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The β-Subunit of Human Chorionic Gonadotropin Contains *N*-Glycosidic Trisialo Tri- and Tri'-antennary Carbohydrate Chains

JAN B L DAMM[1], HANS VOSHOL[1], KARL HÅRD[1], JOHANNIS P KAMERLING[1], GIJS W K VAN DEDEM[2] and JOHANNES F G VLIEGENTHART[1]

[1]*Department of Bio-Organic Chemistry, Utrecht University, Transitorium III, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands*
[2]*Diosynth B.V., P.O. Box 20, NL-5340 BH Oss, The Netherlands*

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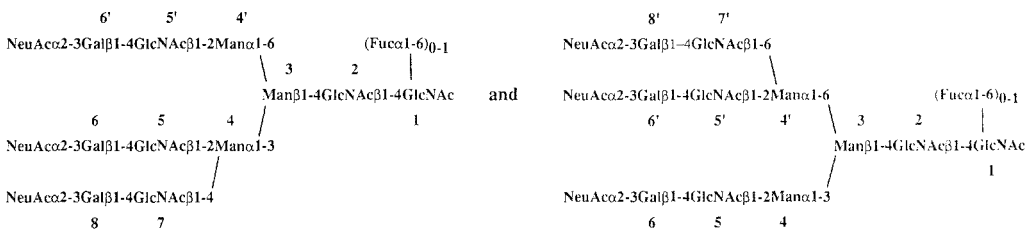
The *N*-linked carbohydrate chains of the β-subunit of highly purified urinary human chorionic gonadotropin have been re-investigated. The oligosaccharides were released enzymatically by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F, and fractionated by a combination of FPLC and HPLC. As a result of the application of improved fractionation methods, apart from the earlier reported carbohydrate chains, also small amounts of trisialo tri- and tri'-antennary oligosaccharides were found. The primary structures of the latter carbohydrate chains have been determined by 500-MHz ^{1}H-NMR spectroscopy to be



Abbreviations: hCG, human chorionic gonadotropin; hCG-β, β-subunit; hCG-α, α-subunit; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F (E.C. 3.5.1.52); endo-F, endo-β-*N*-acetylglucosaminidase-F (E.C. 3.2.1.96); SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; CBB, coomassie brilliant blue R 250; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; Man, mannose; Gal, galactose; Fuc, fucose.

The *N,O*-glycoprotein hormone hCG, containing about 30% carbohydrate, is synthesized in the syncytiotrophoblast of the placenta and consists of two dissimlar, non-convalently linked subunits, designated α (15 kDa) and β (23 kDa) [1]. The carbohydrate chains of hCG are important for the biological functioning of the hormone [2-11]. Several studies have been undertaken to characterize the primary structure of the *N*- and *O*-linked carbohydrate chains of hCG [12-18]. So far, it has been demonstrated that hCG-α contains *N*-linked carbohydrate chains attached at Asn 52 and 78 having the mono-antennary structure N1A and the di-antennary structures N1B (without fucose) and N2B (Table 1). For hCG-β *N*-linked chains at Asn 13 and 30 and *O*-linked chains at Ser 121, 127, 132 and 138 have been found. The *N*-linked chains of hCG-β have the mono-antennary structure N1A and the di-antennary structures N1B, N2A and N2B (Table 1). Furthermore, the *N*-linked oligosaccharides derived from hCG of patients suffering from such trophoblastic diseases as hydatidiform mole [19], invasive mole [20] and choriocarcinoma [19-21] have been investigated. The hCGs purified from the urine of invasive mole and choriocarcinoma patients have been shown to contain significant amounts of tri-antennary carbohydrate chains in addition to the chains mentioned above. Moreover, choriocarcinoma hCG also contains di-antennary chains with a 2,4-disubstituted Manα1-3 residue. It has been stated that these abnormal oligosaccharide structures could serve as markers for the diagnosis of choriocarcinoma and invasive mole. In this paper, however, the occurrence of small amounts of tri- and tri-antennary *N*-acetyllactosamine type of chains in the β-subunit of normal hCG are reported and discussed in relation to the literature data on hCGs from patients with trophoblastic diseases.

