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Human liver and human placenta both contain CMP-NeuAc:Galβ1→4GlcNAc-R α2→3- as well as α2→6-sialyltransferase activity

Martin Nemansky, Wietske E.C.M. Schiphorst, Carolien A.M. Koeleman and Dirk H. Van den Eijnden

Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

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A high pH anion exchange chromatographic (HPAEC) system for the separation of isomeric sialo-oligosaccharide products was developed. Employing this system, using Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc as a substrate, a Galβ1→4GlcNAc-R α2→3-sialyltransferase activity was detected for the first time in human liver. This activity is expressed together with the prevalent α2→6-sialyltransferase. Furthermore, in addition to the major α2→3-sialyltransferase, a low but distinct activity of α2→6-sialyltransferase was detected in human placenta. This activity could not be found by methods based on methylation analysis or high resolution NMR spectroscopy. It is concluded that HPAEC, in combination with the use of the pentasaccharide as an acceptor substrate, is suited for the specific detection of minor, Galβ1→4GlcNAc-specific sialyltransferase activities.

Sialyltransferase; Sialic acid; High pH anion exchange chromatography (HPAEC); Pulsed amperometric detection (PAD); Human liver; Human placenta

1. INTRODUCTION

Individual *N*-glycoproteins from various tissues and species often contain both α2→3- and α2→6-linked sialic acid (NeuAc) at the Galβ1→4GlcNAc termini of their complex-type glycans [1–8]. On the other hand, many glycoproteins are known to contain NeuAc in only one of these linkage types [1,9–14]. Several studies from this and other laboratories have shown that the NeuAc linkage type generally can be explained from the activities of the CMP-NeuAc:Galβ1→4GlcNAc-R α2→3- (α3-sialyltransferase) or α2→6-sialyltransferase (α6-sialyltransferase) in the corresponding tissues [15–17]. However, no α3-sialyltransferase activity has been identified in human liver to date, although structural studies have revealed that the tri- and tetra-antennary glycans of several glycoproteins synthesized by this tissue do contain terminal NeuAcα2→3Galβ1→4GlcNAc sequences (references cited in [2,3]).

Previously, a Galβ1→3(4)GlcNAc-R α2→3-sialyltransferase had been detected in rat liver [18,19]. Although this enzyme prefers type 1 chain (Galβ1→3GlcNAc) acceptors, it was also found to act on acceptors with type 2 termini (Galβ1→4GlcNAc), albeit with lower efficiency [20]. Nevertheless, enzyme-catalyzed transfer of NeuAc to C-3 of the terminal Gal residues of asialo-α_1-acid glycoprotein could not be detected by methylation analysis of the product formed with rat liver [16,17]. Similarly no α3-sialyltransferase activity was found in human liver, when the product formed with Galβ1→4GlcNAc (*N*-acetyllactosamine) as an acceptor was analyzed by HPLC [21].

In an attempt to improve the detection method for α3- and α6-sialyltransferases we developed a procedure using a type 2 chain-based pentasaccharide, Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc [22], as an acceptor, analyzing the reaction product by high pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). The method appeared to have a greatly improved sensitivity as it allowed the specific detection of small amounts of product in the presence of an excess of isomeric material. Using this procedure, we were able to detect α3-sialyltransferase activity in human liver. Moreover, when the method was applied to human placenta, a low but distinct α6-sialyltransferase activity was found in addition to the major α3-sialyltransferase known to be present in this tissue [16].

