International Journal of Mass Spectrometry 278 (2008) 137–142

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Quantification by isobaric labeling (QUIBL) for the comparative glycomic study of O-linked glycans

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ARTICLE INFO

Article history:
Received 18 December 2007
Received in revised form 7 April 2008
Accepted 9 April 2008
Available online 2 June 2008

Dedicated to Professor Robert Cotter, the person who first introduced me to the analysis of carbohydrate by mass spectrometry, on his 65th birthday. Thank you Bob and Happy Birthday!

Keywords:
Glycomics
Isobaric labeling
Stable isotope labeling
Quantitation O-linked glycan

ABSTRACT

Quantification by isobaric labeling (QUIBL) has recently been described as a tool for the relative quantification of N-linked glycans in glycomic studies. In this paper we expand the application of QUIBL to the relative quantification of O-linked glycans. Fetuin, which contains two O-linked glycans, NeuAc-Hex-HexNAc and $NeuAc_2$-Hex-HexNAc, was used to validate this study. The glycans were released by β-elimination and permethylated with the isobaric labeling reagents, $^{13}CH_3I$ and $^{12}CH_2DI$. The exact mass difference between the isobaric labels is minimal (0.00292 Da), but since glycans contain multiple permethylation sites, the mass difference can be resolved with a high resolution mass spectrometer, such as an FTMS. Quantitative data were obtained by comparing the signal intensities from various mixtures of isobarically labeled O-linked glycans of fetuin. Five different ratios of $^{13}CH_3$ to $^{12}CH_2D$ ranging from 10:1 to 1:10 were analyzed for linearity and reproducibility of the QUIBL method on the standard glycan. QUIBL was also evaluated using porcine mucin, a sample with a larger variety of more complex O-linked glycans. These results indicate that QUIBL allows for the relative quantification of O-linked glycans over a linear dynamic range of at least two orders of magnitude, validating QUIBL as an applicable quantitative glycomics approach for both N-linked and O-linked glycans.

1. Introduction

Glycosylation is one of the most common and complex forms of post-translational modifications found on proteins [1–3]. Protein glycosylation is typically referred to as N-linked or O-linked based on its origin of attachment to the peptide backbone. The study of this type of post-translational modification has become of increasing relevance given the functions that glycans have been demonstrated to exhibit in development, differentiation, cell adhesion and growth [1–4]. The potential complexity of glycosylation types requires that each site be structurally characterized. This has led to the emerging field called glycomics, which involves the characterization of glycans at each stage of a biological event, i.e., differentiation, tumor progression, etc. Mass spectrometry plays a key role in glycomics by providing a highly sensitive analytical technique that can offer a rapid and reliable method to distinguish and identify individual glycans in mixtures.

O-linked glycans are a prevalent form of glycosylation found on cell surfaces and secreted proteins. They are highly enriched in epithelial cells due to their role in mucus production [5]. This type of glycan is most commonly attached to the peptide chain through the oxygen residue on the side chains of serine (Ser) or threonine (Thr) residues. There does not appear to be an amino-acid consensus sequence for *O*-glycosylation, nor is there a single characteristic O-glycan core structure, as observed in N-glycans. Mucin-type glycans (the most common type of O-linked glycans) are attached to the peptide backbone via a GalNAc residue. Mucin-type O-linked glycans are thought to have many functions, including blocking the accessibility of the polypeptide backbone to proteases, regulating the serum-half life of chemokines or hormones to attenuate their activity *in vivo*, and modulating the intracellular trafficking of proteins [6].

The first step in the analysis of O-linked glycans is typically to chemically release them from the protein backbone by a β-elimination reaction [5,7–9]. This chemical reaction takes advantage of the slight acidity of the proton on the alpha carbon of Ser and Thr. In an alkaline environment, abstraction of the alpha proton of the glycosylated Ser or Thr residue leads to release of the O-linked carbohydrate that is attached to the beta-carbon via a β-elimination mechanism. The free oligosaccharide contains a carbonyl group as

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doi:10.1016/j.ijms.2008.04.003

a result of the release, which is very susceptible to further elimination, called "peeling". Several methods have been used to prevent peeling, with the most common approach involving the chemical derivatization of the aldose into an alditol with the assistance of a reducing agent such as sodium borohyrdride [10]. Released glycans can be further derivatized by permethylation [9,11]. In this reaction, the hydrogen on both oxygen and nitrogen atoms in the oligosaccharide are replaced by methyl groups, making the oligosaccharide less polar and more hydrophobic [12]. This chemical modification yields more predictable ion products, stabilizes sialic acid residues, and allows oligosaccharides to be separated from salts and other contaminates due to the hydrophobic nature of methylated glycans. In addition, methylated carbohydrates ionize more efficiently, which aids in MS analysis [9,11,13].