Materials and Methods

Materials

Highly purified hCG-β, used in these studies, was obtained from purified hCG isolated from the pooled urine of healthy, pregnant women, provided by Diosynth, Oss, The Netherlands. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)-asparagine amidase-F (PNGase-F) from *Flavobacterium meningosepticum* (E.C. 3.5.1.52) was obtained from Boehringer Mannheim, W. Germany.

Liberation of the N-Glycosidic Carbohydrate Chains

The purity of the hCG-β sample was checked by SDS-PAGE [22]. The *N*-linked carbohydrate chains were released from the protein moiety essentially as described earlier [18]. Briefly, 14 mg hCG-β were dissolved in 0.5 ml 50 mM Tris adjusted with HCl to pH 8.4 and containing 10 mM EDTA, 2% (w/v) SDS, and 1% (v/v) β-mercaptoethanol, and then was kept at 40°C for 1 h. Subsequently, the sample was incubated with PNGase-F (15 U) immobilized on a Sepharose 4B column (1 g dry material) for 40 h at room temperature. The reaction products were eluted from the PNGase-F column with 7 ml 50 mM Tris-HCl, pH 8.4, containing 10 mM EDTA; lyophilized, and desalted on Bio-Gel P-2 (35 × 1 cm column, 200-400 mesh, Bio-Rad, Richmond, CA, USA). Completeness of liberation of the *N*-linked chains from the glycoprotein was checked by SDS-PAGE. A second batch of 15 mg hCG-β was worked up in the same way.

FPLC-Fractionation of the N-Glycosidic Carbohydrate Chains

Fractionation of the enzymatically-released carbohydrate chains according to charge was carried out on Mono Q HR 5/5 (Pharmacia FPLC-system), as described [18]. The fractions monitored at 214 nm were collected, desalted and lyophilized.

HPLC-Subfractionation of the N-Glycosidic Carbohydrate Chains

Subfractionation of the carbohydrate-containing FPLC fractions was carried out with a Kratos Spectroflow 400 HPLC-system (Kratos Analytical, Rotterdam, The Netherlands) using a Lichrosorb-NH_2 10μ column (25 × 0.46 cm, Chrompack, Middelburg, The Netherlands). Samples were dissolved in 0.3 ml of a 35% (by vol) mixture of 30 mM K_2HPO_4/KH_2PO_4, pH 7.0 in acetonitrile. Elutions were carried out isocratically with the same buffer at a flow rate of 120 ml/h at 25°C (SpH 99 column thermostat, Spark Holland B.V., Emmen, The Netherlands). Runs were monitored at 205 nm with a Spectroflow 783 programmable absorbance detector (Kratos Analytical) and peaks were integrated by a Spectra Physics SP 4290 integrator (Spectra Physics Inc., San José, CA, USA). The corresponding HPLC fractions from the two working-up procedures (14 and 15 mg hCG-β) were pooled and desalted on Bio-Gel P-2.

500-MHz ^{1}H-NMR Spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis the desalted samples were repeatedly treated with ^2H_2O, finally using 99.96 atom % ^2H_2O (Aldrich, Milwaukee, WI, USA) at p^{2}H 7 and room temperature. Resolution-enhanced 500 MHz ^{1}H-NMR spectra were recorded using a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, Utrecht University, and SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) at a probe temperature of 27°C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 27°C) with an accuracy of 0.002 ppm [23].

Sugar Analysis

Sugar analysis was carried out by gas-liquid chromatography on a capillary CP-Sil 5 WCOT fused silica column (25 m × 0.34 mm i.d., Chrompack, Middelburg, The Netherlands) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [24].

Results

The purity of hCG-β was checked by SDS-PAGE on a 15% slab gel, and showed one band at M_r = 33 300 (Fig. 1, lane 4). Partially PNGase-F digested hCG-β gave rise to three bands (Fig. 1, lane 3), representing undigested (M_r = 33 300), partially *N*-deglycosylated (*N*-linked chains released from one Asn residue, M_r = 30 100) and completely *N*-deglycosylated (M_r = 27 400) material. Thus, release of the *N*-linked carbohydrate chains from one and two Asn residues results in a decrease of the apparent molecular

