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Rapid method for monitoring galactosylation levels during recombinant antibody production by electrospray mass spectrometry with selective-ion monitoring

Hong Z. Wan, Stacey Kaneshiro, John Frenz, Jerry Cacia*

Department of Manufacturing Sciences, Genentech, Inc., MS#75B, 1 DNA Way, South San Francisco, CA 94080, USA

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Abstract

This report describes a simple and rapid method to determine the relative amounts of glycoforms differing in terminal galactose on a recombinant antibody produced in Chinese hamster ovary (CHO) cells. The method uses a single quadrupole mass spectrometer coupled to an HPLC system to quantify the glycoform amounts found on a recombinant antibody that binds to the human CD20 antigen. Samples from the recombinant antibody process are reduced and injected directly into the HPLC system where the heavy and light chain antibody fragments, as well as host-cell protein contaminants, are separated chromatographically. Mass-selective detection is performed in the selected-ion monitoring (SIM) mode to monitor the most abundant (38^+) ions corresponding to the glycoforms found on the heavy chain of the recombinant antibody. Results obtained using the assay demonstrate good sensitivity, linearity and reproducibility. Comparison to a method using capillary electrophoresis (CE) of the labeled free oligosaccharides demonstrates similar quantitation of the glycoforms in the recombinant antibody. The LC–MS method provides a simple and rapid means for accurately quantifying antibody glycoforms directly from cell culture and other process samples.

Keywords: Mass spectrometry; Antibodies; Glycoproteins; Proteins; Oligosaccharides

1. Introduction

Rituximab is a recombinant mouse/human chimeric monoclonal antibody that binds to the human CD20 antigen [1]. More than 99% of the heavy chains are glycosylated at asparagine 301 with a complex biantennary oligosaccharide. The three glycans typically found on the antibody differ in the number of terminal galactose moieties. The observed structures are identified in Table 1 as G0 (no terminal galactose), G1(1 mol terminal galactose/mol antibody heavy chain), and G2(2 mol terminal galactose/mol antibody heavy chain). The activity of rituximab in vitro varies with the oligosaccharide composition. Biological characterization has demonstrated that an increased G0 glycan level on the molecule is associated with a reduction in biological activity as measured by a complement dependent cytotoxicity (CDC) assay [2]. Variability in glycan structure has been observed in production of recombinant antibodies using a variety of host cell expression systems [3–5]. Monitoring the level of galactosylation observed during cell culture production helps to identify parameters that influence the level

*Corresponding author. Fax: +1-605-225-4436.
E-mail address: cacia@gene.com (J. Cacia).

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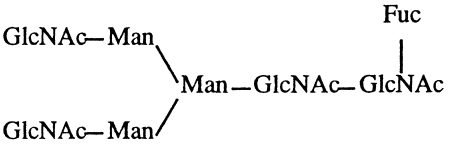
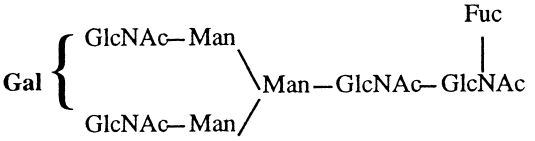
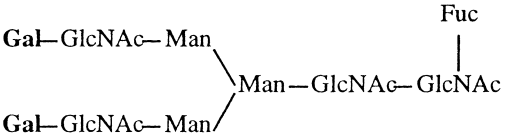
Table 1
Principle oligosaccharide structures found on recombinant rituximab

Identifier	Oligosaccharide structure	Molecular mass of glycosylated heavy chain	m/z of 38^+ ion
G0		50 515	1330.4
G1		50 677	1334.6
G2		50 839	1338.9

of galactosylation in the bulk drug, and so to optimize for manufacture of a consistent product.

