Journal of Chromatography, 488 (1989) 229–236
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4561

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SIALOOLIGOSACCHARIDES AND GANGLIOSIDES

ZIAD EL RASSI[a] and CSABA HORVÁTH*

Department of Chemical Engineering, Yale University, New Haven, CT 06520 (U.S.A.)

and

ROBERT K. YU[b] and TOSHIO ARIGA

Department of Neurology, Yale University School of Medicine, New Haven, CT 06520 (U.S.A.)

SUMMARY

Glycans were cleaved from gangliosides and separated by high-performance liquid chromatography (HPLC). The columns were packed with bonded stationary phases made of microparticulate, macroporous silica with serotonin, phenylpropanolamine or tryptamine as the biogenic amine ligate. The ganglioside oligosaccharides were eluted in the order of increasing number of sialic acid residues in the molecule and their retention decreased with the ionic strength of the mobile phase. Best selectivity was obtained in the pH range from 3.0 to 4.0. The two major sialic acids, N-acetylneuraminic and N-glycolylneuraminic acids, were separated by lectin affinity chromatography using an HPLC column packed with silica-bound wheat germ agglutinin and 10 m*M* phosphate buffer, pH 4.0, as the eluent. Throughout this study, isocratic elution was used and the column effluent was monitored at 195 nm.

INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids. They are found embedded in the plasma membrane and believed to provide recognition sites on the cell surface. A ganglioside molecule is composed of a hydrophilic sialooligosaccharide chain and a hydrophobic moiety, ceramide, which consists of sphin-

[a]Present address: Department of Chemistry, Oklahoma State University, Stillwater, OK 74078-0047, U.S.A.

[b]Present address: Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

0378-4347/89/$03.50

gosine and fatty acid [1]. According to recent studies, the number of sialic acid residues, which determines the density of negative charges on the molecule, play a role in regulating the physiological binding properties of gangliosides [2]. They are known to have important physiological functions such as regulation of cell growth and nerve sprouting and may act as receptors for exogenous ligands such as bacterial toxins, hormones, growth factors, antibodies, viruses and interferons to induce sequential activations of cellular metabolisms [2]. The chemical structure, isolation and analysis of gangliosides have been reviewed by Ledeen and Yu recently [3].

Growing interest in the biological function of sialooligosaccharides has generated a need to develop improved methods for their isolation and analysis; the scope of high-performance liquid chromatography (HPLC) has been extended also to the separation of sialooligosaccharides. The HPLC methods described in the literature employ strong anion-exchange [4] and amino silica [5] columns with gradient elution at increasing salt and organic solvent concentration in the eluent, respectively. Lack of chromophores in the oligosaccharide molecules engender difficulties in their detection due to the relatively low specific sensitivity of conventional UV detection below 200 nm or detection by monitoring the refractive index of the effluent. Recently a triple pulse electrochemical detector was found to be suitable for sensitive detection of oligosaccharides [6,7]. However, the method requires the use of highly alkaline eluents in which silica-based stationary phases are not stable. In this study, the HPLC of sialooligosaccharides was carried out using siliceous stationary phases with either a biogenic amine or the lectin wheat germ agglutinin as the ligate and UV detection at 195 nm.

The rationale for the use of stationary phases with biogenic amine ligates stems from the observation that in several biological systems the N-acetylneuraminic acid (NANA) interacts with serotonin and the suggestion that gangliosides are part of serotonin receptors [8]. Furthermore Wooley and Gommi [9] reported that the addition of gangliosides to neuraminidase-treated muscle restored the contractile response of the tissue to serotonin. Specific serotonin binding glycoprotein was also isolated from rat platelets [10] and certain gangliosides were found to enhance the binding of serotonin to it [11]. These findings prompted the development by Sturgeon and Sturgeon [12] of agarose-bound serotonin as the stationary phase for the chromatography of sialoglycoconjugates. They suggested that serotonin interacts with sialic acids containing N-acetyl but not N-glycolyl group. However, Corfield et al. [13], who successfully fractionated sialic acids and sialoconjugates on serotonin–agarose, found that N-glycolylneuraminic acid also binds to serotonin. Recently a stationary phase with serotonin bound to microparticulate, macroporous silica gel was introduced for the separation of glycoproteins by HPLC [14] and other biogenic amines were found also to be suitable ligates for HPLC of sialooligosaccharides and proteins including glycoproteins [15].