2. MATERIALS AND METHODS

2.1. *Materials*

CMP-[^{3}H]NeuAc (specific radioactivity 28,200 Ci/mol) was obtained from New England Nuclear (Boston, MA) and diluted with unlabelled CMP-NeuAc [23] to the desired specific activity. CMP-NeuAc:Galβ1→4GlcNAc-R α2→6-sialyltransferase (α6-sialyltransferase) was partially purified from bovine colostrum as described

Correspondence address: M. Nemansky, Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Fax: (31) (20) 642 8555.

previously [24]. Asialo-α_1-acid [^{3}H]glycoprotein, specifically labelled in the terminal Gal residues, was prepared as described [17]. Human liver obtained by autopsy of subjects that had died from non-liver disease and human placenta frozen directly after delivery were provided by the Departments of Pathology and Gynaecology and Obstetrics, Academic Hospital of the Vrije Universiteit, Amsterdam. Microsomal fractions were prepared from these tissues as described previously [16,17]. The pentasaccharide, Galβ1→4GlcNAc β1→2Manα1→6Manβ1→4GlcNAc [22], was generously donated by Drs. G. Strecker and J.C. Michalski (Université de Lille, Villeneuve d'Ascq, France), and the trimethylgalactose standards were kindly supplied by Drs. P. and A. Stoffyn (Shriver Center, Waltham, MA). 50% (w/w) NaOH solution (low in carbonate) was purchased from Baker (Phillipsburg, NJ).

2.2. *Methylation analysis of sialylated glycoprotein products*

Incubations of asialo-α_1-acid [^{3}H]glycoprotein and CMP-NeuAc with human liver or human placenta microsomes (2.6 and 8 mg of protein, respectively) were performed as described previously [16]. After isolation, the sialylated products were digested by pronase and the resulting ^{3}H-labelled glycopeptides were isolated by Dowex 50X-16 (H^+) chromatography [17]. Methylation of the glycopeptides was performed as described [16,25]. The methylated materials were subsequently hydrolyzed in 400 μl of 2 M trifluoroacetic acid for 60 min at 120°C. After counting, appropriate aliquots (5–15 μl) containing 100–200 nCi of ^{3}H were spotted together with a mixture of trimethylgalactose references on Silica Gel 60 plates (Merck, Darmstadt). Chromatograms were developed with acetone/4.5 M NH_4OH (500:9) as a solvent system [26]. Radioactive compounds were detected by fluorography, and reference-methylated galactoses were visualized with aniline phthalate.

2.3. *Sialylation of Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc by human liver and placenta*

Incubation mixtures contained in a volume of 50 μl: 50 nmol Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc, 25 nmol CMP-[^{3}H]NeuAc (4.1 Ci/mol), 5.0 μmol of sodium cacodylate, pH 6.7, 50 nl Triton X-100 and either liver or placenta microsomes (1 and 3 mg of protein, respectively). The mixtures were incubated at 37°C for 3 h, whereafter the incubations were stopped by cooling on ice. After the addition of 500 μl ice-cold H_2O, the mixtures were centrifuged for 15 min in an Eppendorf centrifuge at maximal speed. Pellets were washed twice with 200 μl ice-cold H_2O and the pooled supernatant fractions were applied to a column (0.7 × 45 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and room temperature, at a flow rate of 15 ml/h. Fractions (0.7 ml) were collected and ^{3}H radioactivity was determined by liquid scintillation counting. The sialylated products were pooled and lyophilized.

2.4. *Synthesis of sialylated reference compounds*

The α6-sialylated derivative of Galβ1→4GlcNAcβ1→2Man α1→6Manβ1→4GlcNAc was synthesized using bovine colostrum α6-sialyltransferase (190 μU) as described [2,27]. The α3-sialylated analogue was produced using human placenta microsomes (30 mg of protein) as a source of α3-sialyltransferase in a ten-fold scaled up incubation. The products were isolated as described above. Characterization of the latter product was by 400 MHz ^{1}H NMR spectroscopy after treatment of the sample with D_2O [28].

2.5. *HPAEC-PAD of sialylated oligosaccharide products*

The system used for HPAEC-PAD consisted of a Dionex Bio-LC gradient pump, CarboPac PA-1 column (4.0 × 250 mm) and a Model PAD 2 detector. The following pulse potentials and durations were used for detection: E_1 = 0.05 V (t_1 = 480 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = −0.60 V (t_3 = 60 ms). The response time of the detector was set to 1 s. Samples were dissolved in the eluant mixture (see below) and injected via a Dionex Micro Injection valve equipped with a 25 μl sample loop. The chromatographic data were integrated and plotted using a Shimadzu C-R5A integrator. A Dionex Eluant Degas Module was employed to saturate the eluants with helium in order to de-gas and to minimize absorption of CO_2. For the separation of the products, isocratic elution conditions were employed using 0.2 M NaOH/1 M sodium acetate/H_2O (50:4:46, by vol.) as an eluant system, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and counted, and the incorporation of [^{3}H]NeuAc was calculated from the radioactivity in the fractions containing the products.

