Journal of Chromatography A, 1233 (2012) 66–70

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Hydrophilic interaction liquid chromatography of anthranilic acid-labelled oligosaccharides with a 4-aminobenzoic acid ethyl ester-labelled dextran hydrolysate internal standard

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ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 24 January 2012
Accepted 1 February 2012
Available online 11 February 2012

Keywords:
Oligosaccharide
HPLC
Internal standard
Fluorescent
UV

ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) of fluorescently labelled oligosaccharides is used in many laboratories to analyse complex oligosaccharide mixtures. Separations are routinely performed using a TSK gel-Amide 80 HPLC column, and retention times of different oligosaccharide species are converted to glucose unit (GU) values that are determined with reference to an external standard. However, if retention times were to be compared with an internal standard, consistent and more accurate GU values would be obtained. We present a method to perform internal standard-calibrated HILIC of fluorescently labelled oligosaccharides. The method relies on co-injection of 4-aminobenzoic acid ethyl ester (4-ABEE)-labelled internal standard and detection by UV absorption, with 2-AA (2-aminobenzoic acid)-labelled oligosaccharides. 4-ABEE is a UV chromophore and a fluorophore, but there is no overlap of the fluorescent spectrum of 4-ABEE with the commonly used fluorescent reagents. The dual nature of 4-ABEE allows for accurate calculation of the delay between UV and fluorescent signals when determining the GU values of individual oligosaccharides. The GU values obtained are inherently more accurate as slight differences in gradients that can influence retention are negated by use of an internal standard. Therefore, this paper provides the first method for determination of HPLC-derived GU values of fluorescently labelled oligosaccharides using an internal calibrant.

1. Introduction

The post-translational modification of membrane and secreted macromolecules includes N- and O-linked oligosaccharide addition, and the modification of dolichol lipid moieties to form the highly conserved N-linked oligosaccharide precursor glucose$_3$mannose$_9$*N*-acetylglucosamine$_2$-dolichol (Glc$_3$Man$_9$GlcNAc$_2$-dolichol). Additional modifications to phosphatidylinositol that form glycosylphosphatidyl-inositol (GPI)-anchors and ceramide to form complex glycosphingolipids (GSL), are some of the most common and conserved processes in eukaryotic biological systems and makes analysis of the myriad cellular-derived oligosaccharide structures a complex process. The oligosaccharide must be released structurally intact from the protein or lipid moiety using either chemical methods, such as hydrazinolysis [1,2], alkaline borohydride treatment [3] or trifluoromethanesulfonic acid [4], or enzymatically, by using endoglycosidases for protein N-linked oligosaccharides [5,6] or ceramide glycanase for glycosphingolipids [7]. Additionally, multiple methods including high-performance liquid chromatography (HPLC), linkage-specific glycosidase digestions and mass spectrometry, are required to unambiguously assign the released oligosaccharide structures.

Hydrophilic interaction chromatography (HILIC) is a form of HPLC commonly used to separate polar compounds, in particular carbohydrates or oligosaccharides [8,9]. HILIC separates fluorophore-labelled, UV chromophore-labelled and unlabelled oligosaccharides based on increasing polarity and degree of solvation, and is commonly used to analyse oligosaccharides [8–24]. Sensitivity of detection of the oligosaccharide species is dramatically increased by attachment of a fluorescent moiety at the reducing terminus, and a number of different fluorescent reporter groups are reported in the literature [19,22,25,26]. The incorporation of the fluorescent reporter molecule is achieved following reductive amination of the oligosaccharide reducing-terminus. Though HILIC is the most common employed method in oligosaccharide separation, analysis may also be undertaken following weak or strong anion-exchange, porous graphitized carbon and reverse-phase separations. Where possible, the retention times of the oligosaccharides are compared to that of

