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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF GLYCOSPHINGOLIPIDS (WITH SPECIAL REFERENCE TO GANGLIOSIDES)

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SUMMARY

The analysis of mixtures of gangliosides from adult human or bovine brain, supplemented with Tay-Sachs ganglioside, and haematoside from dog erythrocytes by high-pressure liquid chromatography using a moving-wire detector system is described.

The complete separation of six gangliosides within 40 min has been achieved, using silica as the stationary phase and acidified chloroform–methanol–water mixtures as the eluent on a 25-cm column. Neutral glycosphingolipids, *viz.*, the major components from normal human erythrocytes, can be completely separated on the same column, using non-aqueous and non-acidic eluents.

It is shown that the methods described are useful for both analytical and (micro)-preparative purposes.

INTRODUCTION

In this paper, for gangliosides the standard Svennerholm nomenclature[1] is used, while for neutral glycosphingolipids the symbols used by Dawson[2] have been adopted. For structures and synonyms, see ref. 3.

Since the pioneering work of Wagner *et al.*[4] and Jatzkewitz and Mehl[5], the thin-layer chromatography (TLC) of gangliosides and neutral glycosphingolipids has become the method of choice for the analysis of complex mixtures of these lipids from natural sources[6–10]. However, in spite of the simplicity of TLC, this technique has several severe drawbacks. The chloroform–methanol–water mixtures most commonly used as the mobile phase in the TLC analysis of gangliosides create gradients of individual solvent components in both the thin layer and the surrounding gaseous

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phase, as demonstrated by the use of the Sandwich and Vario-KS chambers[11] (*cf.*, ref. 12). Thus, the system in which TLC is carried out in conventional paper-lined tanks is in fact ill-defined and may respond unpredictably to accidental variations in handling and in the surroundings.

Further, quantitative analysis by means of resorcinol staining[13] of the individual ganglioside classes after TLC separation is laborious and relatively insensitive. Such staining, being destructive, prevents the isolation of the separated components by extraction from the plates for further analysis. Although the recent non-destructive use of the universal primuline spray[14] is an important improvement in analysis, there still remains a need for a satisfactory quantitative and micro-preparative assay.

Glycosphingolipids, and gangliosides in particular, have so far withstood high-pressure liquid chromatographic (HPLC) analysis. This difficulty has partially been caused by the fact that a simple conversion of the TLC system to a column system appears to fail (see below) and by the absence of a significant ultraviolet absorption of the solutes, which hampers detection using the commonly available ultraviolet detectors.

As far as we know, only one report has been published[15] in which a column liquid chromatographic separation of glycosphingolipid fractions from normal, Gaucher's and Fabry's plasma and normal erythrocytes is described. However, in that study the glycosphingolipids were converted into their benzoyl derivatives prior to chromatography, in order to permit ultraviolet detection.

Classical column liquid chromatography on silica, followed by off-line detection by means of TLC, has been described for the preparative separation of gangliosides[1,16,17], but the procedure is very time consuming and considerable overlapping between peaks occurs.

In this paper, the complete separation of gangliosides and of neutral glycosphingolipids by means of HPLC within 40 min is described. By using a universal detector, derivatization is unnecessary. Finally, the importance of this technique in future research on these complex lipid classes is outlined.

EXPERIMENTAL

Glycosphingolipids

Gangliosides from adult bovine brain were purchased from Koch-Light (Colnbrook, Great Britain), Sigma (St. Louis, Mo., U.S.A.) and Supelco (Bellefonte, Pa., U.S.A.). These preparations contained essentially all of the principal gangliosides (GM1, GD1a, GD1b and GT1) in different proportions. Preparations, enriched in mono-, di- and trisialogangliosides from normal brain and pure GM2 from Tay-Sachs brain, were obtained from Supelco. Highly enriched preparations of GM1, GD1a, GD1b and GT1 were isolated from adult human brain[16]. Haematoside (GM3) was isolated from dog erythrocyte ghosts[18] by the method of Koscielak[19] with the modification of Hakomori and Strycharz[20]. The crude preparation exhibited only one resorcinol-positive spot with higher mobility than authentic GM2 on TLC. It was purified by treatment with methanolic sodium hydroxide solution and subsequent HPLC, as described below. The purified haematoside showed a molar ratio of glucose, galactose and sialic acid of 1:1:1.

