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Review

High-performance liquid chromatographic separation of carbohydrates on graphitized carbon columns

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Abstract

Graphitized carbon columns (GCC) for high-performance liquid chromatography are relatively new and have a unique ability to resolve isomeric and closely related compounds. The retention mechanism of carbohydrates on GCC is mainly based on adsorption and the flat surface of GCC packings brings about unique selectivity, but also includes hydrophobic interactions. The chromatographic behaviour of monosaccharides, disaccharides, cyclodextrins (CDs), branched CDs, oligosaccharide alditols, chito-oligosaccharides, N-linked oligosaccharides and glycopeptides has been studied, and it has become apparent that the elution patterns are based on the size and planarity of the molecule (position and configuration of linkage).

Contents

1. Introduction

In the field of carbohydrate chemistry, columns packed with charcoal have been used by many researchers mainly for preparative separations of oligosaccharides since Whistler and Durso [1] reported the class separation of oligosaccharides over 40 years ago. Nevertheless, carbon column packings that possessed the appropriate shape, particle size and mechanical

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stability for high-performance liquid chromatography (HPLC) were not available until recently. Several years ago, Shandon Scientific developed a graphitized carbon HPLC column, Hypercarb, and demonstrated that it had a unique ability to resolve isomeric and closely related compounds. Koizumi et al. [2] reported the first use of this column for the separation of carbohydrate compounds, such as mono- and disaccharides and cyclodextrins. Since then, graphitized carbon columns (GCC) have been used for analyses and preparative separations of neutral linear oligosaccharides [3–6], cyclic oligosaccharides [7–9], oligosaccharide alditols containing acetamido sugars and/or sialic acid [10,11], N-linked oligosaccharides [11,12], chito-oligosaccharides [12] and glycopeptides [10–12].

2. Characteristics of GCC

According to the description of the manufacturer (Shandon Scientific), the surface of GCC packings is flat and this flat surface bring about unique selectivity and a matchless ability to resolve isomeric and closely related compounds. Retention on GCC is mainly by an adsorption mechanism. Planar molecules are generally more retained than non-planar molecules. For a series of saccharides having same molecular configuration, the retention increases with increasing molecular size. For example, the elution order of cyclodextrins (CDs) on GCC is α-CD [degree of polymerization (DP) 6], β-CD (DP 7) and γ-CD (DP 8), differing from that on a reversed-phase column (γ-CD, α-CD, β-CD) [13], where the retention mechanism is mainly based on hydrophobic interaction. Although it has been reported that hydrophobic interaction and electron donor–acceptor interaction are also involved in the retention mechanism of GCC, the latter may be negligible for the retention of underivatized carbohydrates.

The hydrophobicity of GCC is greater than those of other reversed-phase materials. Therefore, a greater percentage of organic modifier in the eluent is required and GCC are preferred to octadecylsilyl (ODS) columns for the separation of hydrophilic compounds.

The exceptional chemical and physical stability of a column having highly structured graphite surface allows repeated use without loss of performance or reproducibility.

As GCC are completely unaffected by strongly acidic or alkaline conditions and can be used throughout the entire pH range, separations with a much wider range of solvents become feasible.

The effect of column temperature is small compared with ODS columns. In some cases, retention times (t_R) increase with increase in temperatures, as opposed to the change in t_R on ODS columns. This may be due to the increase in hydrophobic binding with increase in temperature.

3. HPLC of carbohydrates on GCC

3.1. Monosaccharides

Common aldopentoses, aldohexoses, ketohexoses, a sugar alcohol, an amino sugar, and sugar acids were analysed on a Hypercarb column with water as the eluent [2].

Aldoses were weakly retained on this column, and each peak of D-xylose, D-glucose, D-galactose and L-fucose was nevertheless split into anomer peaks (Fig. 1). Aldopentoses other than D-xylose have the possibility of taking two conformations ($^4C_1$ and $^1C_4$) and, in addition, their t_Rs are too short, so the separation of anomers may therefore be difficult. The anomers of aldohexoses having an axial OH at C-4, e.g., D-galactose and L-fucose, showed the best separation, whereas the peaks of aldoses having an axial OH on C-2, e.g., D-lyxose, D-mannose and L-rhamnose, did not split. The doublets disappeared with increasing temperature (ca. 50°C), as the rate of interconversion between the α- and β-anomers is accelerated.

