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Structural analysis of the major urinary oligosaccharides in feline α-mannosidosis

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Two homologous series of urinary oligosaccharides were identified by h.p.l.c. and fast-atom-bombardment mass spectrometry in feline α-mannosidosis. The predominant series has the composition $Man_{2-8}GlcNAc_2$ and a minor series the composition $Man_{2-9}GlcNAc$. The structure of the most abundant oligosaccharide, which accounts for over 80% of the urinary oligosaccharide, was shown to be α-D-Man*p*(1→3)[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc by gas chromatography and mass spectrometry. Such a structure is consistent with the incomplete catabolism of complex *N*-linked glycans due to a deficiency of α-D-mannosidase in tissue lacking an endohexosaminidase activity.

INTRODUCTION

Lysosomal storage diseases result from a genetically or chemically induced deficiency of a lysosomal hydrolase, which leads to the accumulation in tissues and excretion in the urine of partially catabolized metabolites. α-Mannosidosis is the lysosomal storage disease resulting from a deficiency of α-D-mannosidase (EC 3.2.1.24) [1]. It is characterized by the accumultion and excretion of mannose-rich oligosaccharides derived from the incomplete catabolism of the asparagine-linked glycans of glycoproteins. It has been described in humans, cattle and cats, and clinical variants of the human disease have been reported [2]. The storage products in the three species are different, reflecting differences either in the catabolic pathways of glycoproteins or in the specificity of the residual acidic α-D-mannosidase [3, 4]. The storage products in genetically induced human [5] and bovine mannosidosis [6, 7] and in swainsonine-induced mannosidosis in several species have been fully characterized. We have shown previously that the storage products in feline α-mannosidosis contain only mannose and *N*-acetylglucosamine [4], and in the present paper we report on their structures.

MATERIALS AND METHODS

All chemicals used were of analytical grade obtained from sources described previously [6, 8].

Preparative methods

Feline mannosidosis urine was stored at −20 °C until required. After thawing, the samples were deionized and the neutral oligosaccharides were extracted by t.l.c. and gel-permeation chromatography as described in ref. [6].

Analytical methods

Densitometric scanning of stained t.l.c. chromatograms was performed with the Joyce–Loebl Chromoscan 200 instrument fitted with a scan 201 attachment as reported in ref. [9].

F.a.b.m.s.

F.a.b.m.s. of native samples was carried out as reported in ref. [10] and after acetylation of oligosaccharides [11].

H.p.l.c.

Fractions of neutral oligosaccharides isolated by gel-filtration chromatography on Bio-Gel P-4 were reduced and benzoylated [12], and the per-*O*-benzoylated alditols were analysed by h.p.l.c. as in ref. [8]. Sugar composition and structures were assigned by comparing the elution patterns of samples with those of authentic oligosaccharides of known structure [7, 12].

G.c.

Capillary g.c. was carried out on the major component after the permethylation of the alditol acetate derivative by a modification of the procedure of Waeghe *et al.* [13], with potassium dimsyl in the methylation step in accordance with Phillips & Fraser [14]. Peak identifications were assigned by comparison with authentic standards, which were characterized by g.c.–m.s.

RESULTS AND DISCUSSION

Analysis by t.l.c. of the oligosaccharides excreted in the urine of a cat with α-mannosidosis revealed the presence of one major component, accounting for over 80% of the

Abbreviations used: g.c., gas chromatography; f.a.b.m.s., fast-atom-bombardment mass spectrometry.

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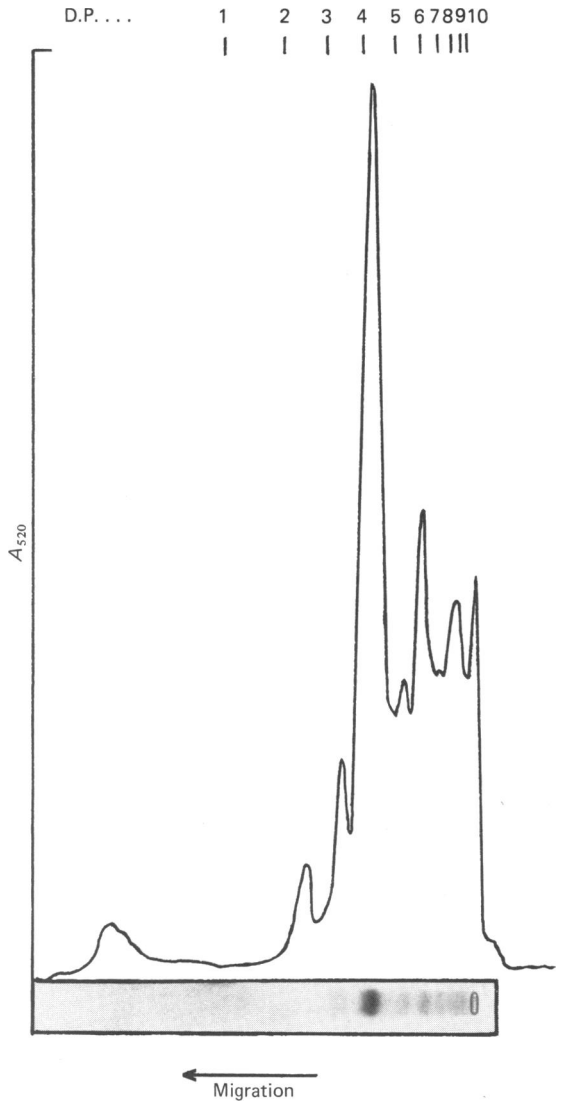