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doi:10.1016/j.chroma.2012.02.007

an external standard, usually consisting of glucose oligomers, and elution is expressed in terms of glucose unit (GU) values. The GU values obtained with differing column matrices have been used to generate oligosaccharide retention chromatography databases [2,15,17,27] that are now available for researchers use (http://www.glycoanalysis.info/ENG/index.html and http://glycobase.nibrt.i.e./cgi-bin/public/glycobase.cgi). These resources are two of a growing number of carbohydrate-related information available to the research community [28]. These elution tables or databases commonly encompass GU values from HILIC separations alone (one-dimensional), or in combination with reverse-phase (RP) separations (two-dimensional), or in combination with reverse-phase and ion-exchange separations (three-dimensional) [15,17,27,29,30]. The external standard can only be applied to HILIC or RP separations as outlined above. Additionally, it is now possible to combine differing chromatographies, hydrophilic interaction and anion-exchange, using a single column [10,18,31,32] or a tandem column arrangement [12]. This has been termed mixed-mode chromatography. It is also now possible to obtain GU values following mixed-mode chromatography using a single Dionex AS11 column [18]; a technique referred to as hydrophilic interaction anion-exchange chromatography (HIAX).

Column calibration, with an external standard that is used when calculating GU values, must be performed following a preset number of sample analyses to ensure no degradation in retention on column has occurred. Additionally, as solvent gradients are used to elute the oligosaccharides, variability is introduced to oligosaccharide elution times. This variability can be observed in the retention times of both the external standard and the samples being analysed. The use of a detectable internal standard while performing HPLC separations would negate these variances, as was common in low-pressure chromatography separation when oligosaccharides, co-injected with an internal standard (dextran hydrolysate), using Biogel P-4 gel filtration [33,34]. We demonstrate in this paper a method to separate fluorophore-labelled oligosaccharides using HILIC with a 4-aminobenzoic acid ethyl ester (4-ABEE)-labelled dextran hydrolysate as an internal standard. The oligosaccharides are detected by fluorescence and the internal standard by UV. The method is applicable to the fluorophore most prevalent in oligosaccharide analysis and is the first report of the use of an internal standard for HILIC separations of oligosaccharides.

2. Materials and methods

2.1. Reagents

Ceramide glycanase was purified in-house from the medicinal leech, *Hirudo medicinalis*. All GSL (at least 95% pure), anthranilic acid and sodium cyanoborohydride were purchased from Sigma (Poole, Dorset, U.K.). Acetonitrile (HPLC-grade, far-UV), 2-propanol (HPLC grade), methanol, chloroform, boric acid (Aristar) and acetic acid were purchased from VWR International (Lutterworth, Leics, U.K.) or Fisher Scientific (Loughborough, Leics, U.K.). Spe-ed Amide 2 (100 mg, 1 ml) solid-phase extraction (SPE) columns were purchased from Pelican Scientific (Tattenhall, Cheshire, U.K.).

2.2. Ceramide glycanase digestion and carbohydrate fluorescent labelling

GSL standards (10 μL, 0.5–2 mg/ml in chloroform/methanol (2:1, v/v)) were dried down under a stream of nitrogen before digestion with ceramide glycanase as described previously [17]. The released oligosaccharides were labelled with 2-AA (2-aminobenzoic acid or anthranilic acid), as described by Neville et al. [17] The reaction mixture was allowed to cool to room temperature and 1 ml acetonitrile/water (97:3, v/v) was added and vortexed. The 2AA-labelled oligosaccharides were purified over Spe-ed Amide-2 columns [18]. The labelled oligosaccharides were eluted with 2 ml × 0.75 ml of water and stored at −20 °C.

Dextran hydrolysate was labeled with 4-ABEE, essentially as described [35] but with the following modifications. An aliquot (40 μl) of 4-ABEE-labelling mix (4-ABEE, 90 mg/ml and sodium cyanoborohydride, 9 mg/ml in methanol/acetic acid (9:1, v/v)) was added to 10 μl dextran hydrolysate (10 mg/ml in water). The reaction was placed at 80 °C for 45 min. The reaction was allowed to cool and 500 μl of water was added. The mixture was extracted 5 times with 0.75 ml of diethyl ether to remove excess 4-ABEE. The aqueous layer was transferred to a new tube, dried using a Speedvac and redissolved in 1 ml of water prior to use in subsequent experiments.

