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RAPID ION-EXCHANGE SEPARATION OF HUMAN BRAIN GANGLIOSIDES

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SUMMARY

An extract of human brain gangliosides was separated on a Spheron 1000-DEAE spherous fine-grain macroporous glycolmethacrylate anion-exchanger. Linear gradient elution using ammonium acetate in methanol resulted in the complete separation of mono, di-, tri-, tetra- and pentasialogangliosides in 35 min at the load of 2.4 mg/ml of ion-exchanger. The ganglioside fractions thus obtained were characterized by thin-layer chromatography on silica gel. In conclusion, the scope of rapid separation of gangliosides is discussed, based on a combination of column chromatographic methods.

INTRODUCTION

Techniques for ganglioside separation are the focus of deep interest as these substances are important membrane receptors [1] and tumour-associated markers [1, 2]. Methods for ganglioside separation using adsorption chromatography [3- 5] did not lead to the isolation of separate pure gangliosides because of their largely diverse nature. The introduction of some of the latest adsorbents for high-performance liquid chromatography (HPLC) did make a distinction between the main common gangliosides possible [6—8], but the

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combination of ion-exchange and adsorption chromatography is still in general use as it permits the isolation of even minor components.

Considering the differences in the levels of sialic acid in mono-, di-, tri-, tetra- or even pentasialogangliosides provided the groundwork for their stepwise elution from diethylaminoethyl (DEAE) anion-exchange column using increasing concentrations of acetate ions in methanol. Mono-, di- and trisialoganglioside groups were subsequently separated using adsorption chromatography in totally porous silica gel, which resulted in the isolation of individual gangliosides in great purity. The introduction of DEAE anion-exchangers in the techniques for ganglioside isolation and separation also permitted the isolation of a number of gangliosides as tumour-associated markers [2]. DEAE-cellulose was used for the initial studies [9]. Stepwise elution with ammonium acetate in methanol resulted in the separation of mono-, di-, tri- and tetrasialogangliosides. Greater use was made of the DEAE-Sephadex A-25 technique, employing the concave gradient of ammonium acetate in methanol [10, 11] in combination with subsequent separation on silica gel Iatrobeads 6 RS-8060. Comparing DEAE-Sephadex, QAE-Sephadex and DEAE-Sepharose, Ivamori and Nagai [12] found the last of the three as giving the best separation and yields. Kundu et al. [13] used DEAE-silica gel for its higher flow-rate and other advantages even though the binding capacity was apparently lower. Fredman et al. [14] recommended macroporous silica gel coated with DEAE-dextran and cross-linked. Using the stepwise elution with potassium acetate in methanol, they achieved the separation of brain gangliosides into mono- up to pentasialogangliosides. All the above-listed carriers, however, were employed for conventional low-pressure chromatography. Separation on these materials lasted a number of hours or even days.

Mansson et al. [15] have recently published their method of rapid chromatography of gangliosides on a strong Mono-Q anion-exchanger. Stepwise elution with potassium acetate in methanol resulted in the separation of mono- up to tetrasialogangliosides. This anion-exchanger has a relatively great binding capacity and separation effect, except that with tetrasialogangliosides the use of a strong exchanger leads to lactonization of groups of sialic acid and to partial elution with monosialogangliosides. Besides, a certain non-specific adsorption of the carrier is an interfering factor, too.

The purpose of the present study was to exploit a macroporous, rigid, purely organic copolymer of the glycolmethacrylate type — medium-basic Spheron 1000-DEAE — for medium-pressure anion-exchange chromatography of gangliosides. Thanks to this, it proved possible to cut the time needed for ganglioside chromatography from a number of hours down to a few tens of minutes, with sufficient capacity preserved, without secondary undesirable modifications of gangliosides.

EXPERIMENTAL

Materials

A Spheron 1000-DEAE anion-exchanger of 17 μm particle size and 1.6 mequiv./g nominal capacity, was acquired from Lachema (Brno, Czechoslovakia). It can be replaced by packing of the same composition,

produced by Laboratorní přístroje (Prague, Czechoslovakia), under the name DEAE-Separon HEMA. Lachema supplied also the pure chemicals of methanol and anhydrous ammonium acetate, as well as the agents for detection and solvents for thin-layer chromatography (TLC). TLC plastic sheet of silica gel 60 (without fluorescent indicator) was acquired from Merck (Darmstadt, F.R.G.). Samples of gangliosides, enriched for higher oligosialogangliosides, were prepared by the method of Svennerholm and Fredman [16].