Optimization and control of a manufacturing operation requires data from analytical monitoring that provides feed-back on the effects of adjustments in process variables. An important consideration in the development of methods to be used for process monitoring is the time constant necessary for obtaining information about the process. Preparation of process samples can add significantly to the time constant for the method, and thus is an important element in the method optimization. Obtaining relevant product information in the analysis of recombinant antibodies can be challenging since they are large and inherently complex. Hyphenated techniques and enzymatic fragmentation of antibodies prior to analysis have become standard practice in the analysis and biochemical characterization of recombinant antibodies [6–8]. Digestion of the antibody prior to analysis can improve resolution and quantitation of relevant components, and simplify the interpretation of data at the expense of rapid availability of the analytical result.

An established assay for the determination of the relative glycan composition in rituximab (G0, G1 and G2) uses a capillary electrophoresis method [9]. Antibody samples are purified using Protein A affinity chromatography, followed by release of the constituent oligosaccharides using PNGase-F. The oligosaccharides are derivatized using APTS (8-aminopyrene-1,3,6-trisulfonic acid), separated by capillary electrophoresis, and detected with laser-induced fluorescence (CE–LIF). This approach has proven useful for routine bulk product testing, for highly purified antibodies at relatively high concentration. The CE–LIF method is not suitable for monitoring cell culture processes due to the requirement of relatively large amounts of purified sample, the labor required to purify and prepare large numbers of samples, together with the time lag between sampling and availability of the data.

The LC–MS method described here requires only minimal sample preparation. A fast RPLC method has been developed to introduce process samples to the mass spectrometer, separating the heavy and light chains from host cell proteins. The RPLC solvent system developed maximizes recovery and ionization efficiency of the antibody fragments. Selective-ion monitoring (SIM) is used to detect and quantify ions corresponding to the three principal glycoforms found on the heavy chain of the recombinant rituximab antibody. An integrated software package

allows for direct quantitation from selectively monitored ions, thus rendering the method useful for rapidly monitoring crude process samples. The technique provides a simple, rapid, and sensitive analytical tool for monitoring glycosylation during cell culture production of recombinant antibodies.

2. Experimental

2.1. Reagents and materials

Acetic acid, formic acid, acetonitrile, and 2-propanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA), HPLC/spectro grade, was from Pierce (Rockford, IL, USA). Dithiothreitol (DTT) was from Bio-Rad Labs. (Richmond, CA, USA). The Poros R1/H reversed-phase HPLC column was from PE Biosystems (Framingham, MA, USA). The HP1100 HPLC system and the electrospray ionization mass spectrometer (model HP1100-MSD) were from Hewlett-Packard (Mountain View, CA, USA; now Agilent Technologies, Palo Alto, CA, USA). Purified rituximab material was manufactured by Genentech (South San Francisco, CA, USA). Process cell culture fluid samples were all from Genentech's South San Francisco manufacturing facility.

2.2. Instrumentation and data analysis

The HP 1100 LC–MS system employed consists of an HP1100 HPLC system and an electrospray ionization single quadrupole mass spectrometry (ESI-MS) system. The mass-selective detector has two operating modes. In the scan mode the instrument scans across a specified mass range and monitors the ion abundance at a specified time interval. The other operating mode is SIM which monitors only the ions specified. The scan mode was used to identify ions suitable for routine monitoring. The SIM mode was used for routine process monitoring to maximize the sensitivity and reproducibility for quantitation of known components in samples. Peak areas from selectively monitored ions were integrated using the HP-MSD ChemStation software package, and glycoform amounts were expressed as the relative percentage of the total peak area. The HP1100-MSD was tuned using the automated tuning program and the pre-mixed calibration solutions provided by the manufacturer.

2.3. Method optimization

The method was optimized with regard to HPLC column selection, mobile phase composition, gradient elution conditions, column temperature, and sample injection mass. The conditions were evaluated for optimal recovery, separation, and ionization efficiency of the light chain and heavy chain fragments of the antibody. Columns tested include Poros R1/H, 100×2.1 mm (PE Biosystems), TSKgel phenyl-5PW (TosoHaas, Montgomeryville, PA, USA), Synchropak RP4, 7.5×0.46 cm (Alltech, Deerfield, IL USA), Zorbax C_4, 150×2.1 mm (Hewlett-Packard) and PLRS, 50×2 mm (Polymer Labs., Amherst, MA, USA).