While MS offers a powerful tool for qualitative glycomics, quantitation can be problematic for a multitude of reasons. For instance, the ionization efficiency of each analyte depends on factors such as its molecular mass, proton/cation affinity, surface activity, the presence of compounds which compete with or interfere with the ionization of the analyte, etc., and thus the intensities of the ions do not directly correlate with concentration. In addition, the instrument's response can vary over time, so that the direct comparison of two or more spectra obtained at different times yields a qualitative, rather than quantitative, indication of the glycan content of the two analyte samples. To compensate for these factors, quantitative measurements are typically performed by adding an internal standard and measuring the analyte's response relative to this standard. One of the best internal standards is an isotopically labeled (^{13}C, D, ^{15}N, etc.) form of the analyte itself, as this often retains the physical–chemical characteristics of the analyte while allowing the mass analyzer to resolve the isotopomers, thus permitting their relative abundances to be determined by comparing the intensity of ions from the analyte to that of the isotopically labeled standard. For comparative studies of complex samples where isotopically labeled standards are not available for all species, an isotopic labeling approach where one of the samples is modified with a "light" tag while the other is derivatized with a "heavy" tag can be used [14].

Stable isotopic labeling has shown widespread application in the proteomics field [14,15], whereas it has shown limited use in the glycomics field. Some advances in the quantitative glycomics field have been shown by Yuan et al. [16] and Hitchcock et al. [17], who used an isotopically varied reductive amination tag for quantification. Bowman et al. synthesized tags that could modify oligosaccharides with four isotope-enriched variants [18]. In these cases, derivatives were added to the reducing terminus of N-linked glycans. This method would become problematic for quantification of O-linked glycans since the reducing end of the glycans are usually reduced as a result of β-elimination. Xie et al. reported a method for quantification of O-linked glycans using sodium borohydride or sodium borodeuteride during β-elimination to incorporate deuterium [19]. While successful for O-linked glycans, this method would require additional chemistry to be applied to N-linked glycans. Another proposed method for comparative isotopic labeling of oligosaccharides uses heavy methyl iodide ($^{13}CH_3$ or $^{12}CD_3$) vs. light methyl iodide ($^{12}CH_3$), which employees standard permethylation conditions, which are routinely used for both N-linked and O-linked glycan analysis [11,20]. While this study was successful for N-linked glycans and shows great potential for O-linked glycans, the large *m*/*z* shift between heavy and light labeled glycans can become problematic in the analysis of complex unknown mixtures where the number of modified sites is not known, thus making it difficult to identify differentially labeled mass pairs.

Here, we evaluate the use of a new approach, know as quantitative isobaric labeling (QUIBL), for relative quantification of O-linked glycans in glycomic studies. We have previously demonstrated the utility of QUIBL for comparative N-linked glycomics [21]. In this previous report, released N-linked glycans were permethylated with either $^{13}CH_3I$ or $^{12}CH_2DI$ and the small mass shift between these two isobaric labels permitted the ions from the two samples to be resolved in the MS, which, in turn, allowed relative quantification of glycans. Since the isobaric labels were introduced during the permethylatyion step, QUIBL should be applicable to the analysis of O-linked glycans without additional sample manipulation. This possibility was evaluated here by the analysis of the two O-linked glycans released from fetal calf fetuin and with porcine stomach mucin. This analysis demonstrates the extension of QUIBL to O-linked glycans, and thus further supports that this approach is useful for comparative glycomic investigations.

2. Experimental

2.1. Materials

Bovine fetuin, type III partially purified porcine stomach mucin, and Dowex ion exchange resin was purchased from Sigma–Aldrich (St. Louis, MO). The 99% $^{13}CH_3I$ and 98% $^{12}CH_2DI$ used during permethylation were purchased from Cambridge Isotopes Inc. (Andover, MA).

2.2. β-Elimination of glycoproteins

β-Elimination was carried on fetuin from fetal calf serum, as previously described by Dell et al. [9]. Briefly, a 1.0 M solution of sodium borohydride in 50 mM NaOH was added to 400 μg of fetuin and incubated for 16 h at 45 °C. After the allotted time, the sample was cooled to room temperature and neutralized by the addition of 10% acetic acid. Sodium ions were removed using an ion exchange resin (Dowex 50 W × 8–100) and the sample was lyophilized to dryness. Next, borate was removed by at least three repeated additions of a methanol/acetic acid solution, which was dried under a stream of nitrogen.

2.3. Permethylation of released O-glycans

Permethylation was performed on the O-linked glycans according to standard procedures [22]. Briefly, dried glycans were suspended in DMSO (200 μL) and stirred until completely dissolved. Approximately 200 μL of 50% sodium hydroxide in DMSO was added and the solution was mixed vigorously. Each sample was divided into two equal portions, which were permethylated separately by the addition 100 μL of either 99% $^{13}CH_3I$ or 98% $^{12}CH_2DI$. After at least 15 min, the methylation reaction was terminated with 2 mL of water. Excess iodomethane was purged from the resulting mixture with N_2 gas. The permethylated glycans were then extracted with dichloromethane and dried for further analysis by mass spectrometry.

