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Stable-Isotope Labeled Hydrophobic Hydrazide Reagents for the Relative Quantification of *N*-linked Glycans by Electrospray Ionization Mass Spectrometry

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Abstract

This study presents the development of stable-isotope labeled hydrophobic, hydrazide reagents for the relative quantification of *N*-linked glycans. The P2GPN ‘light’ (¹²C) and ‘heavy’ (¹³C₆) pair are used to differentially label two *N*-linked glycan samples. The samples are combined 1:1, separated using HILIC, and then mass differentiated and quantified using mass spectrometry. These reagents have several benefits: 1) impart hydrophobic character to the glycans affording an increase in electrospray ionization efficiency and MS detection; 2) indistinguishable chromatographic, MS, and MS/MS performance of the ‘light’ and ‘heavy’ reagents affording relative quantification; and 3) analytical variability is significantly reduced due to the two samples being mixed together after sample preparation. Obtaining these analytical benefits only requires ~4 hours of sample preparation time. It is shown that these reagents are capable of quantifying changes in glycosylation in simple mixtures, and the analytical variability of the reagents in pooled plasma samples is shown to be less than ±30%. Additionally, the incorporation of an internal standard allows one to account for the difference in systematic error between the two samples due to the samples being processed in parallel and not mixed until after derivatization.

INTRODUCTION

Glycosylation is one of the most common post-translational modifications in biological systems, estimated to occur in more than 50% of translated gene products.¹ Furthermore, of the entire translated genome, 0.5–1% of all gene products are involved in glycosylation.² Glycans are ubiquitous in biological processes regulating cell-cell interactions, cellular recognition, cell division and adhesion, immune response, and protein stability.³ Due to the importance of glycosylation, aberrations in the glycosylation patterns are often found to be detrimental to the biological system. In 1969, Robbins and coworkers first reported aberrations in glycosylation by describing the disparity in the size of membrane glycoproteins between healthy and diseased fibroblasts.⁴ Furthermore, aberrant glycosylation was first linked to cancer in 1978 where aberrant glycosylation patterns of α₁-antitrypsin were discovered in lung, prostate, and gastrointestinal cancers.⁵ Since then, numerous studies have provided evidence for the correlation between aberrant glycosylation and cancer,^{6–11} and now, researchers believe that glycans may be possible candidates for

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sensitive and specific disease biomarkers.¹² Recent studies have supported these earlier findings demonstrating aberrant glycosylation in many different types of cancers,¹³ including breast,^{14–16} prostate,^{17–18} liver,¹⁹ ovarian,^{20–24} pancreatic,²⁵ etc. However, there are no ubiquitous methodologies used to detect, profile, and quantify these changes in glycosylation.

Relatively few glycan quantification strategies have been developed; however, since 2007, the relative quantification of *N*-linked glycans via the incorporation of stable-isotope labeling has produced several novel methodologies.^{3, 26} These involve using common glycan derivatization strategies such as permethylation,^{27–29} reductive amination,^{30–32} and hydrazone formation (this study) to incorporate chemically similar but isotopically differentiated labels that can be used to separate the glycans by mass, allowing relative quantification in MS. Additionally, metabolic incorporation of stable isotopes has been shown by Orlando and coworkers³³ where cells can be grown in “heavy media” differentially labeling the glycans and allowing for separation by mass (*vide infra*).