Galactocerebroside (GL1b), a preparation from bovine brain, was obtained

from Koch-Light and glucocerebroside (GL1a), isolated from Gaucher's spleen, from Supelco. Lactosylceramide (GL2a) was a synthetic preparation (Miles Labs., Slough, Great Britain) consisting of the stearoyl derivative only. Authentic digalactosylglucosylceramide (GL3) originated from *post mortem* kidney of a patient with Fabry's disease[21]. GL3 was also isolated, together with GL2a and ceramide tetrahexoside (globoside, GL4), from human erythrocyte ghosts[18–20]. Ceramide, prepared from bovine brain cerebrosides, was a product of Koch-Light.

Solvents

All solvents were of analytical-reagent grade and were used without prior purification.

Column materials

Silica SI 60 (E. Merck, Darmstadt, G.F.R.) with a particle size range of 63–200 μm was ground in an agate mortar and then fractionated by means of an air classifier (Model 100 MZR; Alpine, Augsburg, G.F.R.). The particle size distributions of the classified fractions were determined with a Coulter counter and the fraction of 9 ± 1.5 μm was used for column packing. The columns were packed using a pressurized balanced-slurry technique[22].

Apparatus

The liquid chromatograph was constructed from custom-made and commercial parts and consisted of a thermostated eluent reservoir, a high-pressure pump (DMP 1515, Orlita, Giessen, G.F.R.), a flow-through manometer as damping device, a sampling valve (Rheodyne, Model 7120) and a stainless-steel 316 column. Column tubings of length 10 and 25 cm, I.D. 2.8 mm and O.D. 6.35 mm were constructed from precision-bore stainless-steel tubing. The detector was the improved moving-wire system equipped with a flame-ionization detector (Pye Unicam, LCM2)[23]. The chromatograms were recorded using a linear potentiometric recorder (Philips PM 8220).

The gas chromatograph (Varian Model 2100) was equipped with a flame-ionization detector and an electronic integrator (Infotronics Model CRS-208). A glass column of length 150 cm, I.D. 2 mm and O.D. 6.35 mm was used, packed with Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), coated with 3% SE-30 by an evaporation technique.

Determination of the chromatographic parameters

The capacity ratio, k_i', of a component i was determined from the retention time, t_{Ri}, and the time of a non-retarded compound, t_{Ro}:

$$k_i' = \frac{t_{Ri} - t_{Ro}}{t_{Ro}} \quad (1)$$

The selectivity coefficient, r_{ji}, of two compounds was calculated from their capacity ratios:

$$r_{ji} = \frac{k_j'}{k_i'} \quad (2)$$

Ceramide was used as a non-retarded compound.

Thin-layer chromatography

All separations were performed on pre-coated silica plates (Merck) in conventional TLC tanks lined with Whatman 3 MM paper.

Gangliosides (1–20 μg) were applied as 1-cm long streaks, about 3 mm wide. The plates were developed by two successive runs over 15 cm using chloroform–methanol–water (containing 200 mg/l of potassium chloride)[10] in the volume ratio 60:35:8.5 as solvent. Between the two runs, the plates were dried for exactly 10 min by means of an infrared lamp. Resorcinol staining, which is specific for sialic acid, was used for detection[24]. Neutral glycosphingolipids were separated by two successive runs in the same solvent and rendered visible by staining with orcinol[25] or naphthoresorcinol[26]. Contaminating phospholipids were detected with Zinzadze reagent[27].

Preliminary monitoring was performed by means of a primuline spray[14] and inspection at 350 nm. This procedure did not interfere with the subsequent application of the aforementioned sprays.

Gas chromatography

The individual glycosphingolipid classes were analysed for their sugar content by gas–liquid chromatography. In principle the method of Sweeley and Walker[28] was used, except for the following modifications.

Methanolysis was performed in 0.5 *N* anhydrous methanolic hydrochloric acid for 5 h at 90°, followed by neutralization with silver carbonate. Re-N-acetylation, which is essential for reproducible (high) yields of amino-sugars and sialic acid to be obtained, was performed by reaction with acetic anhydride (0.1 by volume) for 5 h at room temperature. After evaporation at 50° in a stream of nitrogen, the residue was dissolved in water and shaken with chloroform. The aqueous upper layer, containing the methylglycosides, was freeze-dried and the residue was silylated in pyridine with a mixture of 1% (v/v) trimethylchlorosilane (TMCS) in N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) at 50° for 1 h. Aliquots of the contents of the vials were injected into the chromatograph with an injection port temperature of 200°. Chromatography was carried out with a linear temperature programme of 4°/min from 180 to 250°. The reliability of the method was checked with standard glycosphingolipids.