Methods

A spare glass amino acid analyser column, 8 mm I.D., was used for all the column chromatography tests, filled with the ion-exchanger up to 25 cm. The ion-exchanger was first deaerated in water suspension under water pump vacuum, decanted in a ten-fold volume of water from turbidity, precycled by stepwise washing on a fritted disc with 2 *M* hydrochloric acid, water, 2 *M* sodium hydroxide and water, and the anion-exchanger in the OH^- form was washed with methanol up to the elution of water. Then, still on the disc, it was slowly washed with a 2 *M* solution of ammonium acetate in methanol and, following equilibration, the ammonium acetate was eluted with pure methanol until a conductivity of 10 μS or less was reached.

Electrical conductivity was measured with a Type OK 102/1 conductivity meter (Radelkis, Budapest, Hungary). Pure methanol had a conductivity of 1.8 μS; 0.01 *M* ammonium acetate in the same solvent, 254 μS; 0.1 *M* solution, 1.69 mS; 0.2 *M* solution, 2.8 mS; 0.5 *M* solution, 5.5 mS; 1 *M* solution, 7.6 mS; and 2 *M* ammonium acetate in methanol had a reading of 9.6 mS. (Naturally, conductivity is sensitive to the presence of traces of water supplied in the hygroscopic acetate as well as in the dissolved sample.)

The methanol-washed ion-exchanger was then filled into the column using the slurry method in the stepwise pulse packing modification [17], and there again briefly washed with methanol. In cases of repeated separation, the column, following regeneration with 2 *M* ammonium acetate solution in methanol, was washed with pure methanol until a 10-μS conductivity was reached, whereafter a new sample could be supplied.

The apparatus for medium-pressure chromatography (cf. Fig. 4 in ref. 18) consisted of an all-glass mixer for linear gradients with a magnetic stirrer, a laboratory micropump with two reversely operating pistons, fritted discs for the trapping of mechanical impurities, a manometer, an ion-exchange column and a fraction collector (see also Fig. 4.5.1 in ref. 17). The ganglioside samples were stirred in a mixture of water and methanol (the ratios are given in the captions to the figures) to a suspension which, following ice-bath cooling, was applied with a cooled syringe fitted with a short piece of polyethylene tubing under the methanol solution over the top of the ion-exchange column using the method of underlayering. Then a methanol-filled column head was pressed into the top of the column, immediately starting the withdrawal of the first fraction. After the fritted disc of the head had been pressed down to the ion-exchanger and fixed, the pump supplying the solvent from the gradient mixer was turned on.

The fractions thus obtained were measured for conductivity in each fifth test tube. A 400-μl aliquot taken from each test tube was assayed for sialic

acid using Svennerholm's [19] method modified according to Miettinen and Takki-Luukkainen [20], i.e. colorimetry at 580 nm. Also 20-μl aliquots taken from each fraction were applied on a TLC plastic sheet of silica gel without fluorescence indicator (Merck). The technique of "ganglioside mapping" [12] was employed to detect the presence of individual gangliosides in the fractions. A mixture of chloroform–methanol–water (50:45:10, v/v/v) containing 0.02% $CaCl_2 \cdot 2H_2O$ was used for development. Detection consisted in spraying the plate with the resorcinol agent [19] and subsequent heating up to 120° C for 30 min.

RESULTS

In order to find optimum conditions for ganglioside separation on Spheron-DEAE (DEAE-Separon HEMA), we carried out a number of experiments. Of these, we wish to report on the two most successful ones with separation times of less than 1 h, i.e. meeting the requirements of medium-pressure liquid chromatography.

Fig. 1 shows mono- up to tetrasialogangliosides separation reached in 56 min. It appeared useful, after the specimen was applied, to use brief isocratic elution with methanol in order to eliminate any ballast substances possibly present in the ganglioside preparation. This was followed by a combination of linear gradients of ammonium acetate in methanol.

The qualitative composition of peaks as listed in Fig. 1 can be seen from Fig.

