RAPID PREPARATION OF RELEASED N-GLYCANS FOR HILIC ANALYSIS USING A NOVEL FLUORESCENCE AND MS-ACTIVE LABELING REAGENT

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INTRODUCTION

Conventional approaches to the preparation of N-glycans for HILIC-FLR-MS are either laborious, time-consuming, or require compromises in sensitivity.1 In the case of one of the most frequently employed labeling compounds, 2-aminobenzamide (2-AB), the resulting glycans can be readily detected by fluorescence but are difficult to detect by electrospray ionization mass spectrometry (ESI-MS). Variations of conventional approaches for N-glycan sample preparation have been explored, but have not yet presented a solution that combines the desired attributes of simplicity, high MS sensitivity, and high throughput. One example is rapid tagging procedures that yield labeled glycans in a matter of minutes. Cook and co-workers have presented the use of a rapid tagging analog of aminobenzamide (AB).2 In a rapid reaction, the precursor glycosylamines of reducing, aldehyde terminated glycans are modified via a urea linked aminobenzamide. Although this rapid tagging reagent accelerates the labeling procedure, it does not provide the ionization efficiencies needed in modern N-glycan MS analyses.

To address the above shortcomings, we have developed a sample preparation solution that enables unprecedented FLR and MS sensitivity for released N-glycan detection while also improving the throughput of N-glycan sample preparation. A novel labeling reagent has been synthesized that rapidly reacts with glycosylamines following their release from glycoproteins. Within a 5 minute reaction, N-glycans are labeled with Rapifluor-MS, a reagent comprised of an N-hydroxysuccinimide (NHS) carbamate rapid tagging group, an efficient quinoline fluorophore, and a tertiary amine for enhancing ionization. To further accelerate the preparation of N-glycans, rapid tagging has been directly integrated with a Rapid PNGase F deglycosylation procedure involving RapiGest SF surfactant and a HILIC μElution SPE clean-up step that provides highly quantitative recovery of the released and labeled glycans with the added benefit of not requiring a solvent dry-down step prior to the LC-FLR-MS analysis of samples.
METHODS

SAMPLE DESCRIPTION:

N-glycans were prepared using a GlycoWorks RapiFluor-MS N-Glycan Kit (p/n 176003606) and the manufacturer suggested protocols provided in the Care and Use Manual (715004793EN). Samples were either directly analyzed from the collected SPE eluate or analyzed after drying and reconstitution. To compare the response factors of Instant AB™ and RapiFluor-MS labeled glycans, labeling reactions were performed with equivalent molar excesses of reagent, and crude reaction mixtures were directly analyzed by HILIC-FLR-MS in order to avoid potential biases from SPE clean-up procedures. To compare the response factors of 2-AB labeled versus RapiFluor-MS labeled glycans, equivalent quantities of labeled N-glycans from pooled human IgG were analyzed by HILIC-FLR-MS. Column loads were calibrated using external quantitative standards.

For more experimental details and additional results, see Waters Application Notes 720005275EN, 720005344EN, 720005352EN, 720005353EN, and 720005370EN.

Figure 1. RapiFluor-MS Molecular Structure.

Figure 2. Reaction Schematic for RapiFluor-MS Derivatization of an N-Glycosylamine.
**METHOD CONDITIONS (unless otherwise noted):**

**LC Conditions:**
- **LC system:** ACQUITY UPLC H-Class Bio System
- **Sample Temp.:** 5 °C
- **Analytical Column Temp.:** 60 °C
- **Flow Rate:** 0.4 mL/min
- **Fluorescence Detection:**
  - Ex 265 / Em 425 nm (RapiFluor-MS)
  - Ex 278 / Em 344 nm (Instant AB™)
  - Ex 330 / Em 420 nm (2-AB)
  - 5 Hz scan rate
  - Gain =1
- **Column:** ACQUITY UPLC Glycan BEH Amide 130 Å 1.7 μm, 2.1 x 50 mm (p/n 186004740)

**Mobile Phase A:** 50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)

**Mobile Phase B:** ACN (LC-MS grade)

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**MS Conditions**
- **MS system:** SYNAPT G2-S HDMS
- **Analyzer mode:** ESI+, TOF MS, Resolution Mode (~20 K)
- **Capillary voltage:** 3.0 kV
- **Cone voltage:** 80 V
- **Source temp.:** 120 °C
- **Desolvation temp.:** 350 °C
- **Desolvation gas flow:** 800 L/Hr
- **Acquisition:** 500–2500 m/z, 1 Hz scan rate
- **Data management:** MassLynx Software (V4.1)
RESULTS AND DISCUSSION

Rational Design of a New N-Glycan Labeling Reagent

A new labeling reagent for facilitating N-glycan analysis has been synthesized based on rational design considerations (Figure 1) that would afford rapid labeling kinetics, high fluorescence quantum yields, and significantly enhanced MS detectability. Within a 5 minute reaction, N-glycans are labeled with the new reagent under ambient, aqueous conditions to yield a highly stable urea linkage (Figure 2). In addition to rapid tagging capabilities, the new labeling reagent also supports high sensitivity for both MS and fluorescence detection.

