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A Dual-Detection Strategy in the Chromatographic Analysis of 2-Aminoacridone-Derivatized Oligosaccharides

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A protocol has been developed involving the derivatization of glycan mixtures with 2-aminoacridone and co-injection with a dextran ladder derivatized with methyl 4-aminobenzoate (M-4AB). These two derivatizing agents have very different ultraviolet absorbance and fluorescence characteristics. A chromatographic separation using a normal-phase column support followed by in-series UV and fluorescence detection allowed simultaneous analysis of the two mixtures of the separately derivatized carbohydrates without any interference. This new approach uses the M-4AB dextran ladder derivatives as internal standards spanning the whole chromatogram, allowing an accurate and detailed comparison of glycosylation profiles. It also saves much time by avoiding the necessity of "sandwiching" an unknown glycan mixture between two chromatographic runs of a dextran ladder. The use of this technique has been demonstrated in the case of glycans released from ribonuclease B and human IgG.

The role played by oligosaccharides in modulating the physicochemical and structural properties of glycoproteins is now widely accepted.[1,2] This increased understanding in the biological function of these molecules largely stems from the fact that, over the last 10 years, major developments have occurred in the structural analysis of picomole levels of carbohydrate. Although direct analysis of oligosaccharides using ion-exchange chromatography at alkaline pH's and amperometric detection has been used extensively,[3,4] derivatization prior to analysis facilitates detection due to the formation of highly volatile[5] or fluorescent[6] molecules which can be analyzed by gas or high-performance liquid chromatography, respectively.

We have found that 2-aminoacridone (2-AMAC), covalently linked to carbohydrates via reductive amination, is an excellent reagent for the analysis of complex glycan mixtures. Besides being an intense fluorophore, 2-AMAC is highly hydrophobic; it is also neutral over a wide pH range. 2-AMAC had been used originally for the analysis of carbohydrates by slab-gel electrophoresis.[7] However, its physicochemical properties permit a wider scope in the use of this derivatizing agent. In our studies, we have found that 2-AMAC carbohydrate derivatives can be analyzed by a variety of separation (reversed and normal-phase chromatography[8] and micellar electrokinetic capillary chromatography[9−12]) and detection (UV, fluorescence,[8−12] electrospray, and matrix-assisted laser desorption/ionization mass spectrometry[8,13]) techniques.

We now report on the application of 2-AMAC for the preliminary characterization of glycan mixtures on the basis of their retention properties on a normal-phase chromatographic stationary phase, and using an aqueous mobile phase. To enhance the accuracy of our measurements, we have mixed the 2-AMAC derivatized mixtures with a dextran ladder derivatized with methyl 4-aminobenzoate (M-4AB) prior to chromatographic separation. The fluorescence and UV properties of 2-AMAC are complementary to those of M-4AB, allowing the simultaneous and distinctive monitoring of the two sets of carbohydrate derivatives by fluorescence and UV absorbance, respectively. This internal standardization procedure over the whole length of the chromatogram removes the necessity to run dextran ladder standards before and after the chromatographic analysis of a glycan mixture of interest. The methodology we describe thus saves time and ensures that peak movements between one chromatogram and another can be accounted for. It also allows meaningful interpretation of chromatographic profiles before and after enzymic digestion.

EXPERIMENTAL SECTION

Materials. Sodium cyanoborohydride, ammonium formate, dimethyl sulfoxide, trifluoroacteic acid, and methyl 4-aminobenzoate were supplied as >95% pure from Aldrich (Poole, U.K.). Glacial acetic acid was purchased as ANALAR grade from Fisher Scientific (Gillingham, U.K.). Acetonitrile was purchased from Romil (Cambridge,U.K.). Aqueous solutions were prepared using

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distilled−deionized water. N-Linked oligosaccharides, glycan mixtures from bovine ribonuclease B and human IgG, and α-fucosidase (bovine kidney) were obtained from Oxford GlycoSystems (Abingdon, U.K.).

2-AMAC was prepared according to a modification to the procedure of Barnett et al.;[14] ring closure of the 4-acetamidodiphenyl-2-carboxylic acid intermediate in the presence of polyphosphoric acid (rather than concentrated sulfuric acid) at 140 °C gave 2-acetanilacridone, which was in turn hydrolyzed by refluxing in about 52% concentrated sulfuric acid at 130 °C for 2 h. After cooling and neutralization with ammonia solution, the yellow product was collected by filtration and washed thoroughly with cold water before vacuum drying. The final product was a yellow powder which had a purity >98% by HPLC and mass spectrometric analysis.

Spectroscopic Measurements. Absorbance spectra for 2-AMAC and M-4AB were recorded in methanol solution using a 10-mm-path length cell in a Perkin-Elmer Lambda UV/visible/near-infrared spectrophotometer. Fluorescence spectra for the two compounds were also recorded in methanol solution in a 1-cm cell positioned in a Perkin-Elmer LS50B luminescence spectrometer.

