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Rhodamine B amine as a highly sensitive fluorescence derivatization reagent for saccharides in reversed-phase liquid chromatography

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Abstract

6-Rhodamine B amine functions as a highly sensitive fluorescence derivatization reagent for mono- and oligosaccharides; it reacts with the reducing end of saccharides under acidic conditions. The fluorescent derivatives of five monosaccharides can be separated within 25 min by reversed-phase liquid chromatography with isocratic elution. The detection limits (S/N = 3) for mono-, di-, and oligosaccharides are 7–51, 13, and 9–35 fmol/20 μl injection, which correspond to analyte concentrations of 35–255, 65, 45–175 nM, respectively. We have applied this derivatization method successfully to the analysis of the components of oligosaccharides in glycoproteins (ribonuclease B and fetuin) following their acidic or enzymatic hydrolysis. The results from these analyses are in good agreements with the reported values established previously.

Keywords: Derivatization, LC; Food analysis; Rhodamine B amine; Saccharides

1. Introduction

Carbohydrates are distributed widely in plants and animals as structural components of glycoproteins and glycolipids. In recent years, trace amounts of glycoconjugates have been recognized to be associated with important biological processes, such as cell recognition, signal transmission, and cell differentiation [1–3]. In addition, a number of therapeutic proteins produced by recombinant techniques in mammalian cells are expressed having glycan moieties. Since glycosylation affects the biological activity, lifetime, cellular uptake, and specificity of proteins, convenient and highly sensitive methods to assay saccharides are required to clarify the relationship between glycoconjugates' structures and functions, and to control the quality of protein-based drugs.

Various methods have been reported for the analysis of saccharides, such as the use of (capillary) gas chromatography (GC) [4–7], high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) [8–10], liquid chromatography (LC) [11–19], and capillary electrophoresis (CE) [20–25]. Because saccharides possess neither suitable fluorophores nor chromophores, derivatization steps are required inevitably for any highly sensitive detection of them by LC or CE. Of the various derivatization methods, fluorescent labeling methods—reacting the reducing carbonyl group of saccharides with fluorescent amines—have been used most widely because of their high sensitivity and feasibility. The resulting Schiff base derivatives can be detected directly [17–20] or after their reductive conversion to more-stable amines [11–16,21–25]. In particular, 2-aminopyridine [12–15] is a widely used reagent for allowing the precise analyses of sugar chains [13–15]. Unfortunately, most aromatic amine-based reagents emit fluorescence at short wavelengths (350–500 nm), and their fluorescence can be interfered readily by endogenous substances.

In this study, we describe LC-based methods for analyzing mono- and oligosaccharides by using rhodamine B amine (RBA) as a pre-column fluorescence derivatization reagent. RBA exhibits intense fluorescence and a long fluorescence wavelength. Saccharides can be covalently bonded to RBA to form Schiff bases under acidic conditions (Fig. 1). We

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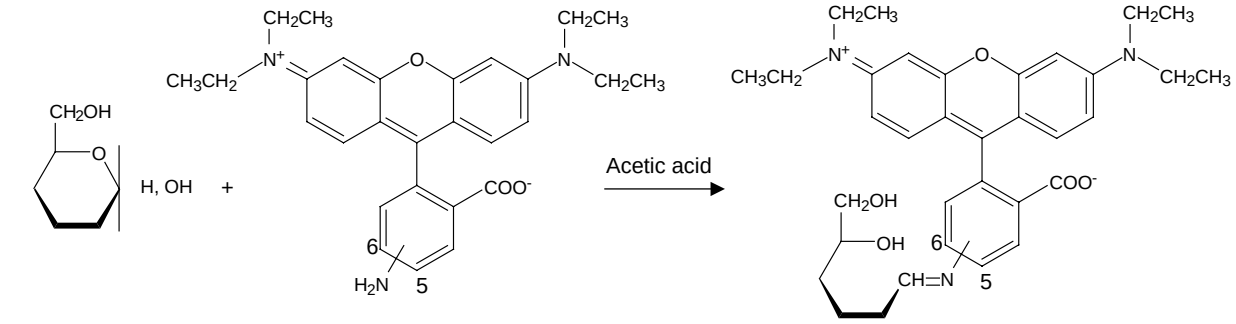


Fig. 1. Fluorescence derivatization of saccharides using 5- and 6-RBA.

optimized the conditions for the pre-column fluorescence derivatization of mono- and oligosaccharides, and, furthermore, applied this method successfully to analyses of the components of the glycoproteins ribonuclease B and fetuin.