Fig. 1. T.l.c. of whole urinary oligosaccharide present in feline α-mannosidosis

The plate was developed in propan-1-ol/water (8:3, v/v) for 5 h and stained with orcinol. Carbohydrates were detected after heating for 5 min at 100 °C as described in the Materials and methods section. D.P. indicates the degree of polymerization of glucose oligomers.

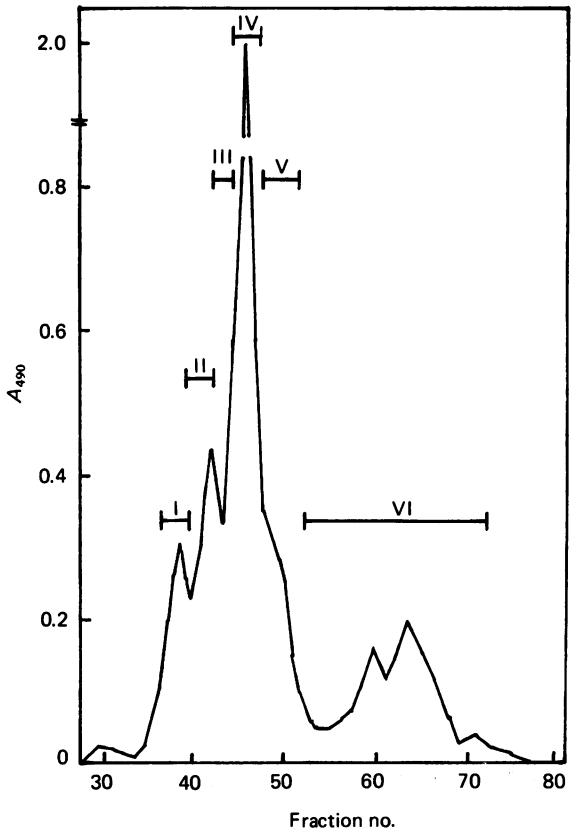

Fig. 2. Gel-permeation chromatography of neutral oligosaccharide (1 mg) from feline mannosidosis urine

The column (1 cm × 100 cm) of Bio-Gel P-4 (–400 mesh) was eluted with distilled water, and 1 ml fractions were collected. A portion of every alternate fraction was assayed for neutral carbohydrate content as described in the Materials and methods section. Fractions I–VI were pooled as indicated and used in the subsequent experimental analysis.

total carbohydrate, and a series of homologues, up to an apparent decasaccharide (Fig. 1). Fractionation of these oligosaccharides on Bio-Gel P-4 resolved only six components, giving the elution profile shown in Fig. 2. Fractions were pooled as indicated, derivatized and analysed by h.p.l.c. (Fig. 3). The compositions of the observed components were tentatively assigned by comparison with the retention times of authentic standards of known structure (Table 1). These results suggested that two homologous series of oligosaccharides are excreted. The predominant series has the probable composition $Man_{2-6}GlcNAc_2$, and a less abundant series has the probable composition $Man_{2-6}GlcNAc$. In general structural isomers of the same composition were not observed. The main component had the identical retention time on h.p.l.c. as an authentic sample of α-D-Man*p*-(1→3)[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc.

Confirmation of the presence of the two series of oligosaccharides and of the sizes of the oligosaccharides was obtained by f.a.b.m.s. of the total neutral oligosaccharides in the urine, after conversion into the peracetyl derivative. Molecular ions were present for oligosaccharides of compositions $Hex_{2-6}HexNAc$ and $Hex_{2-6}HexNAc_2$, thus confirming the h.p.l.c. results. In addition, very small amounts of oligosaccharides with masses corresponding to $Hex_{7-9}HexNAc$ and $Hex_{7-8}HexNAc_2$ were also observed. Presumably these oligosaccharides were eluted before the components in fraction I from the gel filtration (Fig. 2) and therefore not analysed by h.p.l.c., or were present in fraction I at too low concentrations to be detected by h.p.l.c.

