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Note

Separation of *O*-methylhexitols and *O*-methyl-*myo*-inositols by reverse-phase high-performance liquid chromatography

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Methylation analysis is an important tool for carbohydrate characterization[1,2], and several methods are available for separation of the resulting partially methylated monosaccharides. These include gas–liquid chromatography[3,4], paper chromatography[5], thin-layer chromatography[6], and gel filtration[7]. In the present Note, we report that reverse-phase high-performance liquid chromatography (l.c.) is particularly effective for separating partially methylated hexitols and that the method has several advantages that recommend it. The procedure we describe is fast, has high resolving power, allows quantitative recovery of materials on a large scale, and is particularly convenient for quantifying radiolabeled derivatives.

Table I lists the retention times of several partially methylated hexitols that are normally encountered during methylation analysis of glucans[8,9], mannans[10], and mannophosphoinositides[11]. Unreduced methylated sugars may also be separated by this technique, but they usually give double peaks as a consequence of the resolution of the anomers. A similar technique has previously been used to separate partially alkylated oligosaccharides[12].

To achieve optimal separation of the *O*-methylhexitols within a reasonable time, three solvent systems were used. Retention times of >15 min usually resulted in broad and unsymmetrical peaks. Mono- and di-*O*-methylhexitols are separated with water; di- and tri-*O*-methylhexitols with 1% aqueous acetonitrile; and tetra-*O*-methylhexitols and tetra-*O*-methyl-*myo*-inositols with 5% acetonitrile. Excellent resolution is observed and it is notable that 2,3,4- and 2,3,6-tri-*O*-methylglucitol, and 2,3,4,6-tetra-*O*-methylglucitol and 2,3,4,6-tetra-*O*-methylmannitol, are well resolved, in contrast to the lack of separation during gas chromatography on OV-210.

This separation technique is suitable for structural analysis of carbohydrates by methylation analysis. *O*-Methylglucitols obtained by successive methylation, acid hydrolysis, and reduction of the carbohydrate component of the pyruvylated glycolipid (acidic oligosaccharide A) from *Mycobacterium smegmatis*[9] were applied

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TABLE I

SEPARATION OF PARTIALLY METHYLATED HEXITOLS AND *myo*-INOSITOLS BY REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMOTOGRAPHY

O-*Methylhexitol or* myo-*inositol derivative*	*Retention time (min)*			
	Glucitol	*Mannitol*	*Galactitol*	myo-*inositol*
Water[a]				
1-				1.8
2-	2.4			
3-	2.0	2.2	2.1	
4-	2.3	2.2	2.1	
6-	2.7		2.4	
1,3-				2.8
2,3-	4.7			
Aqueous CH_3CN, 1%				
2-	2.0			
1,3-				2.3
2,3	3.2	3.0		
2,4-		5 5		
2,6-	3.4			
3,4-		2.8		
4,6-	5.0	4.8		
2,3,4-	6.9	7.0		
2,3,6-	7.8	8.0		
2,4,6-	11.6	18.5		
3,4,6-		9.2		
1,2,3,5-				8.4
1,3,4,5-				13.9
1,3,4,6-				7.2
1,4,5,6-				20.0
2,3,4,6-	24.4			
Aqueous CH_3CN, 5%				
2-	1.8			
2,3-	2.2			
2,3,4-	2.9			
2,4,6-	3.8	5.6		
1,2,3,5-				3.3
1,3,4,5-				4.4
1,3,4,6-				3.1
1,4,5,6-				5.6
2,3,4,6-	7.2	8.7	7.8	
1,2,4,5,6-				10.0

[a]Glucitol, mannitol, galactitol, and *myo*-inositol all have a retention time of 1.7 min with water as eluant.

and eluted with 1% aqueous acetonitrile. The effluent was monitored by a refractometer and the chromatogram is shown in Fig. 1. The peaks were identified as 2-*O*-methyl-, 2,3-di-*O*-methyl-, 2,3,4-tri-*O*-methyl-, and 2,3,6-tri-*O*-methylglucitol in the molar ratios of 1:1:1:1 (Table II). This is in good agreement with results obtained by analysis with gas chromatography–mass spectrometry[9]. 2,3,4,6-Tetra-*O*-