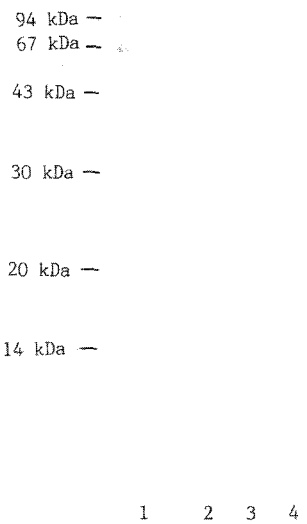


Figure 1. SDS-PAGE of hCG-β on a 15% slab gel.
Sample size 10-25μg. The gel was stained with CBB. Lane 1: molecular mass markers (phosphorylase B, 94 kDa; bovine serum albumin 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa). Lane 2: hCG-β, completely PNGase-F digested. Lane 3: hCG-β, partially PNGase-F digested. Lane 4: hCG-β, intact.

mass of 3 200 and 5 900, respectively. Because the majority of the *N*-linked chains of hCG-β are di-antennae [18] the expected decrease in molecular mass of the glycoprotein upon release of the carbohydrate chains was 2 400 and 4 800, respectively. From the discrepancy between the determined and expected decrease in molecular mass it can be inferred that the oligosaccharides, presumably by partial shielding of the protein moiety, hinder the binding of SDS to the protein. This resulted in a disproportionally decreased mobility of the glycoprotein on SDS-PAGE [25]. Exhaustive PNGase-F digestion of hCG-β (40 h at room temperature) under denaturing conditions led to a nearly complete (>94%) liberation of the *N*-linked chains (Fig. 1, lane 2). With respect to the application of Sepharose-4B-immobilized PNGase-F it should be mentioned that the enzyme activity gradually decreased upon repeated use of the column, probably due to accumulation of SDS. Therefore, it was necessary to check the enzyme activity regularly or, alternatively, to use the enzyme in its free state.

Anion-exchange chromatography of the PNGase-F digest of hCG-β over Mono Q yielded one neutral fraction, denoted NN and four charged fractions, denoted N1, N2, N3, and N4 (Fig. 2). Sugar analysis revealed that, apart from glucose, fraction NN contained no carbohydrate. Fraction N4 represents the *N*-deglycosylated protein-SDS complex [18]. Almost identical patterns were obtained in an earlier study [18], wherein the oligosaccharides from fraction N1 were shown to be monosialo mono- and di-antennary compounds and those from fraction N2 disialo di-antennary compounds (see Table 1).

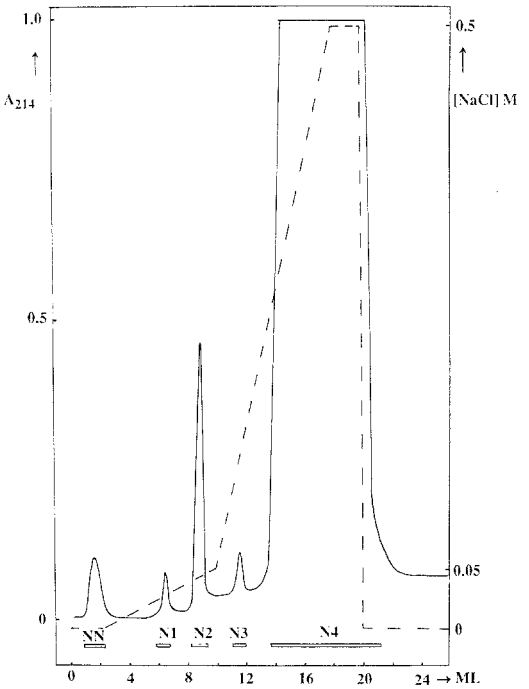
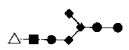
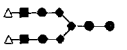
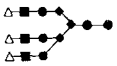
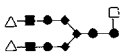
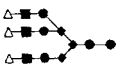
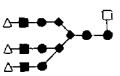
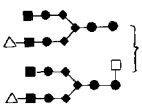
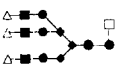


Figure 2. Fractionation pattern of the PNGase-F digestion products derived from hCG-β on an FPLC HR 5/5 Mono Q column.
PNGase-F digested hCG-β was desalted, lyophilized and dissolved in 0.8 ml H_2O (HPLC quality). The column was eluted with a linear concentration gradient (---) from 0-50 mM NaCl in 8 ml H_2O (HPLC quality), followed by a steeper gradient from 50-500 mM NaCl in 8 ml H_2O at a flow rate of 60 ml/h. Injection volume 0.1 ml, detection at 214 nm. Fractions were collected as indicated.