The size and composition of all the oligosaccharides resolved by h.p.l.c. and f.a.b.m.s. correspond to fragments that could arise from the incomplete catabolism of asparagine-linked glycans of glycoproteins resulting from a deficiency of lysosomal α-D-mannosidase. The predominant storage oligosaccharides have two *N*-acetylglucosamine residues at the reducing terminal. This implies that cleavage of the core chitobiosidic linkage by

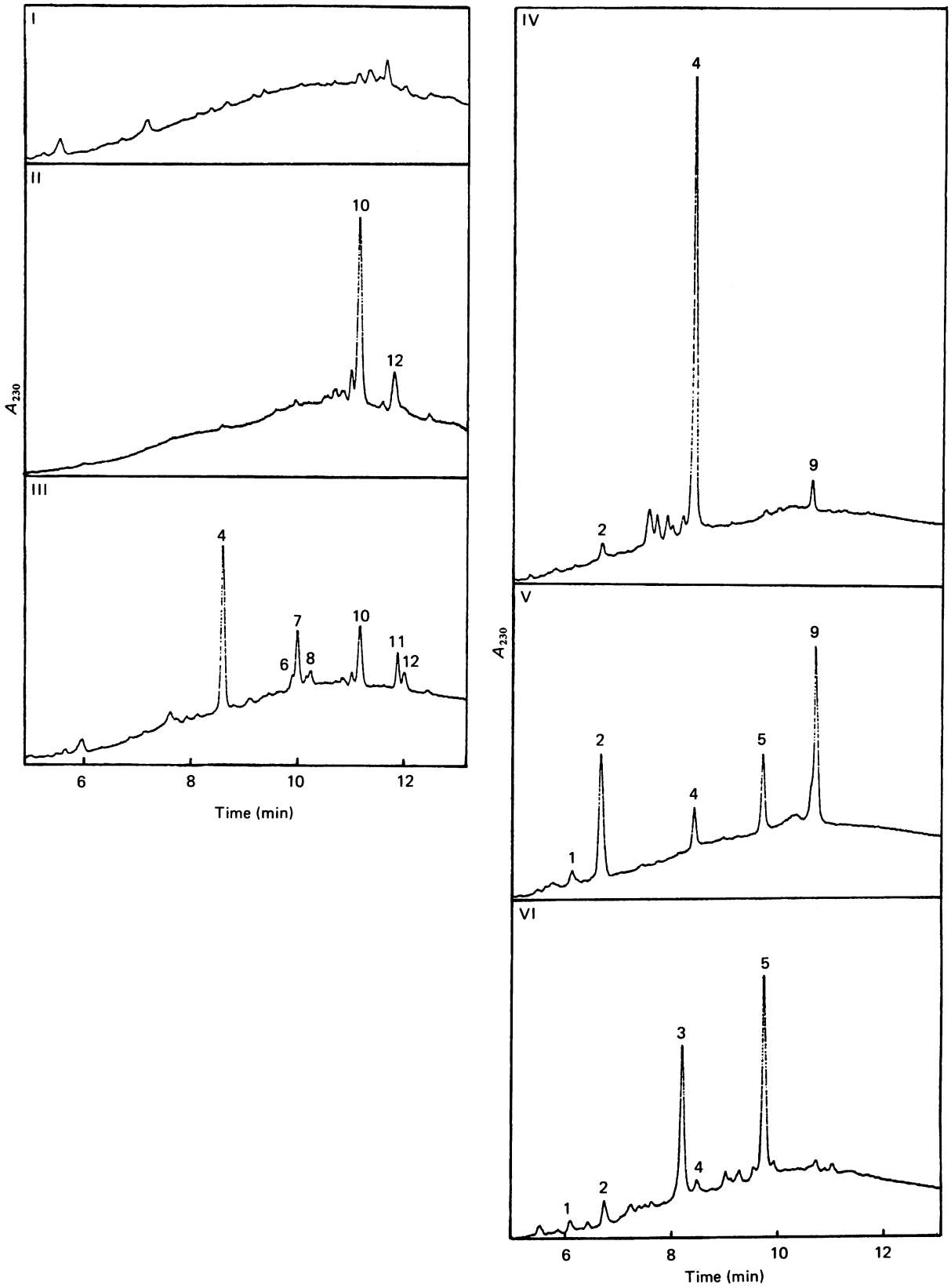

Fig. 3. H.p.l.c. elution profiles of feline urinary oligosaccharide fractions from gel-permeation column