2. Experimental

2.1. Apparatus

The LC system we used comprised a Jasco PU-980 pump (Tokyo, Japan), a Jasco DG-980-50 three-line degasser, a Rheodyne 7125 syringe-loading sample injector (Cotati, CA, USA) equipped with 20-μl sample-loop, a Jasco FP-1520 fluorescence detector, and a Hitachi D-2500 integrator (Tokyo, Japan). The fluorescence detection was operated at excitation and emission wavelengths of 550 and 580 nm, respectively.

Uncorrected fluorescence excitation and emission spectra were measured using a Hitachi F-3010 fluorescence spectrophotometer and a 10 mm × 10 mm quartz cell; spectral bandwidths of 5 nm were used for both the excitation and emission monochromators. ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Jeol JNM-A500 spectrometer (Tokyo, Japan) at 500 MHz with tetramethylsilane as an internal standard.

2.2. Chemicals and solutions

All chemicals and solvents were of the highest purity available and were used as received. Distilled water, purified using a Milli-Q II system (Millipore, Milford, MA, USA), was used to prepare all aqueous solutions. Rhodamine B amine was obtained from Sigma (St. Louis, MO, USA) as a mixture of isomers. Oligosaccharides (lacto-*N*-neohexaose, lacto-*N*-fucopentaose, lacto-*N*-difucohexaose, and disialyllacto-*N*-tetraose; Table 1) were purchased from Seikagaku Corp. (Tokyo, Japan). Ribonuclease B (derived from bovine pancreas) and fetuin (derived from fetal calf serum) were obtained from Sigma. *N*-Glycosidase F (200 units/μl; derived from *Chryseobacterium meningosepticum*) was purchased from Calbiochem (San Diego, CA, USA). Neuraminidase (0.01 units/μl; derived from *Arthrobacter ureafaciens*) was purchased from Sigma.

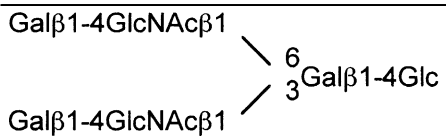
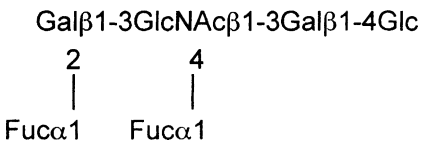
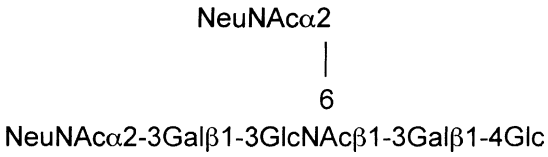
Sample solutions of mono- and oligosaccharides were prepared in water. RBA solution (3 mM) and 0.1 M acetic acid solution were prepared in methanol. *N*-Glycosidase F solution was prepared with 20 mM Tris–HCl buffer (pH 7.5) containing 50 mM sodium chloride and 1 mM EDTA disodium salt. Neuraminidase solution was prepared with 20 mM Tris–HCl buffer (pH 7.5) containing 25 mM sodium chloride.

2.3. Purification of 6-RBA

Commercial RBA (100 mg) is a mixture of 5-amino and 6-amino isomers. Column chromatography (25 cm × 3.5 cm i.d. column) was performed to separate these isomers on silica gel 60 (approximately 120 g, 70–230 mesh; Merck, Darmstradt, Germany) using acetone/methanol (1:1, v/v) as the eluent. The respective fractions containing 6-RBA and 5-RBA were determined visually, collected, and evaporated to dryness under reduced pressure. The resulting powders (5-RBA: 28 mg; 6-RBA: 23 mg) were used without further purification. The purities of these isomers were checked by their fluorescence on silica gel TLC plates (Merck 5554), eluting with the same solvent used for column chromatography, which confirmed that the purities of both 5-RBA (Rf = 0.34) and 6-RBA (Rf = 0.59) were >99%.