2.3. Carbohydrate analysis by HPLC

Purified 2AA- or 4-ABEE-labelled oligosaccharides were separated by normal-phase high-performance liquid chromatography (NP-HPLC) as described [17] with slight modifications. The chromatography system consisted of a Waters Alliance 2695 separations module, an in-line Waters 474 fluorescence detector set at Exλ 360 nm and Emλ 425 nm (2AA) or Exλ 305 nm and Emλ 360 nm (4-ABEE) [36], and an in-line Waters 2487 UV detector set at an absorbance wavelength of 304 nm. All chromatographic separation was performed at 30 °C. Solvent A was acetonitrile. Solvent B was Milli-Q water. Solvent C was composed of 400 mM ammonium hydroxide, titrated to pH 3.85 with acetic acid, in Milli-Q water and was prepared using a standard 5 N ammonium hydroxide solution (Sigma). All chromatographic separation was controlled and data collected and processed using Waters Empower® or Millennium® software. Glucose unit values were determined following comparison with a 2AA-labelled dextran hydrolysate external standard, or with a 4-ABEE-labelled dextran hydrolysate internal standard. The retention time (*x*-axis) was plotted against GU (*y*-axis), from GU 2 upward, and a polynomial curve of the fifth order was fitted. The curve was not fitted through zero and accuracy to 10 decimal places was necessary when generating the polynomial for assignment of GU values. Samples (less than 100 μl) were injected in water, acetonitrile (3:7, v/v). 2AA-labelled dextran hydrolysate was injected as a 1 μl aliquot in water. 4-ABEE-labelled dextran hydrolysate (10 μl) was added to samples prior to injection, while maintaining the 3:7, v/v, aqueous sample to acetonitrile ratio. Additionally, GU values were determined, following comparison with a 2-AA-labelled glucose oligomer ladder external standard, using PeakTime software (developed in-house) or Waters Millennium® software.

Purified 2AA-labelled oligosaccharides were separated by NP-HPLC using a 4.6 mm × 250 mm TSK gel-Amide 80 column (5 μM) (Anachem, Luton, Beds, U.K.) with slight modifications to the published method [17]. Gradient conditions for total GSL and N-linked oligosaccharide analysis were as follows: time – 0 min (*t* – 0), 71.6% A, 28.4% B, 5% C (0.8 ml/min); *t* – 6, 71.6% A, 28.4% B, 5% C (0.8 ml/min); *t* – 45, 46.2% A, 53.8% B, 5% C (0.8 ml/min); *t* – 46, 35% A, 60% B, 5% C (0.8 ml/min); *t* – 48, 35% A, 60% B, 5% C (0.8 ml/min); *t* – 49, 71.6% A, 28.4% B, 5% C (0.8 ml/min); *t* – 51, 71.6% A, 28.4% B, 5% C (1.2 ml/min); *t* – 64, 71.6% A, 28.4% B, 5% C (1.2 ml/min); *t* – 65, 71.6% A, 28.4% B, 5% C (0.8 ml/min).

3. Results and discussion

The available databases and tables of GU values (of known oligosaccharide structures) are based on external calibration of HPLC columns. Pump flow-rate accuracy, temperature variations, and other in-run inconsistencies lead to both sample retention time and GU value variations. For

HILIC separations, this variability can be observed in the standard deviations published for GU values of individual 2AA-labelled GSL-derived oligosaccharides (0.006–0.056 GU) [17], and the database values of 2-aminobenzamide (2AB)-labelled glycoprotein-derived oligosaccharides (0.02–0.25) – reported as values from single analyses or mean values (http://glycobase.nibrt.i.e.:8080/database/show_glycobase.action). No information on the variability of 2-aminopyridine (2-AP) or 4-ABEE-labelled oligosaccharides was found, so it was surmised that mean values are reported. This variability can complicate the initial diagnosis of oligosaccharide structure, in particular where multiple oligosaccharides have near identical GU values. This is a particular problem with HILIC of glycoprotein-derived N-linked oligosaccharides where neutral and negatively charged structures closely elute. As elution time closely correlates with the number of hydroxyl groups present [37], this may be abrogated by the use of HIAX [18]. If HIAX is not possible, the use of an internal standard would overcome the problem of variability in GU values observed due to inter-sample chromatography variations.