Fraction N3, which could not be characterized previously [18] due to an insufficient amount of material, had the same elution volume as reference trisialo tri- and tri-antennary oligosaccharide-alditols obtained from human serotransferrin [26]. Taking into account the structural analysis data for N3 (see below) the Mono Q pattern at 214 nm indicated that the fractions N1, N2 and N3 occur in a molar ratio of 2.5:10:1. The 500-MHz ^{1}H-NMR spectrum of fraction N3 revealed that only α(2-3)-linked *N*-acetylneuraminic acid residues were present (H-3a, δ = 1.802 ppm; H-3e, δ = 2.756 ppm). The reducing *N,N'*-diacetylchitobiosyl unit was demonstrated by the presence of the NAc signals of GlcNAc-**1** at δ = 2.038 ppm and GlcNAc-**2** at δ = 2.080 ppm, the H-1α and H-1β doublets of GlcNAc-**1** at δ = 5.190 ppm and δ = 4.695 ppm, respectively, and the GlcNAc-**2** H-1 signals at δ = 4.605 ppm (αGlcNAc-**1**) and δ = 4.594 ppm (βGlcNAc-**1**), respectively. Similar δ-values have been found for the *N,N'*-diacetylchitobiosyl unit of di-antennary oligosaccharides [18]. A partial α1-6 fucosylation of GlcNAc-**1** is characterized by the set of fucose structural-reporter-group signals, namely H-1 at δ = 4.900 ppm, H-5 at δ = 4.099 ppm, CH_3 at δ = 1.211 ppm (αGlcNAc-**1**) and δ = 1.223 ppm

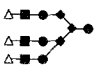
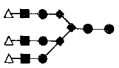
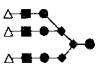
Table 1. *N*-Glycosidic carbohydrate chains isolated from the β-subunit of urinary hCG from healthy pregnant women. Molar ratios of oligosaccharides are given in percentages relatively to each other. For quantification of the oligosaccharides the following procedures were used: (1) The molar ratio of oligosaccharides present in the FPLC fractions N1, N2 and N3 were determined on the basis of the number of C=O groups (absorption at 214 nm) being known after structural identification. (2) The molar ratio of the constituent oligosaccharides within each FPLC fraction was determined on the basis of HPLC peak areas at 205 nm. Compounds are represented by short-hand symbolic notation [23]: GlcNAc, ● ; Man, ◆; Gal, ■ ; NeuAcα2-3, △ ; Fuc, □.

Mono-antennary[a]	%	Di-antennary[a]	%	Tri-antennary	%
	15		37		0.9
N1A		N2B		N3.1	
			37		3.4
		N2A		N3.2A	
					1.3
			4	N3.2B	
		N1B			1.4
				N3.3	
Total	15		78		7

[a] Data taken from [18]

(βGlcNAc-**1**), and the NAc singlet of GlcNAc-**2** at δ = 2.094 ppm [18]. Based on the intensities of the NAc signals of GlcNAc-**2** the ratio of fucosylated to non-fucosylated compounds is approximately 1:2. From the relative intensities of characteristic structural-reporter-group signals it is evident that fraction N3 represents a mixture of tri-antennary (2,4-disubstituted Man-**4**) and tri-antennary (2,6-disubstituted Man-**4'**) compounds in a molar ratio of 1:2. The tri-antennary oligosaccharide is characterized by the H-1 signals of Man-**4** and Man-**4'** at δ = 5.123 ppm and δ = 4.872 ppm, respectively, in combination with the H-2 signals of Man-**3**, Man-**4** and Man-**4'** at δ = 4.249 ppm, δ = 4.201 ppm and

Table 2. ^{1}H-Chemical shifts of structural-reporter group protons of the constituent monosaccharides for the oligosaccharides N3.1 and N3.2A derived from hCG-β, together with those for the reference compounds **1** and **2** (van Pelt *et al.*, unpublished results). Chemical shifts are given at 27°C in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate in ^2H_2O. Compounds are represented by shorthand symbolic notation [23]: GlcNAc, ● ; Man, ◆; Gal, ■ ; NeuAcα2-3, △ ; Fuc, □.