The structures of 5- and 6-RBA were confirmed by their ^{1}H NMR spectra. The values of the chemical shifts (δ) of 5- and 6-RBA aromatic protons are listed. 5-RBA (DMSO-d_6): 6.86 (d, J = 2.44 Hz, 2H), 6.88 (dd, J = 2.44, 8.24 Hz, 1H), 6.95 (d, J = 8.24, 1H), 6.98 (dd, ArH, J = 2.44, 9.46 Hz, 2H), 7.34 (d, J = 2.44, 1H), 7.46 (d, J = 9.46, 1 H); 6-RBA (DMSO-d_6): 6.40 (d, J = 2.14 Hz, 1H), 6.79 (d, J = 2.44 Hz, 2H), 6.85 (dd, J = 2.14, 8.55 Hz, 1H), 6.88 (dd, J = 2.44, 9.46 Hz, 2H), 7.23 (d, J = 9.46 Hz, 1H), 7.84 (d, J = 8.55 Hz, 1H).

Table 1
Structures of the oligosaccharides examined and their abbreviations

Compound	Structure	Abbreviation
Lacto-*N*-neohexaose		LNnH
Lacto-*N*-fucopentaose		LNFP
Lacto-*N*-difucohexaose		LNDFH
Disialyllacto-*N*-tetraose		DSLNT

2.4. Derivatization procedure

A sample solution (10 μl) of the saccharide was placed in a screw-capped tube (100 mm × 13 mm i.d.) and dried under a stream of nitrogen gas. Three millimolar 6-RBA and 0.1 M acetic acid (50 μl each) were added to the residue. The tube was closed tightly and heated at 90 °C for 120 min (mono- and disaccharides and non-sialic oligosaccharides) or 60 min (sialic oligosaccharide). The reaction mixture was cooled to room temperature and diluted 10-fold with the mobile phase and then an aliquot of the resulting solution (20 μl) was injected onto the chromatograph.

2.5. LC conditions

The 6-RBA derivatives of carbohydrates were separated on a reversed-phase column by isocratic elution under the following conditions. The flow rate was 1 ml/min under an ambient column temperature (23 ± 2 °C), respectively. We used a Capcell pak C_{18} UG80 LC column (150 mm × 4.6 mm i.d., 5 μm, Shiseido, Tokyo, Japan) for the analysis of mono- and disaccharides and sialic- and non-sialic oligosaccharides, a Mightysil RP-18 GP aqua LC column (150 mm × 4.6 mm i.d., 5 μm, Kanto Chemicals, Tokyo, Japan) for the analysis of the monosaccharides released from glycoproteins, and a Develosil ODS UG-5 LC column (250 mm × 4.6 mm i.d., 5 μm, Nomura Chemical, Aichi, Japan) for the analysis of the oligosaccharides released from glycoproteins.

The mobile phases were acetonitrile/100 mM aqueous acetate buffer (pH 4.0; 25:75, v/v for mono- and disaccharides and sialic oligosaccharides; 20:80, v/v for the analysis of non-sialic oligosaccharides and the oligosaccharides released from glycoproteins) and acetonitrile/methanol/100 mM acetate buffer (pH 4.0; 20:5:75, v/v for the analysis of monosaccharides released from glycoproteins). The columns were washed at the end of each day's measurements by passing acetonitrile/methanol (1:1, v/v) through them at a flow rate of 1 ml/min for 1 h.

2.6. Component analysis of mono- and oligosaccharides in ribonuclease B and fetuin

2.6.1. Acid hydrolysis procedure

A portion of a solution of ribonuclease B or fetuin (0.1 mM each; 50 μl) was placed in a screw-capped tube (100 mm × 13 mm i.d.) and then dried under a stream of nitrogen gas. Four molar aqueous trifluoroacetic acid solution (200 μl) was added to the residue. The tube was closed tightly and heated at 100 °C for 6–8 h. The reaction mixture was cooled to room temperature and dried under a stream of nitrogen gas. Twenty-five millimolar aqueous sodium carbonate (10 μl) and acetic anhydride (2.5 μl) were added to the residue, and the mixture was left to stand at room temperature for 30 min. After evaporation under a stream of nitrogen, the residue was subjected to the derivatization procedure.