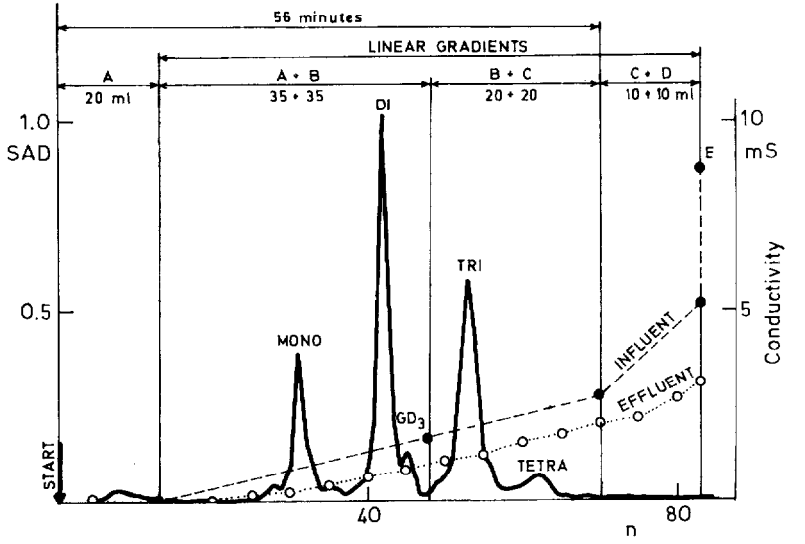

Fig. 1. Rapid anion-exchange separation of human brain gangliosides. Column (25 × 0.8 cm I.D.) of Spheron 1000-DEAE, particle size 17 μm, was equilibrated with 2 *M* ammonium acetate and washed with methanol. Sample (25 mg) was suspended in a mixture of 300 μl of water and 400 μl of methanol, cooled and applied using the underlayering method. Eluents: A = methanol; B = 0.1 *M* ammonium acetate in methanol; C = 0.2 *M* solution; D = 0.5 *M* solution; E = 2 *M* solution. Fractions of 1.8 ml were taken in 48-s intervals. Temperature 25° C, flow-rate 2.25 ml/min, pressure 6 atm, SAD = relative colorimetric value (580 nm) of sialic acid detection.