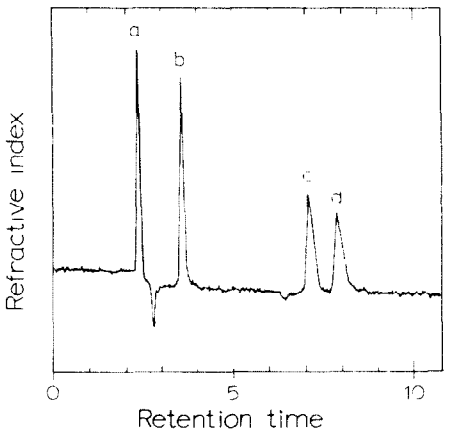

Fig. 1. Separation of *O*-methylglucitols by reverse-phase l c. A sample containing 200 μg of carbohydrate prepared by successively methylating, hydrolyzing and reducing acidic oligosaccharide A [4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-β-D-glucopyranosyl-(1→3)-4,6-*O*-(1-carboxyethylidene)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔1)-α-D-glucopyranoside] was injected and the column was eluted with 1% aqueous acetonitrile and monitored by a refractometer. Peak *a* is 2-*O*-methyl-, *b* is 2,3-di-*O*-methyl-, *c* is 2,3,4-tri-*O*-methyl-, and *d* is 2,3,6-tri-*O*-methylglucitol. Under these conditions, 2,3,4,6-tetra-*O*-methylglucitol is eluted at 24.4 min and is not shown in this figure. The negative peak between *a* and *b* arises from elution of the injected water.

TABLE II

QUANTITATION OF *O*-METHYLHEXITOLS UNDER DIFFERENT CONDITIONS OF ELUTION

O-*Methylhexitol derivative*	*Eluting solvent and conditions*			
	1% CH_3CN[a]	*1% CH_3CN + 5% CH_3CN step*[b]	*5% CH_3CN*[b]	*Water-5% CH_3CN gradient*[b]
			molar ratios[c]	
2-	1.1	1.04	2.18[e,f]	1.02
2,3-	1.0	1.00		1.00
2,3,4-	1.1	1.01	2.09[e,f]	2.14[e]
2,3,6-	1.2	1.04		
2,3,4,6-	[d]	1.03	1.00	1.01

[a]Elution was monitored and ratios were determined with a refractometer. [b]Elution was monitored and ratios were determined by counting radioactivity. [c]Normalized to 1 residue of 2,3-di-*O*-methylglucitol. [d]Not determined. [e]Not separated. [f]Normalized to 2,3,4,6-tetra-*O*-methylglucitol.

methylglucitol was eluted at 24.4 min as a broad and unsymmetrical peak, not shown in Fig. 1.

To reduce the retention time of 2,3,4,6-tetra-*O*-methylglucitol, a gradient had to be applied. This procedure precludes the use of a refractometer and requires radiolabeled compounds and detection by using a liquid-scintillation counter. The partially methylated glucoses from acidic oligosaccharide A were reduced in the