2.6.2. N-Glycosidase F digestion procedure

A portion of a solution of ribonuclease B or fetuin (0.1 mM each; 50 μl) was placed in a micro test tube

(75 mm × 12 mm i.d.) and then dried under a stream of nitrogen gas. The residue was dissolved in 50 mM phosphate buffer (pH 7.5; 50 μl) and then an aqueous denaturant solution (containing 1 M 2-mercaptoethanol, 15% triton X-100 and 2% sodium dodecylsulfate; 2.5 μl) was added. After heating at 100 °C for 10 min, *N*-glycosidase F (2000 units/10 μl) was added and the mixture was incubated at 37 °C for 16 h. The enzymatic reaction was terminated by the addition of glacial acetic acid (2 μl) and acetone (200 μl). After centrifugation at 1000 × *g* for 10 min, the supernatant (200 μl) was placed in a screw-capped tube and dried under a stream of nitrogen gas, and then the residue was subjected to the derivatization procedure or the neuraminidase-meadiated digestion procedure (Section 2.6.3).

2.6.3. Neuraminidase digestion procedure

The residue produced by *N*-glycosidase F digestion was dissolved in 50 mM acetate buffer (pH 5.0; 50 μl) and then neuraminidase (0.1 units/10 μl) was added and the mixture was incubated at 37 °C for 16 h. The reaction solution was dried under a stream of nitrogen gas, and then the residue was subjected to the derivatization procedure.

3. Results and discussion

3.1. Determination of mono- and disaccharides

3.1.1. Derivatization

We used five mono- and disaccharides (Man, Lac, Glc, Xyl, and Rib) for our optimization studies. Commercial RBA is a mixture of its 5-amino and 6-amino isomers. Fig. 1 presents a scheme for the fluorescence derivatization for saccharides using RBAs. The separated isomers were compared for the differences in the fluorescence properties (i.e. wavelengths and intensities) of their resulting saccharide derivatives. The peak areas obtained for the derivatives of 6-RBA were larger than those of 5-RBA (data not shown). There are no great differences between the fluorescence excitation and emission maxima and the spectral shapes of 5-RBA and 6-RBA. Thus, 6- we used RBA in the following experiments. The fluorescence excitation and emission maxima of the RBA derivatives of saccharides appear at 552 and 573 nm, respectively.

The addition of acid accelerates the derivatization reaction. Among the acids we examined (acetic acid, trifluoroacetic acid, trichloroacetic acid, formic acid, and acetic anhydride), acetic acid gave the largest peak areas consistently for all of the saccharides. By varying the acetic acid concentration in the range 0–0.5 M, we found that the maximum peak area was obtained at approximately 0.1 M. The reaction proceeded at temperatures above 45 °C and the rate increased upon increasing the temperature, but the derivatives decomposed slightly at temperatures >100 °C. At 90 °C, the peak areas reached their maximum values after 2 h, after which time they remained constant. From these results, we selected the following conditions: 0.1 M acetic acid, a reaction temperature of 90 °C, and a reaction time of 2 h. The 6-RBA derivatives of saccharides were stable for at least 1 week in the daylight at room temperature. Since the derivatives are sufficiently stable, we did not need to apply any further stabilizing procedure (e.g. reductive amination of the derivatives).

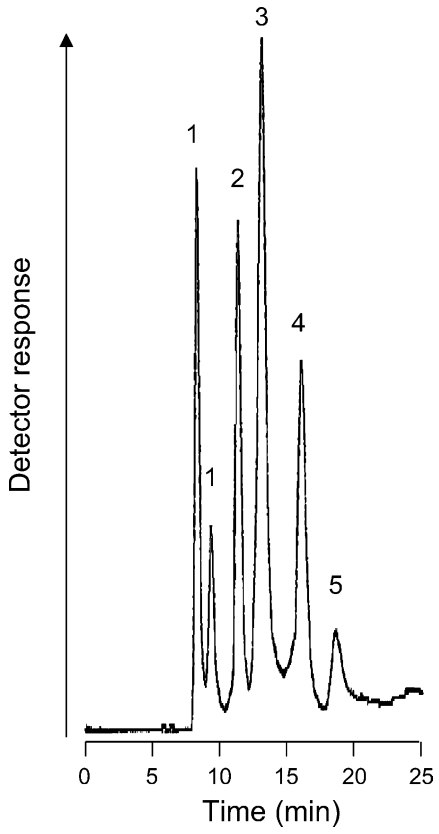


Fig. 2. LC-Elu chromatogram of the 6-RBA derivatives of mono- and disaccharides (10 pmol each on the column). Peaks: (1) lactose; (2) glucose; (3) mannose; (4) xylose; (5) ribose. LC conditions are described in Section 2.5.

