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Notes & Tips

Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization–mass spectrometry

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One of the most widely used methods for glycan analysis is fluorescent labeling of released glycans followed by hydrophilic interaction chromatography–(ultra-)high-performance liquid chromatography [HILIC–(U)HPLC]. Here, we compare the data obtained by (U)HPLC–fluorescence (FLR) coupled to electrospray ionization–mass spectrometry (ESI–MS) for procainamide and 2-aminobenzamide (2-AB)-labeled *N*-glycans released from human immunoglobulin G (IgG). Fluorescence profiles from procainamide show comparable chromatographic separation to those obtained for 2-AB but gave higher fluorescence intensity as well as significantly improved ESI efficiency (up to 30 times that of 2-AB). Thus, labeling with procainamide increases the ability to identify minor glycan species that may have significant biological activity.

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Protein glycosylation is a post-translational modification that influences many protein functions [1,2]. Detailed monitoring and controlling glycosylation is essential in biopharmaceutical development and quality control of drugs that are glycosylated (e.g., erythropoietin) [3,4].

A number of analytical techniques have been developed for glycan characterization, including high-performance liquid chromatography (HPLC),[1] mass spectrometry (MS), and high pH anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD). Each of these techniques can provide different information, and a combination of these techniques in a liquid chromatography (LC)–MS system can greatly improve full glycan characterization [5].

HPLC analysis of released and fluorescently labeled (via reductive amination) *N*-glycans is one of the most common methods; the stoichiometric attachment of one label per glycan allows the relative quantitation of different glycan species based on fluorescence or ultraviolet (UV) absorbance intensity [6]. This approach, combined with specific enzyme digestions, can help to assign glycan structures more fully.

MS and tandem MS (MS/MS) can provide composition and sequence data; however, samples containing isomeric and isobaric glycan structures might not be distinguished by this technique.

An (U)HPLC–fluorescence (FLR) coupled to electrospray ionization (ESI)–MS system can greatly improve glycan analysis by providing specific structural information.

There are several fluorescent labels that have been used for the reductive amination of *N*-glycans such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 1-aminopyrene-3,6,8-trisulfonic acid (APTS), 2-aminopyridine (AP), procaine, and procainamide [7]. 2-AB is the most widely used label providing relative quantitation of *N*-glycan structures through efficient labeling and the production of highly stable labeled glycans. However, a major drawback of 2-AB is poor ionization efficiency when analyzing *N*-glycan structures by ESI–MS [5].

Yoshino and coworkers [8] and Takao and coworkers [9] reported a 1000-fold increase in sensitivity over free oligosaccharides for glycans labeled with procaine [2-(diethylamino)ethyl 4-aminobenzoate] in matrix-assisted laser desorption/ionization (MALDI)–MS and ESI–MS. However, Pabst and coworkers [7] found that the performance of procaine in normal-phase (NP)–HPLC was disappointing. Considerable differences between elution times and separation of glycans labeled with procaine and those labeled with 2-AB were observed.

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; UV, ultraviolet; MS/MS, tandem MS; FLR, fluorescence; ESI, electrospray ionization; 2-AB, 2-aminobenzamide; (U)HPLC, (ultra-)high-performance liquid chromatography; IgG, immunoglobulin G; BPC, base peak chromatogram; EIC, extracted ion chromatogram.

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Here we present data comparing two fluorescent labels for quantitative *N*-glycan profiling and identification by (ultra-)high-performance liquid chromatography [(U)HPLC]–FLR coupled to ESI–MS and MS/MS. *N*-Glycans from human polyclonal immunoglobulin G (IgG) were released, 2-AB-labeled or procainamide [4-amino-*N*-(2-diethylaminoethyl) benzamide]-labeled, and purified on a liquid-handling robotic platform.

All reagents and kits for release, *N*-glycan labelling, and cleanup were obtained from Ludger (Oxford, UK). Acetonitrile (Romil, 190 SpS for UV gradient quality) was obtained from Charlton Scientific (Charlton, Oxon, UK). Human IgG was obtained from Ludger, and all other reagents were obtained from Sigma–Aldrich (Dorset, UK).

Glycan release, 2-AB labeling, procainamide labeling, and labeled glycan purification were performed on a Hamilton Microlab STARlet liquid-handling robotic platform [10].

Samples were denatured by heating for 7 min at 99 °C in 2% SDD/1 M β-mercaptoethanol before overnight incubation at 37 °C with PNGase F (Ludger) in the presence of Triton X-100. The released *N*-glycans were converted to aldoses with 0.1% formic acid [11], filtered through a protein binding plate (Ludger), and dried.