Solvent systems tested were: (A1) 0.1% aqueous TFA–(B1) acetonitrile; (A2) 0.2% aqueous formic acid–(B2) acetonitrile; (A3) 0.2% aqueous propionic acid–(B3) acetonitrile; (A4) 0.2% aqueous acetic acid–(B4) acetonitrile–2-propanol (70:30, v/v); (A5) 2% aqueous acetic acid–(B5) acetonitrile–2-propanol (70:30, v/v).

The fragmentor voltage was the only MS setting optimized for ionization of the antibody heavy and light chains. Voltage settings tested ranged from 90 to 220 V. The antibody injection mass was assessed and ranged from 0.005 to 12 μg.

2.4. Sample preparation and LC–MS analysis of antibody samples

Antibody samples were treated with 1: 10 (v/v) 1 *M* DTT, and incubated for 30 min at room temperature before injection. An injection mass of 0.5–10 μg produced good separation and ionization, as well as reproducible quantitation. Sample injection volumes up to 100 μl were used. A Poros R1/H, 100×2.1 mm, was operated at a flow rate of 0.5 ml/min. The optimal mobile phase tested was 2% acetic acid (solvent A), and acetonitrile–2-propanol, (70:30, v/v) (solvent B). The column compartment temperature was set at 60°C. The gradient used to separate the light chain and heavy chain fragments from host cell proteins was 10–75% B in 30 min.

The flow was directed through the UV detector for the entire analysis, where the effluent is monitored at 280 nm. A valve diverts the column effluent into the MS system 12 min after sample injection. The column effluent is diverted back to waste 18 min after sample injection. For optimal ionization, the MS fragmentor voltage was set at 120 V. The spray chamber parameters were set as follows: drying gas flow 10 L/min, drying gas temperature 350°C, nebulizer pressure 40 p.s.i. and capillary voltage 4000 V (1 p.s.i.=6894.76 Pa).

2.5. Assay reproducibility, linearity, and accuracy

Reproducibility was assessed by injecting purified rituximab and harvested cell culture fluid (HCCF) samples 12 times after individual treatments with 100 m*M* DTT for 30 min. Assay reproducibility was further determined by injecting purified rituximab 23 times over a time period of 4.5 months. To demonstrate the robustness and reproducibility of the LC–MS assay, the percent G0 was calculated using 5 different selectively-monitored ions within the ionization envelope for 8 different rituximab HCCF samples.

The linear range of the assay was determined by injecting seven reference material samples within a mass range of 0.5–10 μg. The accuracy for measurement of G0 levels in rituximab antibody samples was determined by volumetric dilution of two HCCF samples with high and low G0 content. The measured result was compared to the calculated G0 content, and the percent recovery determined for each point in the assay range. Data presented for assay performance is the % G0 relative to the total glycoform species present (G0, G1, and G2).

3. Results and discussion

3.1. Optimizing recovery and ionization of antibody fragments

Various sample preparation approaches were considered in developing a method suitable for rapid analysis of large numbers of antibody samples. The glycosylation attachment site in the recombinant antibody is located on the heavy chain of the molecule. The molecular mass of the glycosylated heavy chain fragment is approximately 50 000 (Table 1). The antibody samples were treated with 100 m*M* DTT, and incubated for 30 min at ambient temperature to reduce the disulfide bonds found on the heavy and light chain antibody fragments. Reduced antibody samples were analyzed using a variety of reversed-phase HPLC conditions. Table 2 summarizes the results for method variables tested in the development of the LC–MS assay.