EXPERIMENTAL

Materials

Phenylpropanolamine, 5-hydroxytryptamine (serotonin), tryptamine, *p*-nitrophenyl-α-D-N-acetylglucosaminide, *p*-nitrophenyl-β-D-N-acetylglucosami-

nide, *p*-nitrophenyl-α-D-N,N′-diacetylchitobiose, N-acetylneuraminic acid (NANA), N-glycolylneuraminic acid (NGNA) and wheat germ agglutinin (WGA) were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade sodium hydroxide, sodium dihydrogen phosphate, phosphoric and hydrochloric acids and methanol (HPLC grade) were obtained from Fisher (Pittsburgh, PA, U.S.A.).

Instrument

The liquid chromatograph was assembled from a Model 750 solvent delivery pump with a Model 753 ternary solvent mixer and a Model 740 control module (Micromeritics, Norcross, GA, U.S.A.). Samples were injected by a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve with 50-μl sample loop. A Kratos (Ramsey, NJ, U.S.A.) Model 770 R variable-wavelength UV detector was used to monitor the column effluent at 195 nm. Chromatograms were recorded and the peak areas measured with Model C-R3A integrator (Shimadzu, Columbia, MD, U.S.A.).

Columns

Phenylpropanolamine, serotonin, tryptamine and wheat germ agglutinin were covalently bound to 7-μm Nucleosil 300-Å silica gel supplied by Macherey-Nagel (Düren, F.R.G.) by a method described elsewhere [15]. The surface coverage of the former stationary phases as calculated from the analysis on nitrogen content (MultiChem Labs., Lowell, MA, U.S.A.) was 0.15, 0.21 and 0.22 μmol/m^2 with serotonin, phenylpropanolamine and tryptamine, respectively. The various stationary phases were packed into 100 mm×4.6 mm No. 316 stainless-steel tubes (Handy and Harman, Morristown, PA, U.S.A.) at 544 bar from a slurry in methanol in the case of silica-bound biogenic amines or from a slurry in 25 m*M* phosphate buffer containing 0.5 *M* sodium chloride and 50% (w/v) sucrose in the case of silica-bound WGA.

Chromatographic conditions

All experiments were carried out with 100 mm×4.6 mm I.D. columns packed with 7-μm Nucleosil-based bonded phase. The flow-rate and temperature were 1 ml/min and 25°C, respectively.

For quantitative analysis of oligosaccharides cleaved from a mixture of gangliosides, calibration curves were made by injecting 50-μl samples containing 0.125, 0.250, 0.500, 1.00 or 2.00 μg of the individual oligosaccharides into the serotonin column. After elution with 13 m*M* phosphate, pH 3.7, peak areas were measured and plotted against the amount of the individual oligosaccharides. In the analysis of oligosaccharides cleaved from gangliosides isolated from the gray matter of human brain, an aliquot of 30 μl of the extract described below was diluted to 1.0 ml with the above mobile phase and 50 μl of this solution were chromatographed on the serotonin column.

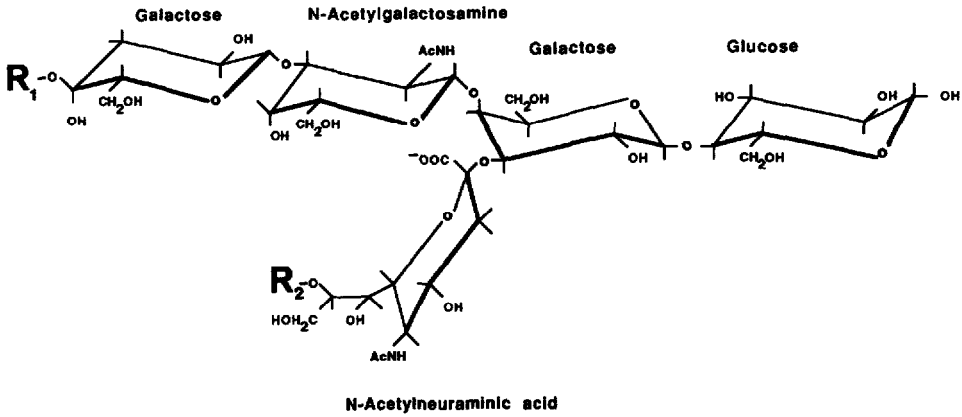

Fig. 1. Structures of ganglioside oligosaccharides. The legends for their codes are as follows. GA1, no sialic acid at the galactose residue; GM1, $R_1 = R_2 = H$; GD1a, $R_1 =$ NANA (N-acetylneuraminic acid), $R_2 = H$; GD1b, $R_1 = H$, $R_2 =$ NANA; GT1b, $R_1 = R_2 =$ NANA. For explanation of the symbols see text.

Isolation of gangliosides and ganglioside oligosaccharides

The gangliosides were isolated from human and bovine brains and purified as described previously [3, 16]. The individual gangliosides gave a single spot in thin-layer chromatography [17].

