Rapid HPLC Analysis of Monoclonal Antibody IgG_1 Heavy Chains Using ZORBAX Poroshell 300SB-C8

Application

Biochemical

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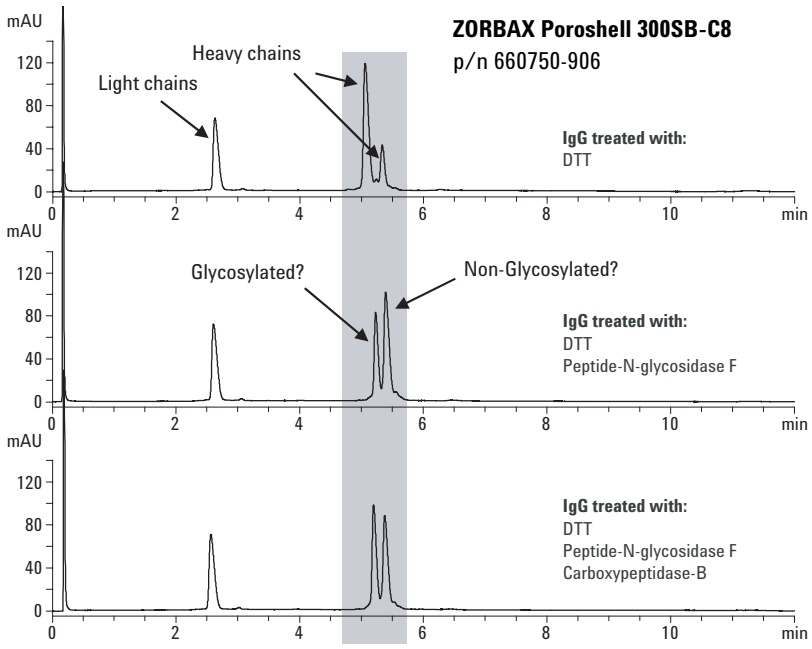
Antibodies represent a class of proteins that are the key to immunological interaction; they bind to an antigen (protein domain, glycoprotein, DNA, etc.) with extreme specificity. This makes antibodies extremely valuable for use in diagnostics, general research, and for therapeutics. IgG is a class of antibodies having two heavy (50 kDa each) and two light chains (25 kDa each), attached through disulfide bridges. While their carboxy-terminal domains are conserved, the amino-terminal domains are variable in amino-acid sequence, producing the molecule's specificity and diversity. Most antibodies are glycosylated, further increasing molecular diversity. Treatment of intact antibodies with various chemicals and enzymes causes specific results: DTT breaks disulfide bridges, resulting in free heavy and light chains. Peptide-N-glycosidase F removes carbohydrate moieties. Finally, carboxypeptidase-B results in C-terminal cleavage. In this manner, one may specifically study the general structure of an antibody. The rapid HPLC method development and analyses possible using ZORBAX Poroshell technology are valuable tools for this type of structural work with antibodies.



Conditions

Column	**ZORBAX Poroshell 300SB-C8** (2.1 × 75 mm, 5 µm) p/n 660750-906
Temperature	70 °C
Flow rate	1.0 mL/min
Detection	UV (210 nm)

Highlights

- High-velocity separations using ZORBAX Poroshell technology result in high-resolution analysis of antibodies with a short run time
- Method development is also more rapid, since these run times are shortened using ZORBAX Poroshell technology
- ZORBAX Poroshell 300SB columns come in many internal diameters and bonded phases. This gives a wide variety of choices for the optimal fast separation of proteins and peptides
- The choice of which ZORBAX Poroshell column to use will depend on the molecular weight (MW) and heterogeneity of the protein sample

Mobile phase

A = H_2O-ACN (90:10)

B = H_2O-ACN (10:90)

Both A and B contain 0.1% TFA and 3 mL/L of PEG 300.

Gradient timetable

Time (min)	% Solvent B
0.00	25.0
10.00	40.0
10.10	25.0
12.00	25.0

The chromatogram shows a rapid reversed-phase analysis optimized for the separation of antibody heavy-chain forms (peaks eluting at 5–6 min). Optimization of the gradient profile allows one to distinguish between various forms of heavy chains (50 kDa). The upper chromatogram of the figure shows IgG antibody sample separated on ZORBAX Poroshell 300SB-C8, after treatment with DTT to break disulfide bonds. Two IgG heavy-chain forms are observed. There are clearly different amounts of absorbancy, or mass, of the two forms.

Subsequent treatment of the IgG sample with a carbohydrate-cleaving enzyme (peptide-N-glycosidase F) followed by chromatographic separation under the same conditions reveals two heavy-chain forms eluting at new positions and in a different ratio. One possibility is glycosylated and nonglycosylated forms; another is different conformers.

The bottom chromatogram shows separation of the sample after its subsequent treatment with carboxypeptidase-B (for C-terminal cleavage of the IgG heavy chains). This last treatment caused little change to the chromatographic elution pattern, but some change to the peak-area ratio. We see here that rapid, high-resolution analysis can be used to quantitate the chain forms, follow additional enzymatic reactions and modifications, or isolate antibody chains for downstream use. While we obtain some initial information about this particular pair of heavy-chain forms, further analytical work would help to elucidate antibody structure and function.

ZORBAX Poroshell technology facilitates rapid analysis and method development of large molecules by use of its shortened diffusion path and tolerance of high linear velocity (high relative flow rate). ZORBAX Poroshell particles consist of a solid silica core covered by a thin totally porous crust. Large molecules, which diffuse very slowly compared to small molecules, can move into and out of the thin crust in a very short time. Flow rate can be increased to reduce run time, without significant peak broadening. ZORBAX Poroshell columns are typically used at flow rates five to ten times those used with a column of the same dimensions containing totally porous particles. Note that the analysis is complete in less than 10 min. The resolution achieved is similar to that of a 50-min run on a column having totally porous particles. Finally, the short column length and 5-µm particle diameter function together to keep back pressure within acceptable limits.

Separations on ZORBAX Poroshell often benefit from increased flow rate because this increases gradient volume and reduces the gradient slope, resulting in increased relative retention (k') and resolution (Rs). The resolution of a 50-min run is achieved in 10 min! The additional benefit of using a rapid, high-resolution separation in method development should not be overlooked. A series of scouting runs may be made in, for example, one-fifth the usual time. This gives the analyst more time to achieve the type of resolution required for separation of heavy- (or light-) chain forms. For an example of a rapid ZORBAX Poroshell separation optimized for IgG light-chain analysis, see Agilent publication number 5989-0030EN.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.
Search "Poroshell".

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