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Analysis of sialic acid-containing mucin oligosaccharides from porcine small intestine by high-temperature gas chromatography–mass spectrometry of their dimethylamides

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ABSTRACT

Acetylation of sialic acid-containing oligosaccharides lactonises the sialic acid residue quantitatively for all oligosaccharides studied except for 6′-sialyl-lactose. The modified, unsulphated, sialylated and sulphated oligosaccharides can then be fractionated by anion-exchange chromatography. Ammonolysis of the lactones followed by methylation yielded the dimethylamides, which are amenable to g.l.c.–m.s. and give intense and informative mass spectra. This approach has been used to characterise the sialic acid-containing O-linked oligosaccharides obtained from the mucin glycopeptides of the small intestine of the pig. At least 28 structures were found, having NeuAc or NeuGc 6-linked to the HexNAc attached to the peptide core or to a Hex 3-linked to HexNAc. Four different disialylated oligosaccharides were found having NeuAc or NeuGc on the Hex residue 3-linked to HexNAc and 6-linked to HexNAc.

INTRODUCTION

Sialic acid is present in glycosphingolipids and glycoproteins[1]. The O-linked oligosaccharides found in mucins often have a relatively large content of sialic acid[2] which contributes to their polyanionic character. Sialic acid occurs as *N*-acetyl-(NeuAc) and *N*-glycoloyl-neuraminic acid (NeuGc), depending on the species[1] and sometimes on the strain.The gangliosides of the epithelial cells from the small intestine have both NeuAc and NeuGc in most strains of rat[3].

In order to study the structure of mucins in health and disease, we have developed techniques for the rapid screening of small amounts of oligosaccharides. Oligosaccharides, up to decasaccharides[4,5], can now be analysed by g.l.c. and g.l.c.–m.s. The strategy is based on the fractionation of the mixture of oligosaccharides released on treatment with alkaline borohydride into neutral and acidic components. After acetylation, the latter components are fractionated into (*a*) species that contain sialic acid and (*b*) those that have unlactonised sialic acid or at least one sulphate group, an approach used for glycosphingolipids[6]. Methylation of sialylated oligosaccharides having a HexNAcol

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terminus with the solid sodium hydroxide procedure[7] gave low yields. The yield was increased if lactonisation of the sialic acid residues by acetylation was followed by ammonolysis and methylation to give the dimethylamide, g.l.c.–m.s. of which gives intense mass spectra, suitable for structural assignments.

EXPERIMENTAL

The 3′- and 6′-sialyl-lactose were purchased from BioCarb. DEAE-Sephadex A-25 and Sephadex LH-20 from Pharmacia, and AG 50W-X8 resin from BioRad.

Preparation of the mucin glycopeptides.— The mucosa of the small intestine of the pig was scraped off and the mucin glycopeptides were prepared as described[5]. The procedure included digestion with papain, DNase, and RNase, gel chromatography, and isopycnic density-gradient centrifugation.

Release of the oligosaccharides from mucin glycopeptides.— The pH of a solution of the lyophilised glycopeptides (9 mg) in water (4.5 mL) was adjusted to 9 before the addition of a solution (4.5 mL) of 2M $NaBH_4$ in 0.1M KOH. The mixture was incubated for 16 h at 45°, the reaction was stopped by the addition of glacial acetic acid, and the salt was removed on a column of AG 50W-X8 resin (13.5 mL) by elution with water (70 mL). The water was evaporated, the residue was dissolved in methanol with 2 drops of glacial acetic acid, and the solvent was evaporated. This procedure was repeated 5 times before a solution of the residue in water with 1% of 1-butanol was applied to a column of DEAE-Sephadex A-25 (0.5 mL/mg of glycopeptide). The neutral oligosaccharides were eluted with water containing 1% of 1-butanol and the acid components with M pyridinium acetate (pH 5.4). The oligosaccharides were recovered by lyophilisation.

Acetylation of the acidic oligosaccharides. — The fraction containing the acidic oligosaccharides or standards was treated overnight with acetic acid anhydride (0.5 mL) and pyridine (1 mL) in tubes with Teflon-faced screw caps. The solvent was evaporated in a stream of nitrogen after the addition of methanol and toluene. A solution of the acetylated oligosaccharides in methanol was applied to a column of DEAE-Sephadex A-25 (0.5 g/10 mg of starting glycopeptide). The lactones were eluted with methanol (100 mL/g of gel) and the bound oligosaccharides with M pyridinium acetate (pH 5.4, 100mL/g of gel).

