Carbohydrate Research, 119 (1983) 303–309
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

A high-pressure liquid chromatography method for the detection of *O*-β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-D-glucose α-(2→3)- and α-(2→6)-sialyltransferases

DAVID H. JOZIASSE, WILLEM M. BLANKEN, PIET L. KOPPEN, AND DIRK H. VAN DEN EIJNDEN
Department of Medical Chemistry, Vrije Universitet, van der Boechorststraat 7, 1007 MC Amsterdam (The Netherlands)

(Received September 21st, 1982; accepted for publication, February 14th, 1983)

By use of a micromethod based on methylation[1], various sialyltransferases linking sialic acid to acceptors containing the terminal structure β-D-Gal*p*-(1→4)-D-GlcNAc have been detected in several tissues[2]. This method allows the unequivocal determination of the specificity of such sialyltransferases present in crude tissue preparations, but is rather time-consuming. Therefore, its use for sialyltransferase purification procedures, where a rapid determination of the enzyme specificity is often essential, is limited.

Recently, we have developed a rapid method for the separation of sialic acid-containing oligosaccharides by liquid chromatography under elevated pressure (h.p.l.c.)[3]. This method was adapted to study the specificity of *O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose (*N*-acetyllactosamine) sialyltransferases in tissue homogenates, as well as enzyme preparations at different stages of purification. The products obtained by sialylation *in vitro* of *N*-acetyllactosamine were separated by h.p.l.c. and characterized by comparison with standards of sialyl-*N*-acetyllactosamine. In the present study, we describe the use of this procedure in establishing the specificities of sialyltransferases in several tissues.

Analysis of the sialyl-N-acetyllactosamine standards. — Methylation and hydrolysis of the product of the reaction of CMP-*N*-acetylneuraminic acid (CMP-NeuAc) and β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc catalyzed by a human placenta homogenate yielded only one tri-*O*-methyl-D-[^{14}C]galactose, which migrated identically to the 2,4,6-isomer (Fig. 1, Lane a). This indicated that an oligosaccharide of the structure α-Neu*p*Ac-(2→3)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc was formed by the enzymic incubation. This result is in agreement with the earlier established specificity of human placenta β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl sialyltransferase[2]. Methylation analysis of the product of sialylation of β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc by a partially purified sialyltransferase preparation from bovine colostrum yielded 2,3,4-tri-*O*-methyl-D-[^{14}C]galactose as the only radioactive methylated sugar (Fig. 1, Lane b). Hence, the product of this incuba-

0008-6215/83/$ 03.00

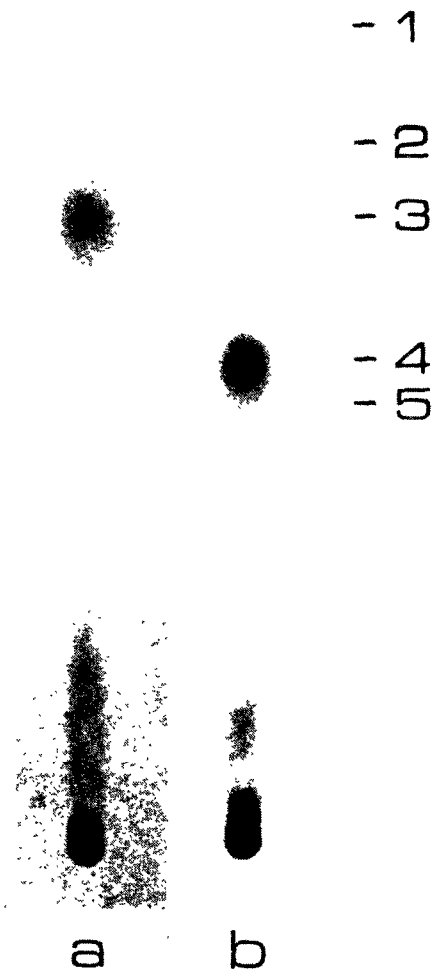

Fig. 1. T.l.c. of methyl ethers of D-[^{14}C]galactose derived from sialyl-*N*-acetyllactosamine synthesized by: (a) a homogenate of human placenta and (b) a partially purified bovine colostrum enzyme. Radioactivity was detected by autoradiography and reference compounds were detected with aniline phthalate. References were: (1) 2,3,4,6-tetra-*O*-tetramethyl-D-galactose, (2) 2,3,6-tri-*O*-methyl-D-galactose, (3) 2,4,6-tri-*O*-methyl-D-galactose, (4) 2,3,4-tri-*O*-methyl-D-galactose, and (5) 3,4,6-tri-*O*-methyl-D-galactose.

