ISOLATION AND CHARACTERISATION OF 4-*O*-β-D-MANNOPYRANOSYL-2-ACETAMIDO-2-DEOXY-D-GLUCOSE FROM THE URINE OF A PATIENT WITH MUCOLIPIDOSIS II AND ITS OCCURRENCE IN NORMAL AND PATHOLOGICAL URINE

Marguerite LEMONNIER+, Christian DERAPPE+, Livia POENARU*, M. Alan CHESTER, Arne LUNDBLAD, Sigfrid SVENSSON and Per-Arne ÖCKERMAN

*+Chargé(e) de Recherches INSERM U 180, UER Biomedicale des Sts.-Pères, 75006 Paris, *Institut de Pathologie Moléculaire, 24 rue du Faubourg St-Jacques, 75014 Paris, France* and *Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden*

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1. Introduction

A number of neutral oligosaccharides have been isolated from human urine. The excretion of some of these oligosaccharides depends on the donor's blood group [1–3], dietary status [2,4], or some physiological [5–7] and pathological [1,2,8–15] conditions. Patients with lysosomal storage diseases in which the degradation of glycoproteins is affected excrete abnormally large quantities of oligosaccharides which are presumably the substrates of the defective enzymes.

Mucolipidosis II (I-cell disease, ML II) and mucolipidosis III (ML III) probably result from defective post-translational processing of lysosomal enzymes in certain cells [16,17], which cause a multiple lysosomal hydrolase deficiency and increased excretion of sialic acid-containing oligosaccharides [11]. Apart from the sialylated oligosaccharides there has been no report of increased amounts of neutral oligosaccharides in urine from these patients. However, routine gas chromatography–mass spectrometry (GLC–MS) screening of human urinary oligosaccharides showed that a hitherto undescribed neutral disaccharide is also excreted by patients with ML II and ML III and to a lesser extent by normal individuals and patients with other lysosomal storage disorders. We now describe the isolation and structure of this disaccharide.

2. Materials and methods

Urine was collected without dietary restriction from normal individuals and patients with various metabolic disorders. The samples were immediately frozen and stored at −20°C until used. Ultrafiltration of filtered urine was performed at 4°C using an Amicon Hollow Fibre HP 05 system. Samples were concentrated by rotary evaporation at ≤25°C. Gel chromatography was done in a column (10 × 110 cm, V_0 3260 ml) of Sephadex G-15 fine (Pharmacia, Uppsala) eluted with distilled water containing sodium azide (0.02%) at a flow rate of 4 ml/min. Column fractions and whole urine samples were deionised by passage through columns packed with AG 50W-X8 (100–200 mesh), H^+ form, and AG 3-X4A (100–200 mesh), OH^- form (Biorad Labs, Richmond CA). Whatman no. 3 papers were used for descending chromatography using the following systems:

(a) Ethyl acetate/pyridine/water, (2/1/2, by vol., upper phase);
(b) Ethyl acetate/acetic acid/water, (3/1/1, by vol.);
(c) Propan-1-ol/ethyl acetate/water, (6/1/3, by vol.);
(d) Ethyl acetate/pyridine acetic acid/water, (5/5/1/3, by vol.);
(e) Ethyl acetate/pyridine/water, (12/5/4, by vol.).

Papers were stained with a silver dip reagent [18]. A colorimetric method was used for determining total hexose in fractions eluted from gel chromatography columns [19]. Optical rotations were determined using a Perkin-Elmer 2411 polarimeter. Sugar analysis was performed by gas–liquid chromatography (GLC) and mass spectrometry (MS) [20,21]. Methylation analysis was done as in [22]. Analysis of permethylated disaccharide was done using a glass capillary column (25 m × 0.25 mm) wall coated with SE-30. The separation was performed between 230°C (isothermal,

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20 min) and 330°C (2°C/min). Isomaltotetraose (IM_4 200 μg) was added as an internal standard. The identification and quantitation of the urinary disaccharides were carried out by GLC–MS as in [23]. Urinary creatinine was also determined [24].