Isolated oligosaccharides (I–VI in Fig. 2) were reduced with $NaBH_4$ and benzoylated as indicated in the Materials and methods section. The per-*O*-benzoylated alditols were dissolved in solvent and subjected to h.p.l.c. on a 3 μm octyl-silica column (4.6 mm × 100 mm) (C8 Microsorb Shortone; Rainin Laboratories, Woburn, MA, U.S.A.). Benzoylated oligosaccharides were eluted with a linear gradient from acetonitrile/water (4:1, v/v) to pure acetonitrile at a flow rate of 1 ml/min and detected by their absorbance at 230 nm [8]. Peak identifications (Table 1) were assigned by comparison with authentic standards of known structure [7].

Table 1. Analysis of the main storage oligosaccharides

Oligosaccharide structures were assigned by comparison with retention times of fully characterized authentic samples [7]. Abbreviation: N.D., not determined.

Peak no. from h.p.l.c.	Tentative composition (M = Man; G = GlcNAc)	F.a.b.m.s. m/z ($M+H^+$) molecular ion of peracetyl derivative*	Oligosaccharide structure
1	M_2G	924	Manα1–6Manβ1–4GlcNAc
2	M_2G_2-I	1211	Manα1–6Manβ1–4GlcNAcβ1–4GlcNAc
3	M_3G	1212	Manα1–6 Manβ1–4GlcNAc Manα1–3
4	M_3G_2-Ia	1499	Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc Manα1–3
5+6	M_4G	1500	Manα1–6 Manβ1–4GlcNAc Manα1–2Manα1–3 and other isomer
7+8	M_4G_2	1787	Manα1–6 Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc Manα1–3 and other isomer
9	M_5G	1788	Manα1–6 Manβ1–4GlcNAc Manα1–2Manα1–2Manα1–3
10	M_5G_2	2075	Manα1–6 Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc Manα1–3 Manα1–3
11	M_6G	2076	N.D.
12	M_6G_2	2363	N.D.

* These ions were observed in the spectrum of the unseparated neutral fraction; individual h.p.l.c. fractions were not analysed by f.a.b.m.s. except for M_3G_2-Ia.

Table 2. Methylation analysis of main urinary oligosaccharide M_3G_2-Ia

Peak identifications were based on retention times and mass spectra of authentic M_2G_2-I and partially permethylated α-methyl mannoside. Molar ratios are expressed as mol/mol of 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol.

Methylated derivative	Molar ratio	Peak no.
Mannitol		
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl-)	2.19 (2)	1
3,4,6-Tri-*O*-methyl-(1,2,5-tri-*O*-acetyl-)	Trace (< 0.1)	2
2,4,6-Tri-*O*-methyl-(1,3,5-tri-*O*-acetyl-)	Trace (< 0.1)	3
2,3,4-Tri-*O*-methyl-(1,5,6-tri-*O*-acetyl-)	Trace (< 0.1)	4
2,4-Di-*O*-methyl-(1,3,5,6-tetra-*O*-acetyl-)	1.00 (1)	6
2-*N*-Methylacetamido-2-deoxyglucitol		
1,3,5,6-Tetra-*O*-methyl-(4-mono-*O*-acetyl-)	0.98 (1)	5
2-Di-*N*-acetylamino-2-deoxyglucitol		
1,3,5,6-Tetra-*O*-methyl-(4-mono-*O*-acetyl-)	0.15	7
2-*N*-Methylacetamido-2-deoxyglucitol		
3,6-Di-*O*-methyl-(1,4,5-tri-*O*-acetyl-)	0.85 (1)	8