3. RESULTS

Methylation analysis of the product obtained by incubating asialo-α_1-acid [^{3}H]glycoprotein and CMP-NeuAc with human liver microsomes yielded a spot of 2,3,4-trimethyl-[^{3}H]galactose as the only trimethylated sugar (Fig. 1, lane a). No such spot was detected when the product of sialylation was digested with neuraminidase prior to methylation or when heat-inactivated microsomes were used (Fig. 1, lanes b and c). This indicates that by this method α6-sialyltransferase is the only activity that can be detected in human liver. With new born liver an identical result was obtained (not

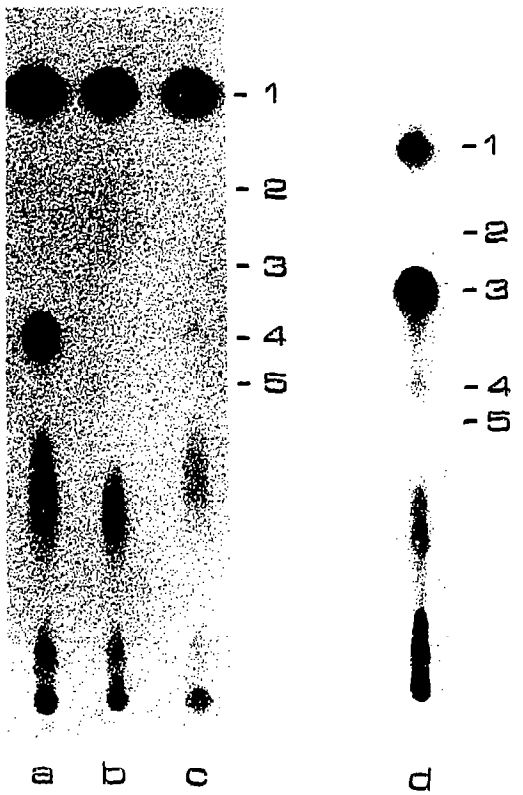

Fig. 1. Thin layer chromatography of methylated galactoses. Methylated ^{3}H-labelled galactoses were derived from asialo-α_1-acid [^{3}H]glycoprotein sialylated in vitro by: (a) human liver microsomes; (b) human liver microsomes followed by neuraminidase treatment; (c) heat-inactivated human liver microsomes; (d) human placenta microsomes. The migration of the following references is indicated: (1) tetramethylgalactose; (2) 2,3,6-trimethylgalactose; (3) 2,4,6-trimethylgalactose; (4) 2,3,4-trimethylgalactose; (5) 3,4,6-trimethylgalactose. ^{3}H-Labelled compounds were detected by fluorography, and reference methylated galactoses by aniline phthalate.

