to measure the purity levels of BoNT 2 and 3. The final method comprises the following conditions:

- Column: Water Acquity BEH SEC 200 1.7µm, 4.6 x 150mm;
- Mobile phase: 115mM sodium phosphate, 200mM NaCl and 300mM arginine at pH 7.0 ± 0.1;
- Injection volume: 10µL;
- Column temperature: 25 °C;
- Detection: UV at 214 nm for detection of very low molecular weight peptides and 280 nm for quantification;
- Flow rate: 0.2 mL/min;
- Running time: 12min;
- Samples concentration: validated to 0.05-0.5 mg/mL;
- Gradient: isocratic.

An IEC method was developed for BoNT 2, based on strong anion exchange. During the development, it was shown that BoNT 2 is not stable at temperatures higher than 30 °C, likely due to denaturation.

The definitive screening design evaluated the flow rate, NaCl and Tris concentration, pH and temperature, in order to identify the main factors and the relationship to resolution and recovery. The pH and temperature were confirmed as key factors for optimal IEX separation.

The custom design generated a response surface for resolution and total area in relation to temperature and pH. The results indicated that, for a maximized resolution, the most important factor is the pH between 7.8 to 8 as optimal range for IEX separation. However, highest recovery is achieved with the combination of low temperature and a pH close to 7.5. But, it should be noted that the area model was poor, therefore, the combination of pH close to 8 and a temperature of 25 °C gave the best resolution and acceptable recovery for BoNT 2.

The final method comprises the following conditions:
Column: Protein-Pak Hi-Res Q 4.6x100mm 5µm; • Mobile phase A: 30 mM Tris pH 8.0 • Mobile phase B: 30 mM Tris, 0.5M NaCl pH 8.0 • Injection volume: 20µL • Column temperature: 25 ºC; • Detection: UV at 214 nm; • Flow rate: 0.3 mL/min; • Running time: 60min; • Samples concentration: 0.5 mg/mL; • Gradient: linear 0-25% salt in 40 min. The obtained IEX results are satisfactory, but should be qualified to demonstrate it is a suitable method to quantify the isoforms of BoNT 2.

IEC fractionation and intact mass results refuted the hypothesis that the source of the different isoforms is due to the presence of the GIR residue on the light chain. Therefore, peptide mapping must be performed to characterize the different isoforms.

A. Future work
For HPSEC method, intermediate precision must be performed and the final conditions have to be confirmed with endopositive (active) material.

The next steps for IEC development could be, inter alia: the comparison between the results of pH 7.5 and 15 ºC versus pH 8 and 25 ºC; the salt gradient optimisation step could also be further evaluated; and, another possibility, is to screen different strong cation exchange and weak anion exchange columns.

In this study, the IEX was performed with salt-gradients. However, elution using a pH-gradient could be evaluated. The utilization of pH-gradients could provide advantages, such as resolution improvements; lower the salt concentration in collected fractions and the possibility to correlate isoelectric point with elution profiles [21][22].

As already mentioned, peptide mapping for the different isoforms is a near future work to understand the source of the different BoNT 2 charge isoforms.

REFERENCES