The asialo- (GA1), monosialo- (GM1), disialo- (GD1a or GD1b) and trisialooligosaccharide (GT1b), which were cleaved from the gangliosides, are shown in Fig. 1. The samples used were prepared either from the ganglioside oligosaccharide mixture or from individual gangliosides using a previously described procedure [3] which is summarized as follows. The ganglioside sample (5–10 mg) was dissolved in 25 ml of a 3:1 (v/v) mixture of methanol–*n*-hexane, and ozone gas was bubbled through the solution at 0 °C for 30 min. The solvent was evaporated at room temperature, the residue dissolved in 10 ml aqueous sodium carbonate, pH 10.0, and the solution was allowed to stand in room temperature for 18 h. Subsequently, the solution containing the liberated oligosaccharides was lyophilized, the residue redissolved in 0.5 ml of 0.05 *M* acetic acid and the solution was chromatographed on a Sephadex G-50 column (86 cm × 1.6 cm) with 0.05 *M* acetic acid as the mobile phase and the oligosaccharides fractions were collected. Analysis by thin-layer chromatography on silica gel with *n*-butanol–acetic acid–water (2:1:2) showed no intact gangliosides present in the fractions.

RESULTS AND DISCUSSION

Silica-based bonded stationary phases with biogenic amine ligates facilitated HPLC of the isolated oligosaccharides of gangliosides by using isocratic elution with an aqueous buffer of low salt concentration and thus sensitive detection of the oligosaccharides at a detector setting of 195 nm. Fig. 2 illustrates typical chromatograms obtained on serotonin and tryptamine columns at two different pH values.

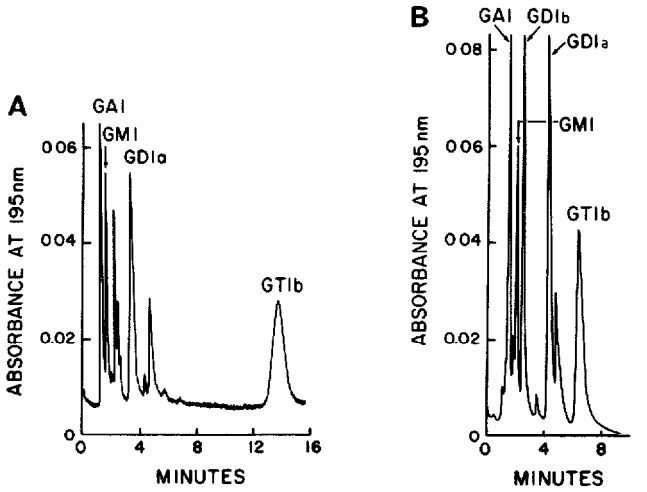

Fig. 2. Separation of ganglioside oligosaccharides on tryptamine (A) and serotonin (B) bonded stationary phases with 15 m*M* phosphate buffer, pH 3.2, and 10 m*M* phosphate, pH 6.3, as the eluent, respectively.

The retention of the sialooligosaccharides on the biogenic amine-bonded stationary phases strongly depended on the ionic strength of the mobile phase and decreased with increasing salt concentration of the eluent in the pH range studied. The asialooligosaccharide, GA1, was not retained on any of the stationary phases under investigation and the sialoglycans were retained in the order of increasing number of sialic acid residues. These findings suggest that the retention of sialooligosaccharides is largely due to coulombic interactions although the existence of some biospecific interactions between the biogenic amine ligates and the sialic acid moiety of the eluites cannot be excluded. In essence, the biogenic amine stationary phases are weak anion exchangers of relatively low phase ratio. As a result, the sialooligosaccharides can be eluted isocratically with mobile phases of low salt concentration so that their detection with the UV detector at low wavelength is possible. This is not the case when conventional ion-exchange columns are used [18] because of the high salt concentration in the eluent required for their elution.

Although GA1, the neutral oligosaccharide from ganglioside, was not retained on any of the biogenic amine columns, other neutral sugars containing phenyl moieties were retained. For instance on tryptamine column with 10 m*M* phosphate buffer, pH 7.0, the retention factors of *p*-nitrophenyl-β-D- and -α-D-N-acetylglucosaminides and *p*-nitrophenyl-α-D-N,N′-diacetylchitobiose were 0.30, 0.46 and 0.49, respectively. These results suggest that besides their weak anion-exchange properties the biogenic amine stationary phases are also weakly hydrophobic [15].