Derivatization of the Dextran Ladder with 2-AMAC and M-4AB and Glycans with 2-AMAC Only. Reductive amination of the carbohydrate mixtures was carried out in DMSO/glacial acetic acid mixture, and in the presence of sodium cyanoborohydride, as detailed by us previously[12] except for the following modifications. The time and temperature for derivatization with 2-AMAC have been changed to 70 °C for 2 h. The derivatization with M-4AB requires more stringent conditions, namely, 90 °C for 1 h in the presence of 30 μL of M-4AB and the reducing agent at molar concentration.

Enzymic Hydrolysis of Glycans. The lyophilized 2-AMAC-derivatized glycan pool from IgG was treated with α-fucosidase (bovine epididymis) according to the following procedure: an aliquot (20 μL) of the enzyme solution, prepared by dissolving 0.1 unit of dried enzyme (1 unit is defined as the amount of enzyme that will hydrolyze 1 μmol of pNP-α-fucoside per minute at pH 6.0 and 37 °C) in 100 μL of 100 mM sodium citrate (pH 6.0), was added to the glycan residue and incubated at 37 °C for 18 h. The reaction solution was then freeze-dried.

Hydrophilic Interaction Liquid Chromatography. Separation of glycan mixtures was performed using a Waters Alliance 2690 instrument, which was fitted with an Oxford GlycoSystems GlycoSepN column (3.9 mm i.d. × 250 mm). About 1.5 μg of the 2-AMAC and 4 μg of the M-4AB derivatives were injected simultaneously onto the column and detected using a Waters 474 scanning fluorescence detector (volume, 5 μL; path length, 1 mm) connected in series to a Kratos Spectroflow 757 UV absorbance detector (volume, 12 μL; path length, 8 mm). The excitation and emission wavelengths of the fluorescence detector were set at 428 and 525 nm, respectively, and the absorbance wavelength at 305 nm.

Three different mobile phases were used. These varied in the rate of change in the ratio of acetonitrile (eluent A) to 250 mM ammonium formate, pH 4.40 (eluent B) with time.

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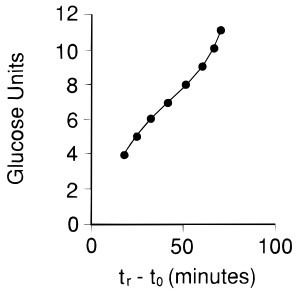

Figure 1. Regression analysis for the 2-AMAC dextran ladder run using nonlinear gradient 3 elution conditions.

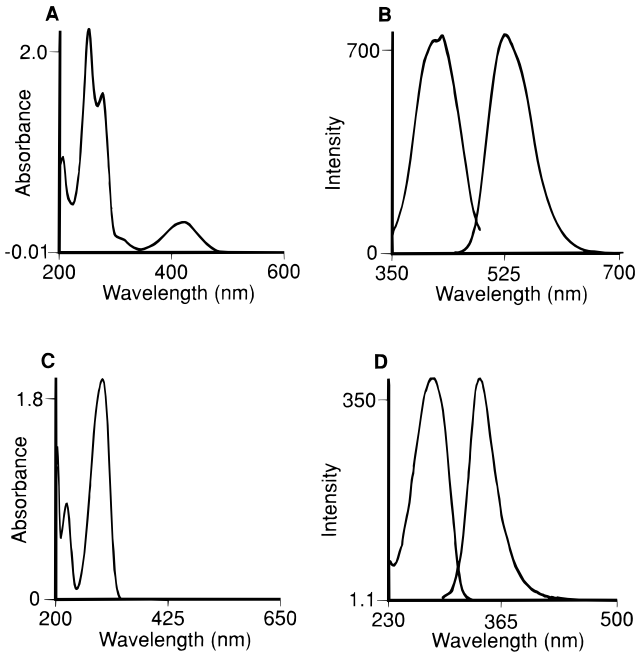

Figure 2. UV and excitation and emission spectra for 2-AMAC (A and B) and M-4AB (C and D) in methanol. Concentrations used were as follows: (A) 5×10^{-5}, (B) 2×10^{-6}, (C) 1×10^{-4}, and (D) 1×10^{-6} M.

Gradient 1. Step 1, linear gradient from 35% to 53% B for 72 min (flow rate 0.4 mL/min); step 2, linear gradient from 53% to 100% B for 3 min (flow rate 0.4 mL/min); step 3, isocratic at 100% B for 2 min (flow increased to 1 mL/min); step 4, isocratic at 100% B for 15 min (flow rate 1.0 mL/min); step 5, equilibration of the column at 30% B for 5 min (flow rate 1.0 mL/min); step 6, equilibration of the column at 30% B for 5 min (flow rate 0.4 mL/min).