Previously, a dextran hydrolysate internal standard was commonly employed when separating neutral oligosaccharides, either sodium borotritiide-reduced [34,38,39] or 2-aminobenzamide-labelled [24,33,40], using a Biogel P-4 gel-filtration column. The internal standard was detected using a refractive index detector, and the sample by either a radioactivity or fluorescence detector, respectively. As two separate detectors are employed, the time delay between the detectors must be allowed for in calculating relative retention, or GU, values. Therefore, we investigated the use of 4-ABEE-labelled dextran hydrolysate as an internal standard while performing HILIC separations of fluorophore-labelled oligosaccharides.

4-ABEE has been used as a UV chromophore, detected at 304 nm, to tag GSL-derived and glycoprotein-derived oligosaccharides [15,20,35]. It is also a fluorophore with a reported excitation wavelength (Exλ) of 320 nm and an emission wavelength (Emλ) of 350 nm [19], or Exλ of 305 nm and Emλ of 360 nm [36]. These wavelengths do not overlap with those reported for many commonly used fluorophores used to label oligosaccharides [19,25,26]. Aliquots of 4-ABEE-labelled dextran hydrolysate (4-ABEE-Dex) and 2-aminobenzoic acid-labelled dextran hydrolysate (2AA-Dex) were subjected separately to HILIC over a TSKgel-Amide 80 column and detected by both UV and fluorescence. The fluorescent spectra were recorded using the conditions for detecting 2AA-labelled samples, and the UV conditions for 4-ABEE-labelled samples. 2AA-Dex was detected by fluorescence alone (Fig. 1A) but not by UV (Fig. 1B), whereas 4-ABEE-Dex was detected by UV alone (Fig. 1D) but not by fluorescence (Fig. 1C), as expected. This demonstrated that no interfering fluorescent signals from the 4-ABEE-Dex would be observed while analysing 2AA-labelled oligosaccharides. The corollary was also true, in that the 2AA-labelled samples produced no extraneous UV signals. The non-UV-absorbing nature of 2AA-labelled oligosaccharides, at 304 nm, was confirmed following a ten-fold increase of amount of 2AA-Dex being analysed (Fig. 2). No UV signal was found for the 2AA-labelled glucose oligomers (Fig. 2B). A non-glucose-oligomer peak was detected by fluorescence (Fig. 2A) and UV (Fig. 2B). However, the peak height was eighty times smaller than the peak detected for 4-ABEE-labelled glucose, and did not interfere with sample analysis. Secondly, this peak elutes prior to 2AA-labelled lactose – the smallest oligosaccharide detected when analysing GSL- or glycoprotein-derived