					
Reporter group	Residue[a]	**1**	N3.1	**2**	N3.2A
H-1	GlcNAc-1	—	α5.190 β4.695	—	α5.190 β4.695
	GlcNAc-2	α5.213 βn.d.	α4.605 β4.594	α5.206 βn.d.	α4.605 β4.594
	Man-3	n.d.	n.d.	n.d.	n.d.
	Man-4	5.119	5.114	5.124	5.123
	Man-4′	4.915	4.909	4.874	4.872
	GlcNAc-5	4.560	4.560	4.580	4.577
	GlcNAc-5′	4.576	4.577	4.590	4.588
	Gal-6	4.546	4.545	4.545	4.545
	Gal-6′	4.546	4.545	4.545	4.545
	GlcNAc-7	4.546	4.545	—	—
	GlcNAc-7′	—	—	4.545[b]	4.545[c]
	Gal-8	4.546	4.545	—	—
	Gal-8′	—	—	4.557[b]	4.560[c]
H-2	Man-3	α4.226 β4.217	4.209	α4.262 β4.249	4.249
	Man-4	4.221	4.219	4.198	4.201
	Man-4′	4.112	4.109	4.104	4.106
H-3	Gal-6	4.119	4.117	4.116	4.117
	Gal-6′	4.119	4.117	4.116	4.117
	Gal-8	4.119	4.117	—	—
	Gal-8′	—	—	4.116	4.117
H-3a	NeuAc	1.802[d]	1.802[d]	1.801[d]	1.802[d]
H-3e	NeuAc	2.756[d]	2.756[d]	2.757[d]	2.756[d]
NAc	GlcNAc-1	—	2.038	—	2.038
	GlcNAc-2	2.060	2.080	2.058	2.080
	GlcNAc-5	2.046	2.044	2.054	2.052
	GlcNAc-5′	2.046	2.044	2.039	2.038
	GlcNAc-7	2.074	2.073	—	—
	GlcNAc-7′	—	—	2.039	2.038
	NeuAc	2.031[e]	2.030[e]	2.031[e]	2.030[e]

[a] For numbering of the monosaccharide residues, see text.
[b] Values may have to be interchanged.
[c] Values may have to be interchanged.
[d] Signal stemming from three protons.
[e] Signal stemming from three NAc groups.
n.d. = not dectected.

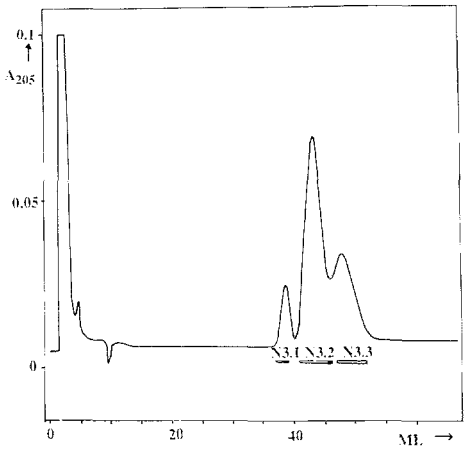
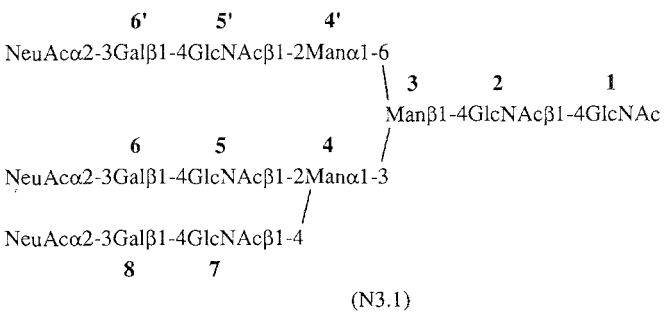


Figure 3. Fractionation pattern of the hCG-β Mono Q N3 fraction on an HPLC Lichrosorb-NH_2 10μ column. The FPLC fraction was lyophilized, desalted and dissolved in 0.3 ml 30 mM K_2HPO_4-KH_2PO_4, pH 7.0/acetonitrile (35/65, by vol). The column was eluted isocratically with the same buffer at a flow rate of 120 ml/h at 25°C. Injection volume 0.1 ml, detection at 205 nm. Fractions were collected as indicated.