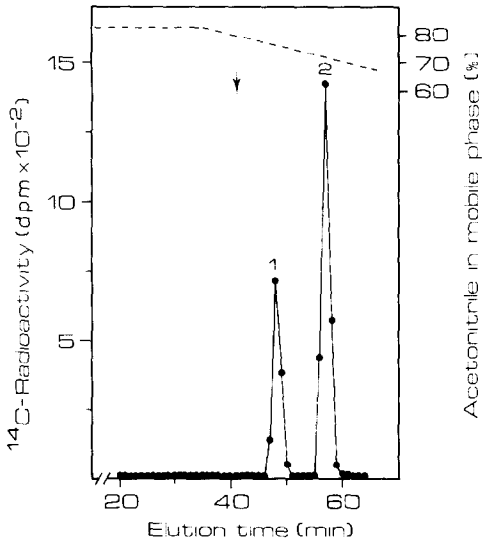

Fig. 2. H.p.l.c. of sialic acid and sialylated *N*-acetyllactosamines. A mixture of α-NeuAc-(2→3)- and α-NeuAc-(2→6)-β-D-[^{14}C]Gal-(1→4)-D-GlcNAc standards (0.5 and 1.0 nCi, respectively) was injected together with unlabeled sialic acid. Separation and detection of the standards was performed as described in the Experimental section. The elution positions of the standards are: (1) α-NeuAc-(2→3)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc; (2) α-NeuAc-(2→6)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc. The arrow indicates the elution position of sialic acid.

tion could be identified as α-NeuAc-(2→6)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc. Previous studies on the specificity of the colostrum sialyltransferase with different high- and low-molecular-weight acceptors have also indicated that this enzyme transfers sialic acid to O-6 of the terminal D-galactopyranosyl groups[4,5]. The two isomeric sialyl-*N*-acetyllactosamine standards could be excellently separated from sialic acid as well as from each other in our h.p.l.c. system (Fig. 2). When injected separately, each isomer gave one single peak in the chromatogram.

Sialyltransferase specificities in various tissues. — The high-pressure liquid chromatographic system was used to distinguish between *N*-acetyllactosamine α-(2→3)- and α-(2→6)-sialyltransferase activities in a microsomal membrane preparation from human liver, and in homogenates of fetal calf liver and normal human-blood platelets. [^{14}C]Sialylated *N*-acetyllactosamine, obtained with the human liver preparation, had the same retention time as standard α-NeuAc-(2→6)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc (Fig. 3A). No trace of radioactivity was found in the fractions in which the other sialyl-*N*-acetyllactosamine isomer was eluted. Thus, human liver shows only α-(2→6)-sialyltransferase activity with *N*-acetyllactosamine as an acceptor, a result that is consistent with that of previous experiments using asialo-α_1-acid [^{3}H]glycoprotein as the acceptor for the human-liver sialyltransferase[6]. The small peak of ^{14}C-radioactivity eluted with a retention time of 30–32 min (Figs. 3 A–C) is probably due to a contaminant present in the CMP-