The two ketohexoses, D-fructose and L-sorbose exist nearly 100% in the β- and α-form, respectively, and therefore they each appeared as a single peak. Although it has been reported that fructose would exist in ca. 75% pyranose

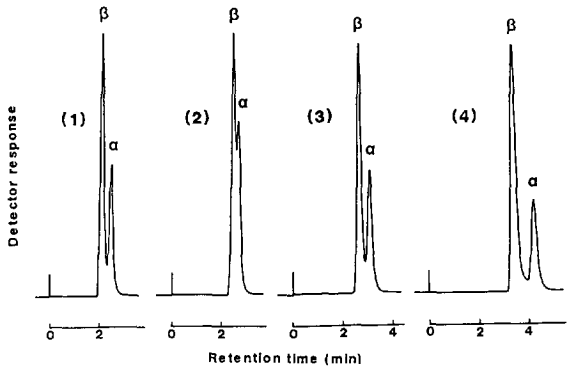

Fig. 1. Chromatograms of monosaccharides: (1) D-xylose, (2) D-glucose, (3) D-galactose and (4) L-fucose. Chromatographic conditions: column, Hypercarb (100 × 4.6 mm I.D.); eluent, water; flow-rate, 1 ml/min; detector, Shodex SE-61 refractive index; temperature, 15°C.

and 25% furanose forms, the separation of pyranose and furanose forms was not observed.

D-Glucitol was also weakly retained, but D-glucosamine hydrochloride was not retained, whereas both gluconic and glucuronic acids were retained too strongly on the column for practical chromatography.

Although the retention times of monosaccharides were too short for their separation, it was demonstrated that this type of chromatography could be used as a convenient method for studying the anomerization of some monosaccharides.

3.2. Disaccharides

All glucobioses, lactose and sucrose were adequately eluted from the Hypercarb column with acetonitrile–water (4:96) or methanol–water (15:85), and their peak shapes were generally better with the former [2]. All reducing disaccharides examined each showed split peaks with the latter eluent below 30°C, whereas with the former eluent the peak of laminaribiose did not split even at 10°C, and the peak of sophorose showed band broadening due to the partial separation of the anomers at 15°C, split at 30°C and became a single peak with a shoulder again at 45°C. Although most of the doublets became single peaks at 60°C, kojibiose and lactose showed split peaks at 60°C and the peak of lactose had shoulder even at 70°C.

Fig. 2 shows elution profiles of glucobioses with acetonitrile–water (4:96) at 15°C. Three configurational isomers of trehalose, trehalose (α,α-linked), isotrehalose (β,β-linked) and neotrehalose (α,β-linked), were also well resolved from each other on this column with acetonitrile–water (3:97) [3]. The elution order of glucobioses [α,α-(1,1), β,β-(1,1), α,β-(1,1), α-(1,6), α-(1,2), α-(1,4), α-(1,3), β-(1,6), β-(1,2), β-(1,4) and β-(1,3)] depends on the linkage form and may suggest rankings of relative degrees of planarity of their molecules.

The characteristic shapes of split peaks were useful for the identification of glucobioses: HPLC analysis was applied to the structure elucidation of glucosyllactoses obtained by transglucosylation to lactose from soluble starch with

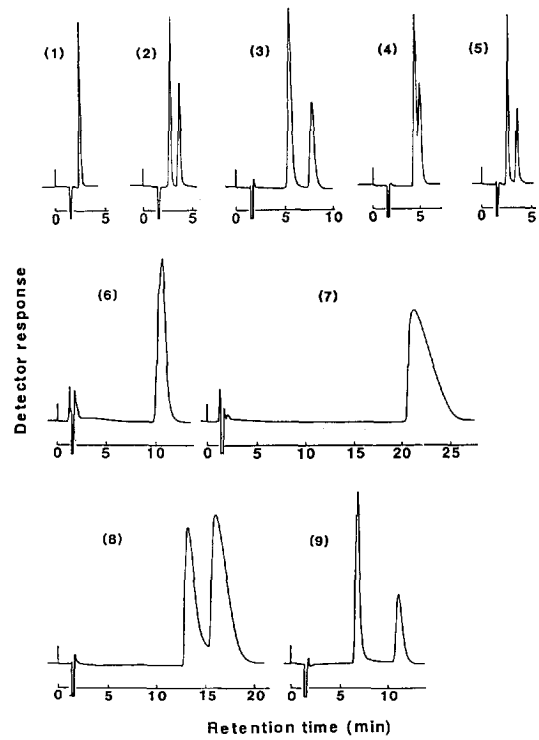

Fig. 2. Chromatograms of glucobioses: (1) trehalose, (2) kojibiose, (3) nigerose, (4) maltose, (5) isomaltose, (6) sophorose, (7) laminaribiose, (8) cellobiose and (9) gentiobiose. Chromatographic conditions: eluent, acetonitrile–water (4:96); other conditions as in Fig. 1.