Two *N*-linked glycan quantification strategies involve variations in permethylation: 1) stable-isotope labeling via deuteriomethyl iodide³⁴ and 2) QUantitation by IsoBaric Labeling (QUIBL) using ¹³CH₃I and ¹²CH₂DI.²⁸ Novotny and coworkers utilize permethylation of glycans using either methyl iodide or deuteriomethyl iodide in order to differentially label two glycan samples.²⁹ Because the methyl or deuteriomethyl groups are incorporated at each hydroxyl, amino and carboxylic acid group, each glycan will have a 3 Da mass shift per functional group methylated. Orlando and coworkers also exploit permethylation for the quantification of glycans; however, ¹³CH₃I or ¹²CH₂DI are used to differentially permethylate samples.²⁸ In this case the two derivatization groups have the same nominal mass, but an exact mass difference of 0.002922 Da is observed per methylation site. Since all *N*-linked glycans have at ≥ 20 methylation sites, the mass shift is ≥ 0.058 Da, which high resolving power instruments (≥ 30,000) can distinguish. Both of these methods are used to relatively quantify glycans from two separate samples in the same LC-MS analysis. Additionally, both samples are prepared individually and then mixed together prior to LC-MS. This technique affords the advantage that ionization and measurement variability from run to run by ESI or MALDI MS analysis is identical for both samples. A disadvantage present in these studies is that the permethylation efficiency of each sample must be identical. Due to the numerous hydroxyl, amino, and carboxylic groups present in oligosaccharides, a 0.1% change in the permethylation efficiency between two samples can result in a 3–10% efficiency difference (depending on glycan size). Thus, the two samples must be permethylated with equal efficiency in order to confidently quantify glycans. Additionally, both of these methods use deuterium labeling, which is known to have a different chromatographic shift than hydrogen in LC.^{35–37} Thus, a multiple deuterio-labeled glycan can elute from a liquid chromatography column at a different time and in different mobile phases introducing analytical variability into the analysis, which negates the usefulness of mixing the two samples together before LC MS.

Two other relative quantification strategies involve derivatization by stable-isotope labeled reductive amination reagents, GRIL³² and tetraplex stable-isotope coded tags.^{30–31} Cummings and coworkers have developed Glycan Reductive Isotope Labeling (GRIL) utilizing [¹³C₆]aniline and [¹³C₆]aniline in order to differentially label glycan samples, combine 1:1, and analyze in the same MS.³² A comparable method has been developed by Zaia and coworkers,^{30–31} where a reductive amination reagent has been developed such that 4 tags can be used all with a different number of deuterium atoms incorporated (+0, +4, +8, +12). This is the first reagent that has the ability of tetraplex quantification of glycans. The authors were able to quantify the relative amounts of *N*-linked glycans from the plasma of four different species in the same mass spectrum. However, the mass shift of only 4 Da per

tag is an inherent disadvantage to this method and creates overlapping isotopic distributions, which involve theoretical simulations to extract the correct ion abundances of the tetraplex quadruplets. Additionally, deuterium is used which can cause a chromatographic shift, introducing ionization and mass spectrometric variability (*vide supra*).

Orlando and coworkers have developed a novel method to metabolically label glycans in cell culture, Isotopic Detection of Aminosugars With Glutamine (IDAWG).³³ In glycan biosynthesis, the only method for the incorporation of nitrogen into glycan molecules is through the hexosamine biosynthetic pathway. Here, the side chain of glutamine is the sole donor source of nitrogen for the production of aminosugars and sugar nucleotides, molecules which transfer individual monosaccharides to glycans during glycan biosynthesis. By doping only ¹⁵N-labeled glutamine into a glutamine deprived media, one can grow cells with glycans that are differentially labeled at all monosaccharides containing nitrogen and incorporate a 1 Da mass shift per HexNAc, GalNAc, and NeuAc into the glycans. This method has a significant advantage over all previous stable-isotope labeled techniques in that after the cells are grown, they are mixed together, and all subsequent sample preparation steps are performed in the same vial, significantly reducing the sample preparation variability between the two samples. However, the primary drawback is that this study can only be performed in a living organism that is capable of being metabolically labeled with ¹⁵N, which is presently limited to cell culture.