RapiFluor-MS Enables High Sensitivity Fluorescence and MS Detection

The response factors of RapiFluor-MS labeled glycans have been benchmarked against those observed for glycans labeled with alternative reagents. The most closely related, commercially available alternative to RapiFluor-MS is an NHS carbamate analog of aminobenzamide, known as Instant ABTM. Figures 3A and 3B present HILIC fluorescence and base peak intensity (BPI) MS chromatograms for equivalent quantities of N-glycans released from a murine monoclonal antibody (Intact mAb Mass Check Standard, p/n 186006552) and labeled with RapiFluor-MS and Instant AB, respectively. Based on the observed chromatographic peak areas, response factors for RapiFluor-MS and Instant AB labeled glycans.

To summarize our observations, we have plotted the response factors of Instant AB and 2-AB as percentages versus the response factors of RapiFluor-MS (Figure 4).

Rapid Deglycosylation with a Novel Formulation of Rapid PNGase F and RapiGest SF Surfactant

A novel formulation of Rapid PNGase F and RapiGest SF surfactant has been used to completely deglycosylate glycoproteins in an approximately 10 minute procedure. RapiGest SF, an anionic surfactant, is used to ensure that N-glycans are accessible to Rapid PNGase F and that the glycoproteins remain soluble upon heat denaturation. Most importantly, RapiGest is an enzyme-friendly reagent and can therefore be used at high concentrations without hindering the activity of Rapid PNGase F. In the developed method, a glycoprotein is subjected to a high concentration of RapiGest (1% w/v) and heated to 80°C for 2 minutes. Subsequently and without any additional sample handling, Rapid PNGase F is added to the solution and the mixture is incubated at an elevated (50°C) temperature for 5 minutes to achieve complete, unbiased deglycosylation for most glycoproteins. The use of this rapid deglycosylation in combination with RapiFluor-MS labeling significantly accelerates the preparation of N-glycan samples for HILIC analysis (Figure 5).
Facilitating the Routine Use of Glycan MS Detection

Mass spectrometric detection of glycans has historically been a non-trivial exercise. Accordingly, it was rarely desirable to employ MS detection during routine N-glycan profiling, particularly because previous approaches have been dependent on relatively expensive instruments exhibiting wide mass acquisition windows. With the use of RapiFluor-MS labeling, however, it is possible to more easily adopt MS as a hyphenated technique with HILIC-fluorescence given that challenges with glycan ESI-MS are alleviated by the fact that the RapiFluor-MS label dramatically increases both ionization efficiency and ESI charging. To demonstrate this, we compared ToF mass spectra for RapiFluor-MS labeled glycans to those labeled with 2-AB (Figure 6). Notice that signal intensity improved dramatically with RapiFluor-MS. In addition, RapiFluor-MS shifted the charge states of the glycans. As shown, FA2 shifted to a [M+2H]^2+ charge state, while a more complex structure began to populate both a [M+2H]^2+ and [M+3H]^3+ charge state. Interestingly, these higher charges states afforded by RapiFluor-MS are within the acquisition range of a miniaturized quadrupole mass detector, known as the ACQUITY QDa (Figure 7).

The combination of RapiFluor-MS labeling and HILIC-FLR-MS with the ACQUITY QDa Detector now makes it possible to add mass confirmation to glycan peaks observed during even routine fluorescence-based N-glycan profiling experiments. The application of HILIC-FLR and orthogonal, online mass detection to the analysis of two biotherapeutic products is demonstrated in Figure 8.

Figure 5. Workflow for the rapid preparation of N-glycans using the RapiFluor-MS N-Glycan Kit.

Figure 6. Charge States of RapiFluor-MS Labeled N-Glycans. Time-of-flight ESI+ mass spectra for two N-glycans labeled with RapiFluor-MS and 2-AB, respectively.

Figure 7. A miniaturized quadrupole mass detector. The ACQUITY QDa Detector.

Figure 8. HILIC-FLR-MS of RapiFluor-MS Labeled Glycans with On-line QDa Mass Detection using a 2.1x150 mm column. (A) Fluorescence chromatogram of N-glycans from Regeneron product A along with corresponding mass spectral data (B). (C) Fluorescence chromatogram of N-glycans from Regeneron product B along with corresponding mass spectral data (D).
CONCLUSIONS

- Preparation of labeled N-glycans (from glycoprotein to analysis ready sample) in 30 minutes using a simple, streamlined protocol

- Unprecedented sensitivity for labeled N-glycan HILIC analysis (2 and nearly 800 fold increases to fluorescence and MS signal compared to Instant AB)

- Ability to use a miniaturized quadrupole mass detector (the ACQUITY QDa Detector) in routine glycan HILIC analyses because of the ESI properties of RapiFluor-MS labeled N-glycans

References