cyclodextrin glycosyltransferase (CGTase) from *Bacillus stearothermophilus* [4]. A mixture of glucosyllactoses was hydrolysed by β-galactosidase and then the reaction product was analysed by HPLC on a Hypercarb column. The elution profile of the main glucobiose in the hydrolysates suggested that it was neotrehalose, and a minor reducing glucobiose was identified as kojibiose. Consequently, the original glucosyllactoses were ascertained to be α-D-glucopyranosyl β-lactoside and 2^{G}-O-α-D-glucopyranosyllactose.

As GCC can be used throughout the entire pH range, the elimination of double peaks of saccharides anomers was achieved by addition of alkali to the eluent. Needless to say, this treatment cannot be applied to silica gel-based ODS columns. Fig. 3 shows the simultaneous separation of nine glucobioses on a Hypercarb column by gradient elution with 1 m*M* sodium hydroxide solution containing 1.5–5.0% acetonitrile. After the column eluate had been made alkaline using an anion micromembrane suppressor, glucobioses in the effluent were detected with PAD 2.

Fig. 4A shows the elution profile of positional

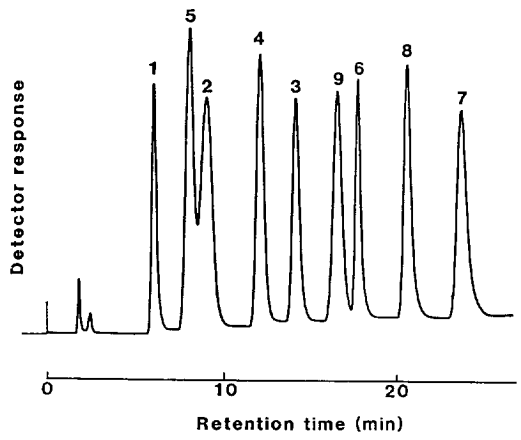

Fig. 3. Simultaneous separation of glucobioses on a Hypercarb column by gradient elution with 1 m*M* sodium hydroxide solution containing 1.5–5% acetonitrile. Numbers of glucobioses as in Fig. 2. Chromatographic conditions: eluent A, 1 m*M* sodium hydroxide solution containing 5% acetonitrile; eluent B, 1 m*M* sodium hydroxide solution; linear gradient, 0–15 min, 30–100% eluent A; flow-rate, 1 ml/min; detector, Dionex PAD 2; temperature, ambient.

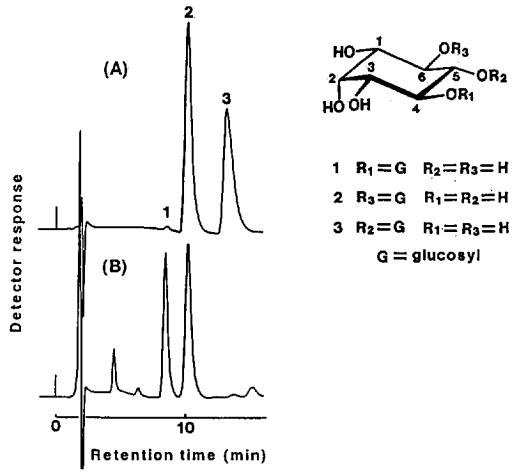

Fig. 4. Elution profiles of positional isomers of glucosylinositol in (A) enzyme-catalysed and (B) synthesized products: **1** and **2** = 1L- and 1D-4-O-α-D-glucopyranosyl-*myo*-inositol, respectively; **3** = 5-O-α-D-glucopyranosyl-*myo*-inositol. Chromatographic conditions: eluent, acetonitrile–water (1:99); temperature, 50°C; other conditions as in Fig. 1.

isomers of glucosyl-*myo*-inositol synthesized by transglucosylation of the CGTase from *Bacillus ohbensis* using *myo*-inositol as the acceptor and β-cyclodextrin as the donor [5]. By ^{13}C NMR spectroscopy the structure of one of the two isomers (**3**) isolated was confirmed to be 5-O-α-D-glucopyranosyl-*myo*-inositol and another (**2**) was presumed to be 1D- or 1L-4-O-α-D-glucopyranosyl-*myo*-inositol. Later, the absolute configuration of **2** was confirmed by chemical synthesis to be 1D-4-O-α-D-glucopyranosyl-*myo*-inositol [6]. The diastereoisomers (**1** and **2**) obtained by chemical synthesis were also well resolved (Fig. 4B).