3.1.2. LC separations

Fig. 2 presents a typical chromatogram obtained when using a standard mixture of the mono- and disaccharides. We achieved good separation of the 6-RBA derivatives within 25 min on the ODS column by isocratic elution with acetonitrile/100 mM acetate buffer (pH 4.0; 25:75, v/v) as the mobile phase. Each 6-RBA derivative of these saccharides presented a single peak, except for that of lactose, which is a disaccharide of glucose and galactose. At first we believed that the second peak is either a 6-RBA-derivatized glucose or galactose that is formed by acidic hydrolysis, but the retention time of the second peak is different from that obtained for either monosaccharide derivative. Therefore, a simple hydrolysis does not provide the reason for the appearance of the second peak in the derivatization of lactose;

Table 2
Detection limits and retention times for mono- and disaccharides

Compound	Detection limit[a] (fmol)	Retention time (min)
D-Maltose	8	8.3
D-Lactose	13[b]	8.4[b]
D-Cellobiose	7	8.5
D-Galactose	13	10.6
D-Glucose	13	11.5
D-Mannose	7	13.2
D-Arabinose	14	15.8
D-Xylose	19	16.2
D-Lyxose	18	17.7
D-Ribose	51	18.8
D-Fucose	16	20.6
N-Acetyl-D-galactosamine	9	11.9
N-Acetyl-D-glucosamine	16	12.8
D-Sorbose	ND[c]	–
D-Fructose	ND[c]	–

[a] Defined as the amount in the injection volume (20 μl) giving a signal-to-noise ratio of 3.
[b] Calculated from the first lactose peak.
[c] ND means "not detected".

the true cause remains unknown. Under the selected LC conditions, 6-RBA itself was retained strongly on the column and did not interfere with the analyses of the saccharides, but the over-accumulation of 6-RBA on the column causes a large, broad peak to appear in the chromatogram or a gradual increase in the height of the baseline. Therefore, we washed the column daily as described in Section 2.5. We also examined the effects of the pH of the mobile phase on the chromatograms in the range from 2.0 to 8.0. The fluorescence peak areas for all the mono- and disaccharides increased upon decreasing the value of pH, but decreasing the pH caused a broadening of the peaks; thus, we selected pH 4.0 as a compromise value. Table 2 lists the retention times and detection limits for the mono- and disaccharides when using the present method. In this approach, aldose-type reducing sugars can be analyzed with highly sensitively, but ketose-type reducing sugars, such as sorbose and fructose, cannot be detected.

3.1.3. Calibration graph, precision, and detection limits

The relationships between the peak areas and the amounts of the individual mono- and disaccharides were linear over concentration ranges from 0.1 to 100 pmol/20 μl injection volume, which correspond to analyte concentrations of 500 nM to 500 μM. The linear correlation coefficients were greater than 0.991 for all of the saccharides we studied. The precision was established by repeated determinations ($n = 5$) using a mixture of the five saccharides (50 μM). The relative standard deviations (R.S.D.s) were 1.2 (Lac), 2.3 (Glc), 1.8 (Man), 3.8 (Xyl), and 5.4% (Rib), respectively. The detection limits (S/N = 3) were 13 (Lac), 13 (Glc), 7 (Man), 19 (Xyl), and 51 (Rib) fmol/20 μl injection, which correspond to analyte concentrations of 65 (Lac), 65 (Glc), 35 (Man), 95 (Xyl), and 255 nM, respectively. These values are approximately five times lower than those reported previously to have been obtained using an LC/fluorescence system [19]. Furthermore, the detection limit values, with respect to analyte concentrations, of the present method are about the same as those described in a previous paper when using a CE/laser-induced fluorescence detection system [22].

3.2. Determination of oligosaccharides

3.2.1. Derivatization and separation conditions

The derivatization and separation conditions for oligosaccharides were optimized using three non-sialic oligosaccharides (lacto-*N*-neohexaose (LNnH), lacto-*N*-fucopentaose (LNFP), and lacto-*N*-difucohexaose (LNDFH)) and one sialic oligosaccharide (disialyllacto-*N*-tetraose (DSLNT)) (Table 1), as in the case with mono- and disaccharides. The optimum derivatization conditions for non-sialic oligosaccharides were almost the same as those for the mono- and disaccharides, with the exception that DSLNT reacted more rapidly with 6-RBA and its desialylation occurred after 1 h under the derivatization conditions.