Preparation of the sialic acid amides and methylation. — The acetylated samples were each dried under vacuum for 1 h, dry methanol (3 mL) was added, ammonia gas was bubbled gently through the methanol for 10 min, and the mixture was left overnight in a sealed vessel. The solvent was evaporated in a stream of nitrogen, and the residue was dried under vacuum for 30 min, then methylated[5,7] by the addition of dimethyl sulfoxide (1 mL), iodomethane (0.2 mL), and NaOH powder (40 mg). The mixture was stirred at room temperature for 10 min, water (4 mL) and chloroform (2 mL) were added, and the chloroform phase was washed at least 4 times with water. The chloroform phase was transferred to new tubes and the solvent was evaporated in a stream of nitrogen. The resulting methylated oligosaccharides were purified on a column (7 × 500 mm) of Sephadex LH-20 by elution with methanol (0.25 mL/min).

G.l.c. and g.l.c.–m.s. — Fused-silica columns (10 m × 0.25 mm i.d.), HT-polyimide-coated and deactivated (Chrompack), were statically coated with 0.04 μm of PS 264 (Fluka) and cross-linked according to Blomberg *et al.*[8]. Capillary g.l.c. was carried out on a Carlo Erba 5160 or Hewlett–Packard 5890A gas chromatograph. Hydrogen was used as carrier gas (80 cm/s at 70° and 0.7 bar) with an oxygen trap (Oxypurge, Alltech). The flame-ionisation detector had a temperature of 400°. An aliquot (1 μL, 1–100 ng/component) of a solution of each sample in ethyl acetate was injected on-column at 70°. The temperature program was 40°/min to 170°, then 10°/min up to 400°.

G.l.c.–m.s. was carried out on a Carlo Erba 4160 gas chromatograph interfaced to a VG ZAB-HF mass spectrometer equipped with a VG 11-250 data system. The column was introduced directly into the ion source and the tip was positioned 1–2 mm from the electron beam. The fused-silica column (10 m × 0.25 mm i.d.) was coated with cross-linked PS 264 (film 0.04 μm) and helium was used as carrier gas (90 cm/s at 70° and 0.1 bar). A high-capacity gas purifier and an OMI-1-indicating purifier (Supelco) were used in the carrier gas line. An aliquot (1 μL, 1–200 ng/component) of a solution of each sample in ethyl acetate was injected on-column at 70° and, after 1 min, the temperature

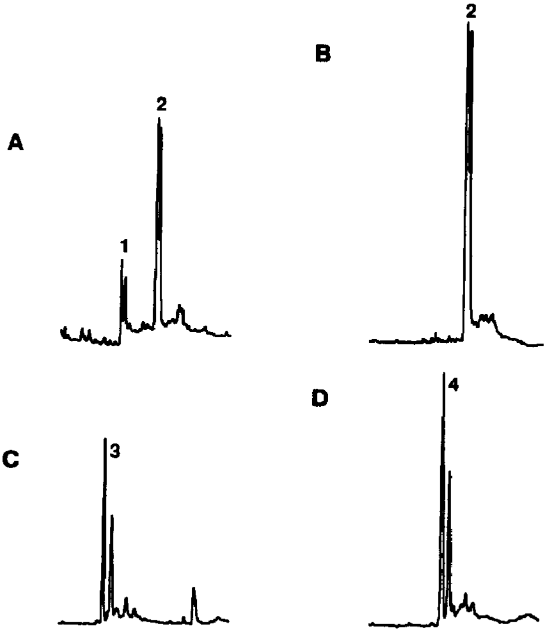

Fig. 1. Gas chromatograms (see Experimental): A, acetylated 3′-sialyl-lactose was treated with aqueous methanolic NH_3 for 15 h, then methylated (1, methyl esters; 2, dimethylamides; and the last the dimethylamide of NeuGc-lactose); B, acetylated 3′-sialyl-lactose was treated with dry methanolic ammonia for 15 h and than methylated. Acetylated 6′-sialyl-lactose was eluted from DEAE-Sephadex with methanol and then M ammonium acetate. Each fraction was treated with dry methanolic ammonia and then methylated; C shows the products (3) of the retarded fraction (methyl ester), and D those (4) from the non-retarded (dimethylamide). Similar amounts were injected in each experiment and all the peaks were eluted at $\sim$300°.

was increased to 390° at 10°/min and held there for 5 min. The conditions for m.s. were as follows: interface and ion-source temperature, 365°; electron energy, 40 eV; trap current, 500 μA; acceleration voltage, 8 kV; mass range scanned, 1600–160 at 2 s/decade; total cycle time, 3.5 s; resolution, 1400; pressure in the ion source region, 10^{-5} mbar.

