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Note

High-pressure liquid chromatography of sialic acid-containing oligosaccharides

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For the definition of the specificity of sialosyltransferases, it is essential to establish the structure of the sialosylated products formed in *in vitro* incubations. For example, when lactose is used as an acceptor in such incubations, a mixture of two isomeric sialosylated structures potentially is formed[1–3]. Likewise, complex mixtures of sialo-oligosaccharides may result from sialosylation *in vitro* of mucin-type glycoproteins, such as ovine submaxillary asialo-mucin[4] and [^{14}C]Gal-GalNAc-protein[5]. For structural studies, these oligosaccharide products generally are isolated by Bio-Gel filtration[4,5], and identified by t.l.c.[4] and p.c.[1,2] techniques. These methods, however, are time consuming and do not always lead to a complete separation of all components.

Therefore, we have developed a high-pressure liquid chromatographic procedure for the separation and isolation of such sialo-oligosaccharides. By use of an isocratic development, base-line separation of sialic acid, and 3′- and 6′-sialyllactose was obtained (Fig. 1). Similarly, an excellent separation of all the sialo-oligosaccharides obtained by β-elimination of the *in vitro* sialosylated mucins was achieved (Fig. 2). For this separation, a linear gradient was applied after 25 min to shorten the analysis time, since, in an isocratic run, β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol would have been eluted after 65 min, and the tetrasaccharide after a few hours. It must be noted that not only the water content in the mobile phase affected the elution times, but also the pH and the phosphate concentration. Raising the pH increased the elution times, whereas they were decreased at higher phosphate concentrations of the solvent.

Since the procedure presented herein does not require pre- or post-column derivatization, the oligosaccharides can be recovered quantitatively from the eluate, thus allowing further structural investigations by such methods as methylation, nuclear magnetic resonance, and periodate oxidation. Furthermore, because of the sensitive detection, very small samples can be handled. It is possible to detect, by

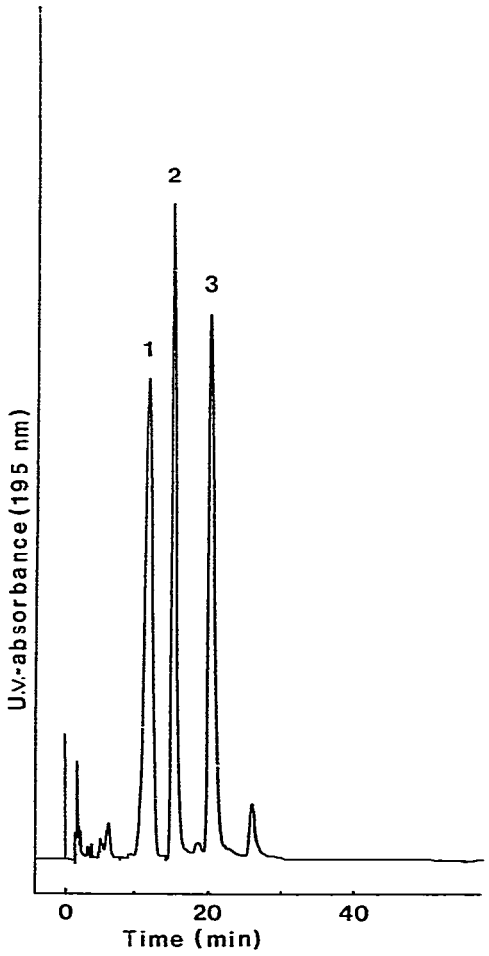

Fig. 1. High-pressure liquid chromatographic separation of a mixture of (1) sialic acid, (2) 3′-sialyllactose, and (3) 6′-sialyllactose. Mobile phase: 18:7 (v/v) acetonitrile–15mM potassium phosphate (pH 5.2). Flow rate: 2 mL/min.

u.v.-absorption measurement at 195 nm, 0.6 nmol of sialo-oligosaccharide at a signal-to-noise ratio of 4:1.

In addition to its applicability to sialyltransferase specificity studies, the present l.c. procedure is of value in the determination of the specificity of neuraminidase. This is clearly demonstrated by the preparation of β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol from α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol by digestion with Newcastle-disease virus neuraminidase. L.c. showed that, under the conditions described, this neuraminidase selectively removes the sialic acid attached to the D-galactosyl residue.

In view of the excellent ability for separation by following the procedure described herein, it may be expected that also sialo-oligosaccharides of higher