Fig. 3 presents a typical chromatogram of the 6-RBA derivatives of non-sialic oligosaccharides. The three non-sialic oligosaccharides were separated within 25 min, each as a single peak. In contrast, we detected sialic DSLNT as two peaks in the chromatogram; they correspond to DSLNT itself and its desialylated product. The intensity of the peak of the desialylated product increased upon changing the reaction time from 1 to 3 h at 90 °C. Therefore, we selected a reaction temperature of 90 °C and a reaction time of 1 h for the derivatization of DSLNT.

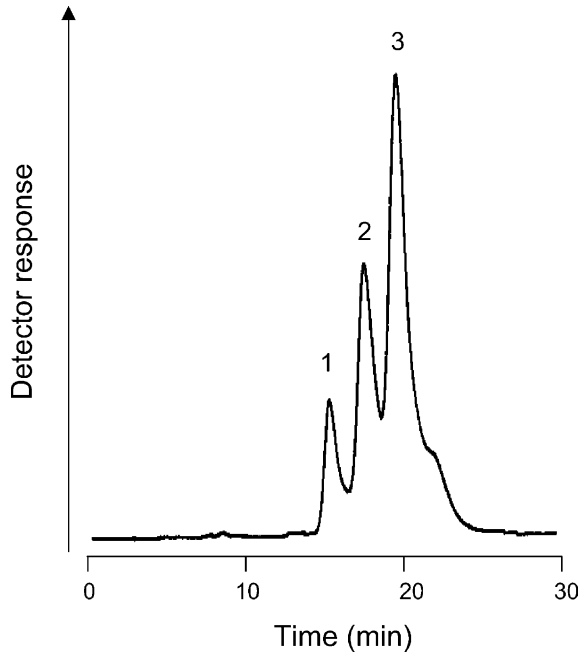


Fig. 3. LC-Elu chromatogram of the 6-RBA derivatives of oligosaccharides (10 pmol each on the column). Peaks: (1) LNnH; (2) LNDFH; (3) LNFP. LC conditions are described in Section 2.5.

3.2.2. Calibration graph, precision, and detection limits

The relationships between the peak areas and the amounts of individual oligosaccharides were linear over a concentration range from 0.1 to 100 pmol/20-μl injection volume, which are values that correspond to 500 nM to 500 μM/10-μl sample solution. The linear correlation coefficients were >0.995 for all of the oligosaccharides we studied. The precision, expressed as R.S.D. values, is 1–3% ($n = 5$) for all four of the oligosaccharides (50 μM). The detection limits (S/N = 3) were 35 (LNnH), 28 (LNDFH), 9 (LNFP), and 13 (DSLNT) fmol/20-μl injection, which correspond to analyte concentrations of 175 (LNnH), 140 (LNDFH), 45 (LNFP), and 65 nM (DSLNT), respectively.

3.3. Compositional analysis of ribonuclease B and fetuin

To investigate the applicability of the present assay method, we applied it to the compositional analysis of model proteins (ribonuclease B and fetuin) that possess structurally well-defined sugar chains. Ribonuclease B (molecular mass: 15,500) has a single *N*-glycosylation site at Asn-34, to which high-mannose-type oligosaccharides, having five to nine mannose residues, are attached (Fig. 4A) [26–28]. Fetuin (molecular mass: 48,000) is an α-globulin and has three *O*-linked sugar chains and three *N*-linked sugar chains. The *N*-linked sugar chains fall into six classes, with each defined by the number of sialic residues (0–5). The major species are di-, tri-, and tetra-sialylated oligosaccharides (Fig. 4B), with most of them having a triantennary structure [29]. Biantennary structures have also been identified [30].