Table 2
Method development for LC–MS analysis of recombinant antibodies

Method variable	Test conditions	Results
Acidic modifier	TFA	Ionization completely suppressed
	Formic acid	Good separation/ionization
	Propionic acid	Good separation/ionization
	Acetic acid	Best separation/ionization
Column chemistry	Silica	Poor separation and recovery
	Polymeric	Good separation and recovery
Column temperature	Ambient	Poor separation and peak shape
	60°C	Good separation and peak shape
Fragmentor voltage	Over 220 V	CID effect
	Under 90 V	Poor ionization efficiency
	110–150 V	Good ionization efficiency
Data acquisition	Scan mode	Erratic component quantitation
	SIM mode	Reproducible component quantitation

The acidic modifier TFA, while yielding good peak shape and recovery on RPLC, was found to suppress ionization of the antibody fragments. The TFA suppression effect is well understood, and has been described for ESI-MS applications [10,11]. A post-column addition of propionic acid prior to MS detection has been shown to mitigate the signal suppression effects of TFA [12]. Several organic acids were tested as mobile phase additives in place of TFA (Table 2). This approach yielded good recovery, peak shape, and ionization efficiency for the antibody fragments, and proved to be much simpler than the post-column addition technique. An important element in eliminating TFA from RPLC mobile phases in the analysis of large protein fragments is the use of polymeric-bead based RPLC columns. Replacement of TFA with other organic acids, such as acetic acid, resulted in poor peak shape and recovery when run on silica-based RPLC columns. The use of polymeric packing materials for introduction of large antibody fragments into the ESI-MS has been described previously [6]. Further, the use of polymeric RPLC columns in conjunction with acetic acid has been used to analyze a large fragment of recombinant human factor VIII by ESI-MS [13]. A recent report has compared the use of various organic acids, including acetic acid, in combination with polymeric stationary phases for the analysis of other standard proteins by HPLC–ESI-MS [14]. This work demonstrated that, while 0.5% acetic acid improved detectability, a considerable loss in separation efficiency was observed. Aside from the expected differences in separation efficiency between proteins, other factors may account for different results observed in the two analytical systems. We have found that the use of 2% acetic acid yields sharper antibody fragment peaks when compared to 0.5% (data not shown). Combining mobile phases containing alternative organic acids with separation on polymeric-bead based RPLC packings has resulted in good recovery and separation of antibody fragments, as well as efficient ionization and detection by ESI-MS. In addition to column and mobile phase selection, the column temperature was optimized to give good peak shape and recovery of heavy and light chain antibody fragments (Table 2).

The fragmentor voltage setting was optimized to produce good ionization efficiency of the antibody fragments, while minimizing effects from collision-induced dissociation (CID). A voltage setting ranging from 110 to 150 V resulted in good ionization of the antibody fragments (Table 2).

A chromatogram representing the optimal conditions for resolution and ionization of antibody heavy chain (HC) and light chain (LC) fragments is shown in Fig. 1. A 2 μg sample of rituximab HCCF was injected, and the bottom profile is the effluent monitored by UV absorbance at 280 nm. Sample contaminants flow through the column, and flow is diverted to waste following UV detection until 12 min after sample injection. The column effluent is directed to the MS system from 12 to 18 min following injection. The top profile in Fig. 1 is the total ion current (TIC) for the MS system operated in the scan mode.

The ionization envelopes for the heavy and light chain spectra, along with the TIC profile are shown in Fig. 2. Reversed-phase separation of the heavy and light chain fragments is essential to the analysis and interpretation of the spectra as there is overlap in the ions observed for the two spectra. In addition, co-elution of the light chain results in suppression of heavy chain ionization. A single molecular species is observed in the light chain spectrum. The light chain of the antibody is not glycosylated, and does not

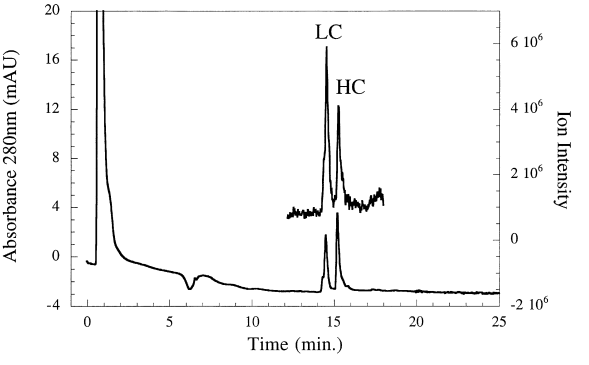


Fig. 1. LC–MS analysis of a recombinant antibody sample taken directly from cell culture. The sample was reduced prior to injection, resulting in separation of light chain (LC) and heavy chain (HC) antibody fragments. The top profile is the total ion current (TIC) trace, and the bottom profile is the absorbance of the effluent monitored at 280 nm. Injection mass 2 μg.