Gradient 2. Step 1, linear gradient from 35% to 40% B for 20 min (flow rate 0.4 mL/min); step 2, linear gradient from 40% to 43.7% B for 30 min (flow rate 0.4 mL/min); step3, linear gradient from 43.7% to 53% B for 30 min (flow rate 0.4 mL/min); step 4, linear gradient from 53% to 100% B over 3 min (flow rate 0.4 mL/min); steps 5−8 are the same as steps 3−6 for gradient 1.

Gradient 3. Step 1, linear gradient from 35% to 39% B for 50 min (flow rate 0.4 mL/min); step 2, linear gradient from 39% to 58% B for 30 min (flow rate 0.4 mL/min); step 3, linear gradient from 58% to 100% B over 3 min (flow rate 0.4 mL/min); steps 4−7 are the same as steps 3−6 for gradient 1.

Calculation of Glucose Unit Equivalents for 2-AMAC-Derivatized Glycans. The first step was to determine glucose

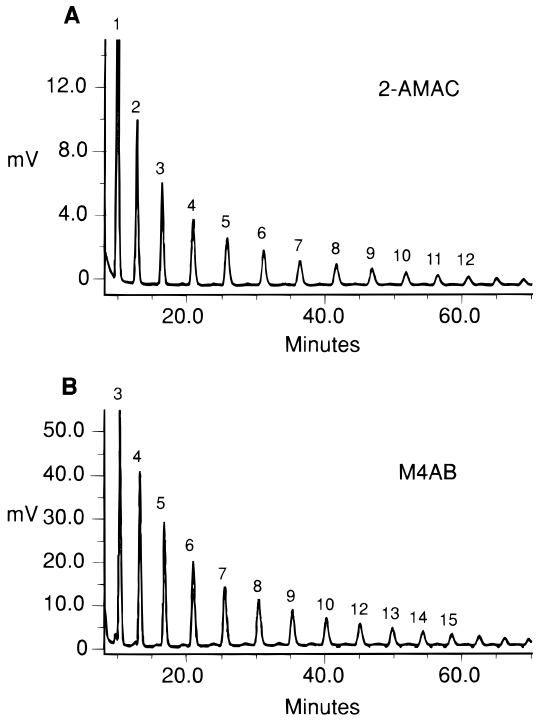

Figure 3. Co-injection of a 2-AMAC-derivatized dextran ladder and an M-4AB-derivatized dextran ladder separated by HILC using linear gradient 1: (A) fluorescence detection at excitation wavelength 428 nm and emission at 525 nm; (B) UV absorbance at 305 nm.

unit (GU) equivalents in terms of 2-AMAC for the M-4AB-derivatized dextran ladder. Figure 3 shows the co-injection of a 2-AMAC dextran ladder (visualized by fluorescence detection) with a M-4AB dextran ladder (visualized from its UV absorbance). This initial separation was used to calibrate the AMAC-derivatized ladder system for all subsequent separations with gradient 1. Calibration of the system was achieved by plotting the retention time of each 2-AMAC-derivatized carbohydrate against its actual glucose unit. The best-fit relationship was determined by applying regression analysis such that the simplest function was used that gave a value of >0.999 for the correlation coefficient, R^2. In reality, the expression for elution gradient 1 was linear ($y = mx + c$); for gradient 2, the polynomial function had the form $y = ax^3 - bx^2 + cx - d$; and for gradient 3, the function was of the type $y = ax^4 - bx^3 + cx^2 + dx + e$. The retention times for the M-4AB-labeled dextran ladder were converted into the equivalent 2-AMAC glucose units from the best-fit curve for the 2-AMAC dextran ladder (for example, see Figure 1). The conversion values are shown in Table 1 (see columns 1 and 3). For example, six glucose molecules labeled with M-4AB have a 2-AMAC GU value of 3.98. From the relative retention times of the two differently labeled dextran ladders, it appears that the 2-AMAC moiety has more interaction with the column than the M4-AB chromophore, hence the longer retention times of derivatives labeled with 2-AMAC.

These calculated GU values, from the UV-labeled dextran ladder of the dual dextran ladder experiment, were then used to internally calibrate subsequent runs by creating regression lines as described previously for the dual dextran ladder run. From these equations, the GU values for the ribonuclease B and IgG glycan pools were calculated. All these manipulations were performed using Microsoft Excel, and an example of the regression curve required for the most complex gradient, gradient 3, is shown in Figure 1.