Table 3 lists the calculated values of the component monosaccharides derived from ribonuclease B and fetuin after their acid hydrolyses. Using our present method, we determined the abundances of mannose and *N*-acetylglucosamine in the ribonuclease B and mannose, *N*-acetylglucosamine, galactose, and *N*-acetylgalactosamine in the fetuin. These calculated values agree well with values reported in the literature [31–33]. It is known that furfural-like compound are formed as by-products of the acid hydrolysis of saccharides, but we conclude that the formation of 2-furfural is negligible under our present derivatization conditions because (1) the corresponding peak of the 2-furfural/6-RBA derivative was not detected in the chromatogram and (2) the quantitativity and the sensitivity of each saccharide are fully preserved.

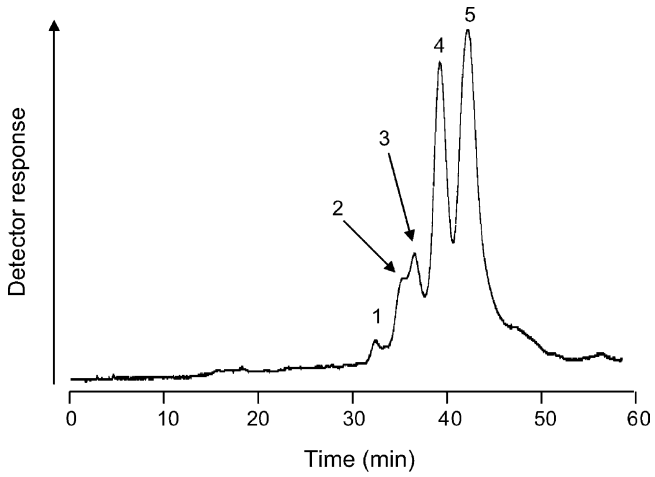


Fig. 5. LC-Elu chromatogram of the 6-RBA derivatives of oligosaccharides released from ribonuclease B (5 nmol) upon *N*-glycosidase F-mediated digestion. Peaks: (1) Man 9; (2) Man 8; (3) Man 7; (4) Man 6; (5) Man 5. LC conditions are described in Section 2.5.

Fig. 5 presents a chromatogram of the 6-RBA derivatives of the oligosaccharides released from ribonuclease B upon *N*-glycosidase F-mediated hydrolysis. *N*-Glycosidase

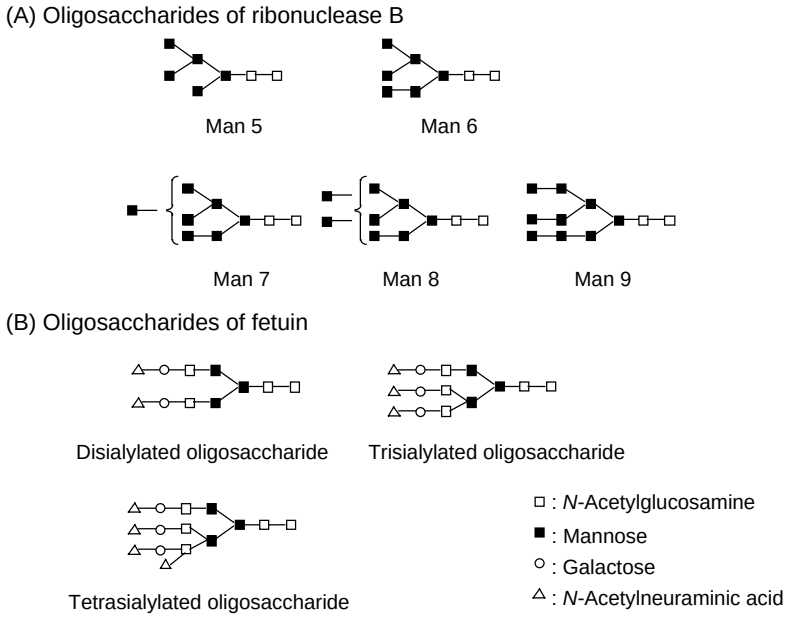


Fig. 4. Structures of *N*-linked oligosaccharides in ribonuclease B (A) and fetuin (B).

Table 3
Monosaccharide compositions of ribonuclease B and fetuin

Glycoprotein	Content (mol monosaccharide/mol protein)			
	Gal	GalNAc	GlcNAc	Man
Ribonuclease B			2.94 (3.8)[a] (2.27 ± 0.3)[b]	6.52 (1.7)[a] (6.84 ± 0.6)[b]
Fetuin	10.4 (2.6)[a] (9.4–9.7)[c]	0.7 (1.4)[a] (1.2–1.85)[c]	6.8 (2.2)[a] (5.8–13.5)[c]	7.1 (2.9)[a] (6.6–7.2)[c]

[a] R.S.D. value (%) established by repeated determinations ($n = 5$).
[b] Ref. [31].
[c] Ref. [32,33].