RESULTS

Sialyl-lactose. — Acetylation of 3′-sialyl-lactose, deacetylation with aqueous ammonia (25%), and methylation gave two major doublet peaks in g.l.c. (Fig. 1A). The first doublet (1), which was retarded on DEAE-Sephadex before methylation, was identified by g.l.c.–m.s. as methylated 3′-sialyl-α- and -β-lactose methyl ester. The second doublet (2) was due to methylated 3′-sialyl-α- and -β-lactose dimethylamide.

If the acetylated 3′-sialyl-lactose was deacetylated under anhydrous conditions with methanolic ammonia, methylation then gave only the second doublet (2) (Fig. 1B). The mass spectrum (Fig. 2) of this fraction had a base peak at *m/z* 756, due to $[M-CONMe_2]^+$, a fragment ion typical of this derivative. The fragment ion of the dimethylamide derivative of NeuAc is found at *m/z* 389, 13 m.u. higher than the corresponding methyl ester. The results showed that 3′-sialyl-lactose was quantitatively converted into the lactone on acetylation, to the amide on ammonolysis, and to the dimethylamide on methylation. The small doublet eluted after the major peak in Fig. 1

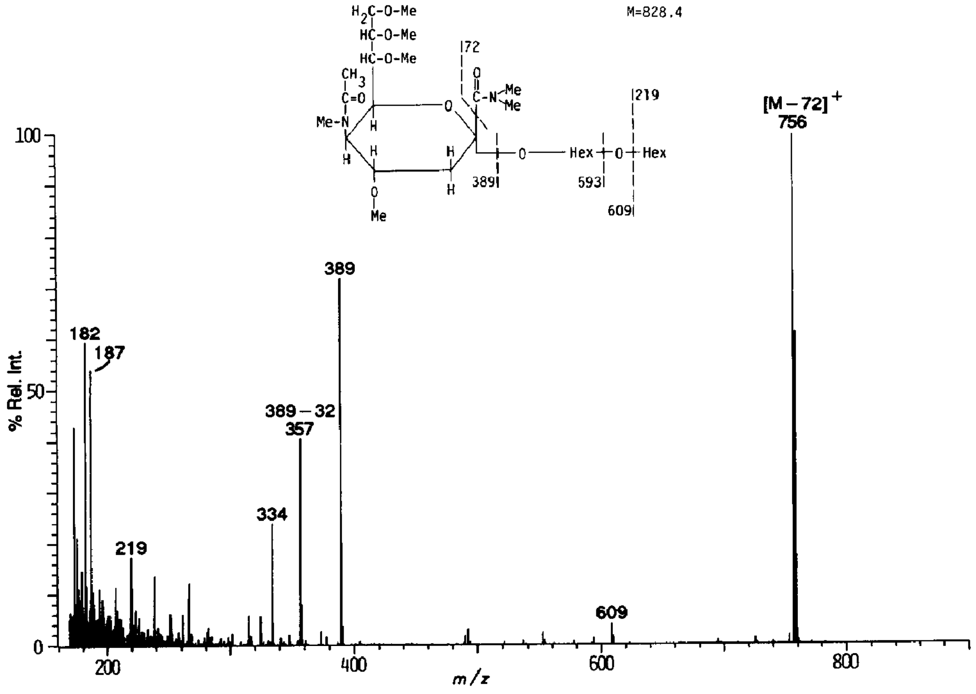

Fig. 2. Mass spectrum from g.l.c.–m.s. of methylated 3′-sialyl-lactose dimethylamide (see Experimental).

(A and B) was due to contamination of the original sample with 3′-sialyl-lactose having NeuGc instead of NeuAc.

6′-Sialyl-lactose was not converted quantitatively into the lactone on acetylation and ~30% of the product still contained a carboxylic group and was retarded on DEAE-Sephadex. After ammonolysis and methylation, g.l.c. showed peaks for the α and β anomers (Fig. 1C, 3), and g.l.c.–m.s. indicated that they were the methyl esters. The material not retarded on DEAE-Sephadex gave, after ammonolysis and methylation, two peaks that eluted later than the methyl esters (Fig. 1D, 4) and their mass spectra showed that they were due to the dimethylamides.

Sialic acid-containing oligosaccharides. — The oligosaccharides released by treatment with alkaline borohydride of the mucin glycopeptides, isolated from the small intestine of the pig, were fractionated into the neutral and acidic components. The latter oligosaccharides were acetylated, and those not retarded on DEAE-Sephadex were subjected to ammonolysis, methylation, and analysis by g.l.c. and g.l.c.–m.s.