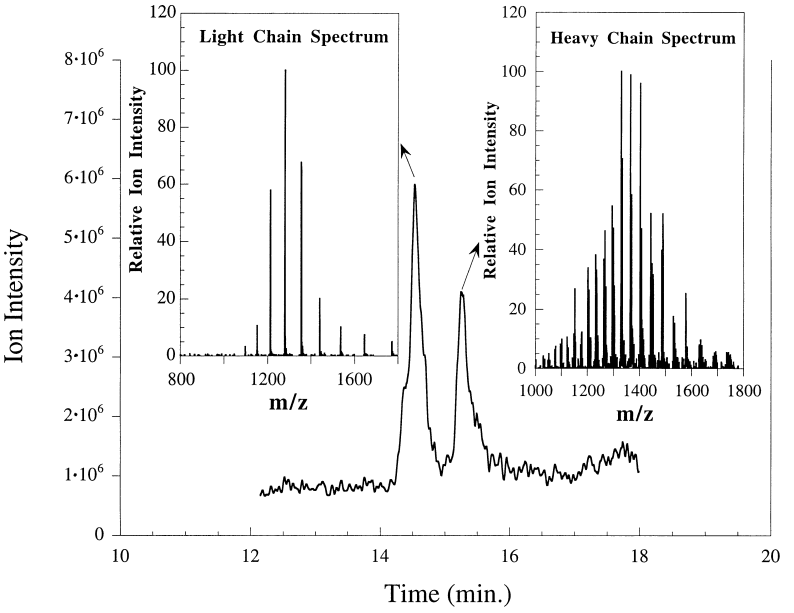


Fig. 2. Mass spectrum taken from light and heavy chain antibody fragments described in Fig. 1.

contain heterogeneity that is detectable by ESI-MS. The heterogeneity observed in the heavy chain spectrum represents the oligosaccharide structures shown in Table 1.

3.2. Monitoring in the SIM mode

Analysis of the heavy chain spectrum observed when operating in the scan mode reveals that the most abundant ions corresponding to the G0, G1, and G2 glycoforms of the antibody are 1330.4, 1334.6, and 1339.0, respectively (Table 1). These ions were chosen for monitoring in the SIM mode during routine analysis of antibody samples. Fig. 3 shows the TIC for the selectively-monitored ions corresponding to the 38^{+} charged state of the antibody glycoforms found on the heavy chain. The TIC profile results in a single peak corresponding to the elution of the heavy chain. Ion peaks are not observed at other points in the elution profile, indicating that there are no other components containing ions coincident with those monitored. The relative ion intensity for the individual G0, G1 and G2 ions is shown on the inset for Fig. 3. Resolution of the ions corresponding to the glycoform components is sufficient to permit direct analysis of reduced antibody samples due to the sizeable mass difference (162 amu).

For routine analysis, extracted ion chromatograms (EICs) were obtained for the three glycoform components. The EIC signals are taken over a 1 amu interval for the maximum ion observed in the scan mode. Peak areas are calculated by integration of the

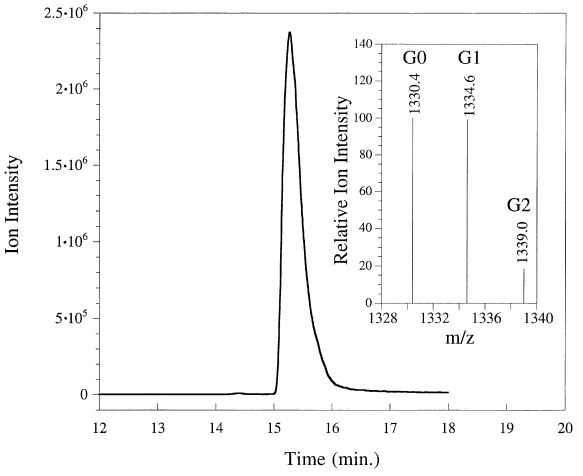


Fig. 3. Total ion current trace for selectively monitored ions shown on the inset.