Table 1. Comparison of Retention Times (t_R) and Calculated GU Values for Seven of the Components of a Dextran Ladder Labeled with M-4AB[a]

	gradient 1		gradient 2		gradient 3	
$(glucose)_n$	t_R (min)	GU	t_R (min)	GU	t_R (min)	GU
$n = 6$	20.93	3.98	20.92	4.02	22.67	4.03
$n = 7$	25.50	4.88	25.45	4.97	29.00	5.04
$n = 8$	30.37	5.83	30.37	5.90	36.57	6.02
$n = 9$	35.33	6.80	35.73	6.82	45.20	6.89
$n = 10$	40.32	7.78	41.57	7.75	54.83	7.79
$n = 11$	45.17	8.73	47.73	8.67	63.40	8.81
$n = 12$	49.83	9.65	54.17	9.63	68.80	9.73

[a] Details of the mobile-phase conditions used in gradients 1−3 are given in the Experimental Section.

RESULTS AND DISCUSSION

As the stationary phase ages with time due to storage or continued use, both retention times and signal characteristics (peak height and peak width) can vary. Moreover, slight variations in the composition of the mobile phase may also add to uncertainty in qualitative and quantitative analysis. To minimize the possibility of inaccurate measurements when comparing chromatograms, it is common practice to add an internal standard to each run. Criteria for the selection of suitable internal standards are that they have run times similar to those of the analytes of interest and that the two sets of compounds have physicochemical properties which are close in semblance. Sometimes these constraints lead to lack of resolution of the internal standard from the analyte. However, this situation does not arise if the two sets of compounds can be distinguished by the use of two detection systems.

The UV/visible absorbance spectrum of 2-AMAC is characterized by three maxima at wavelengths of 250, 276, and 421 nm (Figure 2A), whereas excitation of the same fluorophore at a wavelength of 421 nm gives rise to the emission spectrum shown in Figure 2B. The absorbance of 2-AMAC between 305 and 340 nm is low. This wavelength region provides a window for the use of a derivatizing agent such as M-4AB which absorbs strongly over the wavelength range 270−310 nm (Figure 2C) but does not fluoresce at 525 nm (Figure 2D), the wavelength for maximum fluorescence of 2-AMAC.

The complementarity in the spectroscopic properties of 2-AMAC and M-4AB has allowed us to devise a strategy whereby analytes of interest are derivatized with 2-AMAC and the components in a dextran ladder are derivatized with M-4AB. Both mixtures are simultaneously injected onto the column. The 2-AMAC-derivatized glycans are detected by fluorescence, whereas the M-4AB-derivatized components in the dextran ladder mixture are detected by their UV absorbance (Figure 3A,B). The latter provide adequately spaced and structurally defined internal standards along the whole chromatographic run time. The values obtained for seven glucose chains, $n = 6-12$, using the three gradients are given Table 1. Although retention times are different, calculated GU values are in excellent agreement from gradient to gradient; as the chains increase by one glucose residue, the GU value increments by 0.9 to 1.

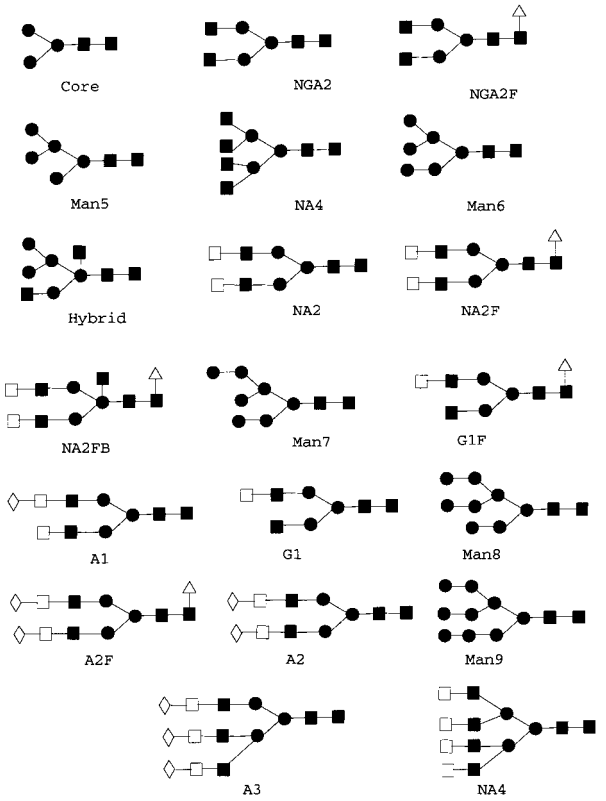

Figure 4. Structures of glycans for which GU values were determined. For simplicity, no linkages have been included. The following symbols have been used to identify the various sugar residues: (■) *N*-acetyl glucosamine, (●) mannose, (□) galactose, (△) fucose, and (◊) sialic acid.