The total-ion chromatogram is shown in Fig. 3 and the corresponding oligosaccharides are listed in Table I. Oligosaccharides with both NeuAc and NeuGc were found as their methylated dimethylamides, as shown by the mass chromatogram of *m/z* 389 for the NeuAc and *m/z* 419 for the NeuGc (Fig. 4). No evidence for the presence of any methyl esters was found in this fraction or in the methylated sulphate-containing fraction. Most of the oligosaccharides were monosialylated and were found as pairs with either NeuAc or NeuGc. The simplest structures (1 and 2) had the sialic acid 6-linked to HexNAcol. This type of linkage was found in several other oligosaccharides,

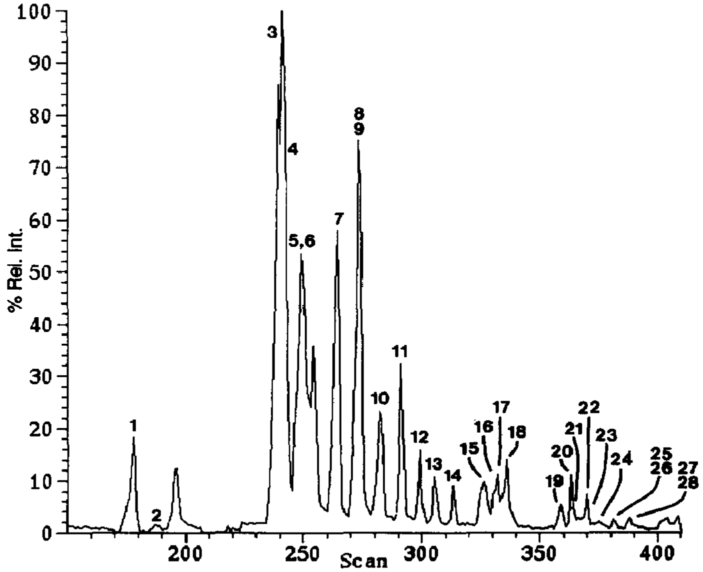

Fig. 3. Total ion chromatogram (see Experimental) from g.l.c.–m.s. of methylated dimethylamides of sialic acid-containing oligosaccharides from mucin glycopeptides of the small intestine of the pig. The numbers refer to structures listed in Table I. The temperature was 275° at scan 200, and 390° at scan 400.

with neutral oligosaccharides 3-linked to HexNAcol (Table I). Compounds 4 and 6 had NeuAc or NeuGc attached to Hex that was 3-linked to HexNAcol. The mass spectrum of 4 (Fig. 5) showed an intense ion at *m/z* 813 for $[M-CONMe_2]^+$. In most of the oligosaccharides, both the C-3 and C-6 of the HexNAcol were substituted, except for 1, 2, 4, 6, 13, and 14, each of which gave a fragment ion at *m/z* 276 (Fig. 4).

Four of the structures identified contained Fuc, as shown in Fig. 4 by the mass chromatogram of *m/z* 189. The mass spectrum (Fig. 6) of 20, having a blood group H-type terminal sequence, showed the NeuAc to be 6-linked to HexNAcol as indicated by the fragment ion at *m/z* 650, and to have Fuc–Hex–HexNAc attached to C-3 (*m/z* 841). The $[M-CONMe_2]^+$ ion is found at *m/z* 1233. The peak in scan 254 is due to a non-sialic acid-containing oligosaccharide contaminant (Figs. 3 and 4).

Four compounds were found to contain either two NeuAc (19), two NeuGc (23), or one of each (20 and 24), all having an identical core of –Hex–3HexNAcol6–. The mass spectrum of 19 is shown in Fig. 7. The two NeuAc residues are shown by the intense