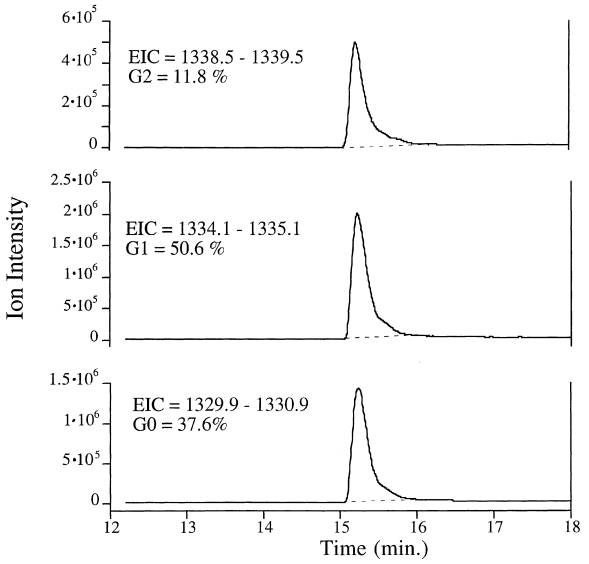


Fig. 4. Extracted ion chromatograms used to quantify G0, G1, and G2 levels in rituximab process samples.

resulting traces and the relative glycoform amounts are calculated from the total EIC peak area (Fig. 4).

3.3. Establishing reproducibility, accuracy, linearity, and robustness of LC–MS assay

A key element to component analysis and quantitation by ESI-MS is establishing that the ionization efficiency is identical for each of the analytes. Characterization studies on human plasma-derived [15,16] and recombinant [17,18] IgG_1 antibodies indicate that the oligosaccharides attached to asparagine 301 of the heavy chain are non-sialylated, complex biantennary structures. The lack of charge on the oligosaccharide structures, while making a charged-based separation technique difficult, creates an opportunity for separation and detection by ESI-MS. To establish similar ionization efficiencies for the G0, G1, and G2 components, mixing studies were performed to represent an appropriate range of G0 levels. Two cell culture process samples containing relatively high and low G0 levels were diluted to the same protein concentration, mixed volumetrically and analyzed using the LC–MS method in the SIM mode (Table 3). A comparison of the measured and expected G0 levels was used to determine the % recovery across a range from 25.8 to 72.9% G0. The sample recoveries observed in Table 3 suggest that the ionization efficiency for the G0, G1, and G2 components is similar enough to permit direct quantitation. The accuracy of the method is important to discern relatively small differences in G0 content between process samples. The accuracy of the assay is excellent as the recoveries averaged 101% with a relative standard deviation of 2% for the analysis (Table 3).

Table 3
Accuracy of LC–MS assay as demonstrated by recovery calculated from measurements performed on volumetrically mixed samples

Mixing volume (sample 1:sample 2)	Measured %G0	Expected %G0	Recovery (%)
1: 0	72.9		
4: 1	64.3	63.5	101
3: 1	61.9	61.2	101
2: 1	56.8	57.3	99.2
1: 1	48.9	49.4	99.1
1: 1	48.4	49.4	98.0
1: 2	41.8	41.5	101
1: 3	39.2	37.7	104
1: 4	36.1	35.2	103
0: 1	25.8		
Average			101
RSD (%)			2.0

Process samples may contain a range of antibody concentrations, creating a need to determine the assay range with respect to mass of antibody injected. A rituximab standard was injected seven times over a mass range from 0.5 to 10 μg of antibody injected (Table 4). The relative standard deviation for G0 levels measured using the LC–MS assay with respect to mass injection was calculated to be 1.1%. A 2 μg sample of the same rituximab standard was injected onto the LC–MS 23 times over a 4.5 month time period. The reproducibility of the assay resulted in a RSD of 1.1% for G0 measurement, demonstrating the stability of the instrument in this application (Table 4). Several hundred injections of cell culture process samples were performed during the same 4.5 month time period. The ionization source for the MS system did not require cleaning, maintenance, or re-calibration, further demonstrating the robustness of the assay and instrumentation.

