Online 2D-LC Concepts for Complex N-Glycan Analysis from Biopharmaceuticals
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Introduction

Erythropoietin (EPO) is a 30400 dalton (Da) glycoprotein hormone that regulates the production of red blood cells (erythropoiesis). The glycosylation of EPO is highly variable because it contains multiple glycosylation sites, each of which can have a wide variety of glycan structures. This results in a huge complexity of glycan structures that is referred to as microheterogeneity. Detailed characterization of the glycan profile of biopharmaceuticals is important as differences in glycosylation can affect both the pharmacodynamics and pharmacokinetic behavior in the human body. Therefore, it is necessary to develop advanced analytical technologies for efficient and detailed glycan analysis.

The method of choice for the analysis of released glycans is typically hydrophilic interaction chromatography (HILIC) after labelling with 2-aminobenzamide (2AB) for sensitive fluorescence detection. Whilst HILIC efficiently separates glycans according to hydrodynamic radius, it is insufficient to fully resolve the complex mixture of branched glycan structures that are present in samples such as EPO. Fortunately, weak/strong anion exchange chromatography (WAX/SAX) provides a highly orthogonal separation that depends on the number and arrangement of acidic monosaccharides in the glycan. A combination of WAX/SAX and HILIC has a huge potential to enhance separation power to the two-dimensional (2D) LC separation technique.

The Agilent 1290 Infinity 2D LC Solution enables online 2D LC workflows for either comprehensive or (multiple) heart-cut analysis. Comprehensive 2D LC analysis, using two sample loops within a 2 position-/4-port-duo valve, leaves no peak missed from the 1st dimension. If higher resolution is desired in the 2nd dimension, the 1290 Infinity Multiple Heart-Cutting 2D LC Solution enables more flexibility, for example longer cycle times or columns.

Results and Discussion

The Agilent AdvanceBio Glycan Mapping Column demonstrated excellent resolving power for different glycoforms, like monoclonal antibodies and others. However, the separation within a one dimensional HILIC setup is not sufficient to resolve all N-glycans of EPO, see Figure 1. To improve resolution and to enhance peak capacity, a combination of WAX and HILIC separation was used for a highly orthogonal separation. A comprehensive HILIC/WAX separation was used in an online 2D LC setup, maintaining the highly orthogonal separation.

Figure 2 shows the 2D LC image from the HILIC/WAX 2D run. The 2D separation provides high peak capacity and resolution, and many of the co-eluting peaks from the HILIC dimension are well separated by WAX. The 2nd dimension separation group glycans by their charge. The neutral glycans, which elute immediately with the injection peak, are shortly followed by the singly charged glycans. More clearly separated, the double, triple, quadruple and few quintuple (fetuin) charged glycans elute with increasing salt gradient in the 2nd dimension. EPO isoforms are classified according to their net charge (epitope alpha, beta, etc.). This setup enables the user to charge profiling in combination with a well resolved glycan peak pattern.

In contrast to the comprehensive 2D LC solution, the multiple heart-cutting approach allows by HILIC in the 2nd dimension. This is, because the multiple heart-cutting approach allows the user of lower gradient and re-equilibration times in the 2nd dimension. In this experiment, a gradient time of 3.5 minutes and re-equilibration time of 1.4 min. The glycans were retained on the short HILIC column and a good 2D resolution was achieved, see figure 3. Six examples are shown to demonstrate the resolving power of the HILIC separation within the multiple heart-cutting setup (peaks 1, 4, 5, 8, 9, and 10). Areas that are only visible as shoulders in the 1st dimension, e.g. peak 8, revealed as eight peaks in the 2nd dimension. Under most of the peaks, which are only showing one major peak in the 1st dimension, several underlying peaks were detected and resolved.

Conclusions

Different online 2D LC concepts are presented for the high resolution analysis of complex N-linked glycans of EPO using the Agilent 1290 Infinity 2D LC Solution with fluorescence detection. EPO has a complex glycosylation pattern with differently branched and charged glycans. Although current HILIC columns have a high resolving power for glycans, the mixture of glycans on therapeutic EPO is so complicated that full resolution cannot be achieved by a one dimensional HILIC separation. A combination of hydrophilic interaction chromatography (HILIC) with weak anion exchange chromatography (WAX) enables highly orthogonal separation for excellent peak capacity.

Comprehensive 2D LC analysis with HILIC in the 1st and WAX in the 2nd dimension provides 2D chromatography together with simultaneous charge profiling. Complete automation of the 2-dimensional analysis enables a run time of only 110 minutes when compared to a much longer run time for offline analysis. Meanwhile, multiple heart-cutting 2D LC analysis combining WAX and HILIC separation provides a flexible alternative whereby the user can select multiple peaks to be analyzed in the 2nd dimension and, moreover, run longer gradients in the 2nd dimension.

Reference:
2. S. Schneider, Edgar Napel, Sonja Kröger, Online 2D LC Analysis of Complex N-Glycans in Biopharmaceuticals Using the Agilent 1290 Infinity 2D-LC Solution, Agilent Application Note 2015. 0911-8250EN.