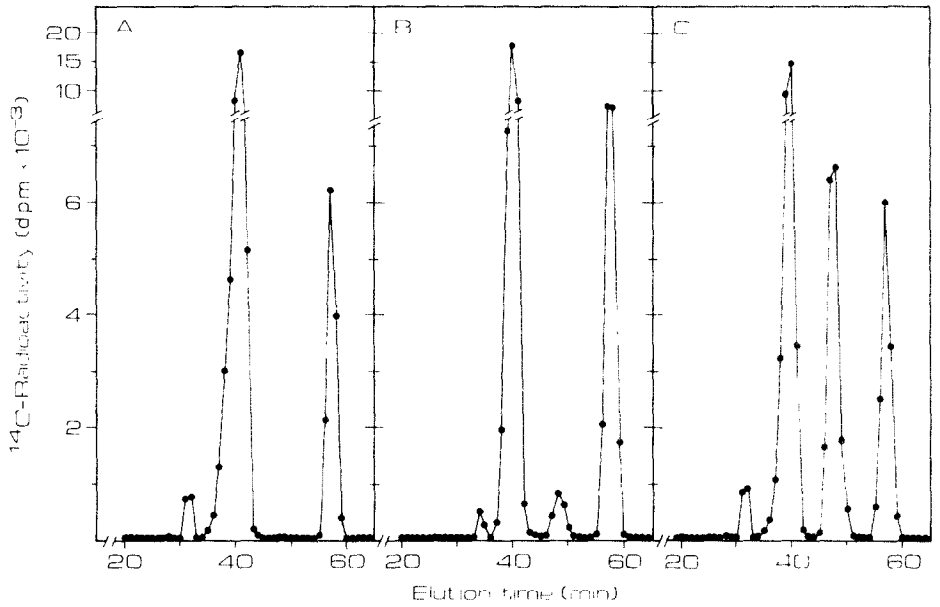

Fig. 3. Sialyltransferase specificities in various tissues. A microsomal preparation from human liver (panel A), and homogenates of fetal calf liver (panel B) and human-blood platelets (panel C) were used to transfer [^{14}C]sialic acid from CMP-[^{14}C]NeuAc to *N*-acetyllactosamine. After precipitation of protein, the products containing [^{14}C]sialyl-*N*-acetyllactosamine were separated by high-pressure liquid chromatography as described in the Experimental section. [^{14}C]NeuAc (derived from the precursor CMP-[^{14}C]NeuAc) and [^{14}C]sialyl-*N*-acetyllactosamine were detected by counting the radioactivity in the eluted fractions.

[^{14}C]NeuAc used. Blank runs in which the enzyme was omitted showed that 1–3% of the radioactivity was eluted in this position, the exact proportion varying with the batch of CMP-[^{14}C]NeuAc used.

In contrast, the incubation of CMP-[^{14}C]NeuAc and β-D-Gal*p*-(1→4)-D-GlcNAc with fetal calf liver homogenate yielded two radioactive sialylated products which, on h.p.l.c., showed the same retention times as those of the 3′- and 6′-sialyl-*N*-acetyllactosamine standards, respectively (Fig. 3B). Indeed, with asialo-α_1-acid [^{3}H]glycoprotein as an acceptor, α-(2→3)- as well as α-(2→6)-sialyltransferase activity had previously been demonstrated in the latter tissue[5].

With human-blood platelets also, both sialyl-*N*-acetyllactosamine isomers were formed (Fig. 3C), a result that was expected since, with asialo-α_1-acid [^{3}H]glycoprotein as acceptor, β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucoside α-(2→3)- as well as α-(2→6)-sialyltransferase activities appeared to occur in these cells[7]. Recently, it was shown that human platelets are capable of transferring sialic acid in an α-(2→3)- and an α-(2→6)-linkage to *p*-nitrophenyl β-D-galactopyranoside[8]. The enzymes involved in these transfers, however, may be different from the sialyltransferases detected with *N*-acetyllactosamine and asialo-α_1-acid [^{3}H]glycoprotein.

In all instances investigated, the qualitative results obtained with the h.p.l.c. method described herein were not different from those previously established by methylation analysis of sialylated asialo-α_1-acid [^{3}H]glycoprotein. The relative activities of the α-(2→3)- and α-(2→6)-sialyltransferases towards *N*-acetyllactosamine observed with the h.p.l.c. method, however, are not necessarily identical with those found in our former studies in which asialo-α_1-acid [^{3}H]glycoprotein was used as an acceptor. This is illustrated in particular by the results obtained for fetal calf liver. With the high-molecular-weight acceptor, a ratio of 9:11 could be estimated for the α-(2→3)- and α-(2→6)-sialyltransferase activities[2], whereas α-sialyl-(2→6)-*N*-acetyllactosamine was the main product where *N*-acetyllactosamine was the acceptor (Fig. 3B). Differences in K_m or V_{max}, or both, with different acceptors are likely to influence the relative reaction rates.

In conclusion, the method described herein is rapid and convenient for establishing the specificities of the β-D-galactopyranosyl-(1→4)-*N*-acetylglucosamine sialyltransferases present in various tissues. The whole analysis, including the sialyltransferase incubation, can be completed in less than 4 h.