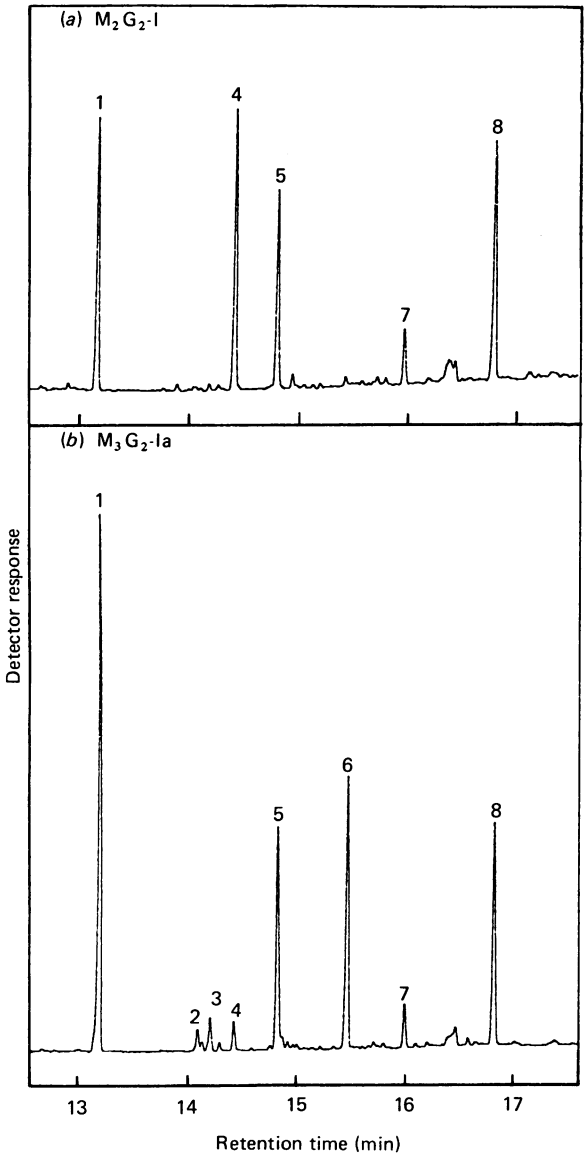

Fig. 4. Capillary g.c. of (*b*) the main feline mannosidosis urine component (M_3G_2-Ia) and (*a*) an authentic standard of known structure (M_2G_2-I) isolated from bovine mannosidosis urine [7]

Capillary g.c. was carried out on the permethylated alditol acetate derivatives [13]. The analysis was performed on a Hewlett–Packard 5890 gas chromatograph with a 30 m × 0.25 mm DB-1 bonded phase column (J & N Scientific) with H_2 as the carrier gas [240 kPa (20 lbf/in² gauge)]. Column temperature was initially at 60 °C with a linear temperature gradient to 220 °C at a rate of 10 °C/min. Detection was by flame ionization. Peaks were assigned (Table 2) by comparison with the retention times of authentic standards.

an endohexosaminidase is not a major step in the catabolic pathway of glycoproteins in cats. However, the presence of oligosaccharides with a single *N*-acetylglucosamine residue indicates that this activity is not completely absent from all or some cells of the cat. A similar situation has been observed in swainsonine-induced mannosidosis in sheep [15], genetic bovine α-mannosidosis [6] and genetic caprine β-mannosidosis [16]. In contrast, in humans, rats and guinea pigs [17, 18] cleavage of the core chitobiosidic linkage appears to be universal, although endohexosaminidase activity cannot be detected in lysosomes in some human and rat cells and has recently been reported to have a cytoplasmic localization [19].

The major storage product, peak 4 on h.p.l.c. (Fig. 3, III and IV), was purified by gel-filtration chromatography and preparative t.l.c. Its composition was shown to be Man,GlcNAc (1.5:1) by g.c. and its size was determined by f.a.b.m.s. to be 910, indicating a molar structure of $Man_3GlcNAc_2$. H.p.l.c. of a partially purified sample (IV) suggested that it consisted almost exclusively of one structural isomer (Fig. 3). This was verified by h.p.l.c. of the purified oligosaccharide, which was co-eluted with an authentic sample of α-D-Man*p*-(1→3)[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc (M_3G_2-Ia).

Evidence supporting this structure was obtained by g.c. of the permethylated alditol acetates obtained on hydrolysis of the sample (Fig. 4). Identification of the peaks was by comparison with authentic standards characterized by g.c.–m.s. (Table 2). During hydrolysis the reducing terminal glucosaminitol undergoes partial demethylation and hence forms the di-*N*-acetyl derivative during the acetylation step [20].

The branched tri-mannose structure of the major storage product is the product expected from the catabolism of complex asparagine-linked glycans in the absence of lysosomal α-D-mannosidase and the cytosolic endohexosaminidase. The higher homologues containing four or more mannose residues arise from high-mannose glycans. As in both human and bovine mannosidosis [3, 6], there is also an oligosaccharide with only two mannose residues (peak 2, Fig. 3). H.p.l.c. suggests that this has the structure α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-GlcNAc, the same as the most abundant storage products in bovine α-mannosidosis [7]. This could be produced by either a residual mutant activity or a form of lysosomal α-D-mannosidase unaffected in this disorder. It is notable that the differences found in the storage products among these diverse mammalian species possibly reflect unique and subtle differences in their catabolic pathways.

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