Characterization of a *scyllo*-inositol-containing sialyloligosaccharide from normal human urine

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Three inositol-containing sialyloligosaccharides were isolated from normal human urine. Structural studies including gas–liquid chromatography of mono- and disaccharide derivatives, methylation analysis, mass spectrometry and glycosidase treatments indicated the structure NeuAc (α2–3)Gal(β1–0)*scyllo*-inositol for one of the oligosaccharides isolated. This provides the first evidence for the natural occurrence of a *scyllo*-inositol glycoside in biological material. The two other oligosaccharides isolated were identified as two isomers of NeuAc(α2–3)Gal(β1–0)*myo*-inositol, which have not been identified in normal urine before.

Scyllo-*inositol* Myo-*inositol* *Sialyloligosaccharide* *Urine*

1. INTRODUCTION

Numerous neutral and acidic oligosaccharides are known to be excreted in the urine under different physiological and pathological conditions [1–3]. Studies on these oligosaccharides have revealed new structures and have thus given insight into the structures and metabolism of the sugar chains of glycoconjugates. Therefore an effort has been made to further characterize the sialyloligosaccharides from normal human urine. This paper describes the isolation and structural characterization of 3 inositol-containing sialyloligosaccharides, one of which was identified as a new type of oligosaccharide, a sialylated *scyllo*-inositol galactoside.

2. MATERIALS AND METHODS

Urine was collected from a healthy male not subjected to any dietary restriction. A crude urinary oligosaccharide fraction was obtained by charcoal adsorption followed by gel filtration as in [4]. The monosialyloligosaccharides were separated from the crude oligosaccharide fraction by ion-exchange chromatography on DEAE-Sephadex A-25 [5] and subjected to descending paper chromatography on Whatman no. 3 MM paper with the solvent pyridine–ethyl acetate–H_2O–acetic acid (5:5:3:1, by vol.) for 2 days. Further purification of the inositol-containing sialyloligosaccharides was achieved by high pressure liquid chromatography (APLC) as in [6], as described in the legend of fig.1. A Waters model 6000A solvent delivery system equipped with a model U6K injector (Waters Associates) was used.

Sialic acid in chromatographic effluents was determined colorimetrically [7]. Sialyloligosaccharides were analyzed by thin-layer chromatography (TLC) on Silica Gel 60 HPTLC plates (Merck) with the solvent ethanol–*n*–butanol –pyridine–H_2O–acetic acid (100:10:10:30:3, by vol.) [8]. For the detection of oligosaccharides the plates were sprayed with resorcinol reagent [9]. The molar ratios of monosaccharides were determined by gas–liquid chromatography [10]. The sialic acid residues were characterized as in [11]. Identification of the inositol residues was carried

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out by gas–liquid chromatography of the trimethylsilyl derivatives on columns of 4% SE-30 and 4% OV-17 as in [12]. *Scyllo*-inositol and *epi*-inositol were purchased from Calbiochem and Sigma, respectively. *Muco*-inositol was kindly supplied by Dr Klaus Bock (Technical University of Denmark) and 1L-*chiro*-inositol by Professor Dr H. Paulsen (University of Hamburg). The sequence and anomeric configuration of the sugar residues was determined by treatment with exoglycosidases. Treatment with *Vibrio cholerae* neuraminidase (Calbiochem) was as in [13]. Galactosidase treatment was carried out with 0.025 units of purified *Aspergillus niger* β-galactosidase (supplied by Dr Jonathan Knowles, Technical Research Centre of Finland) in 0.3 ml of 25 mM sodium citrate–phosphate buffer (pH 3.8) containing 0.1 mg/ml bovine serum albumin and 10 μl of toluene at 37°C for 24 h.

Permethylation of oligosaccharides was carried out using potassium *t*-butoxide [14]. Partially methylated hexoses were analyzed after acetolysis as their alditol acetates by gas–liquid chromatography–mass spectrometry [15]. Detection was carried out by mass-fragmentography at *m/e* 117, 161, and 189 [13]. After removal of the sialic acid residues (0.1 M HCl, 80°C, 1 h) the galactosyl-inositols were methylated and analyzed by gas–liquid chromatography–mass spectrometry on 4% SE-30 at 234°C. The mass spectra were recorded by a Hewlett-Packard 5992 spectrometer at 70 eV.