2.4. MS analysis of released O-glycans

The glycans were resuspended in 1 mM NaOH in 50% MeOH and analyzed on a hybrid linear ion trap Fourier transform mass spectrometer, LTQ-FT (Thermo Finnegan, San Jose, CA). Each glycan mixture was directly infused into the LTQ-FT by syringe injection at a flow rate of 0.3 μL/min. The sample was nano electrosprayed through a pulled silica capillary tip (75 μm × 10 cm Polymicro Technologies, L.L.C, Phoenix, AZ) into the MS where the spray voltage was held at 2.0 kV and the capillary temperature at 250 °C.

The $^{13}CH_3$ and $^{12}CH_2D$-labeled glycans from fetuin were analyzed independently to validate that the proper glycans were

present. Quasimolecular ions at 909 and 1275 m/z were assigned as permethylated fetuin glycans. MS/MS experiments were carried out in the ion trap using normalized collision energy of 29% after isolation of the precursor masses in a 5.0 m/z window to fragment the sample. The fragmentation pattern was used to validate the glycan structures assigned to these two quasimolecular ions.

Mixtures of permethylated fetuin glycans containing the isobaric labels were prepared for $^{13}CH_3$:CH_2D in the following ratios: 10:1, 8:3, 1:1, 3:8, and 1:10. Analysis was carried out in the FTMS on the ions at 909 and 1275 m/z with an isolation width of 25.0 m/z. The resolving power was set to 100,000$M/\Delta M$, which provided ample resolution to separate the ^{13}C and D labeled glycans. The study was carried out in triplicate for each ratio of $^{13}CH_3$:CH_2D. Quantification was performed by comparing the sum of the peak intensities for all isotopes from each permethylated glycan pairs.

O-linked glycans from 400 μg of type III partially purified porcine stomach mucin were released and permethylated as described above. A mixture of $^{13}CH_3$:CH_2D was prepared at a ratio of 1:1 and analyzed on the LTQ-FTMS in triplicate. Fragmentation patterns obtained by MS/MS analysis were used to identify structures of the O-linked glycans of mucin. Relative quantification for each differentially labeled glycan pair was accomplished by comparing the sum of all abundances for its ^{13}C-labeled ions to the sum of all abundances for its D-labeled ions.

3. Results and discussion

3.1. Principles of quantification by QUIBL

The International Union of Pure and Applied Chemistry (IUPAC) definition of isobars is "nuclides of equal mass number" [23]. Hence, isobars contain the same mass number (sum of all protons and neutrons) but have a different atomic number (sum of all protons), which, in turn, leads to isobars possessing different exact masses but the same nominal mass. Isobars are clearly different than isotopes, which have the same atomic number with different mass number, and isotones, which have equal number of neutrons but a different number of protons [23].

The IUPAC definition of an isobar is written for atomic species; however, it can be extended to molecular species as well. Commonly encountered molecular isobars in the field of biochemistry are the two amino acids lysine (K) and glutamine (Q), whose chemical formulae are $C_6H_{14}N_2O_2$ and $C_5H_{10}N_2O_3$, respectively. These two species clearly fit the definition of isobars as they both have a molecular mass number of 146, but possess different numbers of protons, K has 80 while Q has 78. Consequently, these two species share the same nominal mass (146 Da), but have different exact masses (146.1882 Da for K vs. 146.1451 Da for Q). Leucine (L) and isoleucine (I) are another pair of amino acids that share the same nominal mass, but in this instance they have identical elemental compositions ($C_6H_{13}NO_2$), molecular mass number (131), and number of protons (72); consequently these two amino acids have the same exact molecular mass (131.174 Da). In this instance, L and I are structural isomers, not isobars, although they are often referred to as such in the literature [24–27]. Leucine and isoleucine, however, are isobars of hydroxyproline ($C_5H_9NO_3$), which has a molecular mass number of 131, a total of 70 protons, and an exact mass of 131.058–0.115 Da below that of L and I. Similar logic has led to the extension of isobars to include two or more compounds possessing the same nominal mass (i.e., total number of protons and neutrons) with different elemental or isotopic compositions [28] and thus different exact masses.