RESULTS AND DISCUSSION

Separation of gangliosides

In order to develop a selective phase system for gangliosides, a number of experiments were carried out. The choice of adsorption chromatography was based mainly on earlier TLC experiments, which showed that a polar solvent had to be used in order to elute these compounds within a reasonable period. Conversion of the TLC phase system, consisting of silica as stationary phase and a chloroform–methanol–water mixture as the mobile phase, to columns led to disappointing results. Whereas in TLC good results with, *e.g.*, chloroform–methanol–water (60:35:8) were obtained, in column chromatography the use of this phase system led to very broad, asymmetric and consequently severely overlapping peaks.

Glycosphingolipids consist of sugar chains of various lengths and compositions, attached to a ceramide molecule. As ceramides are not retarded in this phase system,

it can be concluded that the ceramide moieties of the glycosphingolipids contribute only slightly to their chromatographic properties. The hydrophilic character of the gangliosides, which is due primarily to the presence of sialic acid residues in the molecule, causes a very strong adsorption on silica. The addition of water to the eluent in order to accelerate the separation strongly promotes the dissociation of the sialic acid residues. The consequence is a very strong interaction of the dissociated acid residue(s) of the molecule with the silica surface, which causes the unsatisfactory peak shapes. This effect can be counteracted by adding a strong acid to the eluent, which converts the gangliosides into neutral molecules, leading to significant improvements in peak shape and capacity ratios. In addition, the presence of a strong acid in the eluent increases the selectivity coefficients, because the contribution of the dominating acid residues to the overall retention decreases and the influence of the structure of the solutes becomes more pronounced.

The optimal composition of the phase system with respect to selectivity and speed was investigated more precisely by determining the effect of the type of acid and the water content on the capacity ratio, selectivity coefficient, peak shape and stability of the gangliosides.

As the use of the moving-wire detector system prohibits the use of organic acids, only volatile mineral acids can be applied. Nitric acid was chosen originally, but further TLC analysis of the eluted fractions was hampered owing to the high salt concentrations formed upon neutralization, leaving gas chromatography as the only supporting technique. It was found that use of hydrochloric acid permits subsequent TLC analysis when the eluted fractions are collected in vials containing silver carbonate. In addition to minimizing the time during which the gangliosides are exposed to lower pH values, only low salt concentrations in the fractions are obtained. In general, the type of acid does not influence the capacity ratios and the selectivity coefficients.

The results with different amounts of acid are presented in Table I. At low acid concentrations, almost no improvement in the peak shape was noticed. The reliability of the data with 0.003 *M* hydrochloric acid is therefore relatively low. On the other hand, the low stability of gangliosides in highly acidic solvents tends to reduce the reliability and reproducibility of the data with 0.05 *M* hydrochloric acid.

TABLE I

EFFECT OF THE ACID CONTENT OF THE MOBILE PHASE ON THE CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME GANGLIOSIDES ON SILICA (LICHROSORB SI 60)

Eluent: chloroform–methanol–aqueous HCl (60:35:5).

Component	*Final HCl concentration (M)*									
	0.003		*0.005*		*0.01*		*0.02*		*0.05*	
	k_1'	r_{j1}	k_1'	r_{j1}	k_1'	r_{j1}	k_1'	r_{j1}	k_1'	r_{j1}
GM1	2.32	—	2.14	—	1.71	—	1.58	—	1.45	—
GD1a	3.37	1.45	3.11	1.45	2.48	1.45	2.30	1.46	2.04	1.41
GD1b	4.66	1.38	3.97	1.28	3.16	1.27	2.96	1.29	2.81	1.38
GT1	6.59	1.41	5.93	1.49	4.68	1.48	4.12	1.39	3.62	1.29

It is known that gangliosides lose their sialic acid residues in aqueous solution at acid concentrations of 0.05 *M* and above, leaving lower gangliosides and/or asialo derivatives, especially at higher temperatures[29]. Therefore, a final hydrochloric acid concentration of 0.01 *M* was chosen as a compromise between the demands of reasonable peak shape, time of analysis and decomposition.