δ = 4.106 ppm, respectively [23]. The presence of the tri-antennary GlcNAc-**7'** residue was indicated by its NAc singlet at δ = 2.038 ppm. The tri-antennary compound is reflected by the H-1 signals of Man-**4** and Man-**4'** at δ = 5.114 ppm and δ = 4.909 ppm, respectively, in combination with the H-2 signals of Man-**3**, Man-**4** and Man-**4'** at δ = 4.209 ppm, δ = 4.219 ppm and δ = 4.109 ppm, respectively. The occurrence of the tri-antennary GlcNAc-**7** residue is proved by its NAc signal at δ = 2.073 ppm [23].

To obtain additional evidence for the composition of fraction N3, a subfractionation was carried out by HPLC on Lichrosorb-NH_2, giving rise to three peaks (Fig. 3), denoted N3.1 ($R_{N3.1}$ = 0.91 × $R_{N3.2}$), N3.2 ($R_{N3.2}$ = 1.00) and N3.3 ($R_{N3.3}$ = 1.11 × $R_{N3.2}$), respectively. The subfractions were investigated by 500-MHz ^{1}H-NMR spectroscopy and the structural-reporter-group signals of N3.1 and N3.2A, together with those of reference compounds **1** and **2**, are compiled in Table 2.

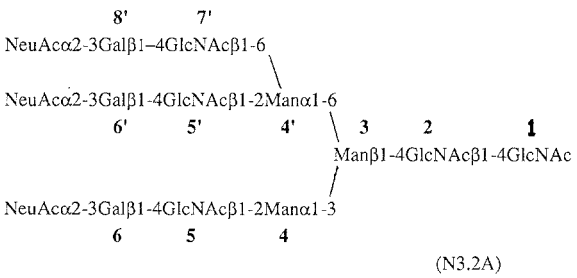
The ^{1}H-NMR spectrum of N3.1 demonstrates the occurrence of the following tri-antennary carbohydrate chain as a single constituent:



(N3.1)

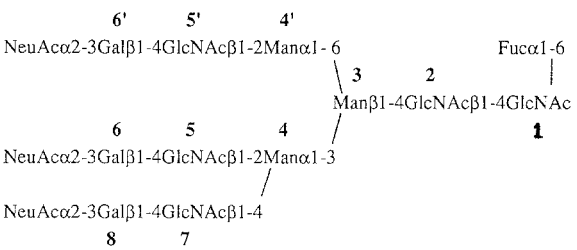
Taking into account the presence of *N,N'*-diacetylchitobiose, the position of the NAc signals of GlcNAc-**5**, GlcNAc-**5'** and GlcNAc-**7** match completely those in **1**.

The major fraction N3.2 represents a 2.5:1 mixture of the non-fucosylated tri-antennary compound N3.2A and the fucosylated tri-antennary compound N3.2B:



(N3.2A)

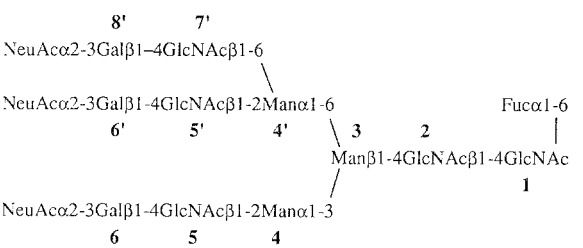
and



(N3.2B)

The tri-antennary compound N3.2A is reflected by the characteristic structural-reporter-group signals of Man H-1 and H-2 and the GlcNAc-**5**, GlcNAc-**5'** and GlcNAc-**7'** NAc signals at δ = 2.052 ppm, δ = 2.038 ppm and δ = 2.038 ppm, respectively (cf. reference compound **2**). The NAc signal of GlcNAc-**2** is visible at δ = 2.080 ppm. The presence of the tri-antennary oligosaccharide N3.2B is demonstrated by a second set of structural reporters, namely H-1 and CH_3 of fucose (δ = 4.893 ppm and δ = 1.211/1.223 ppm, respectively), NAc of GlcNAc-**2** (δ = 2.094 ppm), H-1 of Man-**4** (δ = 5.114 ppm) and Man-**4'** (δ = 4.903 ppm) and H-2 of Man- **3** (δ = 4.209 ppm) and Man-**4** (δ = 4.224 ppm). From the intensity of the two sets of signals it can be deduced that the molar ratio of N3.2A to N3.2B is 2.5:1.