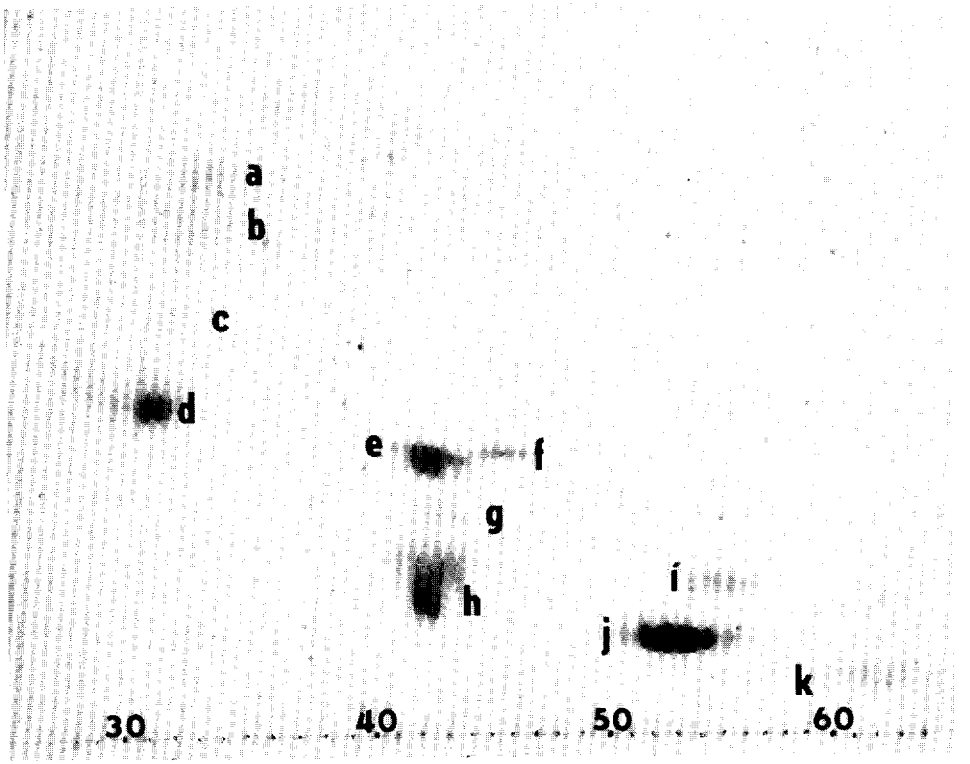

Fig. 2. Ganglioside map of separated fractions. (For conditions of separation, see Fig. 1.) A 20-μl volume of each fraction 28—64 was spotted on a TLC plastic sheet of silica gel 60 (without fluorescent indicator). Solvent system: chloroform–methanol–water (50:45:10) plus 0.02% (w/v) $CaCl_2 \cdot 2H_2O$. Detection: by spraying the plate with resorcinol · HCl reagent [19] and subsequent heating at 120° C for 30 min. Monosialogangliosides: a, G_{M_4}; b, GM_3; c, GM2; d, G_{M_1}. Disialogangliosides: e, $G_{D_{1a}}$; f, G_{D_3}; g, G_{D_2}; h, $G_{D_{1b}}$. Trisialogangliosides: i, $G_{T_{1a}}$; j, $G_{T_{1b}}$. Tetrasialogangliosides: k, G_Q. The nomenclature in based on Svennerholm's system [21].

2, illustrating the results of TLC obtained by the application at the start of 20-μl shares of each fraction. The results show each peak as corresponding to monosialogangliosides (fractions 28—35), disialogangliosides (fractions 41—47), trisialogangliosides (fractions 50–56) and tetrasialogangliosides (fractions 57—63). Partial separation of ganglioside G_{D_3} was noted in the fraction of disialogangliosides.

In our search for optimum separation in the Fig. 1 experiment, the effluents from both moderate linear gradients became virtually linearized although the influents were not exactly linearizable. For that reason, we combined the two gradients in a single long one with a 0–0.2 *M* ammonium acetate concentration range. At a greater column loading in the last experiment (cf. Fig. 3), the fraction of pentasialogangliosides was detected as a small but discernible peak in the wake of tetrasialogangliosides. Using TLC for the fraction's evaluation, we obtained a pattern of separation similar to that in Fig. 2. When 20-μl shares were applied at the start of the chromatogram (out of a total fraction volume of 2 ml), pentasialogangliosides could not be demonstrated in the fraction

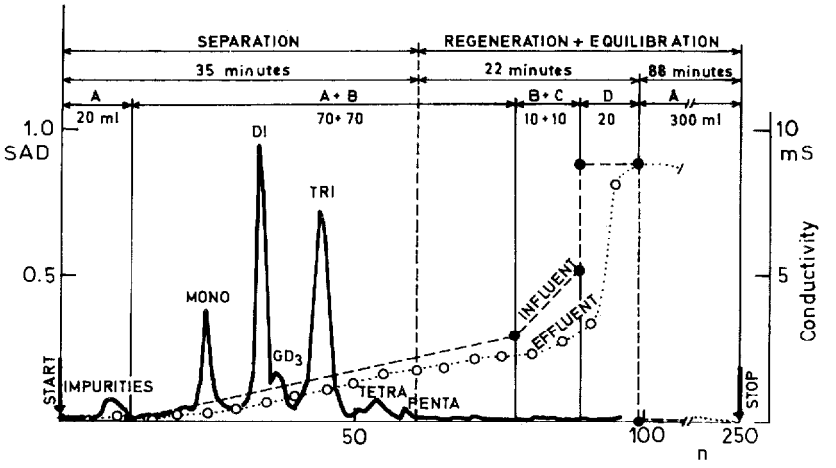

Fig. 3. The whole cycle of repeated accelerated ion-exchange chromatography of gangliosides. The column was as in Fig. 1. A 30-mg volume of sample was suspended in a mixture of 400 μl of water and 400 μl of methanol, and applied as in Fig. 1. Eluents: A = methanol; B = 0.2 *M* ammonium acetate in methanol; C = 0.5 *M* solution; D = 2 *M* solution. Fractions of 2 ml were taken in 35-s intervals. Temperature 25° C, flow-rate 3.4 ml/min (i.e. 6.8 ml/cm^2 min), pressure 13 atm. After washing with methanol, the column is ready for the next sample. SAD, see caption to Fig. 1. Fraction assessment using TLC on silica gel produced a pattern similar to that in Fig. 2.

corresponding to their peak. It was only after the concentration and application of the rest of the share of this fraction that a small but distinct spot of characteristic pentasialoganglioside motility appeared on the chromatogram. This is due to the low concentration of pentasialogangliosides in the brain tissue. Using this technique, we succeeded in the elution of all mono- up to pentasialogangliosides.