Released *N*-glycans were fluorescently labeled by reductive amination in 10 µl of water with either 10 µl of 2-AB labeling solution as per the Ludger 2-AB glycan labeling kit containing 2-picoline borane or 10 µl of procainamide labeling solution as per the Ludger procainamide glycan labeling kit containing 2-picoline borane. Samples were incubated at 65 °C for 1 h.

The 2-AB-labeled *N*-glycans were cleaned up using Ludger Clean T1 Cartridges. 2-AB-labeled *N*-glycans were eluted with water (1 ml). The samples were dried by vacuum centrifugation and resuspended in water (100 µl) for further analysis.

The removal of unreacted procainamide dye was performed using a Ludger Clean plate for cleanup of procainamide-labeled glycans. The procainamide-labeled *N*-glycans were eluted with water (200 µl). The samples were dried by vacuum centrifugation and resuspended in water (100 µl) for further analysis.

2-AB- and procainamide-labeled samples were analyzed by LC-FLR-ESI-MS. Here, 25 µl of each sample was injected into an ACQUITY UPLC BEH–Glycan column (1.7 µm, 2.1 × 150 mm) at 40 °C on a Dionex Ultimate 3000 UHPLC instrument with a fluorescence detector (λ_{ex} = 250 nm and λ_{em} = 428 nm for 2-AB; λ_{ex} = 310 nm and λ_{em} = 370 nm for procainamide) attached to a Bruker Amazon Speed ETD. The running conditions used were as follows: solvent A was 50 mM ammonium formate (pH 4.4) made from Ludger Stock Buffer (Ludger), and solvent B was acetonitrile. Gradient conditions were as follows: 0 to 38.5 min, 76 to 58% B; 38.5 to 40.5 min, 58 to 40.5% B at a flow rate of 0.4 ml/min; 40.5 to 42.5 min, 40% B at a flow rate of 0.25 ml/min; 42.5 to 44.5 min, 40 to 76% B at a flow rate of 0.25 ml/min; 44.5 to