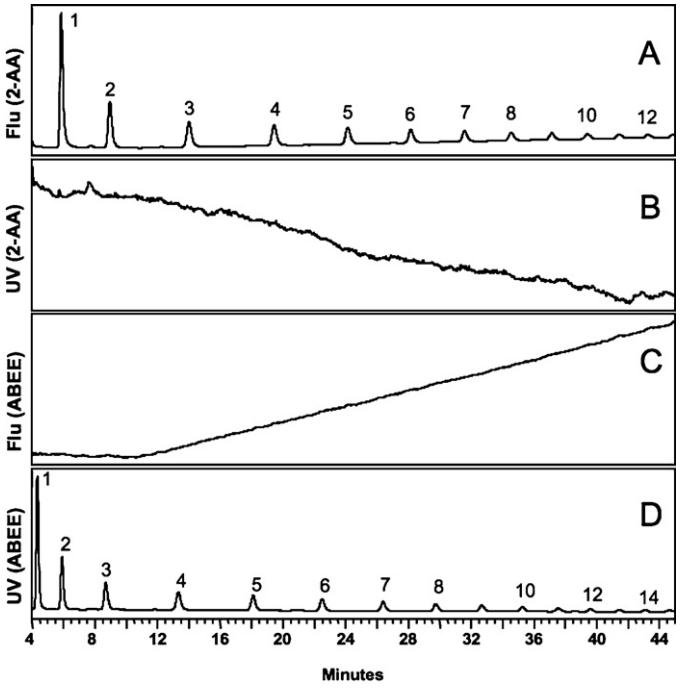

Fig. 1. HILIC separation of 2-AA-Dex and 4-ABEE-Dex with detection by fluorescence and UV. (A) 2-AA-Dex fluorescent trace using 2-AA-labelled oligosaccharide detector conditions, Exλ 360 nm and Emλ 425 nm; (B) 2-AA-Dex UV trace using 4-ABEE-labelled oligosaccharide detector conditions at 304 nm; (C) 4-ABEE-Dex fluorescent trace using 2-AA-labelled oligosaccharide detector conditions, and (D) 4-ABEE-Dex UV trace using 4-ABEE-labelled oligosaccharide detector conditions at 304 nm. The GU values for detected peaks are indicated.

Table 1
Inter- and intra-day variation in retention times for ABEE-labelled dextran oligomers.

Glucose unit (GU)	Same day injections ($n = 5$)			Different day injections ($n = 5$)		
	Retention time (min) – fluorescence	Retention time (min) – UV	Time difference (min)	Retention time (min) – fluorescence	Retention time (min) – UV	Time difference (min)
2	6.096 ± 0.004	5.972 ± 0.004	0.125	6.097 ± 0.016	5.973 ± 0.016	0.124
3	8.987 ± 0.010	8.860 ± 0.010	0.128	8.988 ± 0.025	8.861 ± 0.024	0.128
4	13.656 ± 0.018	13.528 ± 0.018	0.127	13.653 ± 0.027	13.525 ± 0.028	0.128
5	18.428 ± 0.016	18.299 ± 0.015	0.129	18.411 ± 0.032	18.281 ± 0.032	0.130
6	22.793 ± 0.016	22.664 ± 0.017	0.129	22.763 ± 0.035	22.634 ± 0.035	0.129
7	26.632 ± 0.014	26.503 ± 0.014	0.129	26.600 ± 0.035	26.472 ± 0.034	0.129
8	29.966 ± 0.014	29.856 ± 0.014	0.130	29.958 ± 0.036	29.829 ± 0.035	0.129
9	32.931 ± 0.011	32.803 ± 0.011	0.129	32.900 ± 0.038	32.769 ± 0.035	0.131
10	35.523 ± 0.011	35.393 ± 0.012	0.131	35.489 ± 0.038	35.357 ± 0.039	0.132
11	37.818 ± 0.013	37.689 ± 0.012	0.129	37.789 ± 0.037	37.661 ± 0.040	0.128
12	39.876 ± 0.010	39.745 ± 0.011	0.134	39.860 ± 0.034	39.723 ± 0.036	0.138
13	41.736 ± 0.015	41.606 ± 0.010	0.134	41.726 ± 0.036	41.587 ± 0.036	0.138
14	43.421 ± 0.013	43.289 ± 0.011	0.131	43.405 ± 0.031	43.275 ± 0.033	0.130
	Average time difference = 0.130 ± 0.003			Average time difference = 0.130 ± 0.004		