A number of standard glycans (Figure 4) were derivatized with 2-AMAC and co-injected with M-4AB-labeled dextran ladder. The GU values were calculated, and these were used to determine the "monosaccharide rule",[15] that is, the increment in GU value per additional sugar unit. These values are given in Table 2. Figure 5 shows the direct correlation between the published GU values for glycans derivatized with 2-aminobenzamide (2-AB)[15] and GU values estimated in this study for the corresponding 2-AMAC derivatives. From the linearity of this plot and the high correlation coefficient, it is clear that GU values are independent of the derivatizing agent. In fact, it is comforting to find out that GU values obtained by either the 2-AB or the 2-AMAC methods are, within experimental error, indistinguishable. This finding allows calibration of the normal-phase chromatography system with either of the two fluorescent tags, allows correlation with previous acquired data with 2-AB, and permits the subsequent use of a derivatizing agent which is most suitable for use with other complementary or hyphenated analytical techniques, such as mass spectrometry, capillary electrophoresis, and reversed-phase chromatography.

The separation of the 2-AMAC mannose-rich components from ribonucleaseB, that is, Man5−Man9, using gradient 1 conditions is shown in Figure 6A. Simultaneously injected with this mixture was a dextran ladder derivatized with M-4AB, shown in Figure 6B. The five high-mannose 2-AMAC derivatives were identified

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Table 2. (a, Columns 1 and 2) Standard Glycans and Corresponding GU Values and (b, Columns 3−5) GU Values for Individual Sugar Residues, Calculated from Differences in the GU Values of the Individual Standard Glycans[a]

GUs of standard glycans[a]		calculated GUs of monosaccharides		
glycan	GU	sugar residue	formula used in GU estimation	estimated GU
M3N2	4.23	fucose	A2F − A2	0.41
NGA2	5.19	fucose	NA2F − NA2	0.47
NGA2F	5.66	fucose	NGA2F − NGA2	0.46
NA2	7.03	galactose	(NA2 − NGA2)/2	0.92
NGA4	6.32	galactose	(NA2F − NGA2F)/2	0.92
NA2F	7.49	galactose	(NA4 − NGA4)/4	0.87
NA2FB	7.58	bisecting GlcNAc[b]	NA2FB − NA2F	0.09
hybrid	6.95	GlcNAc	(NGA2 − M3N2)/2	0.48
NA4	9.80	GlcNAc	(NGA4 − M3N2)/4	0.52
A1	8.31	mannose	(Man5 − M3N2)/2	0.89
A2	9.48	mannose	(Man6 − M3N2)/3	0.91
A3	10.79	mannose	(Man7 − M3N2)/4	0.91
A2F	9.89	mannose	(Man8 −M3N2)/5	0.91
Man5	6.00	mannose	(Man9 − M3N2)/6	0.87
Man6	6.95	sialic acid	A2 − A1	1.17
Man7	7.89	sialic acid	A2F − (A1 + fucose)	1.14
Man8	8.79	constant term	M3N2 − (3Man + 2GlcNAc)	0.53
Man9	9.45			

[a] Values obtained using gradient 1. [b] GlcNAc between arms containing at least two more sugar residues than the core structure.

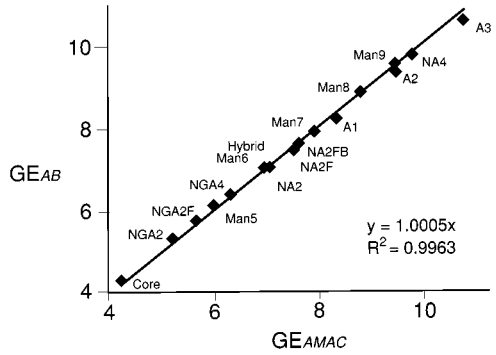

Figure 5. Comparison of glucose unit equivalents for standard glycans derivatized with 2-AMAC and those derivatized with 2-AB.[15]

by collection of fractions and MALDI-MS analysis (data not shown). Components of the M-4AB-derivatized glucose oligomers from a standard dextran hydrolysate ladder have been denoted by the number of glucose units. The separation of both sets of derivatives is dependent on size; thus, the smallest (most hydrophobic) component elutes first, and the biggest (most hydrophilic) component elutes last. Comparison of parts A and B of Figure 6 shows that the use of in-series UV absorbance and fluorescence detectors allows the detection of the M-4AB and 2-AMAC derivatives with minimum interference. As M-4AB does not fluoresce at the excitation and emission wavelengths of 2-AMAC, the chromatogram of the mannose-rich mixture was found to be identical to that run on its own (data not shown). Figure 6C,E shows the high-mannose-rich components from ribonuclease B separated using gradients 2 and 3, respectively. Figure 6D,F shows the corresponding co-injected M-4AB-derivatized dextran ladders. As the gradient slows from gradient 1 through to gradient 3, the retention times of the 2-AMAC-