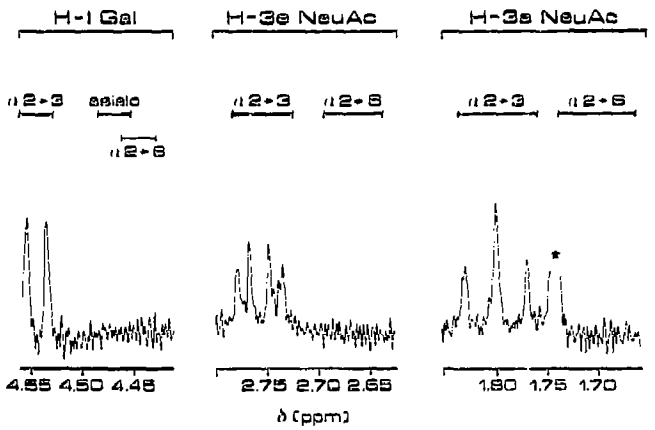

Fig. 2. Characteristic resonance patterns of the galactose H-1 atoms and of the NeuAc H-3e and H-3a protons in the 400 MHz ^{1}H NMR spectrum of Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4GlcNAc α3-sialylated using human placenta microsomes. In addition to the signals of the above mentioned protons of the α2→3-sialylated pentasaccharide, the positions of the resonances of its α2→6-sialylated isomer and of the asialo substrate (H-1 of Gal) are indicated. The asterisk denotes an impurity.

shown). By contrast, the incubation with placental microsomes yielded a product that resulted in a spot of 2,4,6-trimethyl-[^{3}H]galactose upon methylation (Fig. 1, lane d), confirming that this tissue contains an active α3-sialyltransferase.

In all instances, upon prolonged exposure of the fluorograms, weak spots could be seen on each trimethylgalactose position. Although it cannot be excluded that these spots reflect low levels of other sialyltransferases, they are most likely due to some undermethylation or to de-methylation of tetramethylgalactose during acid hydrolysis [16].

Examination of the 400 MHz ^{1}H NMR spectrum of the oligosaccharide used as a substrate in the sialylation reactions showed this material to consist of Galβ1→4GlcNAcβ1→2Manα1→6*Man*β1→4GlcNAc as a major compound with some (9%) contaminating Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc (data not shown). Analysis of the product isolated from the incubation with placenta microsomes as a preparation of α3-sialyltransferase appeared to contain only α2→3-linked NeuAc (H-3eq NeuAc δ = 2.757 ppm; H-3ax NeuAc δ = 1.798 ppm; NAc NeuAc δ = 2.031 ppm; H-1 Gal δ = 4.547) [29]. No indication for the presence of α2→6-linked NeuAc in this product was apparent from the spectrum (Fig. 2). The α2→6-sialylated pentasaccharide, NeuAcα2→6Galβ1→4GlcNAc β1→2Manα1→6Manβ1→4GlcNAc, was obtained using the well-defined Galβ1→4GlcNAc-R α2→6-sialyltransferase from bovine colostrum, which was proven to catalyze the addition of NeuAc into Galβ1→4GlcNAc-structures in α2→6-linkage exclusively [2,27,28].

Using HPAEC-PAD, elution conditions were elaborated by which baseline separation of the reference compounds, NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc and NeuAcα2→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc, was obtained (Fig. 3A). These materials also separated well