3. RESULTS

3.1. *Isolation of the inositol-containing sialyloligo-saccharides*

The monosialyloligosaccharide fraction of human urine was subjected to preparative paper chromatography and the different fractions were analyzed for sialic acid content and monosaccharide composition. Most of the sialyloligosaccharides were recovered from the area between the R_f-values of 0.6 and 1.5 relative to *N*-acetylneuraminyl-(α2–3)lactose (submitted). The area between the corresponding R_f-values of 0.4 and 0.6 was found to contain mainly sialic acid, galactose, *myo*-inositol and an unknown component. In HPLC this paper chromatography fraction separated into 3 sialic acid-containing peaks,

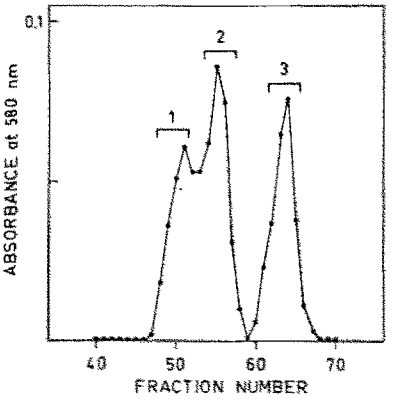

Fig.1. High pressure liquid chromatographic separation of inositol-containing sialyloligosaccharides. The sample recovered after paper chromatography of the urinary monosialyloligosaccharide fraction was injected onto a NH_2-μBondapak column. Mobile phase: 76:24 (v/v) acetonitrile-15 mM potassium phosphate (pH 5.2). Flow rate 2 ml/min. Fractions were analyzed for sialic acid and compounds *1*, *2* and *3* were recovered by pooling the fractions indicated.

designated compounds *1*, *2* and *3* (fig.1). All 3 compounds migrated as single bands in TLC and gave one disaccharide peak in gas–liquid chromatography after desialylation and methylation (fig.3A).

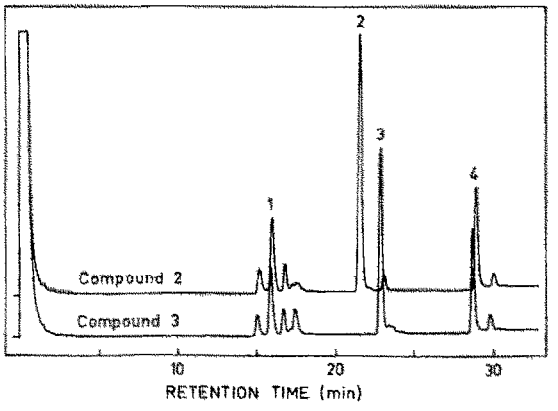

Fig.2. Gas–liquid chromatography of monosaccharides from compounds *2* and *3*. The oligosaccharides were subjected to methanolysis and the components were analyzed as their trimethylsilyl derivatives. Liquid phase: 4% SE-30. Temperature gradient: 130–260°C, 4°C/min. Peaks indicated are: 1, galactose; 2, *scyllo*-inositol; 3-*myo*-inositol; 4, *N*-acetylneuraminic acid.

3.2. *Structural characterization of the compounds*

Monosaccharide analysis revealed the presence of sialic acid, galactose and an unknown component in compound *2* (fig.2). The unknown component was found to be an inositol by comparing the mass spectrum of its trimethylsilyl derivative with that of trimethylsilyl *myo*-inositol. This inositol isomer was identified as *scyllo*-inositol by gas–liquid chromatography under conditions which are known to separate *scyllo*-inositol from all other inositol isomers (table 1, [12]). The monosaccharide analysis of compound *2* indicated the proportions of 1.0:1.0:1.1 for sialic acid, galactose and *scyllo*-inositol, respectively. Compounds *1* and *3* were both found to contain equimolar proportions of sialic acid, galactose and *myo*-inositol (fig.2). The monosaccharide composition of the 3 compounds remained unchanged after reduction with $NaBH_4$, which indicated that the inositols occupied a terminal position in the oligosaccharides. The sialic acid residues were identified as *N*-acetylneuraminic acid in all 3 compounds.

In gas–liquid chromatography of the desialylated and methylated compounds, a different retention time was observed for each derivative (fig.3A). The mass spectrum of each methylated asialo-compound was similar to those reported before for various methylated galactosyl-*myo*-inositols (fig.3B, [3,16]). Methylation analysis of the intact compounds showed that galactose was substituted at C-3 in all compounds (not shown).