Hydrazone formation is an attractive alternative derivatization strategy which, until now, has not been exploited for the incorporation of stable-isotope labels and relative quantification of glycans. Comparable to reductive amination (GRIL,³² *vide supra*), hydrazone formation reacts at the reducing terminus of the glycans and is capable of incorporating a fixed mass shift for the differential mass labeling of two samples. However, hydrazone formation is a more facile method for derivatization that does not involve reduction with sodium borohydride,^{38–42} a method which requires a significant cleanup step, increasing time and analytical variability. A detailed method for hydrazone formation derivatization of *N*-linked glycans has been recently published.^{43–44} These manuscripts detail the methods, advantages, shortcomings, and results of nanoLC MS analysis of hydrazide derivatization of *N*-linked glycans vs. native *N*-linked glycans. Briefly, it has been shown that the addition of a hydrophobic, hydrazide reagent can significantly increase the ion abundance of glycans in ESI.^{43–45} Additionally, a plasma glycan sample can be derivatized and directly loaded onto the nanoLC column in <4 hr without a cleanup step. It is shown that the P2GPN reagent increases the ion abundance of all types of glycans in ESI, allowing for a ~10% increase in glycan identifications in human plasma vs. native glycans.⁴³ The reaction efficiency has been meticulously optimized⁴⁴ and is shown to be >95% for model glycans and for glycans cleaved from plasma.⁴³ Further details on the derivatization reaction, effects on the glycans, fragmentation, etc. have been previously reported.^{39–45}

Herein, a relative quantification strategy for *N*-linked glycans via stable-isotope labeled hydrophobic hydrazide reagents is presented which builds upon the methodologies^{23, 46–47} and rapid (<4 hr) derivatization strategy^{43–45} developed by this group for glycan analysis in epithelial ovarian cancer. The P2GPN hydrazide reagent has been synthesized in its natural isotopic form and with the incorporation of ¹³C₆ stable isotopes. The 'light' and 'heavy' reagents have been characterized and applied to a simple mixture of glycans. Additionally, the relative quantification strategy was applied to pooled human plasma, and the analytical variability of the sample preparation, method, and instrument was measured. Furthermore, maltoheptaose was spiked in at the onset of all sample preparation in order to correct for sample preparation variations between the two samples. It is shown that the internal standard significantly reduces the difference in the systematic bias between the two samples due to parallel processing.

EXPERIMENTAL

Materials

Maltoheptaose, (Gal-GlcNAc)₂Man₃GlcNAc₂ (NA2) glycan, (NeuAc-Gal-GlcNAc)₂Man₃(Fuc)(GlcNAc)₂ (A2F) glycan, hydrazine monohydrate, bromobenzene, tetrakis(triphenyl phosphine) palladium(0), triphenyl phosphine, copper(I)iodide, triethyl amine, and palladium on charcoal were all purchased from Sigma Aldrich (St. Louis, MO). Man₉GlcNAc₂ (MAN9) was purchased from Fisher Scientific (Houston, TX). Ethyl 4-ethynylphenylacetate was purchased from Spectra Group Limited, Inc. (Millbury, OH). ¹³C₆-bromobenzene was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Pooled human plasma was purchased from Innovative Research (Novi, MI). IntegraFrit and PicoFrit capillaries were purchased from New Objective (Woburn, MA). These columns were packed in-house with TSK-Gel Amide-80 stationary phase from TOSOH Bioscience (San Jose, CA). Graphitized solid phase extraction columns were purchased from Alltech (Deerfield, IL). Peptide: *N*-glycosidase F (2.5 mU/μL) and the denaturing solution (1 M β-mercaptoethanol and 2% (w/w) SDS) were purchased from Prozyme (San Leandro, CA).

Synthesis of Reagents

The bi-phenyl reagent, 4-phenethylbenzohydrazide (P2GPN), was synthesized in its native and ¹³C₆-isotopically labeled forms. The stable-isotope labeled and native reagents were synthesized by identical methods; however, the incorporation of ¹³C₆ was achieved by starting with ¹³C-labeled bromobenzene. The synthesis mechanism was comparable to the previous report.⁴³ Briefly, the bromobenzene was coupled to ethyl 4-ethynylphenylacetate via a Sonogashira coupling⁴⁸ and the triple bond was hydrogenated. The ethyl ester was then converted to the hydrazide group completing the 4-phenethylbenzohydrazide reagent. Extensive synthesis and characterization data are provided in the supplemental material.

