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SEPARATION BY LIQUID CHROMATOGRAPHY (UNDER ELEVATED PRESSURE) OF BENZYL AND NITROPHENYL GLYCOSIDES OF OLIGOSACCHARIDES*

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ABSTRACT

Liquid chromatography under elevated pressure (l.c.) was employed for the separation of some benzyl and nitrophenyl glycosides of a variety of mono-, di-, tri-, and tetra-saccharides. The separation was conducted on a Waters Carbohydrate Analysis column by use of a mixture of acetonitrile–water as the mobile phase. In general, monosaccharides emerged first from the column, followed sequentially by di-, tri-, and tetra-saccharides. It was observed that the pattern of substitution imparts a noticeable effect on the elution profiles of isomeric oligosaccharides. Also, substitution of a hydroxyl group with a methyl group, or its replacement with a fluorine atom, led to a substantial decrease in retention times of some oligosaccharides. Moreover, resolution was clearly enhanced, and retention times were congruently increased by decreasing the water content of the mobile phase.

INTRODUCTION

Synthetic oligosaccharides that occur as parts of glycoconjugates have proved to be excellent tools in specificity studies of glycosidases[1] and glycosyltransferases[2], by serving either as acceptor substrates or as reference compounds, or both. They have also been instrumental in the discovery of new glycosyltransferases involved in the biosynthesis of mucin[3]. For this reason, it was desirable to develop rapid and reliable procedures for the separation and identification of a variety of carbohydrate structures that are often encountered in such studies. One such procedure that is increasingly being utilized is "high-performance" liquid chromatography (l.c.). This technique has the potential of being used both qualitatively and quan-

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titatively, with the minimal amount of sample. Moreover, being non-destructive in nature, the sample used can be recovered and recycled for additional investigations. However, despite the numerous reports that appeared in the literature on the use of l.c. for the separation of oligosaccharides[4,5], systematic studies on the elution profiles of structurally-related, isomeric oligosaccharides are still scarce, apparently because of the relative inaccessibility of such compounds. Thus, since our laboratory has procured many synthetic oligosaccharides over the past few years[6], it was possible to engage in such a study, and we now report herein a preliminary investigation on the separation (by l.c.) of some benzyl and nitrophenyl glycosides.

RESULTS AND DISCUSSION

Table I summarizes the separations achieved when nitrophenyl glycosides of some mono-, di-, and tri-saccharides were subjected to l.c. on a Waters Carbohydrate Analysis column, with 83:17 (v/v) acetonitrile–water (solvent *A*), or with the exception of a few compounds, with 87:13 (v/v) acetonitrile–water (solvent *B*) as the eluent. In both of the solvent systems, monosaccharides were eluted first, followed by di-, and finally tri-saccharides. However, all three types of compounds experienced an overall increase in retention time in solvent *B*, apparently because of the decrease in the water content of the solvent system. Interestingly, a concomitant increase in resolution, and also in the reproducibility of results (as judged by a smaller standard deviation) was generally observed with solvent *B*. Also, in comparison to all other monosaccharide glycosides examined, both 4-nitrophenyl α-, and β-L-fucopyranoside appeared to be retained the least, in both solvent systems. It was therefore reasonable to anticipate that oligosaccharides bearing an L-fucopyranosyl group (or residue) might exhibit an analogous tendency. This was indeed the case; the retention times for α-L-Fuc*p*-(1→3)-β-D-Gal*p*$OC_6H_4NO_2$(4) (**13**) were 6.57 and 9.65 min in solvents *A* and *B*, respectively, whereas those for β-D-Gal*p*-(1→3)-β-D-Gal*p*$OC_6H_4NO_2$(4) (**20**) were 11.35 and 21.20 min in solvents *A* and *B*, respectively. It should be noted that there were no subtle differences in retention times between most of the nitrophenyl α- and β-anomeric glycosides of the same sugar (*e.g.*, **1** and **2**, or **3** and **4**).