Table 4
Reproducibility and injection mass range for LC–MS assay using SIM of 38^+ charged ion

Experiment	Condition	*n*	Average % G0	RSD (%)
Assay range	0.5–10 μg of antibody injected	7	47.0	1.1
Reproducibility	Standard injected over 4.5 months	23	47.2	1.1

LC–MS allows for the collection of data from multiple ions in the SIM mode. To determine the effect of ion selection on quantitation, data was collected using five ions selected from within the ionization envelope for the antibody glycoforms. The % G0 calculated from eight different cell culture process samples using the five selected ions is shown in Table 5. The average relative standard deviation determined for the eight process samples was 2.2%, demonstrating that the method is insensitive to the ions selected from within the ionization envelope.

3.4. LC–MS in the SIM mode for quantitation of G0 levels in antibody process samples

Levels of G0, G1, and G2 in the purified protein have been measured using a CE–LIF method [8]. The glycoform levels produced at the end of the cell culture process were measured using the LC–MS assay, and compared to the same samples purified by protein-A affinity chromatography and analyzed by CE. A comparison of the measured G0 levels, shown in Fig. 5, reveals the close correlation achieved between the two methods ($R=0.9859$). A linear fit was applied to the data, and the resulting equation can be used to calculate the expected G0 levels measured using the CE assay.

A second data set, shown in Fig. 5, was used to correlate the G0 levels determined by LC–MS at the end of the cell culture process to values obtained in the purified protein bulk using CE. This correlation confirms that the LC–MS method is useful for directly monitoring glycoform levels in cell culture process samples. This analysis also confirms that the glycoform levels observed at the end of the cell culture process do not change during the purification process for the recombinant antibody.

The simplicity, rapid analysis time, and high-throughput nature of the LC–MS assay has enabled more detailed study of galactosylation levels throughout the entire production process. Analysis of the G0 levels during four stages of the cell culture production process is shown in Fig. 6. Several different classes of G0 profiles can be identified from the three different cases reflecting different process conditions. Comparison of cases A and B reveals that the glycan content tracks identically through the first three stages of cell culture, only to diverge in behavior at the final stage. In other cases (case B versus case C), while glycan content tracks similarly throughout all stages of the cell culture process, the

Table 5
Reproducibility of LC–MS method as demonstrated by monitoring G0 levels using different ions while operating under SIM mode

Sample No.	% G0						RSD (%)
	35+ (*m*/*z* 1444.3)	36+ (*m*/*z* 1404.2)	37+ (*m*/*z* 1366.3)	38+ (*m*/*z* 1330.4)	39+ (*m*/*z* 1296.3)	Average	
1	43.4	43.1	42.2	42.7	42.2	42.7	1.2
2	40.7	40.7	40.0	40.2	38.7	40.0	2.1
3	40.7	41.7	38.9	39.2	39.5	40.0	3.0
4	34.2	34.7	35.0	33.2	32.9	34.0	2.7
5	30.7	29.6	30.9	29.7	28.8	29.9	2.9
6	31.4	31.4	31.3	30.0	29.5	30.7	2.9
7	43.2	43.3	42.8	42.8	42.6	43.0	0.7
8	53.1	53.8	54.2	52.3	52.0	53.1	1.8
Average relative standard deviation (%)							2.2