The characteristic of isobars having nearly the same exact mass offers a unique advantage for the introduction of an isotopic label for various quantitative studies. For instance, if two samples were labeled with isobaric reagents, the molecular ion species from these samples would appear as the same ion under low resolution conditions and therefore lead to increased ion intensity as ions from both samples would not be distributed between isotopic species having different m/z values. This reduction in spectral complexity clearly is beneficial for reducing the limit of detection of the compound being analyzed. Similarly, the small mass difference between these isobars allows the molecular ions from the two samples to be simultaneously selected for MS^n analysis, permitting qualitative confirmation of the analyte. Quantification would be achieved at either the MS or the MS^n level by performing the analysis in a high resolution instrument. Consequently, isobaric labeling offers the ability to rapidly switch between high sensitivity qualitative analysis at low resolution and quantitative studies at high resolution using an appropriate spectrometer such as a hybrid ion trap-FTMS system.

The primary obstacle to using isobars for quantitative labeling is their small mass difference. For example the isobaric tags we have selected for this study, $^{13}CH_3I$ and $^{12}CH_2DI$, have exact masses of 142.931 and 142.934 Da, respectively. The mass difference (ΔM) between these isobars is 0.003 Da, and would require a mass resolution ($M/\Delta M$) of over 700,000 in the case of a single label on an analyte with a molecular mass of 2000 Da. This would be difficult to attain routinely on all but the most expensive mass spectrometers, and thus is impractical for general use. These two reagents were selected because methyl iodide is the common reagent for per-methylating glycans, which contain multiple methylation sites (i.e., –OH and NH_2 groups). As the number of methylation sites increases so does the mass difference between a pair of differentially labeled isobaric species. For example, an O-linked glycan with a NeuAcHexHexNAc monosaccharide composition has 14 permethylation sites, resulting in a mass of 886.489 Da when labeled with $^{13}CH_3$ and a mass of 886.530 Da when labeled with $^{12}CH_2D$. The mass difference of 0.042 Da between these two species requires a mass resolution of only ~22,000$M/\Delta M$ to resolve. Furthermore, the mass difference between oligosaccharide isobaric species becomes larger as the size of the structure increases due to the increasing number of permethylation sites, and in this situation, the resolution required to distinguish between the isobaric pairs becomes relatively independent of the size of glycan.

3.2. Validation of QUIBL on O-glycans with fetuin

The O-linked glycans released from the fetal calf serum protein fetuin were used to demonstrate the principles of QUIBL. The fetuin samples were β-eliminated to release the O-linked glycans, permethylated with isobaric labels, $^{13}CH_3$ and $^{12}CH_2D$, and analyzed with a hybrid linear ion trap-FTMS. The high resolution FTMS spectrum of a 1:1 mixture containing both ($^{13}CH_3$ and $^{12}CH_2D$) labeled forms of HexNAc-Hex-NeuAc (glycan 1) contains intense ions at 909.510 and 909.550 m/z, respectively, which correspond to (monoisotopic) sodium adducts of the differentially labeled glycan (Fig. 1A). It is noteworthy that m/z for the quasimolecular ion of the $^{12}CH_2D$-labeled glycan is shifted by 0.040 units compared to its $^{13}CH_3$-labeled counterpart, in good agreement with the shift predicted for the presence of 14 methyl groups on a singly charged ion ($[0.003 \times 14] = 0.042$). Similarly, the 0.055 Da mass shift observed for HexNAc-Hex-$NeuAc_2$ in the high resolution FTMS spectrum (glycan 2, Fig. 1B) agrees well with the calculated shift of 0.057 Da based on the 19 permethylation sites on this glycan. The excellent agreement between the calculated and experimental mass shifts suggests that these can be used to calculate the number of methylation sites in a molecule, thereby aiding in the structural elucidation of unknown glycans.