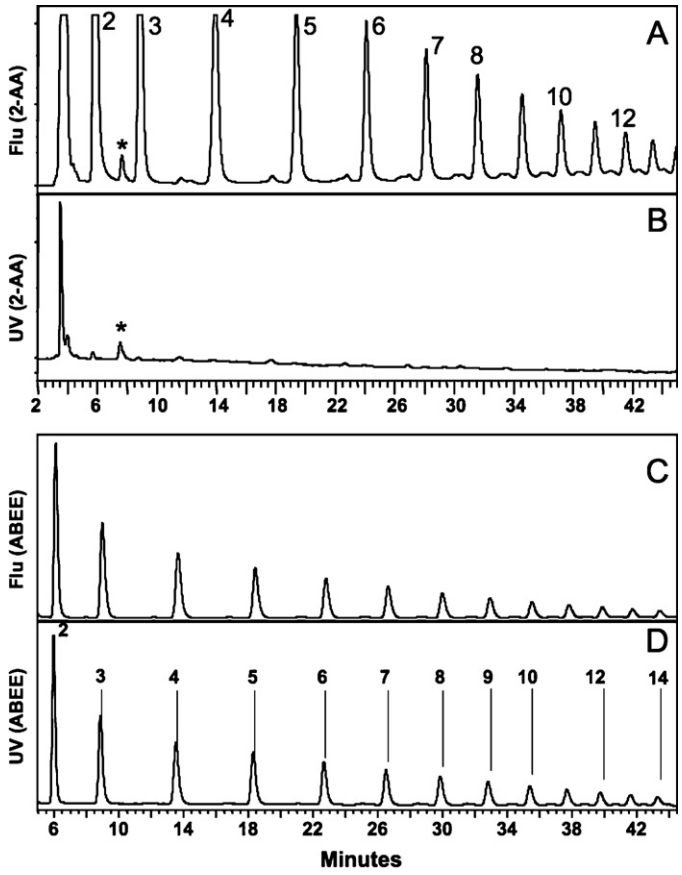

Fig. 2. HILIC separation of 2-AA-Dex and 4-ABEE-Dex with detection by fluorescence and UV. (A) 2-AA-Dex fluorescent trace using 2-AA-labelled oligosaccharide detector conditions, Exλ 360 nm and Emλ 425 nm; (B) 2-AA-Dex UV trace using 4-ABEE-labelled oligosaccharide detector conditions at 305 nm. The asterisk marks a non-glucose oligomer contaminant peak. (C) 4-ABEE-Dex fluorescent trace using 4-ABEE-labelled oligosaccharide detector conditions, Exλ 360 nm and Emλ 425 nm; and (D) 4-ABEE-Dex UV trace using 4-ABEE-labelled oligosaccharide detector conditions at 304 nm.

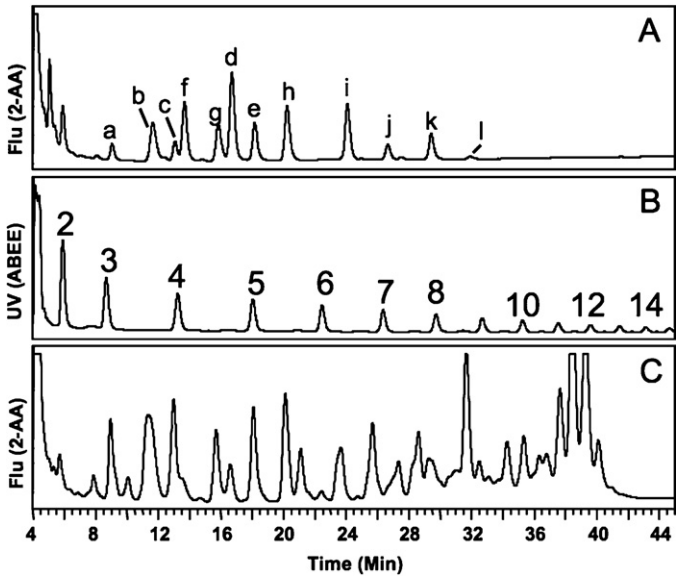

Fig. 3. HILIC separation of 2-AA-labelled oligosaccharides with 4-ABEE-Dex internal standard. (A) Fluorescent trace of 2-AA-labelled GSL-derived oligosaccharides. The letters above each peak correspond to the oligosaccharides listed in Table 2. (B) UV trace of 4-ABEE-Dex internal standard co-injected with the GSL-derived oligosaccharides shown in Panel A. (C) Fluorescent trace of a mix of 2-AA-labelled GSL-derived oligosaccharides and 2-AA-labelled N-linked oligosaccharides derived from ovalbumin and fetuin.

oligosaccharides. Therefore, 4-ABEE was chosen as the internal standard label.