In Table II, the influence of the water content of the eluent on the capacity ratios and the selectivity coefficients is summarized. For all eluents tabulated, the final concentration of hydrochloric acid was maintained at 0.01 *M*. An increase in the water content decreases the capacity ratios of the individual gangliosides, and decreases the selectivity coefficient of GD1a and GD1b. Hence the system containing 4% of water and a final acid concentration of 0.01 *M* appears to be optimal.

TABLE II

EFFECT OF THE WATER CONTENT OF THE MOBILE PHASE ON THE CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME GANGLIOSIDES ON SILICA (LICHROSORB SI 60)

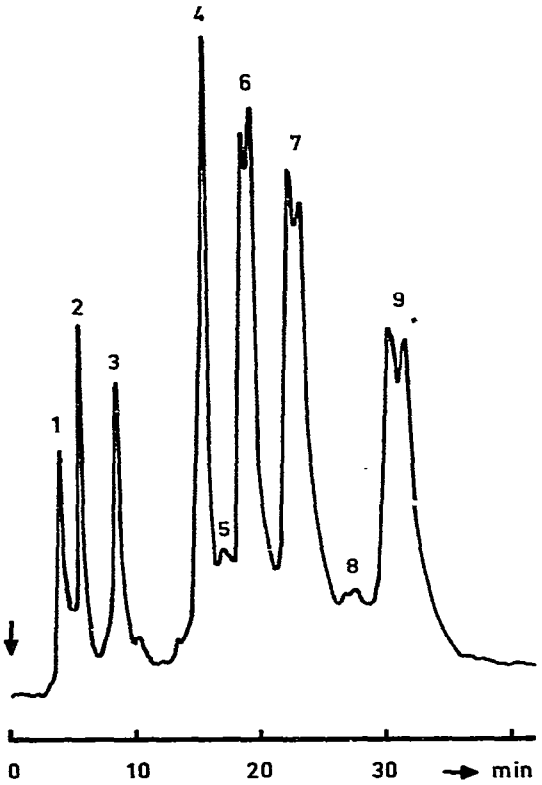
Final HCl concentration: 0.01 *M*. Eluent: chloroform–methanol–aqueous HCl.

Component	*Chloroform–methanol–aqueous HCl*							
	60:35:3		*60:35:4*		*60:35:5*		*60:35:6*	
	k_i'	r_{j1}	k_i'	r_{j1}	k_i'	r_{j1}	k_i'	r_{j1}
GM3	—	—	0.38	—	—	—	—	—
GM2	—	—	0.98	2.54	—	—	—	—
GM1	2.33	—	2.16	2.21	1.71	—	1.64	—
GD1a	3.13	1.34	2.98	1.38	2.48	1.45	2.50	1.52
GD1b	5.02	1.60	4.31	1.45	3.16	1.27	2.93	1.17
GT1	6.67	1.33	6.02	1.40	4.68	1.48	4.52	1.54

Fig. 1 shows a chromatogram of a test mixture of six gangliosides obtained by using this phase system and a 25-cm column. Good separation of the major components was achieved, although the unknown compounds 5 and 8 (apparently impurities) interfered. All of the eluted compounds except 5 and 8 (owing to the considerable overlapping on both sides) were collected and subsequently inspected by means of gas and thin-layer chromatography, as described before. For all collected peaks the expected molar ratios of glucose, galactose, galactosamine (if present) and sialic acid were found. TLC confirmed the identity of the fractions as indicated and showed the high purity of the monosialoganglioside fractions. The purity of the di- and trisialogangliosides was poorer, as judged by visual observation of the primuline-sprayed thin-layer plates. While the liquid chromatogram showed doublets for the higher gangliosides, it appears from gas chromatography that the molar ratios were identical for fractions collected from the front and back of these peaks.