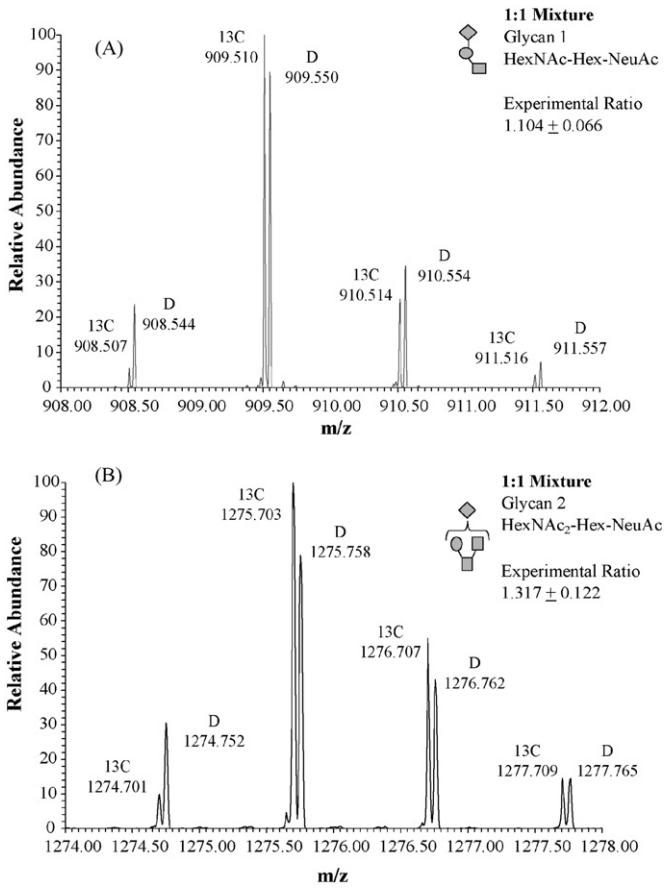

Fig. 1. FTMS spectrum of $^{13}CH_3$- and $^{12}CH_2D$-labeled (A) glycan 1 from fetuin at a 1:1 mixture and (B) glycan 2 from fetuin at a 1:1 mixture. The following carbohydrate residues are represented: square: *N*-acetylhexosamine (HexNAc); circle: Hexose (Hex); and diamond: *N*-acetylneuraminic acid (NeuAc).

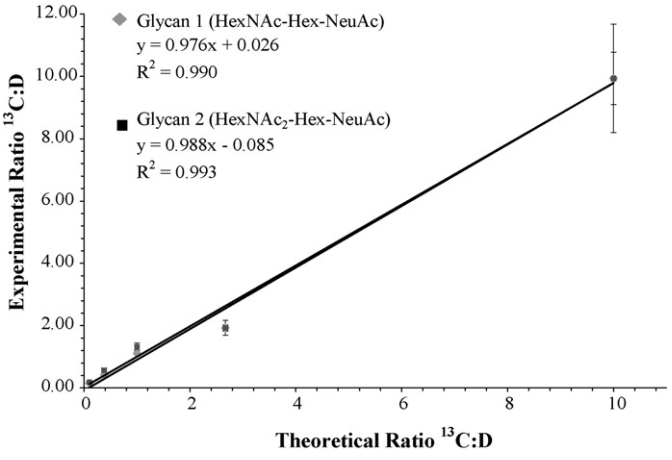

Fig. 2. This graph illustrates the correlation between the theoretical and experimental ratios for quantification of the two O-linked fetuin glycans by QUIBL. In each experiment the $^{13}CH_3$- and $^{12}CH_2D$-labeled glycans were mixed together at the ratios 10:1, 8:3, 1:1, 3:8, and 1:10 ($^{13}CH_3$:CH_2D) and analyzed by FTMS. The calculated expression ratios were determined by comparing the sum of the peak intensities for all isotopes between $^{13}CH_3$- and $^{12}CH_2D$-labeled precursor ions for each glycan. For both glycans a linear correlation was observed over two orders of magnitude with a minimum R^2 of 0.99.

The spectra of the isobarically labeled species (Fig. 1A) also contain a doublet of ions at 908.507 and 908.544 *m*/*z* that is exactly 1 *m*/*z* unit below that observed from the fully labeled species. These ions appear to result from incomplete isotope enrichment of the labeling reagents used, i.e., 99% $^{13}CH_3$ and 98% $^{12}CH_2D$, which also leads to differences in the ion abundances of the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled series. This spectrum demonstrates that in a QUIBL experiment, it is possible to resolve the incompletely labeled ions. This is possible because when one of the