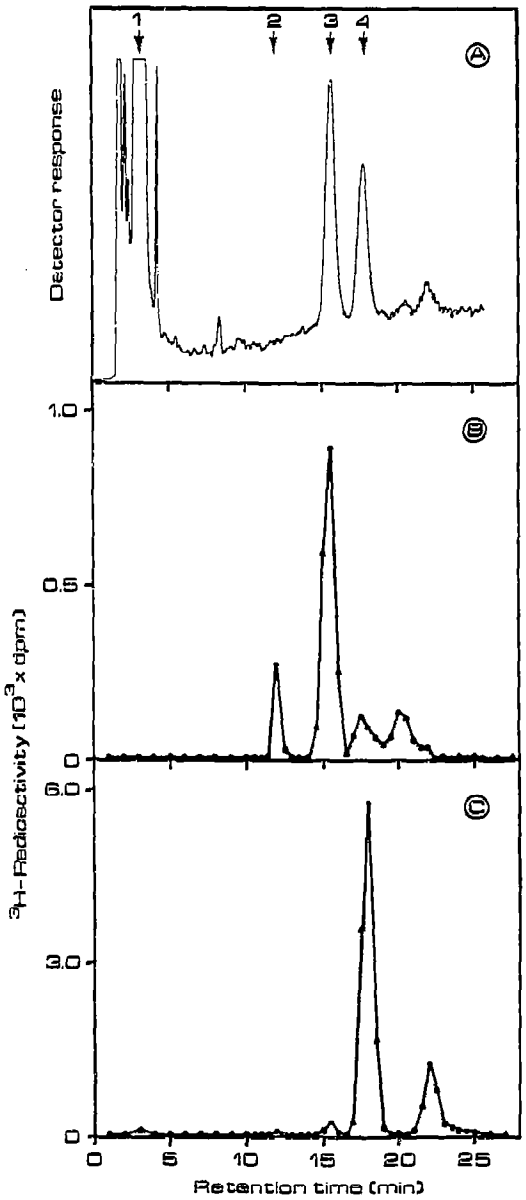

Fig. 3. HPAEC-PAD profiles of the products of sialyltransferases using Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4GlcNAc as acceptor substrate. Elution of (A) references obtained with bovine colostrum α6-sialyltransferase and human placenta microsomes as a source of α3-sialyltransferase, (B) the products of incubation of with human liver microsomes, and (C) the products obtained with human placenta microsomes. The elution positions of the following references are indicated: (1) Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc; (2) NeuAc; (3) NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc; (4) NeuAcα2→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc. Isocratic elution with 0.20 M NaOH/1.00 M sodium acetate/water (50:4:46, v/v) was carried out as described in Materials and Methods. The flow rate was 1 ml/min.

Table 1

Ratios of sialylated products as detected by HPAEC-PAD

Products formed	Retention time (min)	Ratio (%) of products formed with	
		Human liver	Human placenta
NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc	15.6	69.4	2.5
NeuAcα2→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc	17.8	13.1	76.9
NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc	20.5	14.4	<0.8
NeuAcα2→3Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc	21.9	3.1	20.5

Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc was sialylated using CMP-[^{3}H]NeuAc and human liver or human placenta microsomes as described in Materials and Methods. The products were separated using HPAEC-PAD and their relative amounts were estimated by liquid scintillation counting.

from the oligosaccharide substrate and NeuAc. Two satellite peaks could be observed which most likely represent the α6- and α3-sialylated derivatives of Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc, respectively, which are formed from the minor oligosaccharide isomer in the substrate. It is noteworthy that α6-sialylated compounds and oligosaccharides, containing a Manα1→6Man linkage, elute earlier than the isomers having an α3-linkage, since this order is opposite to that obtained by HPLC on amino-bound silica columns [21,30,31].

HPAEC-PAD analysis of the products of sialylation obtained with human liver microsomes showed that several sialylated oligosaccharides were formed (Fig. 3B). The major (retention time 15.6 min) and the minor peak (20.5 min) could be identified as α2→6-sialylated products, together comprising >80% of the total sialylated products (Table I). In addition, minor amounts of α2→3-sialylated oligosaccharides (≈20%) could be detected, with retention times of 17.8 and 21.9 min, respectively (Fig. 3B). By contrast, the incubation with human placenta microsomes was found to yield mainly α2→3-sialylated products (retention time 17.8 and 21.9 min) (Fig. 3C), along with distinct trace amounts (≈3%) of α6-sialylated product (Table I).

4. DISCUSSION

A characteristic structural feature of the di-antennary *N*-acetyllactosamine type glycans of glycoproteins synthesized by the human liver is the predominant occurrence of α2→6 terminally linked sialic acid [1]. By contrast, the tri- and tetra-antennary glycans of these glycoproteins have been shown to contain not only α2→6- but also α2→3-linked sialic acid (references cited in [2,3]). Different methods have been employed to detect the human liver α3-sialyltransferase responsible for the synthesis of the α2→3-sialylated glycans. However, only α6-sialyltransferase and no α3-sialyltransferase activity was found in this tissue using Galβ1→4GlcNAc as an acceptor followed by HPLC analysis of the products [21], nor could we detect α3-sialyltransferase activity by methylation analysis using asialo-α_1-acid [^{3}H]glycoprotein as an acceptor (this study). Moreover, purification of a Galβ1→4GlcNAc-R specific sialyltransferase from human liver yielded a preparation which did not contain α3-sialyltransferase activity [32]. However, using the pentasaccharide, Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc, as an acceptor substrate in the incubation with human liver microsomes and employing HPAEC-PAD for the separation of the α2→3- and α2→6-sialylated oligosaccharide products, we were able to detect a distinct amount of α2→3-sialylated material. This indicates that an α3-sialyltransferase, capable of sialylating Galβ1→4GlcNAc-R acceptor structures, is indeed present in human liver in addition to the more abundant α6-sialyltransferase. This is the first report of such an enzyme in this tissue.