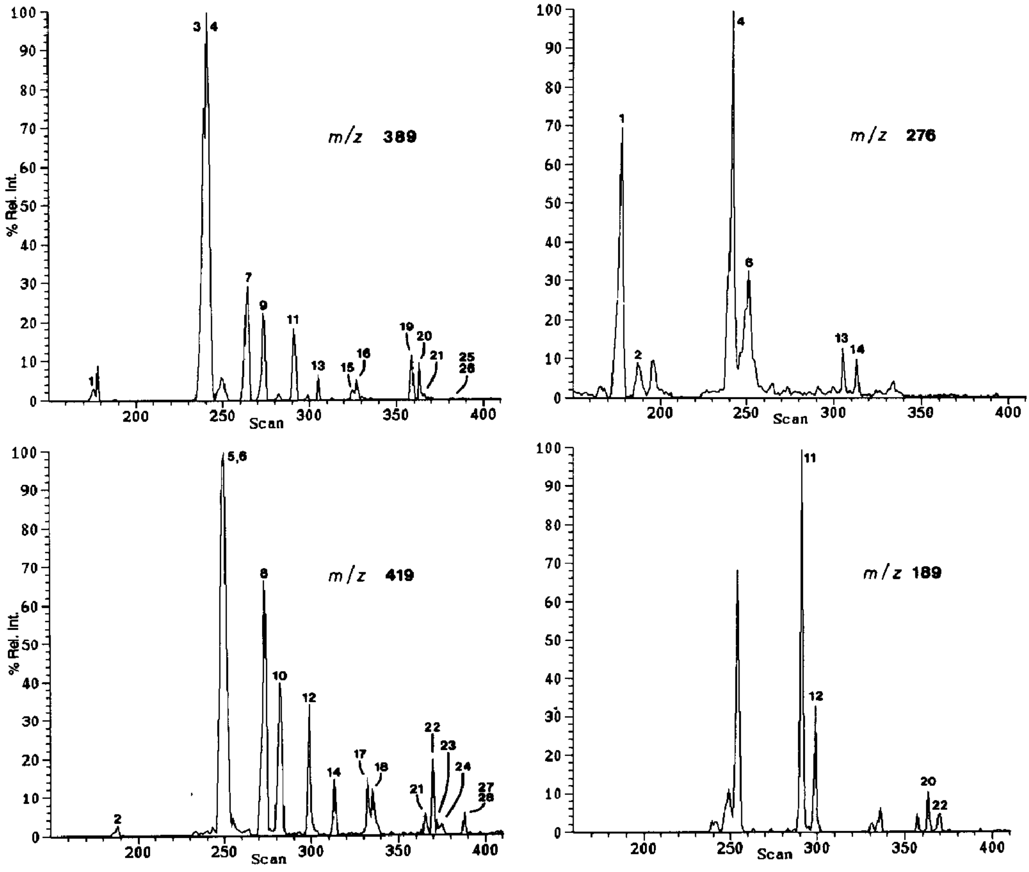

Fig. 4. G.l.c.–m.s. of the methylated dimethylamides of the sialic acid-containing oligosaccharides from the mucin glycopeptides of the small intestine of the pig: *m/z* 389 is due to terminal NeuAc, *m/z* 419 to terminal NeuGc, *m/z* 189 to terminal Fuc, and *m/z* 276 to HexNAcol. From the analysis shown in Fig. 3.

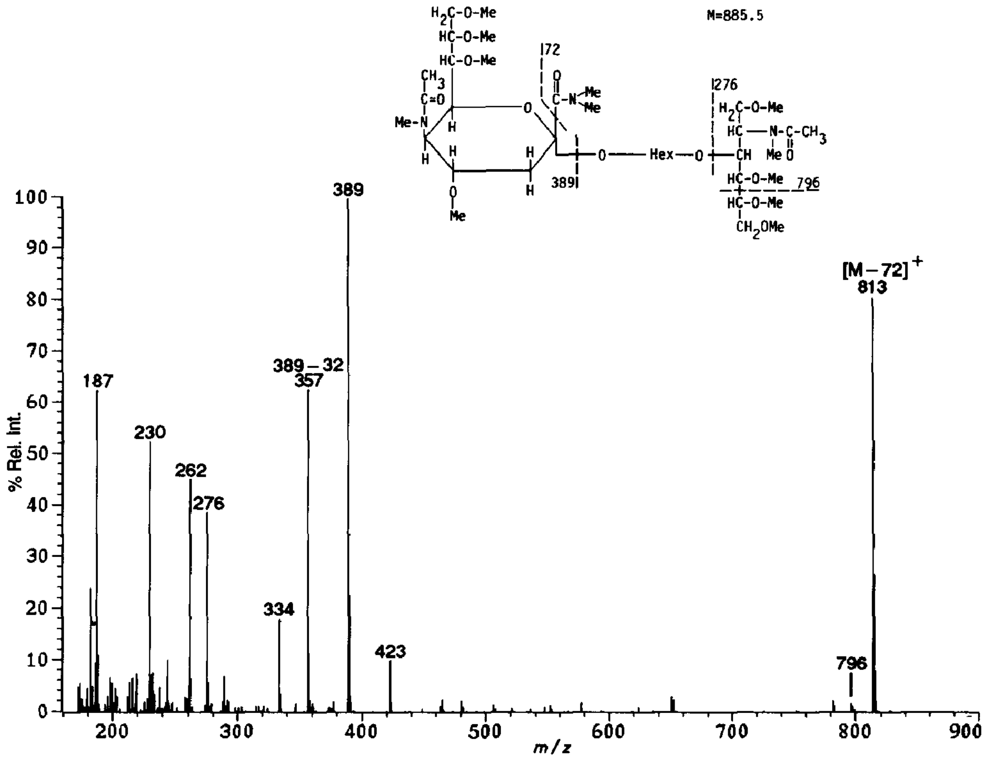

Fig. 5. Mass spectrum of a trisaccharide (4 in Table I) recorded at scan 241 from the g.l.c.–m.s. shown in Fig. 3.