N-linked Glycan Derivatization Procedure

The derivatization reaction was performed as described previously.⁴³ The lyophilized glycans were reconstituted in a 1 mg/ml solution of the phenyl2-GPN reagent in 75:25 (v/v) MeOH:acetic acid solution. This mixture was then incubated for 3.5 hr at 56°C. The reaction solution was immediately placed in a -80°C freezer for 30 min to quench the reaction. The mixture was then lyophilized to dryness and stored at -20°C. The glycans were reconstituted in 50 μL of HILIC mobile phase (initial conditions) for LC-MS analysis. Where relative quantification was performed, the two samples being compared were mixed, vortexed, and analyzed in the same nanoLC MS run.

N-linked Glycan Extraction from Pooled Plasma

In the experiments herein, 100 μL (10 μg/mL) of internal standard (maltoheptaose) was added to 50 μL of pooled human plasma and the solution was lyophilized to dryness. The glycan cleavage procedure has been described in detail previously.^{43, 46} Briefly, the lyophilized plasma containing the internal standard was reconstituted in 250 μL of 50 mM tris-HCl buffer (pH 7.5) and the proteins were denatured at 95°C for 5 min by adding 28 μL of 2% SDS/1M β-mercaptoethanol. The solution was allowed to sit for 20 min until room temperature was achieved, and then 5 μL of PNGase F (2.5 mU/μL) were added to the plasma sample. The solution was then incubated for 18 hr at 37°C and subsequently quenched with 500 μL of 0.1% TFA. The glycans were then separated from the proteins using solid phase extraction (SPE) equipped with a graphitized carbon cartridge as described in detail previously.⁴⁶ The eluted fractions were then dried down, combined, and derivatized (*vide supra*).

nano-Flow Hydrophilic Interaction Chromatography

Derivatized glycans were separated prior to MS analysis via hydrophilic interaction chromatography (HILIC) as described previously.^{43–44, 46} A vented column setup⁴⁹ was used with an Eksigent nanoLC-1D PLUS system equipped with an AS1 autosampler (Dublin, CA). Both the trap and analytical columns were packed in-house with TSK-Gel Amide-80 stationary phase (5 μm particle size). The analytical column consists of a 75 μm I.D. capillary packed to 10 cm, and the trap column consists of a 100 μm I.D. capillary packed to 10 cm. Mobile phase A consists of 50 mM ammonium acetate (pH 4.5) and mobile phase B consists of 100% ACN. Gradient elution was used in which the solvent composition was varied from 20 to 55% solvent A over 37 minutes. The glycans were loaded onto the trap column in 80:20 solvent B:A and an initial wash step was used at 93% B before beginning the gradient, and after the end of the gradient, the column was washed with 85% A giving a total run time, including re-equilibration, of 70 min.

LTQ-Orbitrap Mass Spectrometry

A hybrid linear ion trap Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was used for all LC-MS glycan analysis data presented herein. Ionization of glycans was achieved by applying a 2 kV potential to a zero dead volume union creating an electric field over the capillary and inducing gas-phase ionization of the glycan analytes. Upon ionization, the analytes enter a heated (225°C) capillary and travel through a series of ion guides before being detected. The capillary and tube lens voltages were 42 V and 120 V, respectively, and the AGC in the Orbitrap was set to 1×10^6 ions with a maximum injection time of 500 ms. A resolving power of 60,000 was used in the Orbitrap, allowing for ~ 1 scan/sec. The AGC in the LTQ was set to 8×10^3 with a maximum injection time of 80 ms and normalized collision energy (NCE) of 22. Data dependent mode was used to acquire precursor and fragmentation spectra. Up to 5 MS/MS spectra were acquired per precursor Orbitrap scan based on the most abundant m/z values. A 10,000 count threshold was used and m/z values chosen twice in 30 s were placed on an exclude list for an additional 30 s. Calibration was performed according to the manufacturer protocol and Xcalibur software (v. 2.0.7) was used for data analysis and peak integration.

