Reversed Phase Antibody Separations with Jupiter™300 C4

Greg Scott and Michael McGinley
Phenomenex Inc., Torrance, CA, USA

Introduction

The number of antibody therapeutics has increased dramatically over the last ten years. This is due much in part to the ability of antibodies to target specific biomolecules *in vivo* thereby either blocking or activating specific biological activities[1]. While antibodies have proved incredibly useful as therapeutic agents, there have been many challenges in analyzing and purifying such proteins.

> "antibodies have often proved difficult to analyze by reversed-phase chromatography"

Immunoglobulin G (Ig-G), which is the traditional recombinant antibody used, is a 150 kDa glycoprotein consisting of two heavy chains (~50 Kda each) and two light chains (~25 Kda each) that are disulfide linked forming a "Y"-like structure[2]. Because of its large size and moderate hydrophobicity as well as its non-globular structure, antibodies have often proved difficult to analyze by reversed phase chromatography.

Figure 1.
Reversed phase chromatogram of Ig-G comparing different organic mobile phase conditions

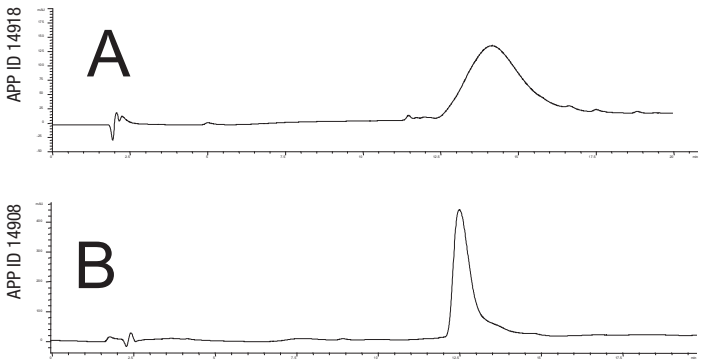

Figure 1: Comparison of dog Ig-G antibody run on Jupiter 300 C4 using either 95% Acetonitrile (A) or 70% Acetonitrile/20% Isopropanol (B) as the organic mobile phase. Note the dramatic improvement in peak shape with the addition of isopropanol.

Antibody protein peaks are often broad and poorly defined with poor recoveries. Such poor chromatography can make separating minor components in an antibody sample difficult. Efforts were undertaken to improve reversed phase chromatography of Ig-G by investigating different mobile phase conditions as well as flow rate and temperature.

Materials and Methods

Analyses were performed using an HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump and UV detector and using HP Chemstation software (Version A.08.03) for data analysis. The HPLC columns used for the analyses were Jupiter 300 C4, C5, and C18 150 x 2.0 mm (Phenomenex, Torrance, CA, USA). Recombinant dog immunoglobulin G (Ig-G), trifluoroacetic acid (TFA), and dithiothreitol were purchased from Sigma Chemicals (St. Louis, MO, USA). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Figure 2.
Comparison of different Jupiter 300 phases (C4, C5, and C18) for reversed phase chromatography Dog Ig-G.

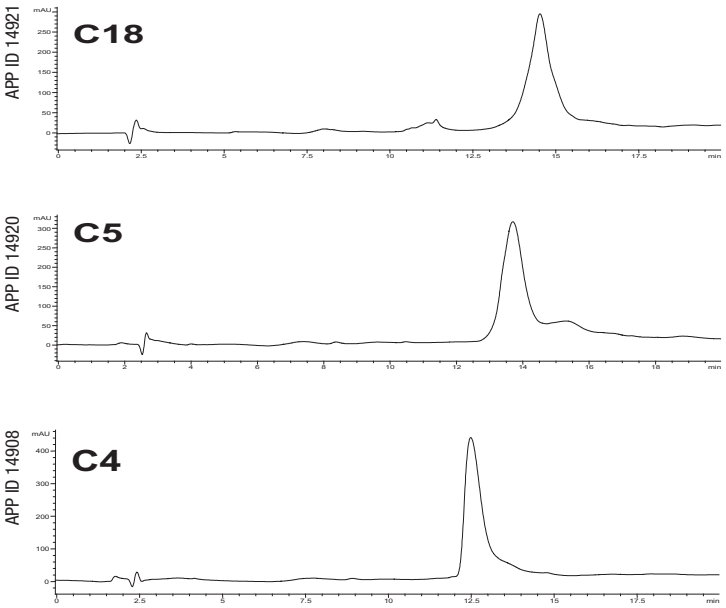

Figure 2: HPLC Chromatograms of the same dog Ig-G antibody run on the Jupiter 300 C4, C5 or C18 phases. Note improved peak shape and reduced retention time with the C4 phase.