4-ABEE is both a UV-absorbing chromophore and a fluorophore. This dual nature is necessary and advantageous, as the delay in signal detection between UV and fluorescence can be accurately determined by exploiting this facet of 4-ABEE. This delay is dependent on flow-rate, tubing internal diameter and flow-cell volume. Once calculated, the elution time of the internal standard is increased, or decreased, depending on whether the UV detector is before, or after, the fluorescence detector. This operation must be performed to enable determination of oligosaccharide GU values for samples analysed using different HPLC systems. The fluorescent (Fig. 2C) and UV (Fig. 2D) spectra obtained for 4-ABEE-Dex are shown. The times at the peaks heights for GU 2 to 14, for both UV and fluorescence, were noted. Table 1 shows the data for both inter- and intra-day experiments ($n = 5$). Though there is a small change, 0.019 ± 0.013 min, in the average retention times of the internal standards, there is no change in the time difference between the fluorescent and UV signals – 0.130 ± 0.004 min. However, there is an increase observed between the standard deviations of all signals when comparing the inter- and intra-day signals, which may lead to greater variance in calculated GUs when using an external standard to calibrate the HPLC column. The same degree of variance should not be observed when using the internal standard to calibrate the HPLC column. The generated formulae and R2 values for each set of data are as follows: five injections in one day, $y = 0.0000003140 \times^5 - 0.0000413513 \times^4 + 0.0022268031 \times^3 - 0.0555944841 \times^2 + 0.8428552605x - 1.4579883438$, $R^2 = 0.9999711769$, and five injections on separate days, $y = 0.0000003114 \times^5 - 0.00004105 \times^4 + 0.0022128464 \times^3 - 0.0552696717 \times^2 + 0.8397277523x - 1.4483065930$, $R^2 = 0.9999708162$.

An aliquot of a standard mix of GSL-derived 2AA-labelled oligosaccharides (Fig. 3A) was co-injected with 4-ABEE-Dex (Fig. 3B). The retention times of the GSL-derived oligosaccharides were adjusted by the calculated delays between the UV and fluorescent signals prior to GU value calculation. The GU values for the oligosaccharides were calculated from the curve-fit equation derived from the 4-ABEE-Dex standard (Table 2, column 4), or from the 2AA-Dex standard (Table 2, column 3). Typically, one 2AA-Dex standard run is performed when calculating the external standard-derived GU values, as this mimics the real-life situation of a single external standard run per preset number of samples that must be analysed.

The calculated GU values for each GSL-derived oligosaccharide, with standard deviations (Table 2, columns 3 and 4) were compared with GU values obtained using two alternative methods (Table 2, columns 1 and 2). Column 1 GU values were obtained using in-house developed software (PeakTime) to determine GU values. Column 2 GU values were obtained Waters Millennium® software to determine GU values. These data were compared to GU values obtained using a polynomial (Table 2, columns 3 and 4), as per Section 2. It was clear that use of the polynomial, using both a 2AA-labelled external standard and the 4-ABEE-labelled internal standard, to determine GU values gave slightly different GU values, but a smaller standard deviation. When GU values, as determined using the internal standard were examined, the standard deviation was on average a log factor less than those obtained using PeakTime. Additionally, when comparing the calculated standard deviations for GU units derived from the polynomial method, the standard deviations were smaller when using the 4-ABEE internal

Table 2
Comparison of GU values for GSL-derived oligosaccharides calculated using different methods.