The effect of eluent pH on the retention of sialooligosaccharides on the three biogenic amine columns was investigated in the pH range from 3.0 to 7.0 and resulting plots of the retention factors versus the pH of the eluent are shown in Fig. 3. In each case, the retention first increased and then decreased with increas-

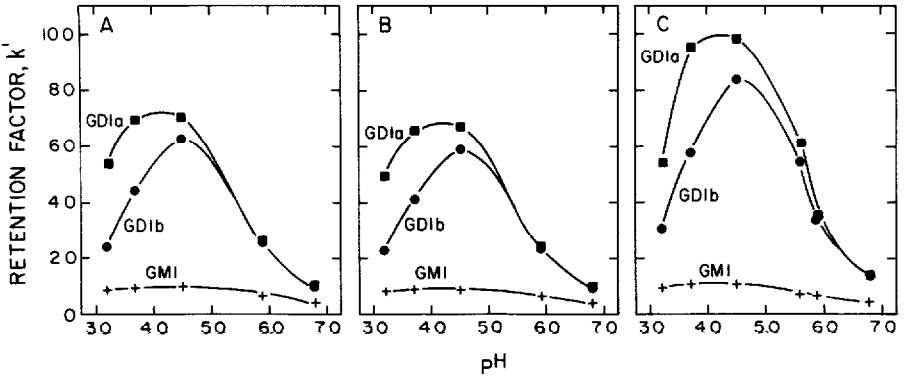

Fig. 3. Plots of the retention factors of ganglioside oligosaccharides against the pH of the eluent as measured on serotonin (A), phenylpropanolamine (B) and tryptamine (C) bonded stationary phases with 10 m*M* phosphate solutions of different pH.

ing pH and reached a maximum between pH 4.0 to 5.0. The increase in retention when going from pH 3.0 to pH 4.5 is caused by dissociation of the carboxylic group in the sialic acid moiety, whereas the decrease in retention with the pH is believed to occur due to gradual deprotonation of the amino groups on the surface of the stationary phase above pH 5.0.

Whereas the retention maximum of oligosaccharides on the serotonin and phenylpropanolamine columns was about the same, the tryptamine column exhibited slightly higher retentivity under the same elution conditions as can be seen in Fig. 3. This is likely due to differences in the 'anion-exchange capacity' at neutral pH, which was calculated from the density of biogenic amines as 0.020, 0.024 and 0.035 mequiv./g for the stationary phases with phenylpropanolamine, serotonin and tryptamine ligates, respectively [15]. As can be concluded from the data shown in Fig. 3, the selectivities of the three columns for the oligosaccharides investigated are slightly different and decrease in the order of tryptamine > serotonin > phenylpropanolamine. In all cases the selectivity for the GD1b and GM1 oligosaccharide pair is the highest at pH 4.5, wheres the selectivity for the GD1a and GD1b pair increases monotonically with decreasing pH in the domain studied here.

Calibration curves of GM1, GD1a, GD1b and GT1b were obtained by the external standard procedure at a detector setting of 195 nm and they were linear in the concentration range 0.1–2.0 μg with correlation coefficients better than 0.997. Ganglioside oligosaccharides from the gray matter of human brain were analyzed for carbohydrate contents using the above chromatographic system. A typical chromatogram is depicted in Fig. 4 along with that of the standard mixture. According to the analytical results, the amounts of GM1, GD1a, GD1b, and GT1b in 30 μl of the sample were 370.0, 132.2, 100.3 and 49.3 μg, respectively.

The term sialic acid stands for a family of neuraminic acid derivatives with acetyl or glycolyl functions at the amino or hydroxyl group. Approximately twenty different sialic acids have been discovered, but only a few of them were found thus far in gangliosides [3]. The two major sialic acids are NANA and NGNA and

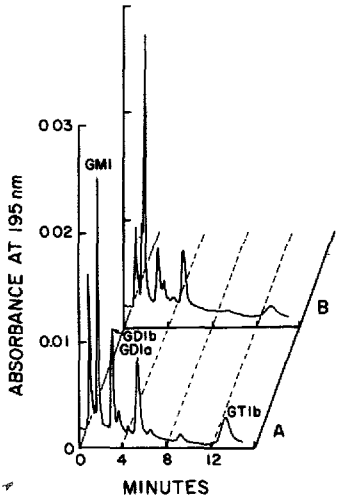

Fig. 4. Chromatograms of ganglioside oligosaccharides obtained on the serotonin column with 15 m*M* phosphate, pH 3.2, under identical conditions. (A) Sample of known composition, (B) human brain extract.