ion at *m/z* 389, and the $[M-CONMe_2]^+$ ion is found at *m/z* 1188. The weak fragment ion at *m/z* 800 is due to the loss of one NeuAc from $[M-CONMe_2]^+$ with the addition of a hydrogen atom.

DISCUSSION

The dimethylamide derivatives of sialic acid-containing oligosaccharides are useful for the characterisation of O-linked structures by g.l.c.–m.s. Acetylation lactonises the sialic acid residues, which makes the glycan neutral. Ammonolysis under anhydrous conditions produces the amide, methylation of which gives the dimethylamide. For 3′-sialyl-lactose, HO-2 of the Gal is most probably involved in the lactone, as found for the ganglioside GM_3-lactone[9].

Using this procedure, the sialic acid-containing oligosaccharides of the mucin glycopeptides obtained from the small intestine of the pig were characterised. These oligosaccharides have the sialic acid residues either 6-linked to HexNAcol (the GalNAc linked to Ser or Thr) or to a Hex (Gal) 3-linked to HexNAcol. These two types of sialic acid linkages have also been found in the porcine submaxillary gland[10]. The sialic acids in the small intestine were either of the *N*-acetyl or the *N*-glycoloyl type as in other pig

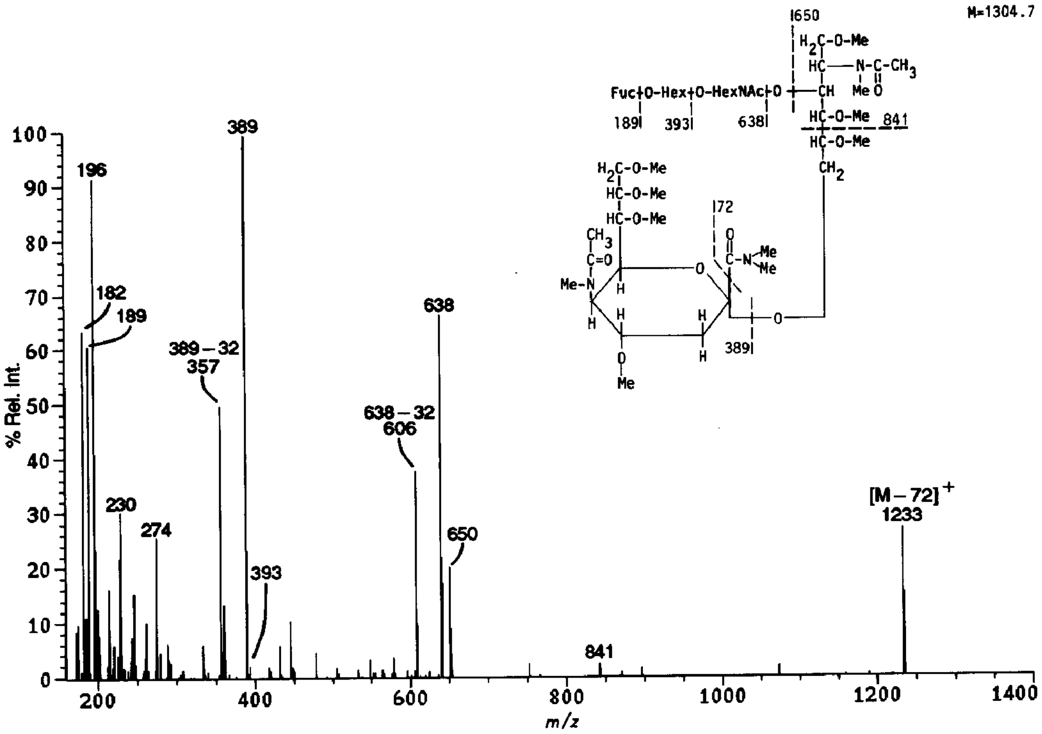

Fig. 6. Mass spectrum of a sialylated blood-group H-type pentasaccharide (20) recorded at scan 363 from the g.l.c.–m.s. shown in Fig. 3.