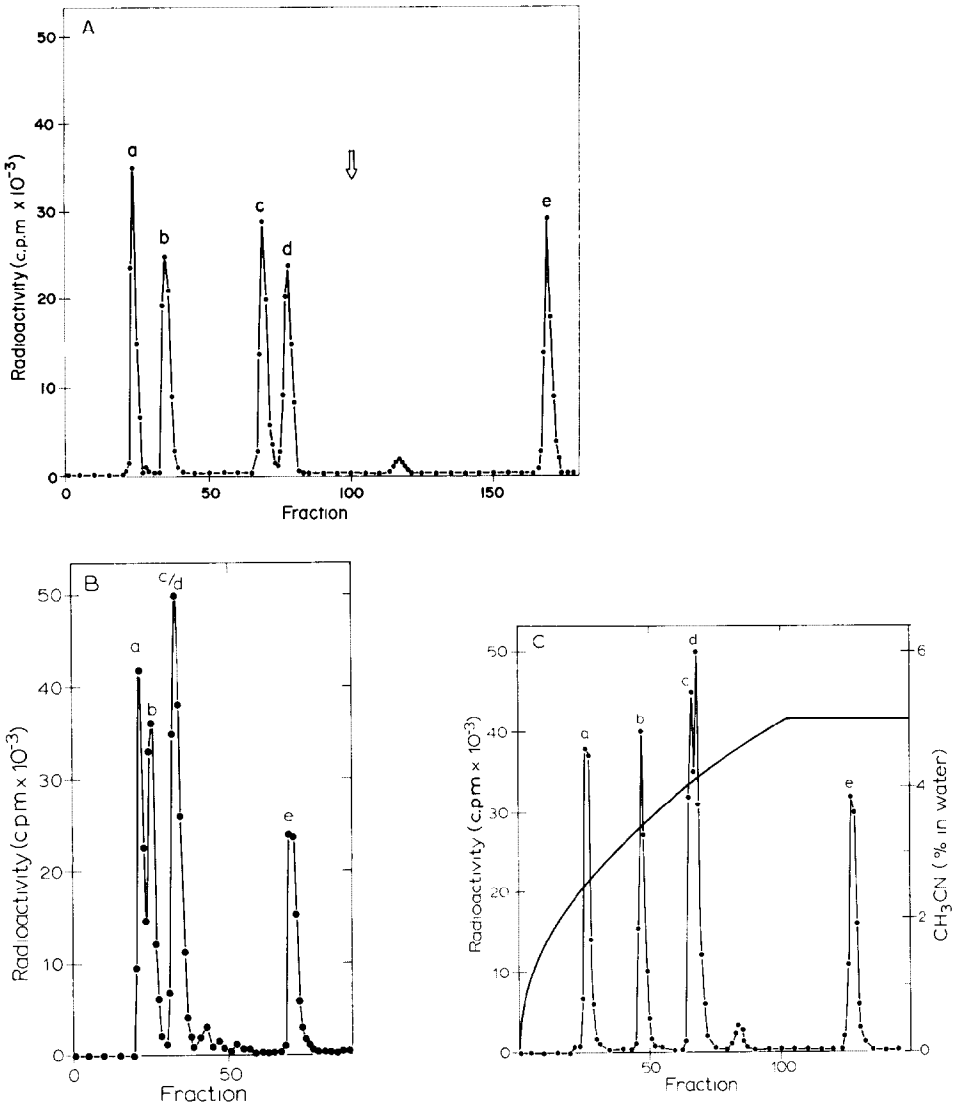

Fig. 2. Separation of ^{3}H-labeled *O*-methylglucitols by reverse-phase l.c. In (A) the compounds were eluted first with 1% aqueous acetonitrile and, after 10 min, with 5% aqueous acetonitrile (as indicated by the arrow), in (B) with 5% aqueous acetonitrile, and in (C) with a gradient between water and 5% aqueous acetonitrile. The effluent was monitored by counting radioactivity. Peak *a* is 2-*O*-methyl-, *b* is 2,3-di-*O*-methyl-, *c* is 2,3,4-tri-*O*-methyl-, *d* is 2,3,6-tri-*O*-methyl-, and *e* is 2,3,4,6-tetra-*O*-methylglucitol. The samples were the same as used in Fig. 1.

presence of $NaBT_4$. The addition of unlabeled sodium borohydride was necessary to obtain uniform labeling of the partially methylated glucoses. If sodium borohydride is not added, the less methylated glucoses incorporate a higher amount of

radioactivity, thus preventing accurate quantitation (data not shown). If accurate quantitation is not required, however, much smaller amounts than were used in this study may be analyzed by using sodium borotritide of high specific activity. Fig. 2A shows the elution profile of the ^{3}H-labeled *O*-methylglucitols with 1% aqueous acetonitrile followed by 5% aqueous acetonitrile as solvent. The molar ratios of the five glucitol derivatives were 1:1:1:1:1 (Table II), again in good agreement with previous results[9]. In Fig. 2B, the same sample was analyzed by elution with 5% aqueous acetonitrile to obtain the 2,3,4,6-tetra-*O*-methylglucitol. Only four peaks were observed because the trimethyl ethers were not resolved. Fig. 2C shows the separation of the sample by a hyperbolic gradient from water to 5% aqueous acetonitrile. The 2,3,4- and 2,3,6-tri-*O*-methylglucitols were only partly resolved, but 2,3,4,6-tetra-*O*-methylglucitol was eluted as a sharp peak. The molar ratios are shown in Table II. Optimal separation between the tri-*O*-methylglucitols was achieved with a linear gradient between water and 5% aqueous acetonitrile, but the retention time of the tetra-*O*-methylglucitol was >18 min (data not shown).

In conclusion, reverse-phase high-pressure liquid chromatography is particularly useful for the separation of ^{3}H-labeled *O*-methylhexitols for quantitative analysis, and it also facilitates preparative recovery of derivatives. Depending on the composition of the sample, complete separation may be achieved by either a step elution or a gradient.

EXPERIMENTAL

Procedures. — High-performance liquid-chromatography was performed with a Waters Associates model ALC/GPC 201 chromatograph equipped with a model 660 solvent programmer and a second model 6000A solvent pump. The reverse-phase column, Supelcosil LC-18 (4.6 × 250 mm), was obtained from Supelco and was operated at a flow rate of 1.8 mL/min.

Samples were dissolved in water and injected in 10-μL portions. Isocratic elution was used with water, 1 and 5% aqueous acetonitrile, or with a gradient between water and 5% aqueous acetonitrile produced by program no. 5 (hyperbolic gradient) of the solvent programmer. The effluent was monitored either by a refractometer or by collecting fractions every 6 s and assaying for radioactivity in a Beckman LC-3150T liquid-scintillation counter after the addition of scintillation cocktail 3a70 (Research Products International).

O-Methylhexoses were prepared by the methylation and subsequent acid hydrolysis of yeast mannans[10], methylglucose lipopolysaccharide[8], and acidic oligosaccharide A (ref. 9), or were from the laboratory stock, and the samples were converted into *O*-methylhexitols as described[1]. The methylated derivatives of *myo*-inositol were from an earlier study[11]. A fraction of the *O*-methylglucoses (0.06 μmol), after methylation and hydrolysis of acidic oligosaccharide A, was reduced by an excess of a mixture of sodium borohydride (2.6 μmol) and sodium borotritide (2 mCi, Amersham) in 50 μL of 10mM ammonium acetate. About 600,000 c.p.m.

were applied and 77% of the radioactivity was recovered in the *O*-methylhexitol peaks.

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