Separation of neutral glycosphingolipids

As neutral glycosphingolipids do not contain sialic acid residues, the hydrophilic character is considerably reduced in comparison with the gangliosides. Chloro-



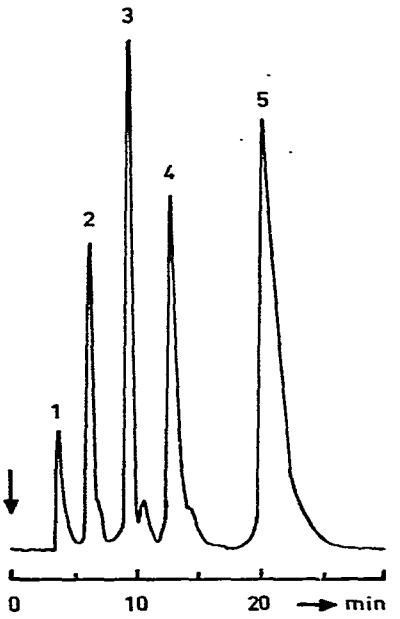


Fig. 1. Separation of a sample mixture of six gangliosides by HPLC. Column: 250 × 2.8 mm I.D. packed with LiChrosorb SI 60 (9 μm). Eluent: chloroform–methanol–aqueous HCl (60:35:4); final HCl concentration, 0.01 *M*. Detection: moving wire with flame-ionization detector. Arrow denotes time of injection. Peaks: 1, 5, 8 = unknown; 2 = GM3; 3 = GM2; 4 = GM1; 6 = GD1a; 7 = GD1b; 9 = GT1.

Fig. 2. Separation of a sample mixture of four neutral glycosphingolipids by HPLC. Conditions as in Fig. 1. Eluent: chloroform–methanol (3:1). Arrow denotes time of injection. Peaks: 1 = unknown; 2 = GL1a; 3 = GL2a; 4 = GL3; 5 = GL4. A thin-layer chromatogram of peaks 2–5 is shown in Fig. 3.

form–methanol (3:1) proved adequate to achieve a good separation of four neutral glycosphingolipids (GL1a, GL2a, GL3 and GL4) (Fig. 2). The pertinent data for the individual compounds in this system are collected in Table III.

All glycolipid fractions were analysed by means of TLC before and after liquid chromatography, as illustrated in Fig. 3. The high purity of the eluted fractions deserves special attention.

It was further shown that the same column can be used for the separation of gangliosides and neutral glycosphingolipids alternately without a severe change in the retention parameters. The silica is reactivated reproducibly after ganglioside analysis by pumping chloroform–methanol (3:1) through the column for 1 h at a flow-rate of 0.5 ml/min.

The chloroform–methanol system thus enables one to separate natural mix-

TABLE III

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF SOME NEUTRAL GLYCOSPHINGOLIPIDS ON SILICA (LICHROSORB SI 60)

Eluent: Chloroform–methanol (3:1).

Component	k'_i	r_{ji}
GL1a	0.63	—
GL2a	1.38	2.19
GL3	2.21	1.60
GL4	4.06	1.84

tures of glycosphingolipids into groups, *viz.*, the neutral glycosphingolipids and the more polar gangliosides, by using a step gradient of chloroform–methanol (3:1) to chloroform–methanol–dilute aqueous acid. The suitability of the method is enhanced by its application to the purification of crude preparations of glycosphingolipids, which contain considerable amounts of contaminating phospholipids and neutral lipids (see below).

Quantitative aspects

In order to improve the applicability of the method described, its use in quantitative analysis was examined. As an example, GL1a as a neutral glycosphingolipid and GM1 as a ganglioside were investigated. The relationships between peak area and amount injected, ranging from 2 to 400 μg for GL1a and from 2 to 200 μg for GM1, were linear, provided that the eluent flow-rate and wire speed were kept constant during the analysis. As the ceramide moieties of the glycosphingolipids differ widely in fatty acid and sphingosine composition within one glycosphingolipid class, the overall carbon content should be known for each compound eluted, prior to final quantification.

