Increased throughput and resolution with optimized Peptide Mapping, SEC and IEX for the analysis of therapeutic proteins

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WCBP 2011

Agilent Technologies

Introduction

In depth physico-chemical characterization of therapeutic proteins is required during all phases of drug development to ensure drug safety and efficacy. Providing proof of protein drug identity and integrity is usually demonstrated by performing several (LC-based) assays such as peptide mapping, size exclusion (SEC) ion-exchange chromatography (IEX). Resolution and robustness is required for all techniques in order to separate and identify potential aberrations that might occur during the production process or during drug formulation in a reproducible manner.

In this study we present data for a 26 kDa recombinantly expressed NBE, protein P128 under development at GangaGen Biotechnologies for human therapy. Peptide mapping, SEC and ion-exchange chromatography are performed for this protein using the new Agilent Bio HPLC columns in combination with a new Agilent 1260 Infinity bio-inert quaternary LC instrument especially developed for the analysis of large bio-molecules.

Experimental

Fig.1 New Agilent 1260 Infinity bio-inert LC with completely metal-free bio-inert sample flow path, wide pH range, 600 bar compatibility.

System

G5611A	1260 Bio-inert Quaternary pump
G5667A	1260 Bio-inert HiP-autosampler
G1330B	1200 Series Thermostat
G1316C	1260 Thermostatted column compartment with bio-inert click-in heating elements
G4212B	1290 DAD with bio-inert with 60 mm Max-Light flow cell or
G1316C	1260 DAD with 10 mm flow cell

System contains bio-inert high-pressure capillaries

Results and Discussion

PEPTIDE MAPPING

UV-based peptide maps are often generated in a QA/QC new biological entity (NBE) environment in order to detect any sequence abnormalities that are caused during the manufacturing or processing of a recombinant protein drug. Highest possible resolution is required for a complex mixture which often results in excessive long analysis times. Using STM particle technology in combination with UHPLC instrumentation allows for shortening analysis time while preserving resolution (Fig. 2). Bio-inert instrumentation has been beneficial especially for hydrophobic peptides and proteins that tend to stick to metal surfaces.

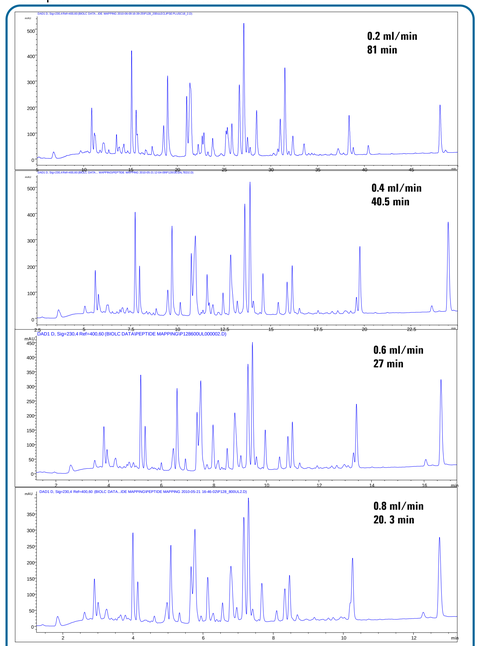

Fig.2. Analysis of a tryptic peptide map for P128 protein. The gradient slope was identical for all experiments. **Column:** Eclipse Plus C18 3.0 X 100 mm, 1.8 . **Mobile phase** A: 0.1% TFA; B: 99.2% ACN, 0.08% TFA

Results and Discussion

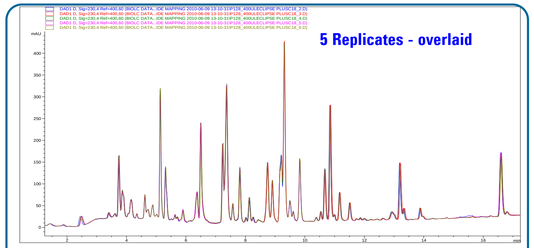

Fig 3. Replicates (n=5) of a tryptic peptide map of a therapeutic protein at 0.6 ml/min.
Column: Eclipse Plus C18 3.0 X 100 mm, 1.8 µm. Conditions were used as described in Fig. 2

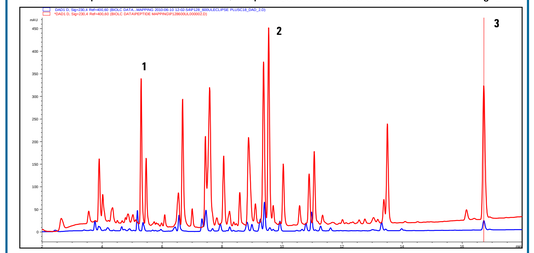

Fig 4. Comparison of a peptide map with detection on a standard DAD (G1315C) with bio-inert flow cell (blue trace) and with the G4212 B with the max-light flow cell (red trace). Columns and chromatographic conditions as described above. Detection was performed at 230 nm with identical detector settings.