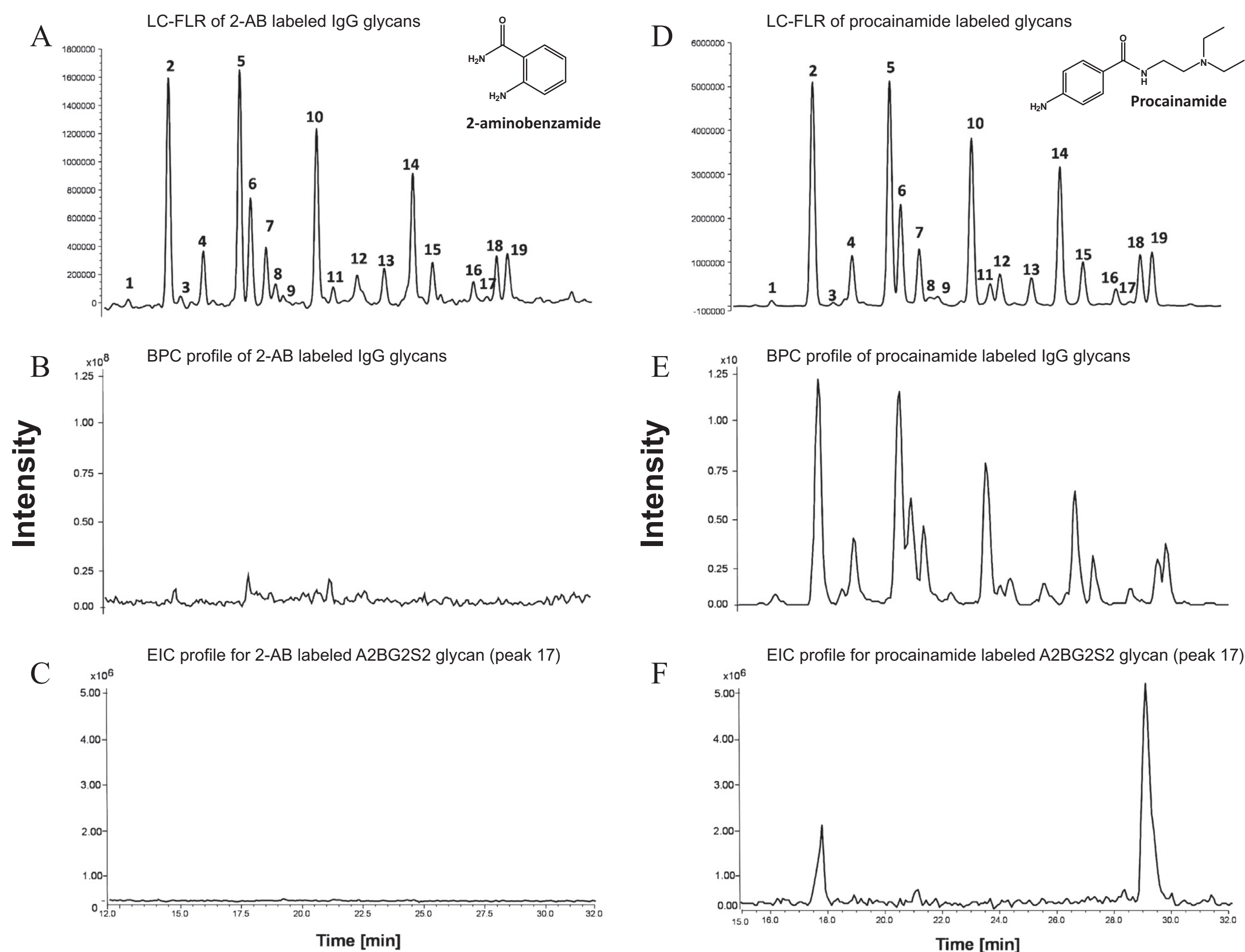

Fig.1. Comparison of fluorescently labeled human IgG glycans: (A) LC–FLR of 2-AB-labeled IgG glycans; (B) LC–ESI–MS BPC profile of 2-AB-labeled IgG glycans; (C) LC–ESI–MS EIC for 2-AB-labeled A2BG2S2 glycan (peak 17 on LC–FLR trace); (D) LC–FLR of procainamide-labeled glycans; (E) LC–ESI–MS BPC profile of procainamide-labeled IgG glycans; (F) LC–ESI–MS EIC for procainamide-labeled A2BG2S2 glycan (peak 17 on LC–FLR trace).

50.5 min, 76% B at a flow rate of 0.25 ml/min; 50.5 to 51.5 min, 76% B at a flow rate of 0.25 ml/min; 51.5 to 55 min, 76% B at a flow rate of 0.4 ml/min. The Amazon Speed settings used were as follows: source temperature, 250 °C; gas flow, 10 L/min; capillary voltage, 4500 V; ICC target, 200,000; Max. accu. time (Maximum Accumulation Time), 50.00 ms; rolling average, 2; number of precursor ions selected, 3; release after 0.2 min; positive ion mode; scan mode, enhanced resolution; mass range scanned, 200 to 1500; target mass, 900. A glucose homopolymer ladder (Ludger), labeled with either 2-AB or procainamide, was used as a system suitability standard as well as an external calibration standard for GU allocation [11].

Release and recovery of *N*-glycans with free reducing termini is important for structural and functional analysis. Using human IgG as a model glycoprotein, we compared two fluorescent labels: 2-AB and procainamide. The *N*-glycans from human IgG were released, labeled, and purified using a liquid-handling robotic platform.

The profiles obtained by (U)HPLC–FLR coupled to ESI–MS from the human IgG samples labeled with 2-AB and procainamide are compared in Fig. 1. Comparable *N*-glycan profiles were obtained for each sample, labeled with 2-AB and procainamide (Fig. 1A and D), as described before [12]. The peak intensity of the fluorescent profiles from the samples labeled with procainamide (the emission and excitation wavelengths were set to λ_{ex} = 310 nm and λ_{em} = 370 nm) were higher than those labeled with 2-AB (λ_{ex} = 250 nm and λ_{em} = 428 nm), allowing better integration of the lower intensity peaks.

The base peak chromatogram (BPC) in positive mode ESI–MS for procainamide-labeled human IgG *N*-glycans showed up to 30 times higher signal intensity compared with 2-AB (Fig. 1B and E). The extracted ion chromatogram (EIC) profiles for the 2-AB-labeled (Fig. 1C) and procainamide-labeled (Fig. 1F) A2BG2S2 glycan (Supplementary Table 1 and Fig. 1A and D, peak 17) showed significantly higher ion intensity for procainamide derivative, enabling identification of low-level glycan species not mass detected using 2-AB. This high sensitivity in positive mode ESI–MS can be explained by of the high proton affinity of the procainamide basic tail [2-(diethylamino)ethyl group].

All 19 peaks that were detected by (U)HPLC–FLR were identified by MS and MS/MS (Supplementary Table 1). One, two, or three charge states were detected for the procainamide derivatives. In some cases, when the MS/MS analysis was performed on singly charged precursor ions, a loss of 73 Da was observed. This observation indicated degradation of the procainamide label during MS/MS fragmentation and corresponds to loss of a diethylamine ion.

In summary, the comparison of the two fluorescent labels presented here demonstrates that procainamide derivatization can be used as a standard method for manual and automated glycan characterization. The procainamide-labeled glycans are suitable for both (U)HPLC–FLR analysis, providing good chromatographic separation for relative quantitation, and ESI–MS analysis, providing more efficient ionization for glycan identification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.06.006.

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