After this, the next stage of column elution with the rest of the slow linear gradient up to 0.2 *M* acetate concentration and the short steep gradient up to 0.5 *M* concentration are designed solely for the detection of any other anionic substances possibly present. Brief, stepwise, 2 *M* acetate elution regenerates the column and restores it in its full acetate form. This regeneration takes 22 min to complete, and the subsequent washing with methanol (88 min) brings the ion-exchanger in a state of readiness for the application of the next sample (after conductivity had been reduced to below 10 μS). All in all, one whole cycle between two samples lasts 145 min.

For the purpose of controlling the whole process of elution, regeneration and equilibration in the ion-exchange column, monitoring the influent and effluent electrical conductivity proved to be an excellent approach. At the same time, we found equilibration of the DEAE-Spheron, both in the region of high buffer concentrations (2 *M*) and in the region of very low concentrations approaching those of the pure solvent, to proceed surprisingly rapidly in methanol in comparison with aqueous solution.

DISCUSSION

The ion-exchanger employed is the DEAE derivative of Spheron, the detailed

characterization of which was described previously [22–24]. This anion-exchanger was used for a series of chromatographic separations of proteins (see ref. 25, review), oligosaccharides, oligonucleotides, as well as oligomers of organic acids. Our study describes its first use for the rapid separation of gangliosides.

The above-described procedures were preceded by preliminary experiments [26], in which glass columns were filled with Spheron 1000-DEAE of larger grain size (25–40 μm), and gangliosides were eluted with sodium acetate concentration ranges. Chromatography proceeded without the use of a pump, by gravity, at a sufficient hydrostatic pressure. Promising separation was achieved but the chromatography took too long, as the flow-rate was a mere 0.17–0.20 ml/min.

Our subsequent experiments were aimed at shortening the chromatographic process by the use of finer beads (17 μm) and increasing the pressure up to 1.5 MPa with a pump. In this way, a separation time of 36 min was achieved. During the experiments the column loading was doubled, but with no effect on the quality of separation. Consequently, we believe the column could well be exposed to even greater loading.

A comparison of the patterns in Figs. 1 and 3 makes it obvious that even after a substantial increase in the flow-rate between the last two experiments, the peaks are equally slender and their distinction shows that there is still some way to go to reach the limits of ganglioside separation on DEAE-Spheron and that the rate could be increased still further. Needless to say, a reduction in the particle size down to 10 μm or less and a still greater pressure increase could accelerate the process further and, apparently, step up the effect of separation. However, according to our opinion, the further development of rapid ion-exchange chromatography of gangliosides should rather follow the course of automatic detection as this is now substantially more time-consuming than rapid chromatography proper.

Table I gives the results of attempts published so far on ion-exchange chromatography arranged according to the diminishing separation times. Studies 1–6 were performed using the technique of conventional low-pressure chromatography. Study No. 7 by Mansson et al. [15] offers a shortening of separation time in 12–50 min at 9.8 μm particle size and pressures of 1–4 MPa. Our technique (No. 8) permitted the separation of gangliosides within 35 min using an ion-exchanger of 17 μm particle size at 1.5 MPa. The last two communications represent a subtantial reduction in separation time.

"Slow" ion-exchangers with polysaccharide matrices offer the advantage of great sorption capacity. In our experiments, we used the loading of 2.4 mg/ml ion-exchanger, a figure representing 21.5% of the greatest published loading of 11.2 mg/ml for low-pressure chromatography (Table I, No. 2) while amounting to 2.400% of the lowest published loading of 0.1 mg/ml, in what was a relatively rapid separation on DEAE-silica gel (Table I, No. 5). If our results are compared with those used by Mansson et al. [15] on Mono-Q (Table I, No. 7), the corresponding loading is ca. 10%.

Even though stepwise elution is an extremely simple procedure, from the experimental point of view, it is no guarantee of perfectly fine ion-exchange

TABLE I

CHARACTERISTICS OF PUBLISHED ATTEMPTS AT ION-EXCHANGE SEPARATION OF GANGLIOSIDES

This table dose not give a complete list of all hitherto published reports on ion-exchange chromatography on soft supports not suitable for pressure chromatography. However, it does list all reports offering scope for the exploitation of medium-pressure LC or HPLC techniques.