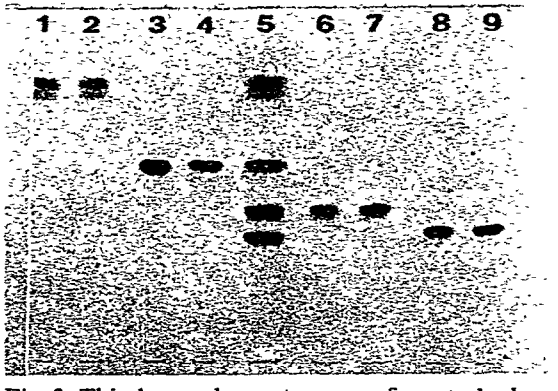


Fig. 3. Thin-layer chromatogram of neutral glycosphingolipids before and after HPLC. Plate: silica pre-coated. Solvent: chloroform–methanol–water (60:35:8.5) containing 200 mg/l of KCl. Development: twice, over 15 cm. Detection: naphthoresorcinol spray. Lane 1, GL1a from Gaucher's spleen; lane 3, synthetic GL2a; lane 5, mixture of GL1a, GL2a, GL3 and GL4 to be separated by HPLC; lane 6, GL3 from Fabry kidney; lane 8, GL4 from human erythrocyte ghosts; lanes 2, 4, 7 and 9, fractions 2, 3, 4 and 5, respectively, from Fig. 2.

Preparative aspects

As the moving-wire detector system renders the derivatization of glycosphingolipids superfluous and its construction is such that only about 1% of the effluent is used for detection, we investigated whether the method can be used on a preparative scale.

Preliminary experiments indicated that the method has to be adapted to the chemical nature of the lipid extract. It is obvious that lipid extracts from the grey matter of brain tissue, in which gangliosides are abundantly present, require less prepurification in order to be suitable for HPLC than extracts from non-neural tissues, in which gangliosides are only minor components[30,32]. In the latter instance considerable amounts of neutral lipids (as cholesterol) and phospholipids are present, of which the latter interfere severely with the separation of gangliosides. We found that treatment of such lipid extracts with methanolic sodium hydroxide solution, by which glycerophospholipids are split and (glyco)sphingolipids left intact, followed by neutralization with methanolic hydrochloric acid gave good results. The fatty acid methyl esters produced are eluted almost unretarded, together with cholesterol, immediately after the solvent front. As only the alkaline-stable sphingomyelin may now interfere, its chromatographic properties were studied in more detail. With chloroform–methanol (3:1) as the eluent it was completely retarded, whereas with chloroform–methanol–aqueous hydrochloric acid as the eluent it was eluted in the midst of the gangliosides. It was found, however, that the capacity ratio for sphingomyelin was affected far more by water content and far less by the acidity of the eluent than were the capacity ratios for gangliosides. Hence the composition of the eluent can be chosen in such a way that sphingomyelin is not co-chromatographed with one of the gangliosides present in the sample.

In micro-preparative applications, we observed lower yields of individual components in the collected fractions than was expected from the quantitative analysis. Small amounts of gangliosides chromatographed on silica with chloroform–methanol–dilute acid as the eluent showed considerable losses of individual gangliosides, apparently due to a loss of sialic acid during evaporation of the solvent at 40° in a stream of nitrogen, as was confirmed by the presence of less hydrophilic compounds in TLC experiments. Even neutralization with silver carbonate did not improve the recoveries. It was observed that decomposition of the gangliosides occurred only during evaporation of the eluent that passed through the column. A mixture of gangliosides, dissolved in freshly prepared (acidic) eluent was stable for at least 12 h at room temperature, while no decomposition was observed in TLC after evaporation of the neutralized solution. Therefore, the interference could be attributed to trace amounts of silica, present in colloidal form in the eluent. It is known that methanol solubilizes silica to some extent[31], especially from very finely ground material. The solubility of silica in ethanol is much lower than in methanol and, for preparative purposes, the replacement of the latter solvent with ethanol leads to a remarkable improvement in the recoveries. Chromatography on silica with chloroform–ethanol–dilute hydrochloric acid (45:40:10) as the eluent was found to be useful in micro-preparative applications. Although the separation of the higher gangliosides, especially GD1a and GD1b, is far less satisfactory than in the methanol system, no loss of gangliosides was observed. It should be borne in mind that in this instance the sample should be introduced in the proper (ethanolic) eluent, as otherwise severe

de-mixing occurs, resulting in ghost peaks near the eluent front containing all components of the sample.

CONCLUSIONS

The proposed method offers good prospects for future research in the fields of, *e.g.*, neurochemistry and membrane biochemistry. By choosing conditions appropriate for the nature of the biological specimen, complete analysis of gangliosides and the lower neutral glycosphingolipids is now possible. Advantages over previously used methods include greater speed and resolution and possibilities of quantitative and preparative applications.

Further research on the glycosphingolipids of plasma membranes from normal liver and hepatomas is at present being carried out[32].

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