F hydrolyzes *N*-linked sugar chains selectively. The 6-RBA derivatives were eluted in the order of their increasing hydrophilicity, with the larger Man 9 eluting first and the smaller Man 5 last. Constituent ratios, which we calculated from the peak areas of oligosaccharides in ribonuclease B, were 55 (Man 5), 30 (Man 6), 14 (Man 7 + 8), and 0.7% (Man 9), respectively. These values are in good agreement with those reported by Fu et al. [34].

Fig. 6 displays chromatograms of the oligosaccharides released from fetuin by (A) *N*-glycosidase F-mediated digestion, (B) *N*-glycosidase F- and neuraminidase-mediated digestions, and (C) *N*-glycosidase F-mediated digestion followed by acid hydrolysis. In Fig. 6A, no desialylated peaks were detected, in contrast with Fig. 6B and C. These results are consistent with the reported data and suggest that present derivatization procedure is applicable for the analysis of sugar chains present in glycoproteins.

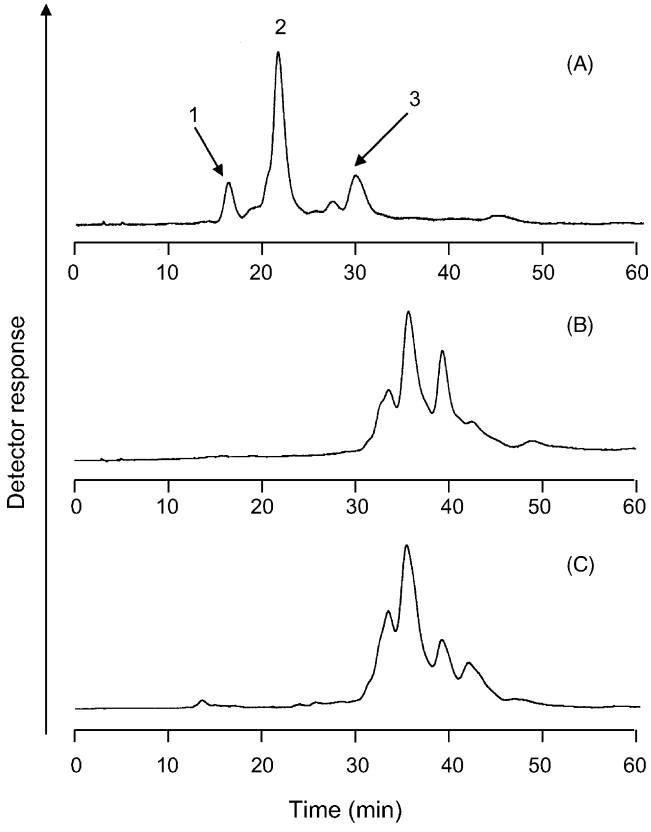


Fig. 6. LC-Elu chromatograms obtained from the (A) *N*-glycosidase F-mediated, (B) *N*-glycosidase F- and neuraminidase-mediated, and (C) *N*-glycosidase F-mediated/acid hydrolysis of fetuin. Peaks: (1) tetrasialylated oligosaccharide; (2) trisialylated oligosaccharide; (3) disialylated oligosaccharide. LC conditions are described in Section 2.5.

4. Conclusions

We have found that 6-RBA is a highly sensitive fluorescence derivatization reagent for reducing sugars. This reagent is isolated readily from commercial RBA, which is a mixture of 5- and 6-RBA, and reacts selectively with mono- and oligosaccharides under acidic conditions. Separation of these saccharide derivatives occurs with high sensitivity when using reversed-phase LC. We have applied this method successfully to the determination of oligosaccharides and the compositional analysis of saccharides in glycoproteins. Our results indicate that this method is a powerful tool for the analyses of mono- and oligosaccharides of various bioactive glycoproteins. Rhodamine derivatives are highly fluorescent and stable and, therefore, rhodamine-labeled saccharides will provide higher sensitivity and resolution to CE/laser-induced fluorescence detection systems. This study is now in progress.

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