The failure to detect this α3-sialyltransferase activity with *N*-acetyllactosamine as an acceptor [21] is probably due to the poor acceptor property of this disaccharide for the α3-sialyltransferase relative to that for the α6-enzyme [20]. Apparently the pentasaccharide used in this study is a much better acceptor substrate in this respect. In addition, the larger size of the products in the latter case, as well as the HPLC system used, make it easier to separate these materials from the excess of CMP-NeuAc, resulting in a better signal-to-noise ratio.

The human liver α3-sialyltransferase might be similar to the previously reported Galβ1→3(4)GlcNAc-R α2→3-sialyltransferase from rat liver [18–20], which was found to preferentially act on type 1 Galβ1→3GlcNAc-R termini, but which can also catalyze the sialylation of Galβ1→4GlcNAc-R (type 2) glycans, albeit with low efficiency [20]. It can not be excluded, however, that the human liver α3-sialyltransferase has a greater preference for type 2 acceptor chains. Type 1 chains have been described on glycoproteins from rat plasma [33], but up until now not on human serum glycoproteins. On the other hand it is highly improbable that the α3-sialyltransferase activity is due to the action of a Galβ1→3GalNAc-R α2→3-sialyltransferase which generally occurs in liver tissue

and is most likely present also in human liver [34]. Purified preparations of this enzyme from porcine submaxillary gland [35], human placenta [36] and porcine liver [37] show virtually no activity with Galβ1–4GlcNAc type acceptors.

It was interesting to find, in addition to the major α3-sialylated products, a small but distinct amount of α2→6-sialylated pentasaccharide as a product from the incubation with human placenta microsomes. This result was even more surprising since prior analysis of this sialylated material by 400 MHz ^{1}H NMR spectroscopy did not reveal NeuAc linkages other than α2→3. In addition, methylation analysis of the product obtained with placenta microsomes using asialo-α_1-acid [^{3}H]glycoprotein as an acceptor did not yield indications for an α2→6-sialic acid transfer either. Based on the latter results and on structural data of sialylated glycoproteins from placental origin (e.g. human chorionic gonadotropin [10,38–40], human β-glucocerebrosidase [41] and human transferrin receptor [42]), revealing the exclusive presence of α3-linked NeuAc, the α2→6-sialyltransferase capable of synthesizing NeuAcα2→6Galβ1→4GlcNAc-R structures was thought to be absent in human placenta. However, more recently acid α-glucosidase from human placenta was found to contain very small amounts of α2→6-sialylated diantennary *N*-acetyllactosamine type glycans [43]. Although other (*N*-acetyllactosaminide and *N*-acetylglucosaminide) α6-sialyltransferases have been detected in this tissue [19,44], none of these are able to act on Galβ1→4GlcNAc-R structures. Therefore the α2→6-sialylated pentasaccharide product reflects the presence of a Galβ1→4GlcNAc-R specific α2→6-sialyltransferase in this tissue. We therefore expect that other *N*-acetyllactosamine type glycoproteins from human placenta may also contain small amounts of α2→6-linked sialic acid.

Finally we conclude that HPAEC-PAD is a convenient method to resolve and sensitively detect small amounts of sialylated oligosaccharide isomers. In combination with appropriate acceptor substrates, this method can be used to reveal the presence of specific sialyltransferases that cannot be detected by methylation analysis or high resolution ^{1}H NMR spectroscopy, because of the higher detection limits of the latter two methods.

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