[APPLICATION NOTE]

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

UPLC TECHNOLOGY FOR THE ANALYSIS OF ANTIBODY GLYCOPEPTIDES

Beth L. Gillece-Castro, Thomas E. Wheat, Jeffrey R. Mazzeo, and Diane M. Diehl Waters Corporation

WATERS CORPORATION, Milford, MA USA

CLICK ON PART NUMBERS FOR MORE INFORMATION

INTRODUCTION

The development of protein biopharmaceuticals requires complete structural characterization. Definition of a protein structure includes the amino acid sequence, storage-induced modifications, and post-translational modifications of the protein. Glycosylation is one of the most important classes of post-translational modifications. Within the human body many circulating proteins are glycosylated, and therefore the most promising candidate drugs for biopharmaceutical use have glycan chains. The oligosaccharide components can directly affect the efficacy and safety of these drugs by influencing binding, immunogenicity, and turnover. It is, therefore, critical to characterize oligosaccharide structure in biopharmaceutical development.

The analysis of glycoproteins is a challenging enterprise because of the extreme complexity of oligosaccharides and the heterogeneity of the cellular products. The molecular weights and branched structure of glycans that have been released from a protein can be determined by applying MALDI or ESI mass spectrometry with collision-induced dissociation (CID). Proposed structures can also be based on elution times from anion-exchange chromatography or from HILIC on amino phases. These analytical techniques do not, however, yield information about the sites of modifications. To approach the modification site question, glycoproteins are subjected to proteolytic cleavage digestion without removal of the glycans. The reversed-phase HPLC peptide maps usually include glycopeptides as broad, asymmetric, poorly resolved peaks. Better chromatographic separations are required for assessing the safety and efficacy of biopharmaceuticals.

Recently, UltraPerformance LC® (UPLC® Technology) has been proposed as a tool for improving resolution in peptide mapping. This technique has now been tested for the analysis of glycoforms of a monoclonal IgG.

EXPERIMENTAL CONDITIONS

Sample Preparation

A monoclonal mouse IgG1 (VICAM division of Waters) was reduced, alkylated, and digested in the presence of 0.1% *Rapi*Gest™ SF surfactant (Waters Part Number:186001860) in a Certified Maximum Recovery vial (Waters, Part Number: 600000670CV). The reducing agent, TCEP (tris[2-carboxyethyl] phosphine, Pierce) was added to a concentration of 10 mM. After 30 minutes of reduction, the alkylation reagent, iodoacetamide, was added to a concentration of 20 mM for 30 minutes. The protein was then digested overnight with Modified Trypsin (Promega) at an enzyme-to-substrate ratio of 1:25.

Figure 1. UPLC Peptide Analysis Solution

LC Conditions

UPLC Chromatography

Instrument:	Waters ACQUITY UPLC® Peptide Mapping System
Detector:	TUV @ 214 nm
Column:	Peptide Separation Technology Column 2.1 x 100 mm, ACQUITY® BEH130 C_{18}, 1.7 µm Part Number: 186003555
Column Temp:	40 °C
Injection Volume:	10 µL
Mobile Phase A:	0.1% TFA in water
Mobile Phase B:	0.08% TFA in acetonitrile
Flow Rate:	100 µL/min