3.3. Cyclodextrins (CDs)

3.3.1. CDs and mono-branched CDs

The mutual separation of three CDs (α-, β- and γ-CD) and that of their 6-O-α-D-glucopyranosyl derivatives (G_1-α-CD, G_1-β-CD and G_1-γ-CD) on Hypercarb [2] and Carbonex (Tonen) columns were achieved with water containing 15 and 16% of acetonitrile at 50°C, respectively. Interestingly, the t_Rs of the CDs and G_1-CDs increased and, moreover, their

peak shapes became much improved with increase in temperature. Therefore, the use of an eluent containing higher concentrations of acetonitrile at higher temperatures is recommended for the separation of CDs and branched CDs. When using methanol as the organic modifier, the effect of temperature on t_R was almost negligible.

The chromatographic behaviour of novel heterogeneous branched CDs [4-O- and 6-O-β-D-galactosylated glucosyl(G_1)- and maltosyl (G_2)-CDs], which were synthesized by trans-galactosylation with β-galactosidases using lactose as a donor and branched CDs as acceptors, was compared [7]. The elution order of each member (**1**, **2**, **3** and **4** in Fig. 5) in three series (α-, β-, and γ-CD) was the same: 6-O-galactosylated G_1-CDs were faster moving than the corresponding 4-O-galactosylated G_1-CDs, whereas 6-O-galactosylated G_2-CDs were slower moving than corresponding 4-O-galactosylated G_2-CDs. This result (elution order **2**, **1**, **3** and **4**) was entirely different from the elution order on an ODS column (**2**, **4**, **3** and **1**), and might reflect their stereochemical disposition.

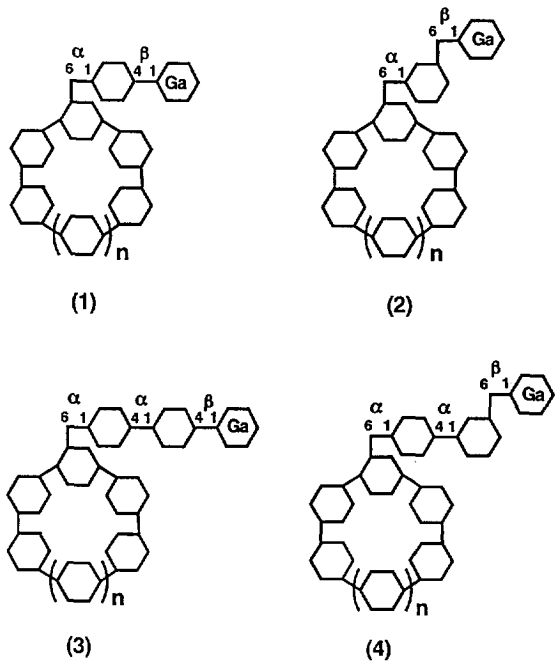

Fig. 5. Abbreviated structural diagrams for *trans*-β-D-galactosylated products from G_1-CDs (**1** and **2**) and G_2-CDs (**3** and **4**): $n=1$, α-CD; $n=2$, β-CD; $n=3$, γ-CD; ○ = glucosyl residue; Ⓖa = galactosyl residue.

3.3.2. *Multi-branched CDs*

The unique resolving power of GCC led to excellent separations of the positional isomers of $6^1,6^n$-di-O-(α-D-glucopyranosyl)-α-CD [8] and $6^1,6^n$-di-O-(α-maltosyl)-β-CD [2] ($n=3$ and 4), and $6^1,6^2,6^n$-tri-O-(α-maltosyl)-β-CD ($n=4$ and 5) [9]. Fig. 6A shows a chromatogram of a mixture of dimaltosyl-β-CDs on an ODS column. Although on this column $6^1,6^2$-di-O-(α-maltosyl)-β-CD (**3**) was well resolved from the isomers, $6^1,6^3$,-di-O-(α-maltosyl)-β-CD (**1**) and $6^1,6^4$-di-O-(α-maltosyl)-β-CD (**2**), the resolution of **1** and **2** was impossible. The resolution of these two positional isomers (**1** and **2**) could be easily achieved on GCC (Fig. 6B).