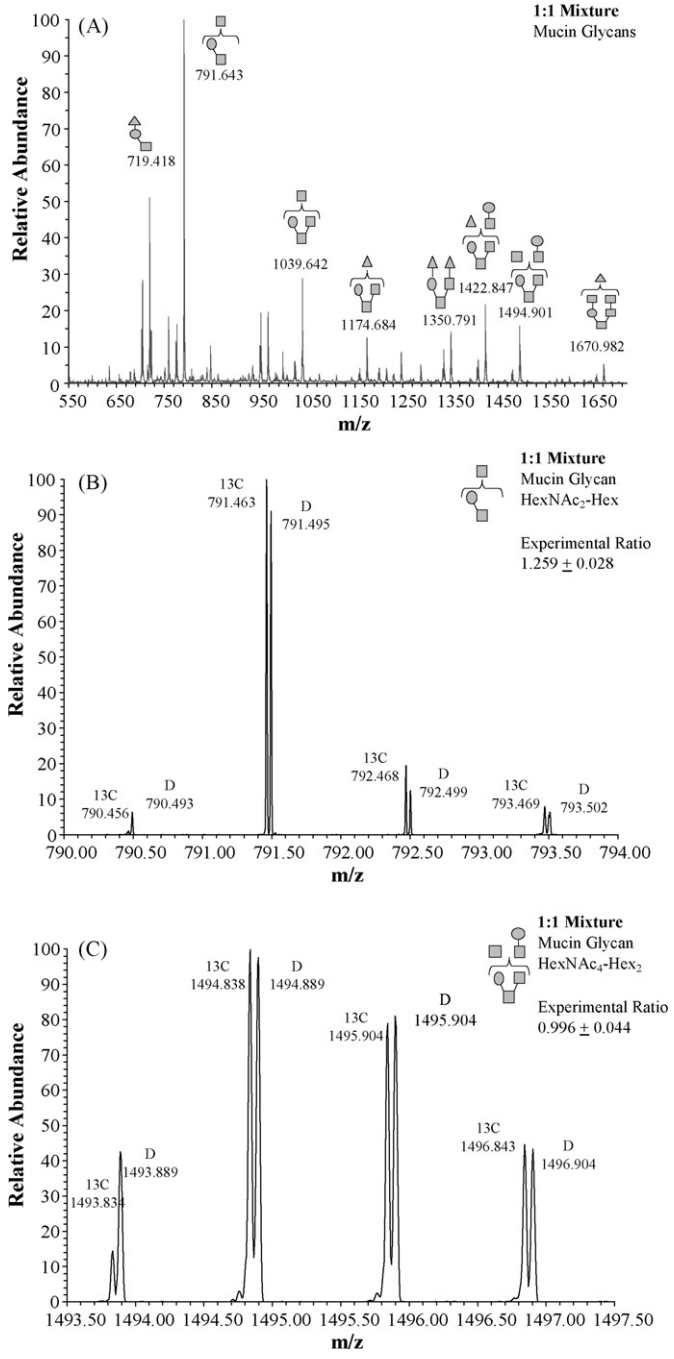

Fig. 3. (A) Full low resolution LTQ spectrum of $^{13}CH_3$- and $^{12}CH_2D$-labeled glycan from mucin at a 1:1 mixture and FTMS spectra of $^{13}CH_3$- and $^{12}CH_2D$-labeled, (B) small glycan and (C) large glycan from mucin at a 1:1 mixture. The following carbohydrate residues are represented: square: *N*-acetylhexosamine (HexNAc); circle: Hexose (Hex); diamond: *N*-acetylneuraminic acid (NeuAc); and triangle: Deoxyhexose (Deoxyhex).

14 ^{13}C atoms is replaced with a ^{12}C atom or one of the 14 ^{13}D (or ^{2}H) atoms is replaced with an ^{1}H atom, the analyte's mass is consequently reduced by approximately 1 Da, however, the resulting ion is detected in the appropriate ($^{13}CH_3$-labeled or $^{12}CH_2D$-labeled) ion series because it still contains 13 of the isotopic labels. This greatly simplifies quantitation, which is accomplished by summing the ion abundances for the $^{13}CH_3$-labeled and $^{12}CH_2D$-labeled series and comparing these two values. This characteristic of QUIBL provides an advantage over traditional isotopic labeling procedures, where the use of incompletely labeled reagents results in overlapping isotopic peaks, i.e., the ion produced by the under incorporated "heavy" species appears at an m/z value that is indistinguishable from an ion produced by the "light" species [11,13,29,30]. The ability of QUIBL to differentiate species from under isotope incorporation greatly simplifies quantitation calculation which simply involves summing the ion intensities for each isobaric distribution.

The quantification capabilities of QUIBL on O-linked glycans were then evaluated by analyzing five different ratios of $^{13}CH_3$ to $^{12}CH_2D$ ranging from 10:1 to 1:10. The glycan mixtures were analyzed by FTMS in technical triplicates. The experimental ratios for the two O-linked glycans were calculated based on the ion intensities and compared to the theoretical ratio. This approach showed good reproducibility with the calculated ratios for each triplicate run being in relatively good agreement. The theoretical and experimentally averaged ratios were compared demonstrating a linear response over two orders of magnitude, as shown in Fig. 2.

3.3. Isotope effects in QUIBL

The method of permethylation used in this analysis involves the deprotonation of the oxygens and nitrogens on the glycan prior to the addition of methyl iodide under strong basic conditions [31]. The addition of methyl iodide is established through an S_N2 reaction where the deprotonated oxyens and nitrogens of the glycan act as the nucleophile (Nu) to displace iodine so that the C—I bond and the formation of the new C—Nu bond occurs simultaneously. The use of isotopes in this reaction could induce a kinetic isotope effect.

A kinetic isotope effect (KIE) is a variation in the reaction rate of a chemical reaction as a result of atoms of a reactant that is replaced by its isotope. When an isotope is introduced, it lowers the minimum vibrational energy of the bond, the zero point energy. As a result, more energy is required to raise the vibrational state of the bond to the point of breakage. The isotope effect is a function of the difference in the vibrational frequencies related to a bond being formed or broken which, in turn, affects the reaction rate. An isotopic substitution can modify the reaction rate, especially if the isotope is directly involved in the chemical bond that is broken and/or formed. The change of rate from the direct isotopic involvement in the reaction is referred to as a primary isotope effect. A secondary isotope effect occurs if the substituted isotope is not directly involved in the bond that is breaking or forming. The relative change in rate associated with the incorporation of these isotopes is a function of the inverse square root of the ratios of the reduced masses of the atoms involved in the bonds based the calculation of the vibrational energy required to break or form a bond.