The ^{1}H-NMR spectrum of fraction N3.3 demonstrates that it represents the α1-6 fucosylated extension of N3.2A:



(N3.3)

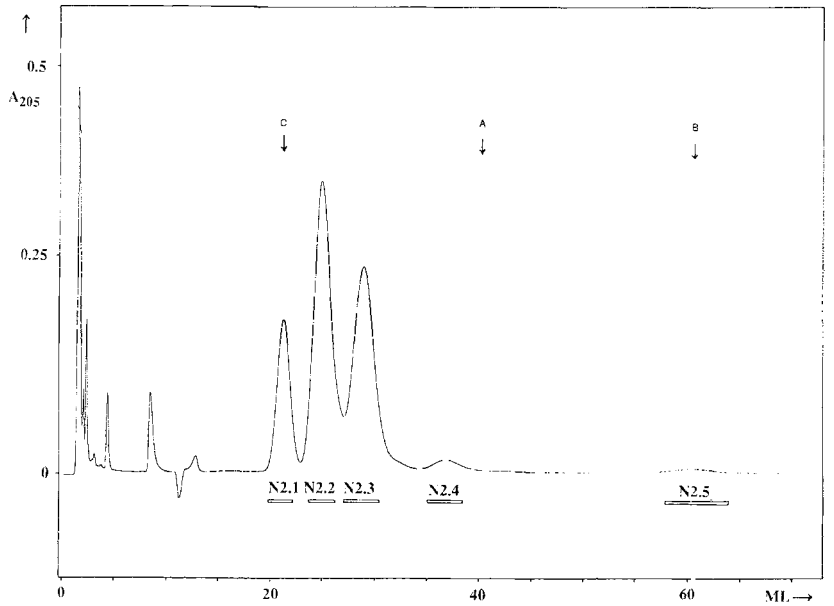


Figure 4. Fractionation pattern of the hCG-β Mono Q N2 fraction on an HPLC Lichrosorb-NH_2 10μ column. For further details, see Fig. 3.
A: Elution volume of reference compound NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc.
B: Elution volume of reference compound NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc.
C: Elution volume of reference compound NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc.

The presence of fucose at GlcNAc-**1** is evident from the typical signals at δ = 2.094 ppm (NAc GlcNAc-**2**), δ = 1.211 ppm (CH_3 of fucose, αGlcNAc-**1**) and δ = 1.223 ppm (CH_3 of fucose, βGlcNAc-**1**). The NAc signals of GlcNAc-**5**, GlcNAc-**5'** and GlcNAc-**7'** are similar to those in N3.2A.

For additional information about the influence of fucose in α(1-6)-linkage to GlcNAc-**1** on the HPLC retention volume, fraction N2 containing disialo di-antennary compounds was also separated on HPLC, yielding five peaks denoted N2.1 - N2.5 (Fig. 4). Because of the low amounts of material the structures of N2.4 and N2.5 could not be determined by 500-MHz ^{1}H-NMR spectroscopy.

Fraction N2.1 represents a degradation product of N2.2/N2.3, lacking the GlcNAc-**1** residue. The presence of this compound is probably due to contamination of the PNGase-F preparation with endo-β-*N*-acetylglucosaminidase-F [27]. N2.3 and N2.2 represent the previously reported fucosylated and non-fucosylated disialo di-antennary oligosaccharides N2A and N2B, respectively (see Table 1). From the retention volumes of N2.2 and N2.3 it is clear that α1-6 fucosylation of GlcNAc-**1** of a di-antennary *N*-acetyllactosamine type of oligosaccharide increased the HPLC retention volume by

a factor of 1.12. An identical effect can be seen for the tri-antennary compounds N3.2A and N3.3 (Fig. 3), namely $R_{N3.3} = 1.11 \times R_{N3.2A}$. As expected from its greater conformational rigidity, tri-antennary N3.1 had a smaller retention volume than tri-antennary N3.2A ($R_{N3.2A} = 1.09 \times R_{N3.1}$). In view of the fucosylation effect mentioned above, it is evident that the α1-6 fucosylated form of N3.1 (N3.2B) coelutes with N3.2A when subjected to HPLC. From the peak areas of the HPLC fractions N3.1, N3.2 and N3.3 in combination with the ^{1}H-NMR data, it can be deduced that N3.1, N3.2A, N3.2B and N3.3 occur in the molar ratio 0.9 : 3.4 : 1.3 : 1.4 (data compiled in Table 1).