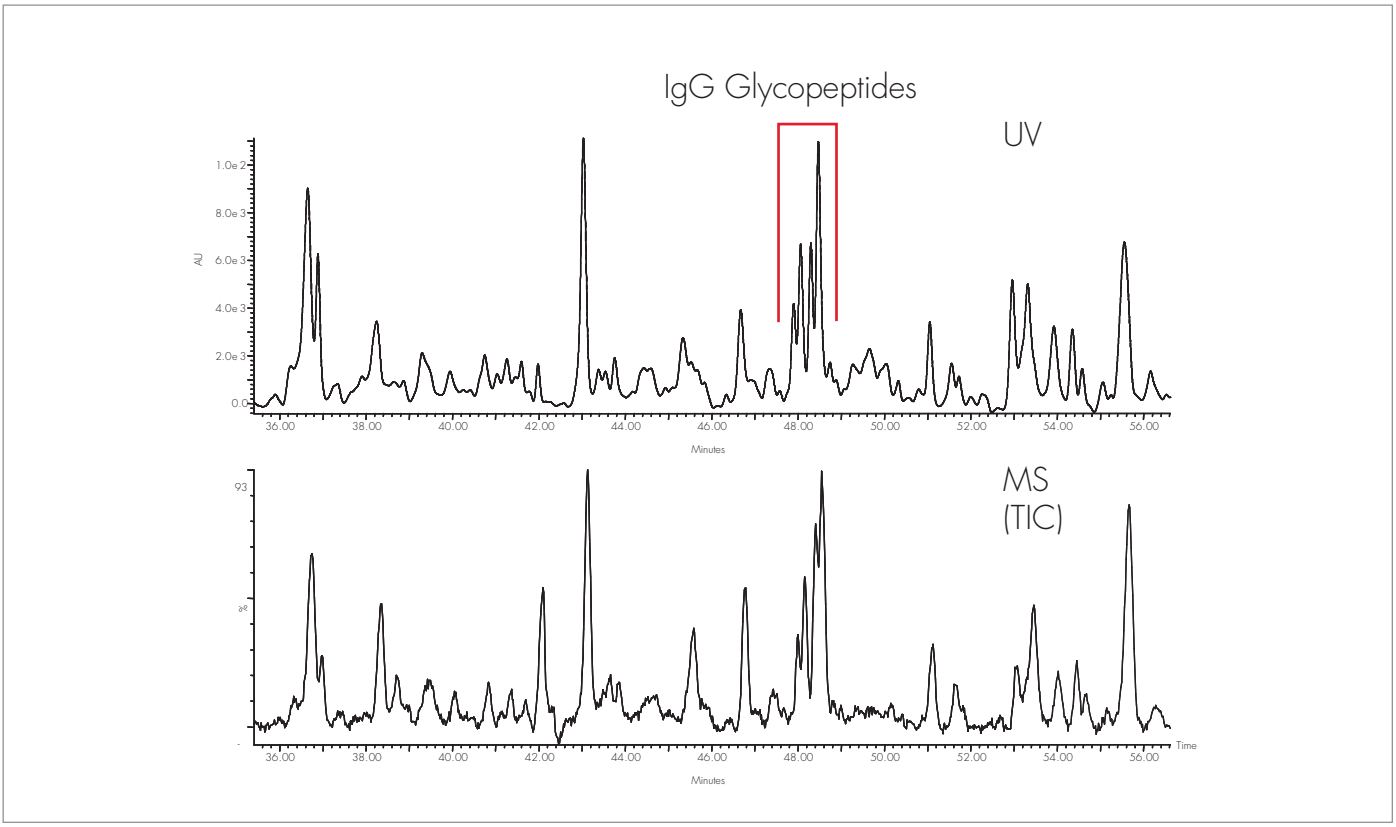
Gradient Table

Time (min)	A	B	Curve
2	100%	0%	*
118	50%	50%	6
122	25%	75%	6
123	0%	75%	6
125	100%	0%	6
143	100%	0%	6

MS conditions

Instrument:	Waters LCT Premier™ Mass Spectrometer
Ionization Mode:	ESI Positive
Capillary Voltage:	3200 V
Cone Voltage:	30 V
Desolvation Temp:	150 °C
Desolvation Gas:	500 L/Hr
Source Temp:	100 °C
Acquisition Range:	50-2000 m/z

Figure 2. UV and MS UPLC chromatograms of a mouse antibody tryptic digest. The selected time range is focused on the glycopeptides.



RESULTS

The tryptic peptides of the reduced and alkylated monoclonal IgG were separated using UPLC Peptide Mapping System, as shown in Figure 2. The same chromatogram could be observed with either UV or MS detection, and the glycopeptides were recognized by their masses as described in Figures 3 and 4.

The cluster of four marked peaks represent the elution of the major glycoforms of a monoclonal IgG. These antibodies have only one conserved glycosylation site. There are two N-linked glycans tucked between the two heavy chains to which they are attached, one to each heavy chain, at the same conserved sequence. They are heterogeneous with respect to the number of terminal galactose residues, so that a given peptide may exist with zero, one, or two galactose residues. The structures of the "G0F", "G1F", and "G2F" glycans are shown in Figure 3.

Extracted ion chromatograms for the three glycopeptide molecular weights, in each case the triply-charged ion, are shown in Figure 4. The G2F glycoform elutes first in the TFA-containing gradient, followed by baseline-separated glycopeptides displaying the two isomers of G1F. Chromatographic peak widths at half height were 0.11 minutes or 6-7 seconds. The improved resolving power allowed the resolution of individual glycopeptide structures.

Detection of four glycopeptide species in the UV trace as shown in Figure 5, can allow relative quantitation of the IgG glycoforms in a peptide map. In this way, glycan fingerprints can be obtained at the same time as oxidation and deamidation analyses.