Glycan Integration and Relative Quantification

The glycan ratios were calculated by integrating the areas under each extracted ion chromatogram (EIC) of the 'light' and 'heavy' labeled glycan. The chromatograms of a specifically labeled glycan were extracted using the monoisotopic peak(s). Because significant ammonium adduction was observed, these m/z values ($[\text{M} + \text{nH}^+ + \text{mNH}_4^+]^{(n+m)+}$) were also summed in the EIC. Additionally, if more than one charge-state was present, then both charge-states' $[\text{M} + \text{nH}^+]^{n+}$ and ammonium adducted peaks were summed in the EIC. For relative quantification, the ratio of the area under EIC of the 'heavy': 'light' for each glycan was calculated. This raw glycan ratio was then 'corrected' by using the 'heavy': 'light' ratio of the spiked-in internal standard, maltoheptaose. The maltoheptaose glycan ratio was calculated using the same EIC and integration as described above. Maltoheptaose serves as an important internal standard to correct for the differences in sample preparation systematic error including derivatization reaction variability, SPE recovery variability, glycan interaction with vials, stationary phases, etc. Additionally, correcting with maltoheptaose takes into account the slightly different isotopic distributions due to the number of possible natural ^{13}C 's in each molecule and the incomplete incorporation of $^{13}\text{C}_6$ (vide infra).

RESULTS and DISCUSSION

The 'light' and 'heavy' forms of the P2GPN reagent were synthesized by identical mechanisms only differing in the source of bromobenzene. The heavy labeled compound was characterized by high resolution FT-ICR MS, and the mass spectrum is shown in Figure 1a. It is seen that ~95% of the compound consists of successful incorporation of the $^{13}\text{C}_6$ isotope labels. Additionally, the data show peaks at $^{13}\text{C}_5$ and $^{13}\text{C}_0$ isotope incorporation corresponding to <5% and <1% relative abundance, respectively. It is paramount in the relative quantification strategy by differential labeling that no cross-reaction occurs after the two samples are mixed together. Because hydrazone formation derivatization does not require the removal of excess hydrazide reagent, there is the possibility of the unreacted 'light' tag reacting with the 'heavy' sample and vice versa. A control study was performed in which an extracted glycan sample from pooled plasma was tagged with the 'heavy' reagent. Additionally, a blank vial was prepared in parallel and tagged with the 'light' reagent. These two samples were then mixed together and analyzed. The result is shown in Figure 1b, where it is observed that less than 1% of the example glycan is derivatized with the $^{13}\text{C}_0$ reagent, indicating that there is $\leq 1\%$ cross-reactivity after mixing. However, the actual cross-reactivity is significantly less because the $^{13}\text{C}_0$ peak is also attributed to the isotopic purity of the $^{13}\text{C}_6$ reagent (Figure 1a).

In order to further characterize the behavior of the 'light' and 'heavy' reagents, glycans were cleaved from two aliquots of a pooled human plasma sample in parallel and were derivatized with the 'light' and 'heavy' reagent, respectively. These two samples were then mixed in a 1:1 ratio and analyzed. Figure 2 displays the near identical behavior of the 'light' and 'heavy' tagged glycans with respect to chromatographic, MS, and collision induced dissociation (CID) performance. It is imperative that the chromatographic performance of the same 'light' and 'heavy' tagged are identical in order to ensure conserved retention times, mobile phases in which the glycans elute, and conditions in the ESI droplet. If these are not identical for the differentially tagged pair, then the ionization efficiency in ESI may be significantly different causing the MS to systematically bias the glycan ratios. For example, Figure 2 shows the EIC of an example Hex₅HexNAc₄Fuc glycan where the 'light' and 'heavy' tagged glycan co-elute from the HILIC column. Thus, there is no isotopic retention effect. A table included in the supplemental material lists the retention times for each pair of detected glycans (Table S1).