Discussion

The occurrence of the *N*-acetyllactosamine type of *N*-linked carbohydrate chains in hCG from pooled urine of normal pregnant women, in the form of α2-3 sialylated mono-antennary oligosaccharides with a terminal Man-**4'** (N1A) and α2-3 sialylated di-antennary oligosaccharides, partially α1-6 fucosylated at GlcNAc-**1** (N1B, N2A, N2B), is well established [15, 16, 18] (see Table 1). In this paper the additional presence of a small amount of fully α2-3 sialylated tri- and tri-antennary chains, partially α1-6 fucosylated at GlcNAc-**1** (N3.1-N3.3) is shown. The finding of these higher branched carbohydrate chains is of interest in view of accumulating data on the possible significance of the structures of the *N*-linked oligosaccharides derived from hCGs of patients with different types of trophoblastic diseases.

Hydatidiform mole, considered to be a benign lesion, may give rise to choriocarcinoma. Analysis of the carbohydrate chains of urinary hCG from patients with this mole gives the same results in terms of mono- and di-antennary structures as reported earlier for urinary and placental normal hCG [19]. On the other hand, investigation of the carbohydrate chains of urinary hCG from patients with the precancerous invasive mole led to the characterization of highly sialylated mono-, di- and tri-antennary structures, partially α1-6 fucosylated at GlcNAc-**1** [20]. Determination of the carbohydrate chains of urinary hCG from choriocarcinoma patients demonstrates, in addition to the partially fucosylated mono-, di- and tri-antennary structures present in invasive mole hCG, partially fucosylated di-antennary structures having a 2,4-branched Man-**4** and a terminal Man-**4'** residue [19, 21]. The degree of sialylation in choriocarcinoma hCG samples varies between 3 and 100%. Both invasive mole and choriocarcinoma hCG are more highly fucosylated than normal and hydatidiform hCG. On the basis of these results it has been concluded that the malignant transformation of the trophoblasts is accompanied by structural changes in the *N*-linked carbohydrate chains of hCG [19-21]. The reduction of sialyltransferase activity might occur only at the advanced stage of choriocarcinoma. The enhancement of fucosyltransferase activity and the expression of *N*-acetylglucosaminyltransferase IV, responsible for the formation of the GlcNAcβ1-4Manα1-3 group leading to the extra antenna, becomes manifest in invasive mole [19-21]. The small amounts of fully sialylated, partially fucosylated tri-antennary carbohydrate chains now reported in normal hCG strongly suggest that the *N*-acetylglucosaminyltransferase IV activity is already present in normal trophoblasts and that the occurrence of larger amounts of tri-antennary oligosaccharides associated with invasive mole is merely due to an increase of *N*-acetylglucosaminyltransferase IV activity. The occurrence of fully sialylated, partially fucosylated tri-antennary carbohydrate chains has not been reported so far for hCG. This study indicates that besides the already mentioned *N*-

acetylglucosaminyltransferase IV activity also *N*-acetylglucosaminyltransferase V activity is present in trophoblasts, supported by the finding of tri-antennary carbohydrate chains in human placental β-glucocerebrosidase [28].

The mentioned endo-F activity in the PNGase-F preparation was not detected in earlier commercially obtained PNGase-F batches. The present finding indicates that it seems to be advisable to check for endo-F activity prior to use. It is noteworthy that no tri-/tri-antennary oligosaccharides with a reducing GlcNAc-**2** were found, which is in line with the earlier reported observation [29] that the *N,N'*-diacetylchitobiosyl unit is resistant to hydrolysis by endo-F when it is part of a tri- or tetra-antennary oligosaccharide-glycopeptide structure. Apparently the stability of the GlcNAcβ1-4GlcNAc glycosidic linkage towards endo-F hydrolysis persists in the free oligosaccharide chain.

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