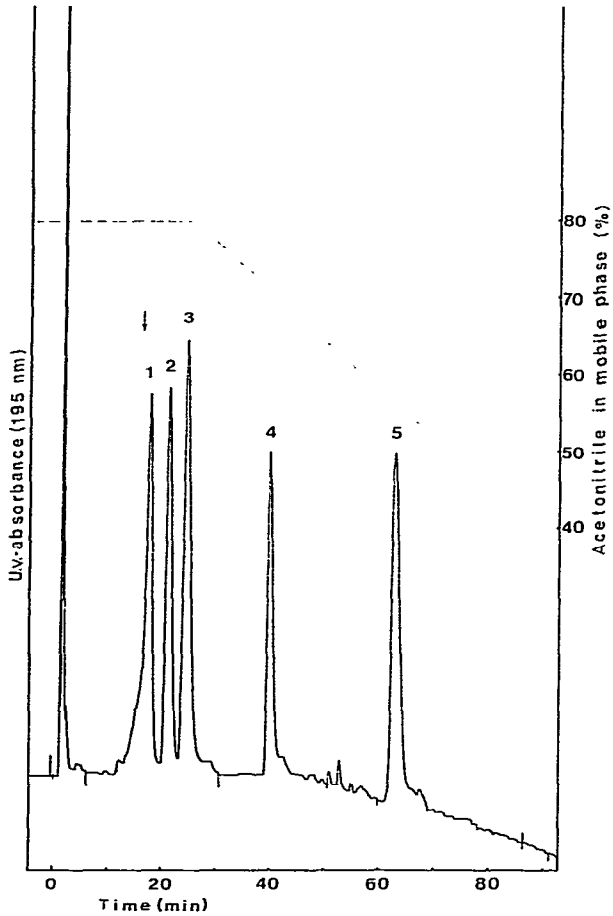

Fig. 2. High-pressure liquid chromatography of mucin-derived sialo-oligosaccharides. The chromatogram was developed isocratically for 25 min with 4:1 (v/v) acetonitrile–15mM potassium phosphate (pH 5.2), after which a linear gradient was applied to decrease the acetonitrile concentration (-----) in the mobile phase. The flow rate was 2 mL/min. (1) Sialic acid, and the digosaccharides, (2) α-NeuAc-(2→6)-D-GalNAcol, (3) α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol, (4) β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol, and (5) α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc(2→6)]-D-GalNAcol were recorded by u.v.-absorption measurement. The radioactive compound α-[^{3}H]NeuAc-(2→3)-D-[^{14}C]Galol (elution time marked by the arrow) was assayed by liquid-scintillation counting of the eluate.

molecular weight can be separated likewise, thus facilitating the preparation of oligosaccharides from milk[6,7], from the urine of sialidosis patients[8], and from other body fluids and tissue extracts.

EXPERIMENTAL

Preparation of oligosaccharides. — α-NeuAc-(2→6)-GalNAcol was obtained by β-elimination of ovine submaxillary mucin as described before[9]. α-NeuAc-(2→3)-

β-D-Gal*p*-(1→3)-D-GalNAcol and α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol were prepared from fetuin[10]. The structure of the trisaccharide was confirmed by 360-MHz ^{1}H-n.m.r. spectrometry[11]. The branched trisaccharide β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol was prepared by a short digestion of the aforementioned tetrasaccharide with neuraminidase from Newcastle-disease virus (strain B1, American Type Culture Collection, Rockville, MD 20852), which was cultured on 10 day-old chicken embryos and isolated from the allantoic fluid according to standard procedures. The incubation mixture consisted of the tetrasaccharide (0.4 μmol), sodium phosphate (80 μmol, pH 7.0), and Newcastle-disease virus particles containing 57 mU of neuraminidase (as assayed with 3′-sialyllactose as substrate[12]) in a total volume of 0.8 mL. After incubation for 5 min at 37°, the mixture was applied to a column (0.6 × 6 cm) of Dowex 1-X8 (AcO^-, 100–200 mesh) at 4°. The column was washed with water and subsequently the acidic compounds were eluted with 4 bed-volumes of M ammonium acetate (pH 5.2). After lyophilization of the eluate, the residue was redissolved in 50mM ammonium acetate (1 mL, pH 5.2), and the sample was applied to a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh), which was equilibrated and eluted with the same buffer. The sialic acid-containing fractions corresponding to the elution volume of a sialo-trisaccharide were pooled and concentrated, and aliquots were chromatographed by l.c. as described in the next paragraph. 360-MHz ^{1}H-n.m.r. spectrometry revealed that the compound was homogeneous and was identical to authentic β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol[11]. The disaccharide α-[^{3}H]NeuAc-(2→3)-D-[^{14}C]Galol was obtained after ^{3}H-sialosylation of β-D-[^{14}C]Gal*p*-(1→3)-β-D-GalNAc–protein by a fetal calf-liver sialosyltransferase preparation. This disaccharide arises as a minor peeling product from the trisaccharide α-[^{3}H]NeuAc-(2→3)-β-D-[^{14}C]Gal*p*-(1→3)-β-D-GalNAc, and can readily be isolated from the other reduced oligosaccharides by gel filtration[5]. The structure assignment of this disaccharide product was based on the following observations: it contained ^{3}H- and ^{14}C-sugar in equimolar amounts; after treatment with neuraminidase (*Clostridium perfringens*, Sigma Chemical Co., St. Louis, MO 63178), the neutral remainder contained only ^{14}C-radioactivity and migrated like galactitol upon high-voltage electrophoresis, whereas all ^{3}H-radioactivity was recovered in the acidic fraction; upon gel filtration, the compound had a molecular size smaller than α-NeuAc-(2→6)-D-GalNAcol, and greater than sialic acid (Sigma); upon t.l.c.[4], it migrated like a sialo-oligosaccharide distinguishable from α-NeuAc-(2→6)-D-GalNAcol.

High-pressure liquid chromatographic separations. — A Perkin–Elmer Series 2 Liquid Chromatograph equipped with a Rheodyne injection valve and with a Perkin–Elmer LC-75 variable-wavelength detector operating at 195 nm was used, and the chromatograms were recorded with a Philips PM 8222 recorder at a 10 mV span. The chromatograph was fitted with a column (4 × 250 mm) of Lichrosorb-NH_2 (particle size 5 μm, Merck). The mobile phase consisted of a mixture of acetonitrile (Lichrosolv grade, Merck) and de-ionized, distilled water containing 15mM potassium phosphate (pH 5.2). The separation of sialyllactose isomers was performed iso-

cratically with an 18:7 (v/v) mixture, and that of mucin-derived oligosaccharides by starting with an isocratic elution with a 4:1 (v/v) mixture for 25 min, after which a linear gradient of increasing water content at a rate of 0.6% per min was applied. Samples (10–20 μL) containing 20–25 nmol per oligosaccharide were injected, and the flow rate of the solvent mixtures was maintained at 2 mL/min.

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