For all separations an aliquot of 4µg of dog Ig-G was injected on selected columns running at a flow rate of 0.25 mL/min. Mobile phase A was 5% acetonitrile/0.1% trifluoroacetic acid in water for all runs. Mobile phase B was either 95% acetonitrile/0.85% trifluoroacetic acid in water or 75% acetonitrile/20% isopropanol/0.85% trifluoroacetic acid in water. A gradient from 20% B to 95% B over 20 minutes was used for all separations.
For HPLC analysis of antibody heavy and light chain mixtures, dog Ig-G was reduced with 2 mM DTT in 8 M Urea at 45° C for 30 minutes[3].

Results

Different organic mobile phase conditions as well as Jupiter phases were evaluated to improve overall peak shape for antibody separations. Figure 1 demonstrates the utility of using low amounts of isopropanol in the organic mobile phase to improve peak shape of hydrophobic proteins. Figure 1A shows the elution of Ig-G on a Jupiter 300 C4 column where Acetonitrile is used as the organic mobile phase. Figure 1B shows the same Ig-G run on the same column using an organic mobile phase that contains 20% isopropanol. Note the dramatic improvement in both recovery and peak shape for the Ig-G peak. The addition of isopropanol to the organic mobile phase improves the efficiency as well as the peak shape for large hydrophobic proteins due much in part to the increased hydrophobicity of the organic mobile phase upon addition of isopropanol.

Different Jupiter 300 phases were evaluated to determine which phase provided the best peak shape and efficiency. The dog Ig-G sample was run on the Jupiter 300 C4, C5 and C18 phases and results are shown in Figure 2. Surprisingly, the Jupiter 300 C5 and Jupiter 300 C18 do demonstrate reasonable peak shape and recovery. However, as expected, the Jupiter 300 C4 phase demonstrated the least retention as well as the best peak shape and recovery of the three phases. This comparison demonstrates that Jupiter 300 C4 phase should be the column of choice for the chromatographic separation of large hydrophobic proteins.

Figure 3.
HPLC Chromatogram of Reduced Dog Ig-G on Jupiter 300 C4

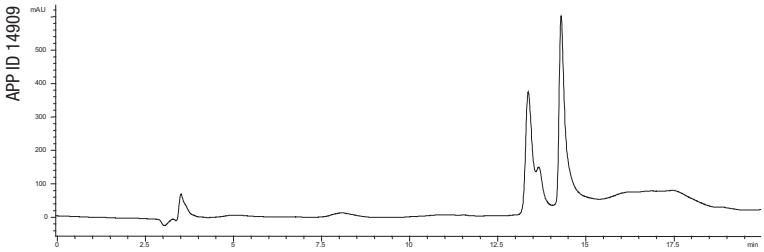

Figure 3: HPLC Chromatogram of dog Ig-G reduced with DTT. Note the baseline separation of the heavy and light chains of Ig-G.

An additional area of application interest for many working with antibodies is in the separation of heavy and light chains of Ig-G. For this example dog Ig-G was reduced with dithiothreitol and injected on a Jupiter 300 C4 column. Results are shown in Figure 3; one can see the heavy and light chains are baseline resolved using the conditions previously described. These results demonstrate the ability of the Jupiter 300 C4 to resolve differences between large hydrophobic proteins for demanding applications such as the separation of immunoglobulin proteins.

References

1. Harris, L.; Larson, S.; Hasel, K.; McPherson, A. Biochemistry 36: 1581 (1997)
2. Clark, M. Chemical Immunology 65, p. 88-110 (1997)
3. Robinson, J.; McGinley, M.; Leidli, J.; Lyons, D.; Lin, C.; Karan-Tamir, K.; Zukowski, M.; Rohde, M. in *Techniques in Protein Chemistry VII* ed. Marshak, D. p. 249-259 (1996)

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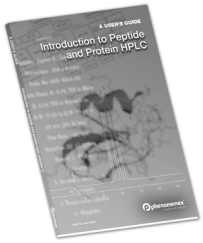

ORDERING INFORMATION

Order Number	Description
00F-4167-B0-TN	Jupiter 300Å 5µ C4 150 x 2.0 mm
00F-4052-B0-TN	Jupiter 300Å 5µ C5 150 x 2.0 mm
00F-4053-B0-TN	Jupiter 300Å 5µ C18 150 x 2.0 mm

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