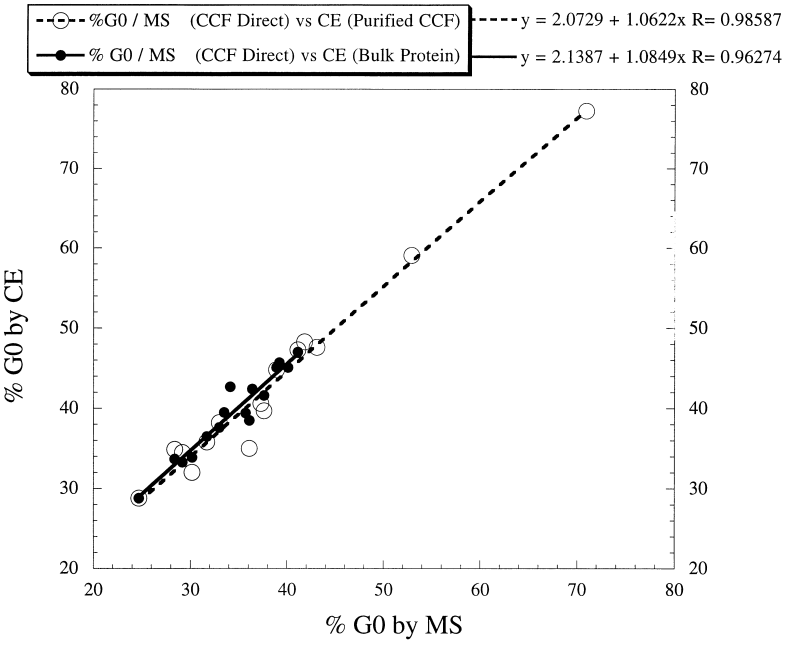


Fig. 5. Correlation between % G0 levels in rituximab samples measured by CE and LC–MS methods. Open circles represent comparison of data from samples analyzed directly from cell culture by LC–MS, and the same samples measured by CE after protein A purification. Closed circles represent data from analysis directly in cell culture by LC–MS, and the resulting purified protein samples analyzed by CE.

difference in the final product G0 content is reflective of the level at the beginning of the process. Measurement of these differences observed at the earliest stages of the cell culture process requires an extremely sensitive method since the antibody concentrations at that stage are very low (<5 μg/ml).

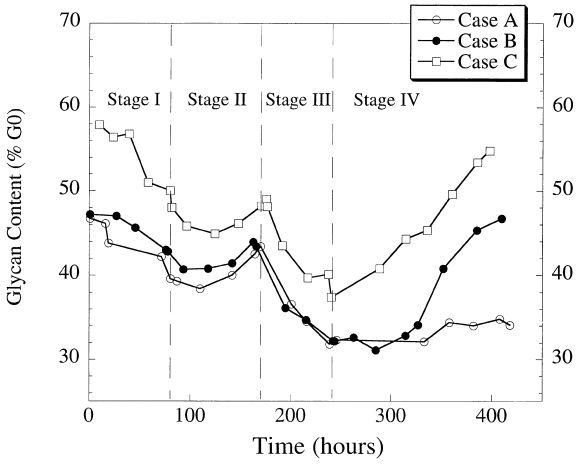


Fig. 6. Rituximab G0 content monitored during the four stages of cell culture production by LC–MS. Glycoform levels monitored directly from cell culture fluid samples.

The LC–MS assay allows real-time analysis of the glycoform content observed during antibody production because the assay time is short enough relative to the culture duration and the rate of change. The assay time, including sample preparation, LC–MS and data analysis, is approximately 1 h per sample. The duration of the process shown in Fig. 6 is much longer than the assay time (approximately 400 h), such that information obtained about glycoform levels could be used to make processing decisions. Cell growth conditions or other operational parameters can be modified to control galactosylation within a targeted range and obtain consistent levels in the final product.

4. Conclusions

A mass-selective detector can be used to quantify component mixtures for protein species that have similar ionization potential. This technique has been

used to directly and rapidly monitor large numbers of in-process antibody samples. The method does not require separation of the glycoform components for analysis since the mass difference is large enough (162 amu, $m/z = 4.2$ amu) to allow for monitoring of selected ions within the ionization envelope of the antibody heavy chain. A reversed-phase HPLC method was optimized to obtain quantitative recovery of antibody fragments, while eliminating the use of TFA to maximize the ionization efficiency. The coupled HPLC is used to desalt, partially purify samples, and separate the heavy and light chain antibody fragments analyzed by MS. Application of this method allows the analysis of large numbers of in-process antibody samples, rapidly providing important product quality information early in the cell culture production process.

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