3. Results and discussion

Routine GLC–MS screening of urinary oligosaccharides frequently indicates the presence of small amounts of a disaccharide with the structure, hexose–*N*-acetylhexosamine (hex-hexNAc). A patient (B. B.) known to suffer from ML II excreted higher than normal amounts of this compound, and since sufficient quantities of this urine were available, the disaccharide was isolated and characterised. The ultrafiltered urine (3 l) was concentrated to 400 ml by rotatory evaporation and applied to the Sephadex G-15 column (maximum load = 80 ml/column). Fractions were tested for hexose content and the disaccharide region was pooled, deionised by ion-exchange chromatography and concentrated. After fractionation by preparative paper chromatography in system (b), one of the fractions ($R_{lactose}$ = 1.5) was purified further by preparative paper chromatography in system (a), yielding 3 mg disaccharide. The purity of the compound was examined by paper chromatography using systems (c)–(e). A single component was observed in all 3 systems, and was shown by GLC–MS of the reduced and permethylated derivative to contain 4-*O*-hexopyranosyl-2-acetamido-2-deoxyhexose. The mass spectrum of this compound is shown in fig.1. The A-series of fragments at *m/e* 219, 187 and 155 indicate the permethylated hexopyranose residue. The ald-J_1 and ald$^+$ fragments at *m/e* 337 and 277 indicate a permethylated acetamido-deoxyhexitol-^{2}H residue. The fragment at *m/e* 131, obtained by cleavage of the alditol chain, indicates a 2-acetamido residue. The presence of an alditol cleavage fragment at *m/e* 175 and the absence of a fragment at *m/e* 133 suggests that the linkage between the sugar units is [1–4].

Sugar analysis indicated mannose and 2-acetamido-2-deoxyglucose in equimolar proportions. The optical rotation (a_D^{20} = –0.04, *c* = 1.55, water) demonstrated that the linkage between the sugar residues is β-, assuming the D-configuration for both sugar units. Methylation analysis of the reduced disaccharide gave 2,3,4,6-tetra-*O*-methyl-D-mannose and 1,3,5,6-tetra-*O*-methyl-2-(*N*-methyl)acetamido-2-deoxy-D-glucitol (and some 1,3,5,6-tetra-*O*-methyl-2-acetamido-2-deoxy-D-glucitol). The latter two derivatives have been identified in [25]. From the above data the structure of the disaccharide is 4-*O*-β-D-Man*p*-GlcNAcp. This compound was obtained from the trisaccharide, α-D-Man*p*-(1–3)-β-D-Man*p*-(1–4)-D-GlcNAc by enzymic digestion using α-mannosidase [25].

GLC of reduced and permethylated urine gives a

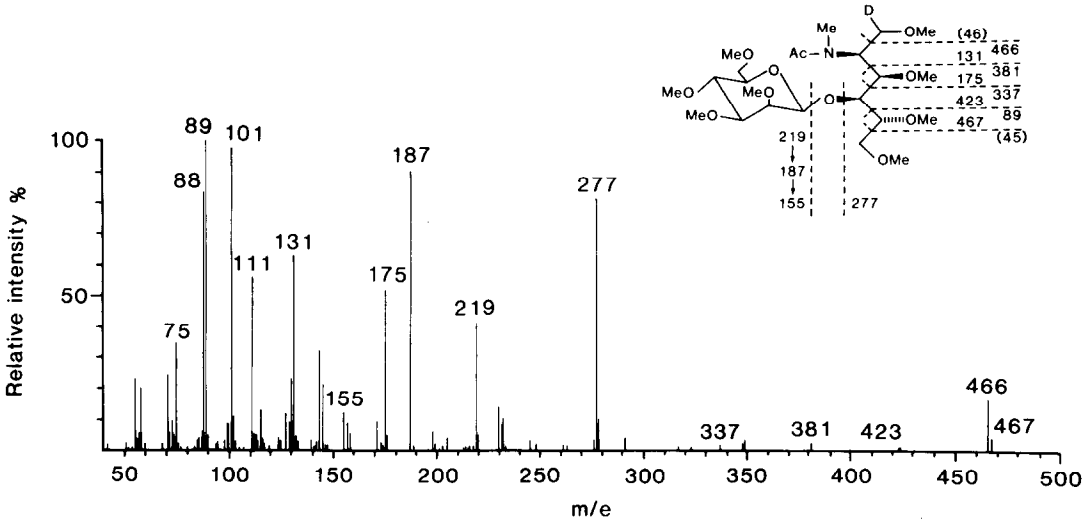

Fig.1. Mass spectrum of reduced (Na B^2H_4) and permethylated Man-β-(1–4)-GlcNAc.