Figure 3. N-linked Glycans of Monoclonal IgG

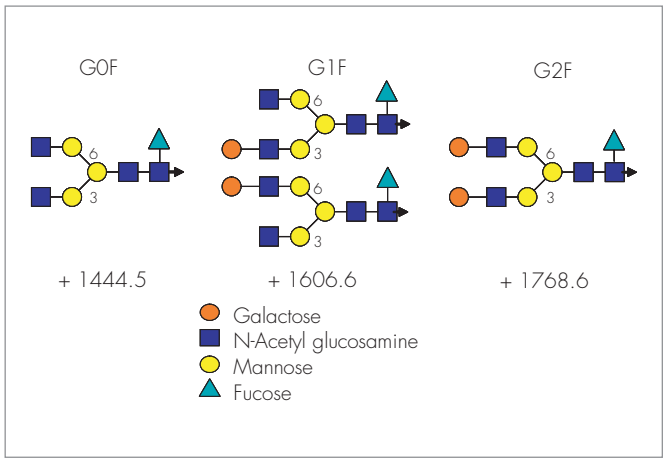


Figure 4. Extracted Ion Chromatograms for the Triply-charged State for Each of the Three Expected Glycopeptides

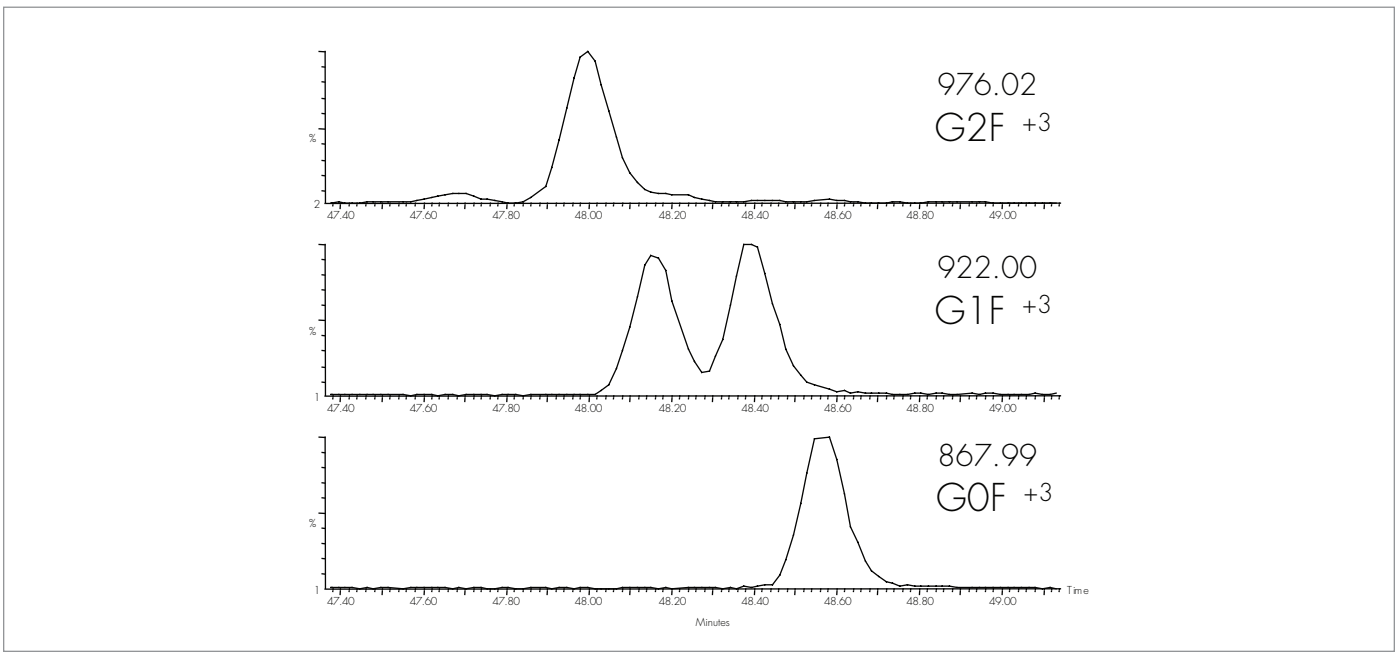
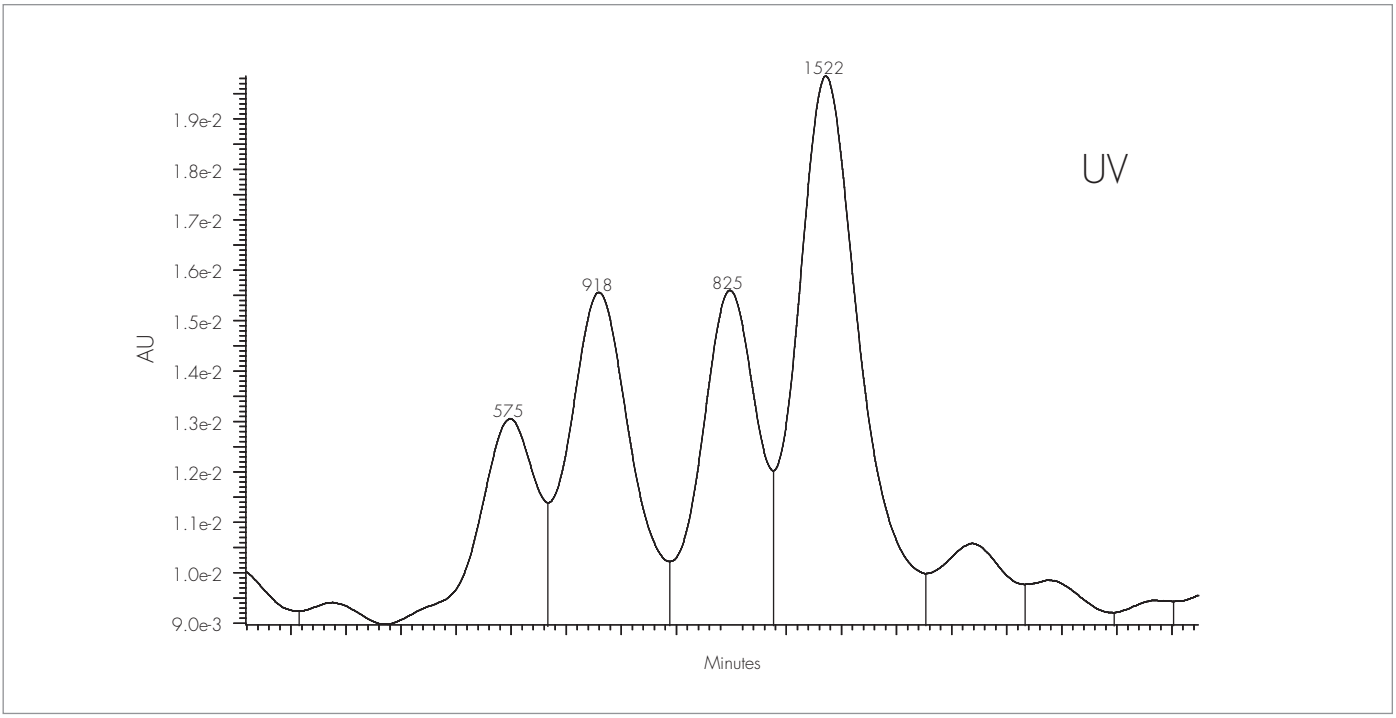