The use of $^{13}CH_3$-I as a labeling reagent would induce a primary KIE during the S_N2 reaction since the ^{13}C is directly involved in the chemical bond breaking and forming. Based on the inverse square root of the reduced mass ratios, the theoretical decrease in reaction rate is approximately 2%, a minimal effect and well within the experimental precision of these measurements. In general, isotope effects involving ^{13}C tend to be much smaller than those involving deuterium. However, the use of $^{12}CH_2D$-I as an isobaric labeling reagent would induce a secondary alpha KIE since the D is not directly involved in the reaction. This secondary alpha effect is also reported [32] to introduce about a 2% decrease in the reaction rate, providing an effect that is similar to the primary KIE due to the presence of ^{13}C. As the effects of these isotopes are small and comparable, any difference in reaction rates for incorporation of the two different isobaric labels is theoretically insignificant. An additional isotope effect to consider could be caused by the steric hindrance from the deuterium during the nucleophilic backside attack of the S_N2 reaction. This hindrance could slow the reaction rate when compared to the S_N2 reaction involving all hydrogens as with the $^{13}CH_3$-I labeling reagent.

If a rate change based on a KIE or steric hindrance occurs during permethylation using these isobaric labeling agents, underlabeling could occur at a greater rate for one sample over the other, affecting quantification. Although the kinetic differential isotope effects are theoretically small for the isobaric labels used in this analysis, avoiding incomplete labeling is important for reliable quantification. Therefore optimization of the reaction conditions for permethylation is a prerequisite for successful application of the QUIBL method.

3.4. Application of QUIBL for the quantification of O-glycans in mucin

To evaluate QUIBL on a sample with a larger variety of more complex O-linked glycans, porcine mucin was subject to β-elimination and permethylated with the isobaric labels. The $^{13}CH_3$- and $^{12}CH_2D$-labeled glycans were then mixed in a 1:1 ratio and analyzed in technical triplicate. After identification of glycans by LTQ analysis (Fig. 3A) and structural determination by MS/MS analysis, quantification was preformed using the FTMS. For each glycan, pairs of differentially labeled ions were generated within the same nominal mass, a major advantage of the QUIBL method. This differs from traditional labeling techniques where large mass shifts between labeled species make identification difficult. Additionally, errors from discrepancies in detection efficiency between ions of very different m/z ratios were avoided using the QUIBL approach. The QUIBL method accurately quantified a broad range of O-linked glycan structures found in mucin from 709 to 1670 m/z. This demonstrates that this method is independent of glycan size or composition of the mucin glycans as illustrated in Fig. 3B and C.

Table 1
Quantification of Various Porcine Mucin Glycans by QUIBL

m/z	Glycan	Experimental ratio (Ave ± 2S.D.)	Theoretical ratio	% Error
719.418	HexNAc-Hex-Deoxyhex	1.013 ± 0.084	1	1.37
791.643	$HexNAc_2$-Hex	1.259 ± 0.028	1	26.43
1039.684	$HexNAc_3$-Hex	1.155 ± 0.062	1	15.04
1174.684	$HexNAc_2$-Hex_2-Deoxyhex	0.971 ± 0.040	1	3.19
1350.791	$HexNAc_2$-Hex_2-$Deoxyhex_2$	0.909 ± 0.026	1	10.02
1422.847	$HexNAc_3$-Hex_2-Deoxyhex	0.809 ± 0.034	1	20.27
1494.838	$HexNAc_4$-Hex_2	0.996 ± 0.044	1	0.91
1670.982	$HexNAc_4$-Hex_2-Deoxyhex	0.920 ± 0.148	1	7.74

The average calculated ratios were determined as shown in Table 1 for each glycan. The maximum observed error for the experimental ratio when compared to the theoretical ratio obtained in the fetuin analysis was 26.43%, and the average error for all glycans analyzed by QUIBL was 10.62%. This does not insure that the analysis of the mucin glycans were not without additional errors associated with the sample preparation as well as the sample itself. For example, the differences in the extent of glycan permethylation for the two reagents would introduce quantitative errors, which can be minimized by optimizing the methylation conditions (i.e., by adjusting the reaction time and reagent amounts).

4. Conclusions

This glycomics study allowed for the validation of quantitative isobaric labeling (QUIBL) on mucin-type O-linked glycans. In this analysis, fetuin was analyzed using the QUIBL approach for relative quantification. These results show that QUIBL allows for relative quantification of O-linked glycans over a linear dynamic range of two orders of magnitude. Additionally, QUIBL was effectively utilized on a highly O-glycosylated species, porcine mucin. In agreement with the results of Atwood et al. [21], QUIBL has proven to be a useful technique for quantitative glycomics of both N- and O-linked glycans without additional manipulation. Additionally, the concept of quantitative isobaric labeling is likely to be pertinent to other quantitative analyses in the *–omics* field.