On examination of Table I, it appeared that, in a series of structurally-related disaccharides, the (1→3)-linked isomer is eluted first, followed by the (1→2)- and the (1→6)-linked isomers. For example, the (1→2)-linked disaccharide **12** had retention times of 8.02 and 12.67 min in solvents *A* and *B*, respectively, whereas the isomeric (1→3)-linked disaccharide **13** had retention times of only 6.57 and 9.65 min in the same two solvent systems, respectively. A similar trend was also observed for compounds **19, 20**, and **21**. Moreover, of the three isomeric disaccharides β-D-Gal*p*-(1→X)-β-D-GlcNAc$OC_6H_4NO_2$(4) where X is 3, 4, or 6, the (1→4)-linked isomer had a mobility intermediate between those of the (1→3)- and (1→6)-linked counterparts; the retention times in solvent *B* for compounds **22, 23**, and **24**, were 18.66, 24.26, and 26.98 min, respectively.

TABLE I

RETENTION TIMES OF SOME NITROPHENYL GLYCOSIDES[a]

Compound[b]	*Structure No.*	*Retention time*[c] *(min) in solvent*		*Ref.*
		A	B	
α-L-Fuc*p*OR	**1**	3.79	4.16	
β-L-Fuc*p*OR	**2**	3.75	4.08	
α-D-Gal*p*OR	**3**	4.89	6.01	
β-D-Gal*p*OR	**4**	4.99	6.02	
α-D-Glc*p*OR	**5**	4.37	5.48	
β-D-Glc*p*OR	**6**	4.40	5.55	
α-D-Man*p*OR	**7**	4.41	5.27	
β-D-Man*p*OR	**8**	4.79	5.80	
β-D-Glc*p*NAcOR	**9**	4.89	6.32	
α-D-Glc*p*NAcOR	**10**	4.40	5.79	
β-L-Fuc*p*-(1→3)-β-D-Glc*p*OR	**11**	5.80	8.23	7
α-L-Fuc*p*-(1→2)-β-D-Gal*p*OR	**12**	8.02	12.67	7
α-L-Fuc*p*-(1→3)-β-D-Gal*p*OR	**13**	6.57	9.65	7
α-D-Man*p*-(1→2)-β-D-Glc*p*OR	**14**	10.05	19.06	7
α-D-Man*p*-(1→6)-α-D-Man*p*OR	**15**	10.17	19.92	8
β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOR′	**16**	10.72	19.74	9
β-D-Gal*p*-(1→6)-α-D-Gal*p*NAcOR′	**17**	11.53	20.62	9
α-D-Gal*p*-(1→2)-β-D-Gal*p*OR	**18**	11.97	21.27	10
β-D-Gal*p*-(1→2)-β-D-Gal*p*OR	**19**	11.97	21.24	10
β-D-Gal*p*-(1→3)-β-D-Gal*p*OR	**20**	11.35	21.20	11
β-D-Gal*p*-(1→6)-β-D-Gal*p*OR	**21**	12.97	24.02	12
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOR	**22**	9.93	18.66	13
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAcOR	**23**	12.54	24.26	13
β-D-Gal*p*-(1→6)-β-D-Glc*p*NAcOR	**24**	13.65	26.98	13
β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAcOR′	**25**	11.42	23.44	7
β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOR′	**26**	10.85	20.77	7
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*OR	**27**	10.34	19.54	14
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOR′	**28**	14.60	32.43	15
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOR	**29**	15.20	34.74	15
α-L-Fuc*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*OR	**30**	15.75	36.38	16
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*OR	**31**	17.94	44.27	17
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*OR	**32**	27.10		7
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOR	**33**	19.81	53.56	18
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOR′	**34**	21.98	62.14	18
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOR′	**35**	25.79		7
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-α-D-Man*p*OR	**36**	27.88		19
β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcOR′	**37**	35.67		7
β-D-Glc*p*NAc-(1→2)-β-D-Man*p*-(1→6)-β-D-Glc*p*OR	**38**	32.60		7

[a]Compounds were analyzed by l.c. as described in the Experimental section with the following solvents (v/v) as the mobile phase: (*A*) 83:17 acetonitrile–water, and (*B*) 87:13 acetonitrile-water. [b]R = 4-nitrophenyl; R′ = 2-nitrophenyl. [c]Retention times represent the average of three separate injections, with standard deviations of generally <0.05.