Table 1

Gas-chromatographic retention times of the trimethylsilyl derivatives of inositols

	Liquid phase	
Inositol	4% OV-17	4% SE-30
Compound *1*	2.02	3.34
Compound *1*	1.65	2.68
Compound *3*	2.03	3.40
myo-	2.03	3.35
scyllo-	1.66	2.68
epi-	1.24	2.50
1L-*chiro*-	1.05	1.96
muco-	0.82	1.65

The samples were analyzed after methanolysis and the values are given relative to the main peak of galactose

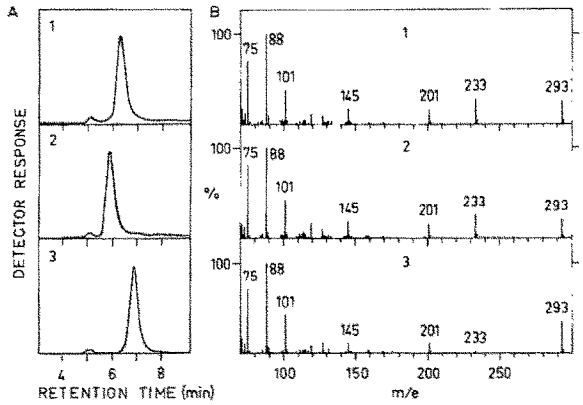

Fig.3. (A) Gas–liquid chromatography of the desialylated and methylated compounds *1–3*. Detection was carried out by fragmentography at *m/e* 88. The first small peak in all samples corresponds to the liberated sialic acid residues. Liquid phase: 4% SE-30. Temperature: 234°C. (B) Mass spectra of the disaccharides.

After neuraminidase treatment the galactose residues were found to be unsubstituted, which indicated that sialic acid was bound with an α2–3 linkage to galactose in all compounds. The desialylated compounds were quantitatively hydrolyzed to galactose and *scyllo*-inositol (compound *2*) or to galactose and *myo*-inositol (compounds *1,3*) by β-galactosidase treatment (not shown). This indicated that the anomeric configuration of the galactosyl linkages was β.

In conclusion, the complete structure of compound *2* was indicated to be NeuAc(α2–3)Gal(β1–0)*scyllo*-inositol. Because all carbon atoms of *scyllo*-inositol are identical [17], no isomers of this structure exist. Compounds *1* and *3* were identified as two isomers of NeuAc(α2–3)Gal(β1–0)*myo*-inositol, which probably differed with regard to the substitution positions of the *myo*-inositol residues. The substitution positions could not be identified due to the small amount of the substances available.

4. DISCUSSION

This paper provides the first evidence for the natural occurrence of a *scyllo*-inositol glycoside in biological material. Free *scyllo*-inositol has been identified in urine and various mammalian tissues in [18,19]. The excretion of *scyllo*-inositol in the

urine of humans was increased after oral administration of *myo*-inositol [20], which together with other observations suggest that this occurred by conversion of *myo*-inositol to *scyllo*-inositol via *myo*-ionose-2 [19]. Various *myo*-inositol glycosides have been identified in mammalian material before. Galactosyl-(β1–6)*myo*-inositol was obtained from rat mammary gland [16] and fucosyl (α1–0)*myo*-inositol from normal human urine [21]. The derivatives of galactosyl (β1–0) *myo*-inositol containing ABO blood-group determinants were identified from pregnancy urine [22]. Recently, a new acidic *myo*-inositol glycoside with the structure NeuAc(α2–3)Gal(β 1–1)-*myo*-inositol was characterized from human pregnancy urine [3]. The presence of the two isomers of NeuAc(α2–3)Gal(β 1–0)*myo*-inositol in the normal urine reported here shows that these structures are not pregnancy-specific.

The acidic *scyllo*- and *myo*-inositol galactosides were identified in the urine samples of several individuals (unpublished), which indicates that they are of common occurrence. The ratio of the *scyllo*-inositol glycoside to the *myo*-inositol glycosides was remarkably high in some urine samples (1 : 2–1 : 4) as compared to the ratio of free *scyllo*-inositol to *myo*-inositol reported for various mammalian tissues (1 : 10–1 : 45) [19]. This may suggest that β-galactosidase or galactosyltransferase, which have been reported to catalyze the synthesis of galactosyl-inositols [23,24], prefer *scyllo*-inositol as a substrate. The possibility that part of the inositol residues in complex lipids of mammalian origin, such as in phosphatidylinositols, might be glycosylated like those found in plants and fungi [25] remains to be elucidated.

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