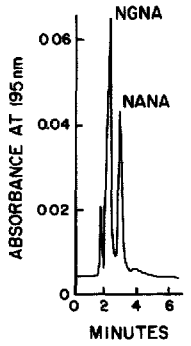

Fig. 5. Separation of N-acetylneuraminic (NANA) and N-glycolyneuraminic (NGNA) acids on silica-bound WGA with 10 m*M* phosphate, pH 4.0. Each peak represents approximately 50 ng of sialic acid.

their physiological role is under investigation. Although ganglioside oligosaccharides can be readily analyzed as their trimethylsilyl derivatives by gas–liquid chromatography [19] the derivatization of free sialic acids is beset with difficulties. Whereas NANA and NGNA can be separated by HPLC using strong anion exchangers [18], attempts to separate the two sialic acids on our biogenic amine columns in a wide pH range were not successful. Since WGA is known for its affinity to sialooligosaccharides and sialoglycoconjugates, a WGA–silica sorbent was prepared and found to be suitable for this particular separation using 10 m*M* phosphate, pH 4.0, as the eluent. It is seen from the chromatogram in Fig. 5 that as little as 50 ng of the two sialic acids can be analyzed by this technique.

The significance of the approach taken in this study for the HPLC analysis of oligosaccharides derived from gangliosides is that it affords isocratic elution with mobile phases having low salt concentration and the concomitant use of ubiqui-

tous variable-wavelength detectors at 195 nm for monitoring the column effluent at relatively high sensitivity. HPLC analysis of intact gangliosides is hampered by the lack of eluents which are optically transparent at such a low wavelength and in which the gangliosides would be soluble. As mentioned above sialooligosaccharides can be separated on strong ion exchangers of sufficiently higher retentive capacity, but gradient elution is required and it precludes the use of a UV detector at such low wavelengths.

The results show that novel stationary phases presented here can facilitate the analysis of closely related sialooligosaccharides by HPLC and are likely to be useful to carry out their isolation and purification. Although the retention mechanism is yet to be elucidated, the ease and quantitative nature of HPLC measurements should make it easier to shed light on the interaction underlying the chromatographic separation process. The information gained from such investigations should not only lead to highly efficient stationary/mobile phase systems for glycan HPLC but also to a better understanding of the physicochemical nature of glycan interactions in physiological systems.

ACKNOWLEDGEMENTS

This work was supported by Grant Nos. GM 20993, CA 21948 and NS 11853 from the National Institutes of Health, U.S. Department of Health and Human Services.

REFERENCES

1 R.W. Ledeen, in A. Lajtha (Editor), Handbook of Neurochemistry, Vol. 3, Plenum Publishing, New York, 2nd ed., 1983, pp. 41–90.
2 S. Ando, Neurochem. Int., 5 (1983) 507.
3 R.W. Ledeen and R.K. Yu, Methods Enzymol., 83 (1982) 139.
4 J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik and G. Stecker, in M.F. Chaplin and J.F. Kennedy (Editors), Carbohydrate Analysis: A Practical Approach, IRL Press, Oxford, 1986, p. 155.
5 M.L.E. Bergh, P. Koppen and D.H. van den Eijnden, Carbohydr. Res., 94 (1981) 409.
6 R.D. Rocklin and A.L. Pohl, J. Liq. Chromatogr., 6 (1983) 1577.
7 S. Hughes and D.C. Johnson, Anal. Chim. Acta, 149 (1983) 1.
8 G.A. Dette and W. Wesemann, Hoppe-Seyler's Z. Physiol. Chem., 358 (1978) 399.
9 D.W. Wooley and B.W. Gommi, Proc. Natl. Acad. Sci. U.S.A., 53 (1963) 959.
10 H. Tamir, R. Belirian, F. Muller and D. Casper, J. Neurochem., 35 (1980) 1033.
11 H. Tamir, W. Brunner, D. Casper and M.M. Raport, J. Neurochem., 34 (1980) 1719.
12 R.J. Sturgeon and C.M. Sturgeon, Carbohydr. Res., 103 (1982) 213.
13 A.P. Corfield, P.J. Winterburn, J.R. Clamp, C.H. Bolton and J. Martin, Biochem. Soc. Trans., 13 (1985) 956.
14 R.A. Pask-Hughes, J. Chromatogr., 393 (1987) 273.
15 Z. El Rassi, Y. Truei and Cs. Horváth, Makromol. Chem., Macromol. Symp., 17 (1988) 305.
16 S. Ando and R.K. Yu, J. Biol. Chem., 252 (1977) 6247.
17 S. Ando, N.-C. Chang and R.K. Yu, Anal. Biochem., 89 (1978) 437.
18 R. Schauer, Methods Enzymol., 138 (1987) 132.
19 R.K. Yu and R.W. Ledeen, J. Lipid Res., 11 (1970) 506.