tissues studied[10–14]. Most of the oligosaccharides identified (Table I) have been described previously in detail in pig and other species, although some of them (14, 17, 18, and 22) only with NeuAc and not NeuGc. In contrast, the structure HexNAc–[NeuAc(Gc)]–Hex–3HexNAcol6–HexNAc (26 and 28) has not been described hitherto. The total ion chromatogram (Fig. 3) shows four peaks (7–10) having mass spectra with the sequence HexNAc–3HexNAcol–NeuAc(Gc). This finding may be due to one pair having terminal GlcNAc and the other terminal GalNAc. Similar types of compounds have been described in human rectal adenocarcinoma[15].

Four disialylated oligosaccharides were found (19, 21, 23, and 24), and those having two NeuAc or two NeuGc have been described earlier. However, no O-linked oligosaccharides appear to have been described with one NeuAc and one NeuGc on the same molecule. These four structures are the only possible ones with one neutral oligosaccharide core and two possible sialic acid linkages.

High-temperature g.l.c. and g.l.c.–m.s. for the analysis of oligosaccharides have, at present, an upper mass limit[5] of 2000. The largest oligosaccharide identified in the present investigation had a molecular mass of 1406. There was evidence for the presence of larger structures, but their low abundances prevent detailed study. The best approach for this would be to purify the larger oligosaccharides by h.p.l.c. before g.l.c. and g.l.c.–m.s. The high resolution of capillary g.l.c. is shown by the fractionation of this

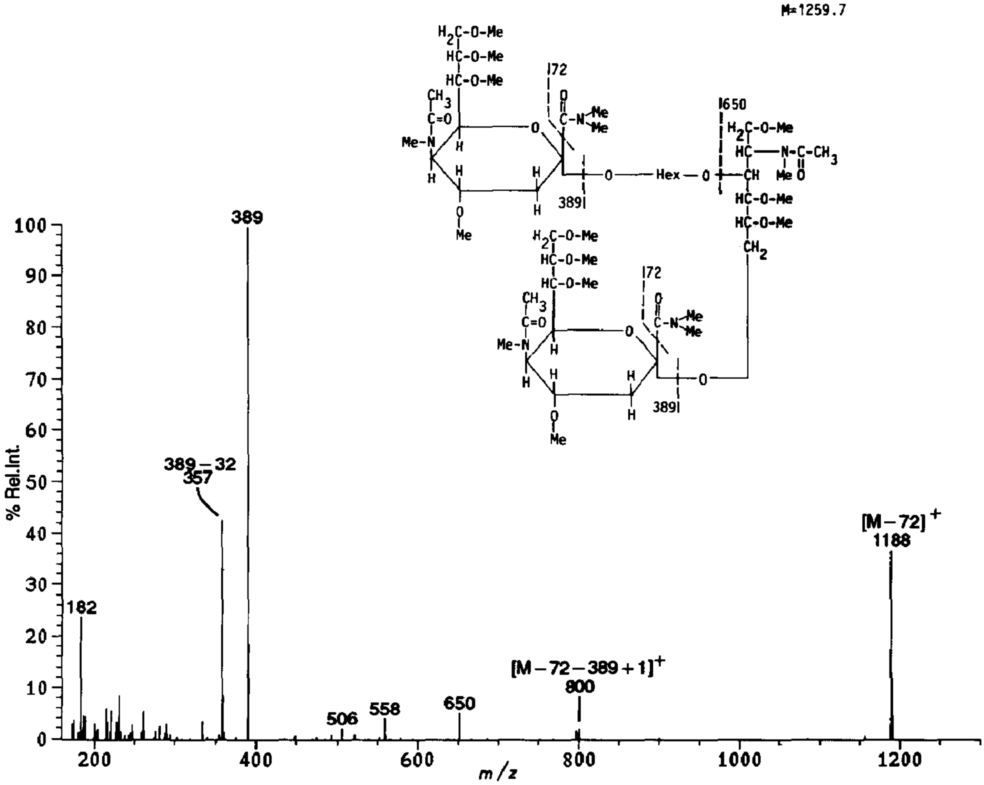

Fig. 7. Mass spectrum of a disialylated tetrasaccharide (19) recorded at scan 359 from the g.l.c.–m.s. shown in Fig. 3.

complex mixture. However, this high resolution is partly lost by the slow scan rate used. Several of the peaks in Fig. 3 contain more than one component that have been resolved by g.l.c. (data not shown).