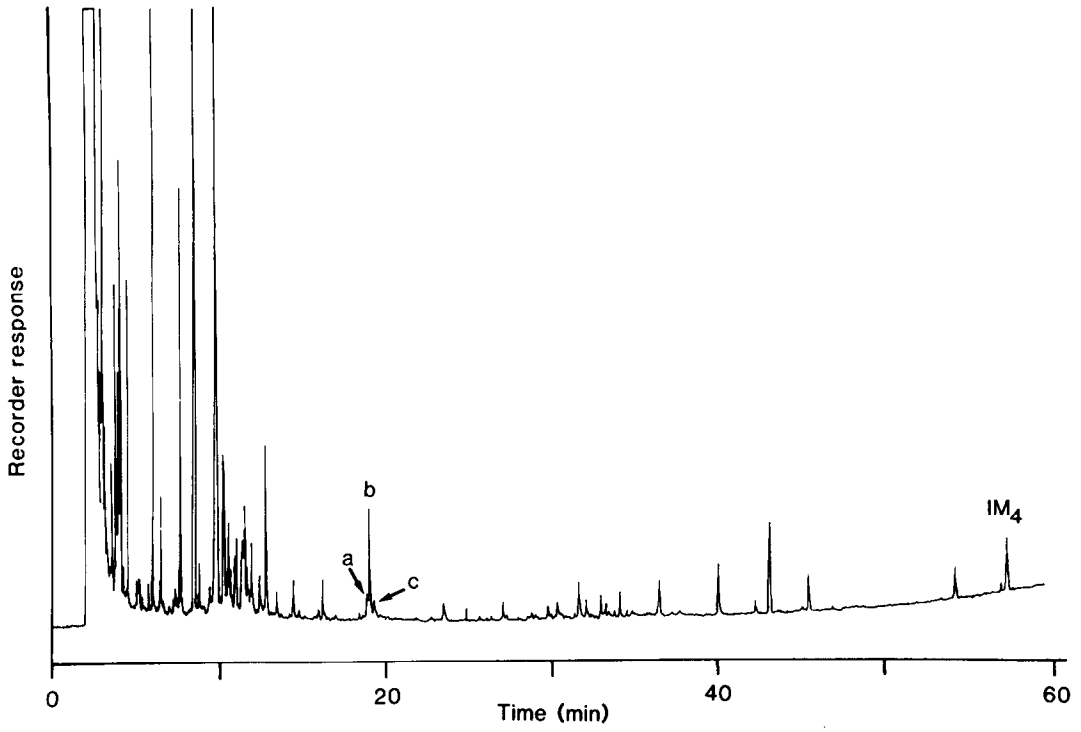

Fig.2. Gas chromatogram of reduced (NaB^2H_4) and permethylated urine from a patient with ML II.

complex picture (fig.2), but the hex–hexNAc region of the trace is usually well separated from other components. However, just one discrete peak in this region is rarely encountered when the GLC is run under optimal conditions. In fig.2, peaks (a,b) have identical retention times to Gal-β-(1–4)-GlcNAc and Man-β-(1–4)-GlcNAc, respectively, and on GLC–MS analysis have mass spectra in accordance with these structures (fig.1). However, peak (c) does not give characteristic carbohydrate fragments. In other urine samples another non-carbohydrate component occurs in variable amounts and has the same retention time as Man-β-(1–4)-GlcNAc. Consequently, quantitation of this disaccharide in urine cannot be performed by GLC alone. We have corrected for the impurity by measuring the relative intensities of the total ion current, and the characteristic ald^+ ion of Man-β-(1–4)-GlcNAc, *m/e* 277, in the samples and the pure compound.

The amount of Man-β-(1–4)-GlcNAc excreted by patients with a number of lysosomal storage diseases is shown in table 1. As can be seen, the patients with ML II and ML III excrete slightly more of this disaccharide than patients with other diseases, although this may not be significant. Urine from normal individuals usually contains <1 mg/mmol creatinine. The only disease in which a significant increase in the excretion of this disaccharide should occur is β-mannosidosis, as yet unknown in man, but described in goats [26].

Table 1
The urinary excretion of Man-β-(1–4)-GlcNAc by patients with lysosomal storage diseases

Patient	Disease	Man-GlcNAc excreted (mg/mmol creatinine)
P. P.	ML I	2.1
B. B.	ML II	4.3
A. S.	ML II	2.4
T. Y.	ML III	5.1
M. O.	ML IV	1.5
A. O.	ML IV	1.0
J. G.	ML IV	0.8
K. A.	Tay Sachs	0.7
C. W.	Sandhoff	0.3
H. K.	α-Mannosidosis	0.3
F. Z.	Fucosidosis	0.2
S. Z.	Fucosidosis	1.0
G. S.	Fucosidosis	0.3
A. J.	Aspartylglucosaminuria	trace
J. S.	Aspartylglucosaminuria	trace

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