EXPERIMENTAL

Materials. — Human placenta and human liver were obtained from the Academisch Ziekenhuis der Vrije Universiteit, Amsterdam. Fetal calf liver was from a local slaughterhouse. Human-blood platelets were obtained from the Centrale Laboratorium van de Bloedtransfusiedienst, Amsterdam. *N*-Acetyllactosamine was generously donated by Drs. S. David, J. Alais, and A. Veyrières, Paris, France. Partially methylated D-galactose standards were a kind gift of Dr. P. Stoffyn, Waltham, Massachusetts. CMP-[^{14}C]NeuAc (213 Ci/mol) was purchased from New England Nuclear, Boston, MA 02118, and diluted with unlabeled CMP-NeuAc[9] to the desired specific radioactivity. All other chemicals were of the best quality available from commercial sources.

Preparation and analysis of sialyl-N-acetyllactosamine standards. — Standards of sialyl-*N*-acetyllactosamine were prepared in the following way: Bovine colostrum 2-acetamido-2-deoxy-D-glucoside β-(1→4)-D-galactosyltransferase (6 mU), purified by a modification[10] of the procedure of Barker *et al.*[11], was incubated with 2-acetamido-2-deoxy-D-glucose (1.6 μmol), UDP-[^{14}C]Gal (160 nmol, specific radioactivity 18 Ci/mol), sodium cacodylate buffer (pH 7.3, 8 μmol), and manganese dichloride (1.6 μmol) in a final volume of 80 μL, for 16 h at 37°. The product, *N*-acetyl-[^{14}C]lactosamine, was separated from UDP-[^{14}C]Gal by ion-exchange chromatography on Dowex 1-X8 (Cl^-, 200–400 mesh) ion-exchange resin and purified by gel filtration on a column (1.6 × 200 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated in 50mM ammonium acetate, pH 5.2.

Part of the β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc was sialylated with a partially purified preparation of bovine colostrum β-D-galactoside α-(2→6)-sialyltransferase[12], and the remainder with a homogenate of human placenta as a source

of β-D-galactoside α-(2→3)-sialyltransferase[2]. The incubation mixtures contained, in a final volume of 50 μL, β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc (12.4 nmol), CMP-NeuAc (27.5 nmol), Tris–maleic acid (pH 6.7, 4 μmol), and α-(2→6)- (2.3 mU) or α-(2→3)-sialyltransferase (25 μU, enzyme activities assayed as described in ref. 4). No detergent was added. After incubation for 180 min at 37°, each reaction was stopped by cooling on ice, and the mixture diluted to 1.0 mL with ice-cold, distilled water and centrifuged at 120 000*g* for 60 min to sediment particulate material. The supernatant solution containing the sialylated product was subjected to ion-exchange chromatography on Dowex AG 1-X2 (AcO⁻, 100–200 mesh) resin to remove the precursor *N*-acetyl-[^{14}C]lactosamine. The column was washed with water, the sialylated oligosaccharide eluted with M pyridinium acetate, pH 5.2, and the eluate lyophilized. Part of each of the products was used as a standard in h.p.l.c. The remainder was subjected to methylation analysis. Methylation and subsequent isolation of the methylated sialyl-*N*-acetyllactosamine were performed as described previously[2]. The methylated samples were hydrolyzed with 2M trifluoroacetic acid (400 μL) for 1 h at 120°, the mixtures lyophilized, and the residues redissolved in 7:3 (v/v) methanol–water. Aliquots were deposited on Silica gel 60 plates and chromatographed in 500:9 (v/v) acetone–4.5M ammonia with appropriate tri- and tetra-*O*-methyl-D-galactose references[13]. Radioactive compounds were detected by autoradiography, and the partially methylated D-galactose standards were made visible with aniline phthalate.