		1	2	3	4
	GSL	GU 2AA-Dex[a]	GU 2AA-Dex[b]	GU 2AA-Dex[c]	GU ABEE-Dex[c]
a	Lac	2.029 ± 0.017	2.008 ± 0.004	2.014 ± 0.003	3.256 ± 0.002
b	GA2	2.571 ± 0.006	2.517 ± 0.027	2.554 ± 0.003	3.927 ± 0.002
c	Gb3	2.819 ± 0.011	2.782 ± 0.021	2.795 ± 0.003	4.205 ± 0.001
e	GA1	3.722 ± 0.010	3.723 ± 0.022	3.750 ± 0.004	5.258 ± 0.001
f	GM3	2.926 ± 0.010	2.933 ± 0.029	2.886 ± 0.004	4.307 ± 0.002
g	GM2	3.291 ± 0.014	3.330 ± 0.014	3.298 ± 0.003	4.764 ± 0.001
h	GM1a	4.121 ± 0.012	4.166 ± 0.015	4.141 ± 0.003	5.687 ± 0.001
i	GD3	4.261 ± 0.015	4.243 ± 0.024	4.274 ± 0.003	5.834 ± 0.001
j	GD1a	4.961 ± 0.020	5.011 ± 0.010	4.944 ± 0.003	6.575 ± 0.002
k	GD1b	5.616 ± 0.015	5.636 ± 0.018	5.578 ± 0.004	7.280 ± 0.004

[a] GU calculated using PeakTime (in-house) software.
[b] GU calculated using Empower (Waters) algorithm as per Section 2.
[c] GU calculated using Excel using polynomial as per Section 2.
For assignment of peaks see Fig. 3.
Lac, lactosylceramide; GA2, gangliotriglycosylceramide; Gb3, globotriaosylceramide; Gb4, globoteraosylceramide; GA1, gangliotetraglycosylceramide; GM3, II^3-α-*N*-acetylneuraminyllactosylceramide; GM2, II^3-α-*N*-acetylneuraminylgangliotriglycosylceramide; GM1a, II^3-α-*N*-acetylneuraminylgangliotetraglycosylceramide; GD3, II^3-α-*N*-acetylneuraminyl-α-2,8-*N*-acetylneuraminyllactosylceramide; GD1a, II^3, IV^3-α, α-Di-*N*-acetylneuraminylgangliotetraglycosylceramide; GD1b, II^3-α-*N*-acetylneuraminyl-α-2, 8-*N*-acetylneuraminylgangliotetraglycosylceramide.

standard. A mix of GSL-, ovalbumin- and fetuin-derived oligosaccharides were also separately analysed to demonstrate that the 4-ABEE-Dex elution profile covers the full range of oligosaccharides separated using HILIC over a TSKgel-Amide 80 column (Fig. 3C). Additionally, it is facile to convert from 4-ABEE-derived GU values to 2AA- or 2AB-derived GU values, as there is a linear relationship between the obtained values.

This paper demonstrates that GU values derived from use of a researcher-calculated polynomial, rather than from proprietary software, gave GU values with smaller standard deviations. Additionally, GU values derived from use of an internal standard gave the most accurate GU values. This ensures that the generated oligosaccharide GU values, when matched to databases with HILIC-derived GU values generated using an internal standard, should remove some of the ambiguity observed with structures that have similar GU values. In particular, this study demonstrates that GU values derived from use of a researcher-calculated polynomial, in combination with use of an internal standard, allows for direct comparison of data generated from multiple laboratories. This method negates any problems that can occur when comparing GU values from data generated from different commercial software packages, and when, as commonly performed, the GU values are calculated by use of an external standard. The choice of 4-ABEE-labelled dextran hydrolysate ensures that the internal standard spectral properties do not interfere with the fluorescence spectra generated by either 2AA- or 2AB-labelled oligosaccharides. The accurate measurement of GU values and the concomitant reduction in GU variability should aid in the identification of oligosaccharides following separation by HILIC.

Acknowledgements

The authors acknowledge the support of the Glycobiology Institute and Prof. R.A. Dwek.

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