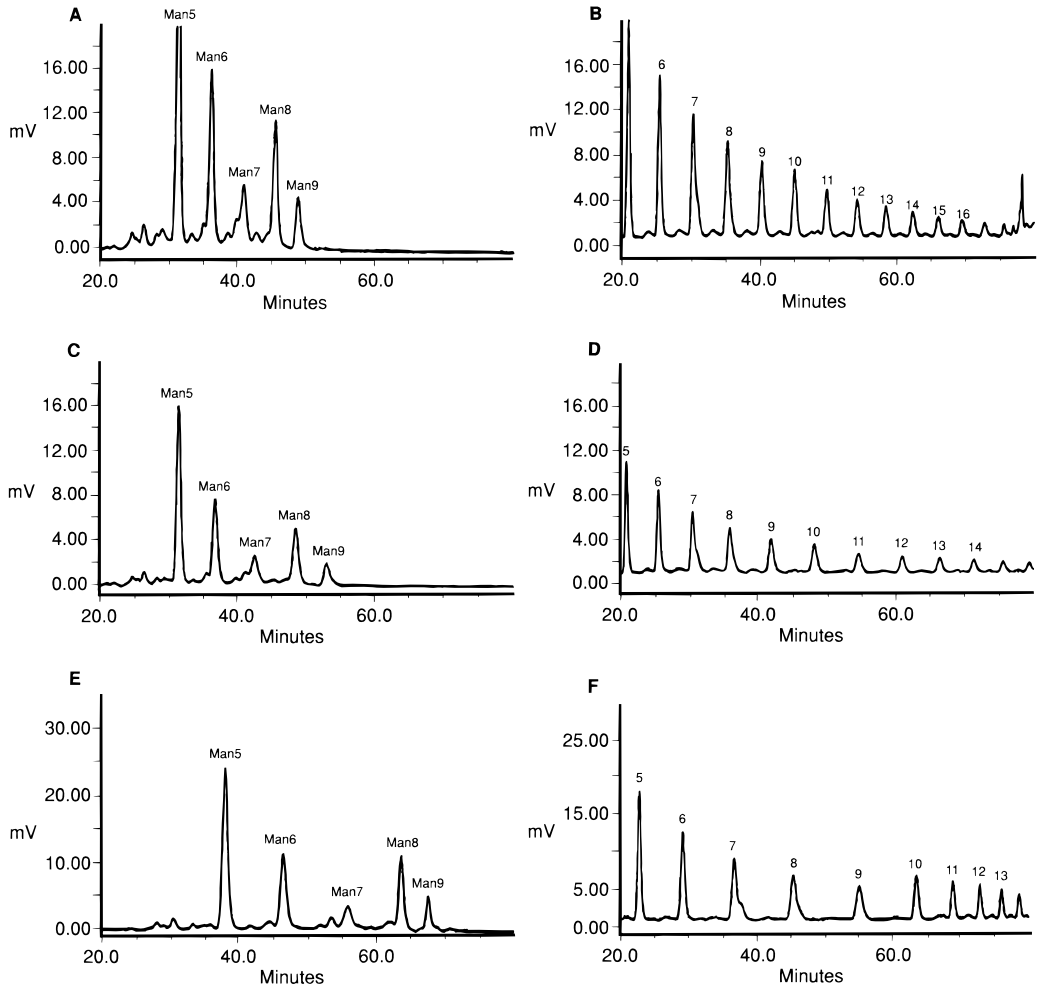

Figure 6. Chromatograms obtained after co-injection of a 2-AMAC-derivatized glycan pool released from ribonucleaseB and a dextran ladder labeled with M-4AB. (A, C, and E) The mannose-rich glycan derivatives were detected by monitoring emission at a wavelength of 525 nm after excitation at 428 nm, eluting from the GlycoSep-N with gradients 1, 2, and 3, respectively. (B, D, and F) The mixture of linear oligosaccharides was detected by UV absorbance at 305 nm, eluting from the GlycoSep-N with gradients 1, 2, and 3, respectively.

derivatized glycans from ribonuclease B and the M-4AB dextran ladder derivatives slow to a similar extent, mirroring the gradients.

To compare chromatograms run under different conditions, the elution position of the 2-AMAC-derivatized glycans was determined in terms of glucose units (GU values) by comparison with the positions of the M-4AB-derivatized components of the dextran ladder. The M-4AB dextran ladder was calibrated by reference to a 2-AMAC-derivatized dextran ladder.