TABLE II

RETENTION TIMES OF SOME BENZYL GLYCOSIDES[a]

Compound	*Structure No.*	*Retention time[b] (min) in solvent*		*Ref.*
		A	B	
α-D-Gal*p*NAcOBn	**39**	4.89	5.44	20
β-D-Gal*p*NAcOBn	**40**	5.40	6.30	21
α-D-Glc*p*NAcOBn	**41**	4.78	5.38	22
β-D-Glc*p*NAcOBn	**42**	5.46	6.48	22
3-OMe-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOBn	**43**	7.81	12.51	7
β-D-Gal*p*-(1→3)-α-D-Glc*p*NAcOBn	**44**	10.98	20.11	23
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOBn	**45**	11.01	20.34	24
β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOBn	**46**	12.14	23.83	25
β-D-Gal*p*-(1→6)-α-D-Gal*p*NAcOBn	**47**	12.68	25.06	9
β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAcOBn	**48**	13.88	28.42	25
β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOBn	**49**	12.42	24.69	26
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAcOBn	**50**	30.60		6
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOBn	**51**	31.31		27
β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→4)]-α-D-Gal*p*NAcOBn	**52**	38.15		28
β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→6)]-α-D-Gal*p*NAcOBn	**53**	42.74		28
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOBn	**54**	41.19		5
β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcOBn	**55**	46.11		25
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOBn	**56**	79.51		29
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→6)]-α-D-Gal*p*NAcOBn	**57**	94.10		28
β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcOBn	**58**	96.68		28
β-D-Gal*p*-(1→3)-6F-α-D-Gal*p*NAcOBn	**59**	7.13	12.08	30
β-D-Glc*p*NAc-(1→3)-6F-α-D-Gal*p*NAcOBn	**60**	5.61	8.13	30
4F-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOBn	**61**	5.99	8.69	31

[a]Compounds were analyzed as described in Table I. [b]Standard deviations were generally <0.05; except for tetrasaccharides **56, 57**, and **58** where they were 0.2.

A reversal of the aforementioned order of elution was observed for the two disaccharides β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAcOC$_6$H$_4$NO$_2$(2) (**25**), and β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOC$_6$H$_4$NO$_2$(2) **26**, which had retention times of 23.44 and 20.77 min in solvent *B*, and 11.42 and 10.85 min in solvent *A*, respectively. A similar behavior was previously reported by Brockhausen *et al.*[4] for the benzyl α-D-glycosides of the same disaccharides (see also Table II).

An elution pattern similar to that of the disaccharide glycosides was also observed for the trisaccharide glycosides. Thus, a trisaccharide containing an L-fucopyranosyl group was less retained on the column, in both solvent systems, than its counterpart containing a D-galactopyranosyl group; the retention times for compound **30** were 15.75 and 36.88 min, whereas those for **31** were 17.94 and 44.27 min in solvents *A* and *B*, respectively. It is also interesting to note that of two related

trisaccharides, which differ by only one glycosyl linkage, the one that possessed a (1→3)-linkage was eluted much earlier than that having a (1→4)-linkage. Thus, the retention times in solvent *A* for β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal-*p*$OC_6H_4NO_2$(4) (**31**) and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal-*p*$OC_6H_4NO_2$(4) (**32**) were 17.94 and 27.10 min, respectively. Furthermore, the order of attachment of the same monosaccharide units (to give a trisaccharide) appears to have a noticeable effect on the retention time of the trisaccharide obtained. Thus, the straight-chain trisaccharide, β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAc$OC_6H_4NO_2$(2) (**35**) had a retention time in solvent *A* of 25.79 min, whereas the branched isomer, β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAc$OC_6H_4NO_2$(2) (**37**) had a retention time of 35.67 min in the same solvent system.