Acknowledgments

This work was funded by the NIH/NCRR-funded Integrated Technology Resource for Biomedical Glycomics (P41 RR018502) and the NIH/NCRR-funded Research Resource for Integrated Glycotechnology (P41 RR005351).

References

[1] J. Bunkenborg, B.J. Pilch, A.V. Podtelejnikov, J.R. Wisniewski, Proteomics 4 (2004) p454.
[2] F. Dall'olio, Clin. Mol. Pathol. 49 (1996) pM126.
[3] Y. Wang, S. Wu, W.S. Hancock, Glycobiology 16 (2006) p514.
[4] N. Taniguchi, E. Miyoshi, J.H. Ko, Y. Ikeda, Y. Ihara, Biochim. Biophys. Acta 1455 (1999) p287.
[5] A. Varki, The Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, 1999.
[6] H.C. Hang, C.R. Bertozzi, Bioorg. Med. Chem. 13 (2005) p5021.
[7] M.D. Carlson, J. Biol. Chem. 241 (1966) 2984.
[8] N.G. Karlsson, N.H. Packer, Anal. Biochem. 305 (2002) p173.
[9] A. Dell, A.J. Reason, K.H. Khoo, M. Panico, R.A. McDowell, H.R. Morris, Methods Enzymol. 230 (1994) p108.
[10] L.G. Wade, Organic Chemistry, Prentice Hall Englewood Cliffs, NJ, 1991.
[11] G. Alvarez-Manilla, N.L. Warren, T. Abney, J.A. Atwood III, P. Azadi, W.S. York, M. Pierce, R. Orlando, Glycobiology 17 (2007) p677.
[12] J. Delaney, P. Vouros, Rapid Commun. Mass Spectrom. 15 (2001) p325.
[13] P. Kang, Y. Mechref, I. Klouckova, M.V. Novotny, Rapid Commun. Mass Spectrom. 19 (2005) p3421.
[14] W.A. Tao, R. Aebersold, Curr. Opin. Biotechnol. 14 (2003) p110.
[15] M.B. Goshe, R.D. Smith, Curr. Opin. Biotechnol. 14 (2003) p101.
[16] J. Yuan, N. Hashii, N. Kawasaki, S. Itoh, T. Kawanishi, T. Hayakawa, J. Chromatogr. A 1067 (2005) p145.
[17] A.M. Hitchcock, C.E. Costello, J. Zaia, Biochemistry 45 (2006) p2350.
[18] M.J. Bowman, J. Zaia, J. Biomol. Tech. 18 (2007) p268.
[19] Y. Xie, J. Liu, J. Zhang, J.L. Hedrick, C.B. Lebrilla, Anal. Chem. 76 (2004) p5186.
[20] P. Kang, Y. Mechref, Z. Kyselova, K. Goetz, M.V. Novotny, Anal. Chem. 79 (2007) p6064.
[21] J.A. Atwood III, L. Cheng, G. Alvarez-Manilla, N. Warren, W. York, R. Orlando, J. Proteome Res. 7 (2008) 367.
[22] I. Ciucanu, C.E. Costello, J. Am. Chem. Soc. 125 (52) (2003) 16213.
[23] A.D. McNaught, A. Wilkinson, IUPAC Compendium of Chemical Terminology: The Gold Book, Blackwell Science, New York, 1997.
[24] R.S. Youngquist, G.R. Fuentes, M.P. Lacey, T. Keough, J. Am. Chem. Soc. 117 (1995) p3900.
[25] B. Casetta, D. Tagliacozzi, B. Shushan, G. Federici, Clin. Chem. Lab. Med. 38 (2000) p391.
[26] B.C. Searle, S. Dasari, M. Turner, A.P. Reddy, D. Choi, P.A. Wilmarth, A.L. McCormack, L.L. David, S.R. Nagalla, Anal. Chem. 76 (2004) p2220.
[27] W. Kulik, L. Van Toledo-Eppinga, R.M. Kok, W.S. Guérand, J. Mass Spectrom. 30 (1995) p1260.
[28] L. Aalberg, C.R. Clark, J. DeRuiter, J. Chromatogr. Sci. 42 (2004) p464.
[29] K. Aoki, M. Perlman, J.M. Lim, R. Cantu, L. Wells, M. Tiemeyer, J. Biol. Chem. 282 (2007) p9127.
[30] P.M. Angel, R. Orlando, Anal. Biochem. 359 (2006) p26.
[31] I. Ciucanu, F. Kerek, Carbohydr. Res. 131 (1984) p209.
[32] H. Simon, D. Palm, Angew. Chem. 5 (2003) p920.