There also cannot be an isotopic bias in the MS detection of the differentially labeled glycans. The precursor ions detected in the orbitrap are shown for the 'light' and 'heavy' forms of an example glycan (Figure 2). A 1:1 ratio is detected; however, it is observed that the isotopic distributions of the 'light' and 'heavy' form have slightly different isotopic abundances for the A+1 through A+5 peaks. This is expected due to the different number of possible natural ^{13}C sites in the tags. Additionally, the product-ion spectra are overlaid for the 'light' and 'heavy' forms of the example glycan. It is shown that the stable-isotope labeled tag does not affect the fragmentation patterns of the glycans any differently than the 'light' reagent. Furthermore, whether or not a fragment is a 'Y' or 'B' ion⁵⁰ can be easily distinguished by determining if the peaks overlap or have a 6 Da shift. If there is a 6 Da shift between the 'light' and 'heavy' tagged fragments, then this means that the tag is still present, thus the reducing terminus is intact and is a 'Y' ion.

In order to test the quantification capabilities of these reagents, a simple mixture of three glycans (NA2, MAN9, and A2F) plus an internal standard, maltoheptaose, were divided into aliquots, and various amounts of NA2 glycan were spiked into each vial so that there were 5 different NA2 'light' to 'heavy' sets (ratios: 5:1, 2:1, 1:1, 1:2, and 1:5). These samples were tagged with either the 'light' or 'heavy' reagent and the two mixtures were combined 1:1.

The results are shown in Figure 3 where the maltoheptaose corrected histogram shows the analytical variability of the method and how well a change in concentration can be detected by mass spectrometry. The analytical variability is shown by the histogram of the 1:1 glycans, which are centered around 0 in the $\log_2(\text{H:L})$ plot. Additionally, it is seen that the expected ratios of the NA2 glycan were able to be recovered. The inset, shown above the base peak chromatogram, displays example spectra of the 'light' and 'heavy' pairs at each of the NA2 ratios. The relative abundance of the $[\text{M}+\text{nH}]^{n+}$ peaks are indicative of the spiked-in ratios, and the $\log_2(\text{H:L})$ histogram shows clear distinction between each of the varied concentrations.

In all *N*-linked glycan quantification experiments, maltoheptaose has been spiked in at the onset of sample preparation in order to account for the difference in systematic error between samples prepared in parallel. The analysis of *N*-linked glycans cleaved from plasma proteins involves numerous sample preparation steps that can lead to different measured abundance ratios in MS. This is unfortunate for the present relative quantification strategy, as samples undergo preparation in parallel but in a different vial with at least slightly different conditions. Though the samples are processed in parallel and efforts are taken to minimize differences in time, temperature, solvent composition, etc., other factors that cannot be controlled also contribute to this difference in systematic error, such as glycan recovery from a specific vial or SPE column, binding to pipette tips, variable reaction efficiency, etc. In order to take into account these variations between samples, an internal standard was introduced into the plasma at the onset of the sample preparation. The internal standard must possess several properties: 1) it must have the same physical properties as the *N*-linked glycans being analyzed, 2) it must not be present on any proteins found in the biological sample of interest, and 3) it must have similar HILIC chromatographic properties as *N*-linked glycans. In these studies, maltoheptaose was used as the internal standard. Maltoheptaose is a straight-chain polysaccharide containing 7 hexose sugar units, and this standard fulfills criteria 2 and 3; however, criteria 1 is only partially fulfilled in that *N*-linked glycans are made up with monosaccharides other than just hexose, such as *N*-acetylglucose amine and *N*-acetylneuraminic acid, residues not present in maltoheptaose.