3.4. *Oligosaccharide alditols containing acetamido sugars and/or sialic acid*

Davies et al. [10] investigated the chromatographic behaviour of oligosaccharide alditols (DP 2–6) containing acetamido sugar(s) and/or sialic acid(s) on a Hypercarb column with 0.05% trifluoroacetic acid (TFA) in 0–25% aqueous acetonitrile as the eluent [10].

The mono-, di- and trisaccharide alditols of D-N-acetylglucosamine (GlcNAc) were satisfactorily separated and the elution times increased with increasing size. Disaccharides consisting of D-galactose (Gal) and GlcNAc were faster eluted than chitobiose consisting of two GlcNAc residues, and three isomers having different linkages were resolved: the elution order was Gal-(1,3)-GlcNAc, Gal-(1,4)-GlcNAc and Gal-(1,6)-GlcNAc. It was shown by comparison of the t_Rs of the linear tetrasaccharide alditols and their fucosylated analogues that the presence of a fucose residue reduced the t_R.

The sialic acid-containing oligosaccharide alditols were the longest retained of the alditols tested and the 2,3-linked compound was retained longer than the 2,6-linked isomer.

Oligosaccharide alditols obtained from base–borohydride degradation of bovine submaxillary

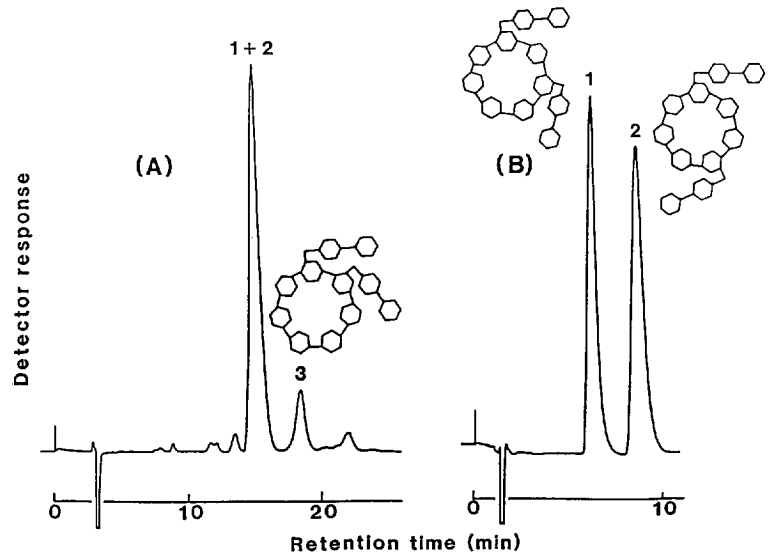

Fig. 6. Separation of positional isomers of dimaltosyl-β-CD on (A) a YMC-Pack A-312 ODS column with methanol–water (6:94) at 35°C and (B) a Hypercarb with acetonitrile–water (17:83) at 50°C. Peaks: 1 = 6^1,6^3-, 2 = 6^1,6^4- and 3 = 6^1,6^2-di-O-(α-maltosyl)-β-CD. Flow-rate and detector as in Fig. 1.

mucin (BSM) were fractionated by gradient elution with 0.05% TFA in water containing acetonitrile. The samples recovered by evaporation were analysed by sensitive liquid secondary ion mass spectrometry (LSI-MS) [11].

3.5. N-Linked oligosaccharides

Sugar chains of high-mannose type glycopeptides, which are hardly retained on an ODS column, are strongly retained on GCC. An oligosaccharide sample prepared by *endo*-β-N-acetylglucosaminidase digestion of ribonuclease B (RNB) could be successfully chromatographed on a Hypercarb column. Two major oligosaccharides of RNB ($Man_6GlcNAc$ and $Man_5GlcNAc$) were separated with a gradient of 8–10% acetonitrile [12]. In order to avoid separation of anomers of the oligosaccharides, 10 m*M* ammonia solution was included in the eluent and the column temperature was maintained at 70°C. $Man_6GlcNAc$ was retained less than $Man_5GlcNAc$. This phenomenon may be attributable to the difference in planarity of these oligosaccharide molecules, although the authors explained the result as follows: "If the interaction between the oligosaccharide and GCC is mainly by the hydrophobic face of the oligosaccharide and the surface of graphitized carbon, the additional Man-α-1,2 residue in $Man_6GlcNAc$ may be masking a part of the hydrophobic region of $Man_5GlcNAc$ with a hydrophobic face".