Table 3 shows the retention times and GU values calculated for the mannose-rich components in the glycan pool released from ribonuclease B for the three gradients. GU values determined by this method are primarily a measure of the relative hydrophilic affinity of sugar residues to the column stationary phase. Although the retention times varied substantially from gradient to gradient, there were relatively only very small changes in the GU values. For example, for Man9, the retention time varied by nearly 19 min from gradient 1 to gradient 3, but the GU values only changed by 1%. Originally, the calibration for glycan analysis had been achieved by bracketing the sample of interest with dextran ladder calibration chromatograms. This is time-consuming, as each run may last for 2 h, depending on the resolution and sensitivity required. The advantage of internal calibration of each run is that it not only saves time but also is potentially more precise. This is an important factor as many of the monosaccharides have very similar GU values, such as core fucose and bisecting GlcNAc (Table 2).

Table 3. Retention Times (t_R) and GU Values of the Mannose-Rich Glycans in the RibonucleaseB Glycan Pool[a]

	gradient 1		gradient 2		gradient 3	
glycan	t_R (min)	GU	t_R (min)	GU	t_R (min)	GU
Man5	31.98	6.00	31.38	6.06	37.97	6.16
Man6	36.03	6.95	36.73	6.95	46.47	6.96
Man7	40.80	7.89	42.47	7.84	55.93	7.88
Man8	45.40	8.79	48.33	8.72	63.70	8.87
Man9	48.78	9.45	52.87	9.39	67.67	9.53

[a] Glycans were labeled with 2-AMAC and Co-Injected with an M-4AB-labeled dextran ladder.

Comparison of the computed GU values in Table 3 also shows that the chromatographic behavior of the branched mannose structures is very similar to that of the glucose oligomers; in fact, the difference in GU values for adjacent glycan components is close to 1. Moreover, there was absolutely no interference from the co-injection of the dextran ladder labeled with M-4AB (Figure

Table 4. Retention Times (t_R) and GU Values of 2-AMAC-Labeled Glycans in the IgG Glycan Pool, Co-Injected with an M-4AB-Labeled Dextran Ladder

	gradient 1		gradient 2		gradient 3	
glycan	t_R (min)	GU	t_R (min)	GU	t_R (min)	GU
G0	26.57	5.13	26.67	5.22	31.23	5.30
G0F	29.05	5.62	29.15	5.69	35.18	5.81
G1	30.80	5.97	30.98	6.03	37.85	6.13
G1F[a]	33.42	6.49	33.80	6.53	42.80	6.66
G1F	*b*	*b*	*b*	*b*	43.87	6.76
G2	35.82	6.96	36.58	7.00	47.75	7.12
G2F	38.40	7.47	39.60	7.50	52.60	7.55
A1	42.52	8.28	44.77	8.30	61.23	8.37
A1F[a]	44.82	8.74	47.80	8.76	64.52	8.77
A1F	*b*	*b*	48.85	8.92	65.50	8.91
A2	48.50	9.46	53.03	9.54	68.80	9.43
A2F	50.97	9.95	56.73	10.09	71.07	9.86

[a] Two isomeric forms, partially resolved. [b] Isomers not resolved using gradients 1 or 2.

Table 5. Comparison of GU Values Obtained for 2-AMAC-Derivatized Glycans Released from IgG, before and after Partial Fucosidase Treatment[a]

		untreated mixture		fucosidase-treated mixture	
peak no.	glycan	t_R (min)	GU	t_R (min)	GU
1	G0	26.57	5.13	26.63	5.15
2	G0F	29.05	5.62	28.93	5.61
3	G1	30.80	5.97	31.07	6.03
4/5	G1F	33.42	6.49	33.47	6.51
6	G2	35.82	6.96	35.98	7.00
7	G2F	38.40	7.47	38.28	7.46
8	A1	42.52	8.28	42.50	8.29
9	A1F	44.82	8.74	44.77	8.74
10	A1F[b]			45.63	8.91
11	A2	48.50	9.46	48.50	9.48
12	A2F	50.97	9.95		

[a] Numbers shown refer to those shown in Figures 6 and 7. Gradient 1 was used. [b] Isomers partially resolved.

4b). The estimation of GU values may also be useful in the acquisition of preliminary information about the structure of glycans in cases where the amounts available may not be enough to gather mass spectrometric data.

The above co-injection methodology was applied to a 2-AMAC-derivatized glycan pool released from human IgG. The glycans released from IgG are complex, and many contain core fucosylation and sialylation. This glycan pool was separated using the three gradients (see Experimental Section). GU values calculated from the three chromatograms are compared in Table 4. Again, despite the considerable change in retention time from gradients 1 to 3, GU values of all 12 components monitored varied to a much lesser extent.