Figure 5. Detection of Four Glycopeptide Species in the UV Trace



CONCLUSION

The Waters UPLC Peptide Analysis Solution was described as a tool for peptide mapping protein characterization. The technique has proven useful for improved resolution and speed in peptide mapping. The significantly improved peptide resolution is now shown to provide separation of glycyopeptides with mixed glycan structures, even the separation of glycopeptide positional isomers. Quantitation of the relative amounts of glycan structures can thus be approached within the peptide map without the cumbersome procedures required for released glycan analysis.

Austria and European Export (Central South Eastern Europe, CIS and Middle East) 43 1 877 18 07, **Australia** 61 2 9933 1777, **Belgium** 32 2 726 1000, **Brazil** 55 11 5094 3788, **Canada** 1 800 252 4752 x2205, **China** 86 21 6879 5888, **CIS/Russia** 7 095 336 7000, **Czech Republic** 420 2 617 1 1384 **Denmark** 45 46 59 8080, **Finland** 358 9 5659 6288, **France** 33 1 30 48 72 00, **Germany** 49 6196 400600, **Hong Kong** 852 29 64 1800 **Hungary** 36 1 350 5086, **India and India Subcontinent** 91 80 2837 1900, **Ireland** 353 1 448 1500, **Italy** 39 02 27 421 1, **Japan** 81 3 3471 7191 **Korea** 82 2 820 2700, **Mexico** 52 55 5200 1860, **The Netherlands** 31 76 508 7200, **Norway** 47 6 384 60 50, **Poland** 48 22 833 4400 **Puerto Rico** 1 787 747 8445, **Singapore** 65 6273 1221, **Spain** 34 93 600 9300, **Sweden** 46 8 555 11 500, **Switzerland** 41 56 676 70 00 **Taiwan** 886 2 2543 1898, **United Kingdom** 44 208 238 6100

All other countries: Waters Corporation U.S.A. 1 508 478 2000/1 800 252 4752

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

October 2007 IH-PDF 720002382EN

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com