Oligosacchardies released by peptide-N-glycosidase F (PNGase F) from fetuin and asialofetuin could be chromatographed under the same conditions [11]. Chromatography on GCC separated biantennary from triantennary non-sialylated oligosaccharides, although the absence of a galactosyl residue from one of the branches could not be distinguished and monosialo-oligosaccharides co-eluted with non-sialylated oligosaccharides.

3.6. Chito-oligosaccharides

Chito-oligosaccharides (DP 1–9) were well separated within 40 min on GCC with 0–25% acetonitrile in 10 m*M* ammonia solution [12]. Their retention increased with increasing size.

However, chito-oligosaccharides were retained much longer than the N-linked oligosaccharides having the same number of sugars. GCC chromatography was used for monitoring the course of partial acid hydrolysis of chitin to optimize di-N,N′-acetylchitobiose production.

3.7. Glycopeptides

Davies et al. [10] reported the HPLC of sialylated glycopeptides in a trypsin digest of fetuin on GCC. In this case, no significant difference between a Hypercarb column (100 × 4.6 mm I.D.) and an ODS column could be found and the glycopeptides were not separated from other peptides, but with the use of another column having a higher performance and larger size [Hypercarb S (100 × 6.4 mm I.D.)], two glycopeptides in a protease digest of fetuin and their peptide moieties obtained after PNGase F treatment were well separated [11].

Several high-mannose type glycopeptides with only one or two amino acids, derived from soybean agglutinin (SBA) and RNB by pronase digestion, were successfully separated [12], although those glycopeptides were barely retained on ODS column.

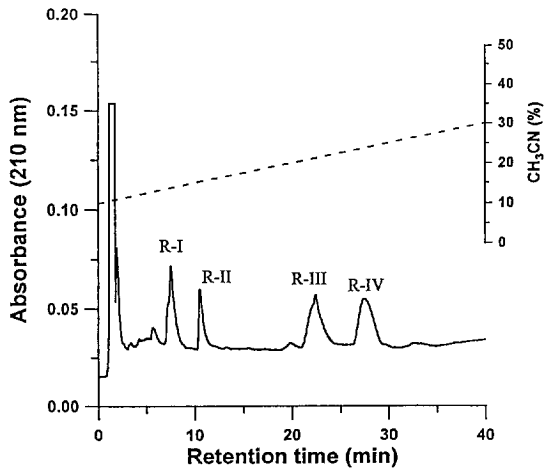

Fig. 7. Separation of RNB-glycopeptides: R-I = $Man_6GlcNAc_2Asn$, R-II = $Man_5GlcNAc_2Asn$, R-III = $Man_6GlcNAc_2AsnLeu$ and R-IV = $Man_5GlcNAc_2AsnLeu$. Chromatographic conditions: eluent A, water; eluent B, acetonitrile; detector, ISCO UV; temperature, 40°C; other conditions as in Fig. 1.

SBA-glycopeptides were easily separated into $Man_9GlcNAc_2Asn$ and $Man_9GlcNAc_2AsnPhe$ by GCC with a gradient of 10–45% acetonitrile in 30 min.

Fig. 7 shows separation of RNB-glycopeptides on GCC. The retention of these glycopeptides on GCC is based on both the carbohydrate and peptide moieties, that is, when the carbohydrate chain is the same, glycopeptides having a single amino acid (R-I and R-II) eluted earlier than those containing dipeptides (R-III and R-IV), whereas glycopeptides involving six mannose residues (R-I and R-III) move faster than those having five mannose residues (R-II and R-IV), but the effect of one mannose is not as dramatic as the effect of one amino acid (Leu).

4. Conclusion

As the retention mechanism of GCC is very unique and GCC possess exceptional chemical and physical stability, they have great potential for the separation and purification of isomeric and closely related carbohydrates. Neutral oligosaccharides, sialylated oligosaccharides, oligosaccharide alditols and glycopeptides can be chromatographed under the same conditions and, moreover, the eluents used for HPLC on GCC do not utilize salts and, therefore, the separated fractions can be used for further analysis (e.g., by LSI-MS) after just evaporation. GCC are especially effective for the separation of less hydrophobic carbohydrates, e.g., small-sized oligosaccharides, and high-mannose type glycopeptides and their sugar chains which are not retained on an ODS column.

Acknowledgement

Fig. 7 was kindly provided by Professor Y.C. Lee (Johns Hopkins University), for which the author is grateful.

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