Previously, we obtained poor yields when methylating sialic acid-containing O-linked oligosaccharides. The formation of lactones, and deacetylation and amidation in dry ammonia gave much higher yields and a dimethylamide in the methylation step. Amides of sialic acid have been produced by treating lactones of gangliosides with ammonia[16] and the formation of the dimethylamides after methylation has been demonstrated[17].

The dimethylamides of methylated sialic acid-containing oligosaccharides are useful for g.l.c., g.l.c.–m.s., and m.s. because their chromatographic properties are similar to those of the methyl esters and they provide intense mass spectra. A strong fragment ion for $[M-CONMe_2]^+$ together with the strong ion of NeuAc (NeuGc) at *m/z* 389 (419) is typical for this derivative. Present also are sequence ions and ions from inductive cleavage at the O-3 of the HexNAcol. However, ions due to cleavage between C-4 and C-5, abundant in neutral oligosaccharides[5], are of low intensity. The $[M-CONMe_2]^+$ ion is useful for establishing the number and type of sugar residues in the oligosaccharide. The intensity of a mass spectrum of the 3′-sialyl-lactose dimethylamide derivative is about twice that of the methyl ester.

TABLE I

Sialic acid-containing oligosaccharides identified by g.l.c.–m.s.

No.[a]	*Structure*	*Molecular mass*
1	HexNAcol6–NeuAc	681
2	HexNAcol6–NeuGc	711
3	Hex–3HexNAcol6–NeuAc	886
4	NeuAc–Hex–3HexNAcol	886
5	Hex–3HexNAcol6–NeuGc	916
6	NeuGc–Hex–3HexNAcol	916
7	HexNAc–3HexNAcol6–NeuAc	927
8	HexNAc–3HexNAcol6–NeuGc	957
9	HexNAc–3HexNAcol6–NeuAc	927
10	HexNAc–3HexNAcol6–NeuGc	957
11	Fuc–Hex–3HexNAcol6–NeuAc	1060
12	Fuc–Hex–3HexNAcol6–NeuGc	1090
13	HexNAc–Hex–3HexNAcol │ NeuAc	1131
14	HexNAc–Hex–3HexNAcol │ NeuGc	1161
15	Hex–HexNAc–3HexNAcol6–NeuAc	1131
16	NeuAc–Hex–3HexNAcol6–HexNAc	1131
17	Hex–HexNAc–3HexNAcol6–NeuGc	1161
18	NeuGc–Hex–3HexNAcol6–HexNAc	1161
19	NeuAc–Hex–3HexNAcol6–NeuAc	1260
20	Fuc–Hex–HexNAc–3HexNAcol6–NeuAc	1305
21	NeuGc–Hex–3HexNAcol6–NeuAc	1290
22	Fuc–Hex–HexNAc–3HexNAcol6–NeuGc	1335
23	NeuGc–Hex–3HexNAcol6–NeuGc	1320
24	NeuAc–Hex–3HexNAcol6–NeuGc	1290
25	NeuAc–Hex–3HexNAcol6–HexNAc–Hex	1335
26	HexNAc–Hex–3HexNAcol6–HexNAc │ NeuAc	1376
27	NeuGc–Hex–3HexNAcol6–HexNAc–Hex	1365
28	HexNAc–Hex–3HexNAcol6–HexNAc │ NeuGc	1406

[a] The numbers refer to Figs. 3 and 4.

The formation of amides can be a useful modification of sialic acid-containing glycoconjugates when studying the carboxyl group in the specificities of toxins, bacteria[18], viruses[19], monoclonal antibodies[20], and cell–cell adhesion molecules[21,22].

The conversion of sialic acid into neutral lactones upon acetylation has been used to separate sialic acid-containing glycosphingolipids from those having sulphate groups[6]. This approach can also be used for oligosaccharides. However, a critical step is the formation of the lactone. By using the anhydrous conditions formed by acetic anhydride, the conversion into lactones was almost complete for gangliosides having sialic acid 3-linked to Hex[6], as well as for the same sequence in 3′-sialyl-lactose. With the sialic acid 6-linked to HexNAcol, the lactone involves HO-5 of the HexNAcol, as shown by m.s. of spontaneously formed lactones followed by methylation (data not given). The formation of this type of lactone is probably facilitated by the flexible nature of the HexNAcol molecule and the lactonisation effected by acetylation is quantitative. In contrast, the sialic acid linked to C-6 of a Gal, as in 6′-sialyl-lactose, gave only ~70% of the corresponding lactone. The formation of lactones upon acetylation can thus vary with the type of sialic acid linkage. The way of obtaining the sialic acid-dimethylamides may be improved by other methods avoiding internal ester formation.

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