The above methodology should be most suitable to allow accurate correlation of the movement of the retention time of analytes after enzymatic treatment, without the worry of retention time changes due to changes in mobile-phase conditions. To test this, we compared the movement of the retention times of the 2-AMAC-derivatized glycan pool from IgG before and after fucosidase treatment. Relative GU values are given in Table 5, whereas chromatograms obtained in each case are compared in

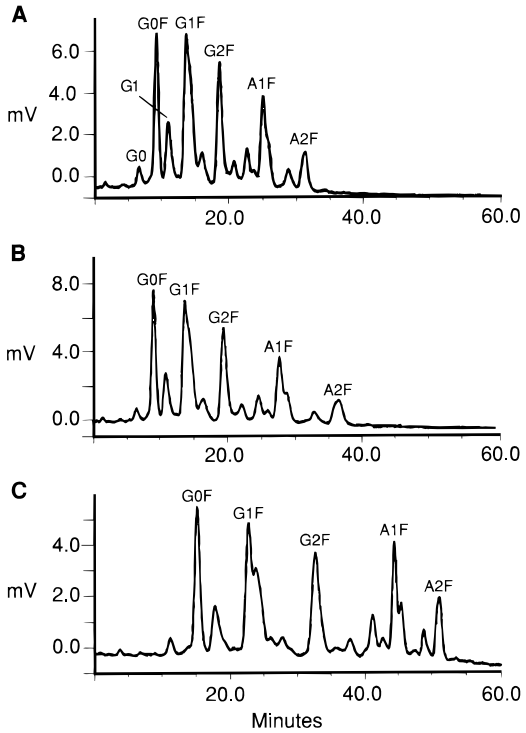

Figure 7. Chromatograms obtained from 2-AMAC-derivatized glycans released from human IgG using (A) gradient 1, (B) gradient 2, and (C) gradient 3. In all three cases, glycan mixtures were co-injected with a dextran ladder derivatized with M-4AB.

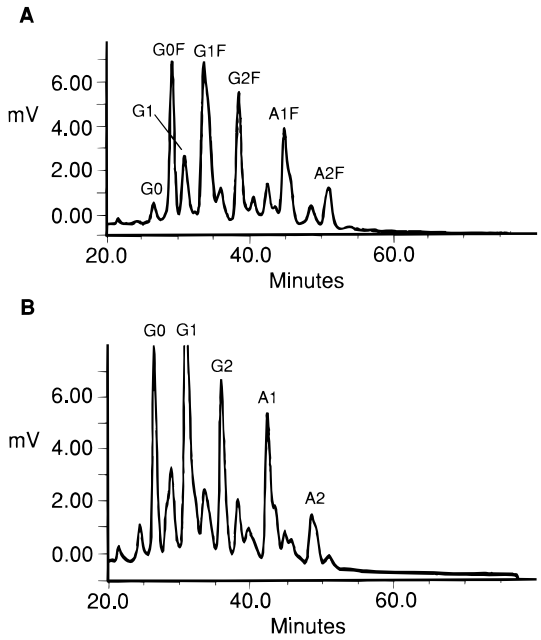

Figure 8. Fluorescence detection of 2-AMAC-derivatized glycans form human IgG before (A) and after (B) fucosidase digestion eluting with gradient 1. The glycan mixtures were co-injected with a dextran ladder derivatized with M-4AB.

Figures 7 and 8. As expected, defucosylation of the IgG glycan mixture followed by derivatization with 2-AMAC led to a shift of most of the peaks to short retention times.

Table 4 shows the GU values obtained for the peaks in Figure 7. Glycans which differ by one galactose residue differ by about 0.9 GU, whereas defucosylated glycans differ from the corresponding fucosylated molecules by about 0.5 GU. The higher

hydrophobicity of a fucose residue compared to those of mannose and galactose is not surprising; a $-CH_2OH$ group in the latter residues has been replaced by a more hydrophobic methyl group. Sialylated species have the longest retention times and the highest GU values. Comparison of GU values obtained for the high-mannose structures to those of the complex biantennary glycans from IgG shows that, in general, mannose structures have higher GU values. This must be due to the different hydrodynamic volumes occupied by the two classes of glycans leading to variations in interaction with the stationary phase.

CONCLUSIONS

We have shown that "spiking" a mixture of 2-AMAC-derivatized glycans with a dextran ladder derivatized with M-4AB allows the gathering of preliminary information regarding the structural identity of a complex carbohydrate. The use of the two derivatizing agents in combination with in-series UV absorbance and fluorescence detection makes possible the analysis of glycans of interest and the internal standards independently from one another with minimal interference. This has been found to save considerable time and to be especially useful when rationalization is made of chromatographic peak movements after enzyme treatment of glycan mixtures.

Finally, our study has laid the foundation for a preliminary identification of the components of an unknown mixture of glycans that are too low in concentration to be identified by other analytical techniques but intense enough to be visualized after 2-AMAC derivatization. To achieve this objective of relating structure to retention characteristics, we are presently delineating the stuctural rules that govern the behavior of 2-AMAC-derivatized glycans under the chromatographic conditions outlined.

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