Table II summarizes the results obtained when some benzyl glycosides were subjected to l.c. under conditions analogous to those employed for their nitrophenyl counterparts (Table I). Under these conditions, no satisfactory separation could be achieved for the benzyl glycosides of 2-acetamido-2-deoxy-D-gluco- and -D-galactose. However, for each of these monosaccharide glycosides, the α and β anomers were readily resolved, with the α anomer being the first to be eluted in both solvent systems. Nonetheless, there was no substantial difference in elution times between the two isomeric disaccharides **44** and **45**, whose retention times in solvent *B* were 20.11 and 20.34 min, respectively. Also, in agreement with the data for the nitrophenyl glycosides (Table I), (1→3)-linked disaccharides were eluted earlier than their (1→6)-linked isomers; the retention times in solvent *B* for **46** and **47** were 23.83 and 25.06 min, respectively. In all cases, however, substitution of the nitrophenyl group with a benzyl group appeared to have a retarding effect on the elution of the pertinent saccharide (see Tables I and II). Moreover, the reversal in the order of elution observed earlier for nitrophenyl glycosides **26** and **27** (Table I), was similarly observed for the benzyl α-glycosides **48** and **49**, which was in agreement with the report of Brockhausen *et al.*[4].

As far as the trisaccharide benzyl glycosides were concerned, their elution patterns were more or less similar to those of their nitrophenyl counterparts. Tetrasaccharides also showed similar trends in elution in so far as the nature of the glycosyl linkage was concerned. For example, β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOBn (**56**) was eluted in solvent *A* at a considerably faster rate (79.51 min) than β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→6)]-α-D-Gal*p*NAcOBn (**57**; 94.10 min).

Finally, the presence of a methyl group at O-3′ of disaccharide **43** rendered it more mobile on the column than its parent compound **44**; compound **43** had retention times of 7.81 and 12.51 min, whereas those for **44** were 10.98 and 20.11 min in solvents *A* and *B*, respectively. A more pronounced decrease in retention time was also observed when a hydroxyl was replaced by a fluoro group. For example, the retention times for β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcOBn (**49**) were 12.42 and 24.69 min in solvents *A* and *B*, respectively, whereas those of its 4′-monodeoxyfluorinated counterpart (**61**) were 5.99 and 8.69 min, respectively.

EXPERIMENTAL

Materials. — All of the monosaccharide nitrophenyl glycosides were purchased from Sigma Chemical Co. St. Louis, MO). Acetonitrile was ChromAR, HPLC grade (Mallinckordt, Inc., St Louis, MO). The remaining compounds were synthesized as described elsewhere[6–31].

Separations were performed on a system purchased from Water Associates, Milford, MA, and consisted of the following components: two Model 510 pumps, UGK Injector, Model 441 U.V. Detector, Model 680 Automated Gradient Controller, Model 740 Data Module, and a Carbohydrate Analysis Column (30 × 3.9 cm, Part No. 84038). Mixtures of acetonitrile and de-ionized water (continuously purged with dry He) were used as the eluting solvents. The flow rate was maintained at 1 mL/min with an operating pressure of 2.6–4.0 MPa. The temperature was maintained constant at 23 ±2° throughout this study. Chromatographic separations were monitored by u.v. absorption at 214 nm. Standard compounds were routinely determined each experimental day. Retention times were determined for three separate injections, and then averaged for each compound. Standard deviation were normally in the range of 0.001–0.05, except for three tetrasaccharides, where the value was 0.2.

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