Robustness and repeatability of analyses are of highest importance when characterizing therapeutic proteins in order to provide proof of integrity and identity of the respective NBE and to fullfil regulatory requirements. Five replicate injections on Eclipse Plus C18 3.0 x 100 cm, 1.8 um particle with the Agilent 1260 Infinity Quaternary LC (Fig 3 demonstrate excellent overlay of all analyses.

Sensitivity increase is of utmost importance especially if minor impurities/modifications need to be detected. The use of the max-light detector technology compared to the standard DAD can therefore result in a up to 10x higher sensitivity (Fig 4). This results also in a wider dynamic range and allows for detection of modifications which might arise if e.g. mutations occur in the expression clone. Using the G4212B DAD with the 60 mm max-light flow cells an 10 x sensitivity increase was observed (Tab.1)

Retention Max-Light (DAD) (min)	Peak ht/ DAD G4212B Max light 60 mm	Peak height/DAD G1315C 10 mm	Sensitivity increase
Peak 1 (5.2 min)	350	47	7.44
Peak 2 (9.5 min)	450	62	7.25
Peak 3 (16.7 min)	375	26	14.4
Average			**9.7**

SIZE EXCLUSION CHROMATOGRAPHY

In order to demonstrate protein integrity or to prove absence of dimer or multimer formation in its native conformation, size exclusion chromatography (SEC) in combination with UV detection is a commonly applied analytical technique. A disadvantage of size exclusion is the lack of resolution. New small particle SEC columns provide superior resolution with minimal secondary interaction providing a powerful tool to detect impurities or multi-mer formation. The same protein used for peptide mapping was used to perform the SEC experiment. Results were compared for two 5 um particle columns and a 3 um particle column. Only with the Agilent SEC 3 um column the 2nd compound could be detected (Fig 5).

Acknowledgement: We are thankful to Faizy Ahmed for his fruitful support and discussions.

Results and Discussion

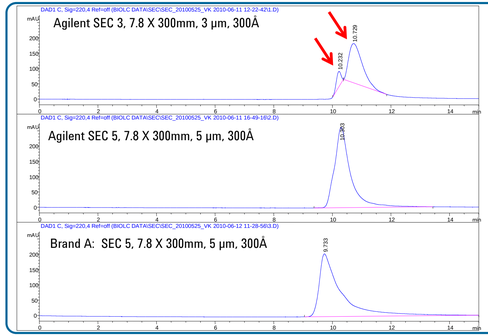

Fig 5. Size exclusion of a P128 therapeutic protein sample performed on the Agilent 1260 Infinity bio-inert LC using different columns. Arrows indicate that the two compounds present in the sample could only to be distinguished when using a 3 µm column. Mobile phase A: 150 mM Sodium Phosphate (pH 7) + 150 mM Sodium Chloride, Flow rates: 1 mL/min, UV detection: 220 nm

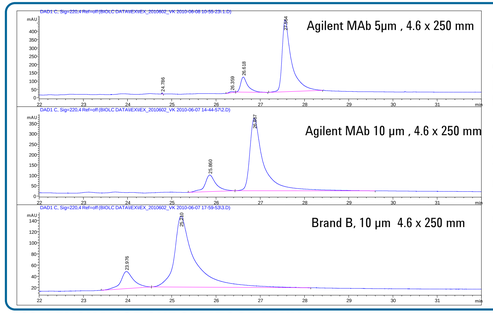

Fig 6. Weak cation exchange chromatography for a P128 therapeutic protein sample on the Agilent 1260 bio-inert LC-system.

Mobile phase A: 20 mM Sodium Phosphate (pH 6.0) B: 20 mM Sodium Phosphate (pH 6) + 1 M Sodium Chloride Flow rates: 0.5 mL/min, UV detection: 220 nm.
Gradient conditions used :0 min,10% B; 35 min 35% B; 36 min 10% B; 45 min,10% B

ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography is applied to separate charge variants of NBEs which might be formed through oxidation, de-amidation or truncation of terminal lysine residues during manufacturing or processing steps. In order to separate basic and acidic variants from the main peak high resolution and minimal secondary surface interaction is required. A sample of P128 protein preparation was separated on the new Agilent 1260 Infinity bio-inert and corrosion resistant LC using the Agilent MAb 5 and MAb 10 column and commerically available column (Brand B) of similar dimensions. Superior peak shape and highest efficiency was achieved on the on the Agilent Mab 5 NP column (Fig6.).

Conclusions

The new Agilent 1260 Infinity bio-inert quaternary LC solution in combination with the Agilent Bio HPLC column portfolio provide a powerful and versatile tool to characterize the physico-chemical properties of a therapeutic protein drug by combining

- **bio-inertness,**
- **superior resolution,**
- **corrosion resistance,**
- **high sensitivity and**
- **fast separation speed**