Figure 4 demonstrates the utility of spiking maltoheptaose into 2 aliquots of plasma samples before sample preparation. The four example glycans shown all have an increase in ion abundance in the 'heavy' sample. However, this is also the case for the internal standard, and if the internal standard ratio is used to correct for the 'light' vs. 'heavy' abundance of the *N*-linked glycans, then the *N*-linked glycan ratios are significantly closer to 1:1. Maltoheptaose is capable of accounting for two types of systematic error in these measurements: 1) the variable sample preparation conditions, efficiencies, and recoveries for each sample and 2) the bias due to the incorporation of $^{13}\text{C}_6$ stable isotopes. Having discussed the possible types of variations derived from parallel processing, the differences in the 'light' and 'heavy' abundance due to the isotopic distributions arise from the incomplete incorporation of the $^{13}\text{C}_6$ isotopes and the statistical probabilities in which natural ^{13}C is incorporated into a molecule. In the former, the ~95% incorporation of the $^{13}\text{C}_6$ isotopes will cause the $[\text{M}+\text{nH}]^{n+}$ peak to be only 95% the abundance of the 'light' reagent. Additionally, since $^{13}\text{C}_6$ has been artificially synthesized into the molecules, there are fewer opportunities for natural incorporation of ^{13}C in the 'heavy' reagent than in the 'light' reagent. These isotope effects cause the $[\text{M}+\text{nH}]^{n+}$ peak of the 'heavy' tagged glycans to have a greater abundance (by ~4.5%) than the $[\text{M}+\text{nH}]^{n+}$ peak of the 'light' tagged glycans. Though these instances arise, these changes in the isotopic distribution are conserved for all *N*-linked glycans and maltoheptaose. Thus, using the maltoheptaose 'light' to 'heavy' ratio will correct for these isotopic differences in addition to a majority of the sample preparation variations. A table has been included in the supplemental material listing the glycan compositions detected and the raw and corrected and ratios (Table S2).

The example glycans in Figure 4 were chosen to represent a majority of the *N*-linked glycan types including high mannose, complex, sialylated, and fucosylated. From these data, the internal standard is able to quantitatively correct for a majority of the difference in systematic error between samples incurred during sample preparation; however, a t-test shows that the 95% confidence interval of the corrected distribution still does not include 0. Thus, it is possible that an internal standard with properties more closely related to *N*-linked glycans can more effectively correct for sample preparation systematic error differences. While maltoheptaose is comprised of the most common hexose monosaccharide units, there are no HexNAc, NeuAc, or Fuc residues present. This might hinder the effectiveness of the internal standard. Also, NeuAc (sialic acid) is a labile residue, and if the conditions in the sample preparation are slightly different, there could be variable peeling that would not be able to be taken into account. Thus, a more comprehensive internal standard, or set of internal standards, may be a more effective strategy in the future to more accurately correct for any sample preparation bias. Furthermore, maltoheptaose elutes much earlier and has a significantly broader (10 $\times$) peak shape than a majority of the *N*-linked glycans. This also is not optimal when using maltoheptaose as an internal standard. However, the cost-effective maltoheptaose has shown to correct for a majority of the differences in systematic error due to parallel sample preparation, and further investigation is needed to determine whether a more expensive internal standard or panel of internal standards will more effectively account for the sample preparation variability.

In order to determine the analytical variability of the relative quantification strategy, two aliquots of a pooled plasma sample were processed in parallel and tagged with the 'light' and 'heavy' reagents, respectively. Biologically identical aliquots were used in order to minimize the biological and sample variability. This will allow one to analyze only the variability in the analytical method, sample preparation, and instrument performance (the variability should be negligible here due to the samples being mixed together before ionization and mass analysis). Figure 5 displays the results of this analysis. The histogram shows two distributions: 1) the raw, uncorrected $\log_2(\text{H:L})$ data (red) and 2) the internal standard corrected $\log_2(\text{H:L})$ data (black). As mentioned previously and further shown here, the internal standard is effective in correcting for a majority of the systematic error, including all classes of *N*-linked glycans (high mannose, hybrid, and complex).