Determination of sialyltransferase specificities. — To study the specificity of sialyltransferases in various tissues, each homogenate or membrane preparation was incubated with CMP-[^{14}C]NeuAc (7.7 nmol, specific radioactivity 13 Ci/mol), Tris–maleic acid buffer (pH 6.7, 4 μmol), and *N*-acetyllactosamine (200 nmol) in a final volume of 50 μL at the bottom of a 100 × 13 mm conical glass centrifuge-tube. After 150 min at 37°, the reaction was stopped by cooling on ice, and protein was precipitated by the addition of 10% trichloroacetic acid (50 μL). The tube content was vigorously mixed with chloroform (500 μL) in order to extract the excess of trichloroacetic acid. After centrifugation, the precipitated protein appeared at the interface, whereas essentially all oligosaccharide material was present in the upper layer (water phase). An aliquot (75 μL) of this layer was added to a 0.2M dipotassium hydrogenphosphate solution (40 μL) resulting in the adjustment of the pH to 4.5–5.0. Samples (20–50 μL) of the mixture were injected into a Perkin–Elmer Series 2 liquid chromatograph, equipped with a Rheodyne injection valve and a Perkin–Elmer LC-75, variable-wavelength detector operating at 195 nm. Chromatograms were recorded with a Spectra-Physics SP-4100 computing integrator. Chromatography was performed on a column (4 × 250 mm) of Lichrosorb-NH_2 (particle size 5 μm, Merck), at a temperature of 20° and a pressure of 11 MPa (1600 p.s.i.). The mobile phase consisted of a mixture of acetonitrile (Lichrosolv grade, Merck) and de-ionized, distilled water containing 15mM potassium phosphate (pH 5.2). Sialic acid and the isomers of sialyl-*N*-acetyllactosamine were separated by starting with an isocratic elution by an 83:17 (v/v) mixture for 35 min, after

which, a linear gradient of increasing water content was applied at a rate of 0.5%/min. The flow rate of the solvent mixtures was maintained at 2 mL/min. The standard compounds, [^{14}C]NeuAc, α-NeuAc-(2→3)- and α-NeuAc-(2→6)-β-D-[^{14}C]Gal*p*-(1→4)-D-GlcNAc, as well as the [^{14}C]sialylated *N*-acetyllactosamine samples obtained from the incubations with crude-tissue preparations, were detected by counting the radioactivity in the fractionated eluate.

REFERENCES

1 D. H. VAN DEN EIJNDEN, P. STOFFYN, A. STOFFYN, AND W. E. C. M. SCHIPHORST, *Eur. J. Biochem.*, 81 (1977) 1–7.
2 D. H. VAN DEN EIJNDEN AND W. E. C. M. SCHIPHORST, *J. Biol. Chem.*, 256 (1981) 3159–3162.
3 M. L E. BERGH, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Carbohydr. Res* , 94 (1981) 225–229.
4 D. H. VAN DEN EIJNDEN, D. H. JOZIASSE, L. DORLAND, H. VAN HALBEEK, J. F. G. VLIEGENTHART, AND K. SCHMID, *Biochem. Biophys. Res. Commun.*, 92 (1980) 839–845.
5 J. C. PAULSON, J. I. REARIK, AND R. L. HILL, *J. Biol. Chem.*, 252 (1977) 2363–2371.
6 D. H. VAN DEN EIJNDEN AND W. E. C. M. SCHIPHORST, *Proc. Int. Symp. Glycoconjugates, 5th,* (1979) 250–251.
7 W. AUGENER, W. E. C. M. SCHIPHORST, AND D. H. VAN DEN EIJNDEN, *Proc. Int. Symp. Glycoconjugates, 6th,* (1981) 405-406.
8 B. BAUVOIS, R. CACAN, B. FOURNET, J. CAEN, J. MONTREUIL, AND A. VERBERT, *Eur. J. Biochem.*, 121 (1982) 567–572.
9 D. H. VAN DEN EIJNDEN AND W. VAN DIJK, *Hoppe-Seyler's Z. Physiol. Chem.*, 353 (1972) 1817–1820.
10 W. M. BLANKEN, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *Eur. J. Biochem.*, 127 (1982) 547–552.
11 R. BARKER, K. W. OLSEN, J. H. SHAPER, AND R. L. HILL, *J. Biol. Chem.*, 247 (1972) 7135–7147.
12 J. C. PAULSON, R. L. HILL, AND W. E. BERANEK, *J. Biol. Chem.*, 252 (1977) 2356–2362.
13 P. STOFFYN, A STOFFYN, AND G. HAUSER, *J. Lipid Res.*, 12 (1971) 318–323.