The histogram comprises the relative quantification of 27 glycan species analyzed in triplicate. The width of the distribution is less than 0.5 on a $\log_2$ scale. This leads one to infer that changes in *N*-linked glycosylation in different biological samples as little as 30% may be able to be detected using the methods presented herein. The mean of the corrected *N*-linked glycan distribution is slightly larger than 0, and a t-test has shown that the 95% confidence interval does not include 0. This is thought to be due to the isotopic distribution overlap in the high molecular weight *N*-linked glycans. In the small glycans, the 6 Da shift is sufficient so that any overlapping isotopic distribution is negligible. However, as the sizes of the glycans get larger, there is more probability that natural ^{13}C is incorporated in the glycans. Thus, the A+6 isotopic distribution peak for the 'light'-tagged glycan begins to become more significant in the spectra and skew the relative quantification data. The internal standard corrects for most isotopic effects, such as the incomplete incorporation of $^{13}\text{C}_6$ into the 'heavy' tag. However, maltoheptaose is a relatively small oligosaccharide, and in the case of overlapping distributions, there is no overlap in the 'light' and 'heavy' forms of maltoheptaose. Therefore, maltoheptaose effectively corrects for variability in the sample preparation, but a panel of standards with varying molecular weights is a possible solution for more accurately correcting for the molecular weight bias.

CONCLUSION

The stable-isotope labeled reagents have been shown to be a viable strategy for the relative quantification of *N*-linked glycans in simple and complex mixtures. The P2GPN 'light' and 'heavy' reagents have near identical properties with respect to chromatographic, MS, and MS/MS performance. These reagents have shown the possibility for relative quantification of *N*-linked glycans from the most complex of mixtures, pooled plasma, and it is hypothesized that the small analytical variability will afford the advantage of detecting subtle changes in glycosylation patterns. These reagents have the potential to quantify the aberrations in glycosylation in numerous studies related to disease and cancer. However, further variability analyses are necessary to determine biologically relevant cutoff values in order to determine significant change. These studies will determine the effectiveness of the stable-isotope labeled hydrazide reagents and how well this quantification method can be applied to biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of the heavy P2GPN reagent: a) the high resolving power mass spectrum of the $^{13}\text{C}_6$ -labeled P2GPN. The $^{13}\text{C}_6$ was incorporated at 95%, while the $^{13}\text{C}_0$ and $^{13}\text{C}_5$ side products account for 1 and 5%, respectively. b) a control study in which one plasma sample was tagged with the 'heavy' reagent and a blank vial was tagged with the 'light'. The samples were mixed together and negligible cross-reaction was observed, as the 1% of 'light' sample was observed from the incomplete incorporation of the $^{13}\text{C}_6$.

Figure 2. Chromatographic, MS, and MS/MS characterization of a 1:1 derivatized human plasma mixture. There is no retention time shift, and the precursor ions are detected in a 1:1 ratio. Additionally, it is shown that there is no isotope effect on the fragmentation of the derivatized glycans.

Figure 3.

A simple mixture of 3 model *N*-linked glycans (NA2, A2F, and MAN9) plus the internal standard, maltoheptaose, in a 1:1 mixture. Also, the NA2 glycan was spiked in at varying concentration ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 (upper inset). The histogram shows the experimental relative quantification data, where glycans with a 1:1 ratio are centered around 0 in the $\log_2(H:L)$ plot, and the NA2 ratios are near the expected values and clearly distinct from each other and the 1:1 glycan samples.

Figure 4.

Maltoheptaose is spiked into all of the samples at the onset of sample preparation. This allows one to track the variations in the sample preparation, since each is prepared in parallel then mixed together after derivatization. In plasma, 4 example glycans have been chosen to show the effect of the internal standard on correcting the glycan ratios. Here, almost a 50% difference in the sample preparation systematic error can be corrected using the ratios of the 'light' to 'heavy' internal standard.

Figure 5.

The histogram of 27 *N*-linked glycans cleaved and quantified using the P2GPN reagent pair from 2 aliquots of pooled human plasma. The red distribution is the raw glycan $\text{log}_2(\text{H:L})$ ratios, whereas the black distribution is the internal standard corrected ratios. The narrow distribution indicates the minimal analytical variability between two 1:1 glycan samples cleaved from a complex mixture. Confidence intervals (95%) are shown above the distributions (Diamonds) along with the quantiles of the distribution (boxes).