No.	Authors	Ion-exchanger type	Elution or gradient type	Column dimensions (length × I.D.) (cm)	Total flow throughout column (ml/min)	Specific flow-rate (ml/cm² min)	Sample loading (mg/ml of ion-exchanger)	Separation time (h)
1.	Fredman et al. [14]	Spherosil coated with DEAE-dextran	Stepwise: methanol, 0.05, 0.1, 0.25, 0.5, 1 *M* potassium acetate in methanol (200, 1000, 2000, 1600, 1000, 600 ml)	28 × 3	1—2	0.32—0.64	6.1	53- 106
2.	Momoi et al. [10]	DEAE-Sephadex	Moderately concave: 0.05, 0.15, 0.45 *M* ammonium acetate in methanol (1200, 1200, 1200 ml)	27 × 4.6	1—2	0.072	4.5—11.2	50
3.	Ledeen and Yu [11]	DEAE-Sephadex	Moderately concave: 0.0, 0.2, 0.5 *M* ammonium acetate in methanol (700, 700, 700 ml)	110 × 1.4	1.0	0.65	5.9	42.5
4.	Winterbourn [9]	DEAE-cellulose	Stepwise: 0.022, 0.04, 0.1, 0.25 *M* ammonium acetate in methanol (2000, 400, 800, 400 ml)	38 × 1.8	2.2—2.5	0.87- 0.98	1.4	24- 27
5.	Kundu et al. [13]	DEAE-silica gel	50 ml methanol, moderately concave: 0, 0.2, 0.5 *M* ammonium acetate in methanol (200, 200, 200 ml)	60 × 2	1.5	0.48	0.1	7.2
6.	Iwamori and Nagai [12]	DEAE-Sepharose	Moderately concave: 0.05, 0.15, 0.45 *M* ammonium acetate in methanol (150, 150, 150 ml)	20 × 2	2.0	0.64	4.0	5.9
7.	Mansson et al. [15]	Mono-Q	Stepwise: 0.05, 0.15, 0.20, 0.225 *M* potassium acetate in methanol	5 × 0.5	1—4	5.1—20.4	35.0	0.2—0.8
8.	Present study	Spheron 1000-DEAE	20 ml methanol, two linear gradients: 0—0.2, 0.2—0.5 *M* ammonium acetate in methanol (70—70, 10—10 ml)	25 × 0.8	3.4	6.8	2.4	0.5

separation in the case of complex mixtures. Gradient elution, as a rule, does not lead to a multiple elution of the same substance in the subsequent fractions (see G_{D1b} in Table I [15]), which was why we gave it preference. The use of linear gradients instead of the usual concave ones makes for simpler instrumentation as well as results evaluation.

The advantage of medium-basic DEAE ion-exchangers over the strongly basic ones (such as a quaternary ammonium derivative of Mono-Q) is that they cause no damage to gangliosides. The use of the Mono-Q ion-exchangers results in lactonization, and tetrasialoganglioside G_{Q1b} appears mostly in the monosialoganglioside fraction. Using the Mono-Q column, the authors concerned report a certain retention of di- and trisialogangliosides so that these do not appear until the tetrasialoganglioside fraction has been reached. The reasons are seen in their non-specific adsorption to the carrier caused by oxidation in the ceramide part of those gangliosides.

As illustrated by Figs. 1–3, ion-exchange chromatography on DEAE derivative separates gangliosides, essentially, solely on the basis of the electric charge, i.e. according to the number of sialic acid moieties. Naturally, it can hardly be expected to lead directly to homogeneous products in cases where the starting mixture is too complex. However, as Fig. 2 suggests, even in such cases there is a major role to play as a prefractionation method, as it simplified and makes it easier to survey the subsequent chromatographic separation of mixtures of these intricate compounds. Exceptionally, in cases of simple starting mixtures, it may, in some fraction, give a relatively homogeneous product, chemically. As a method of prefractionation, however, it takes a significant part in the separation of even complex mixtures of gangliosides. The successive ion-exchange fractions can be separated using subsequent HPLC or medium-pressure liquid chromatography on silica gel columns, e.g. in the sense of the study by Kundu and Scott [7], or a combination with the technique of ganglioside mapping on TLC plates of silica gel [12] can be used for the analytical distinction between gangliosides of the same polarity but different charges.

CONCLUSIONS

The medium-basic DEAE derivative of Spheron was found to be extremely useful for the rapid separation of gangliosides, depending on their sialic acid content. In a matter of 35 min, mono- up to pentasialogangliosides were reliably separated with a simple linear gradient of ammonium acetate concentration in methanol. The time given, far from being a limit for the rapidity of the technique, permits further acceleration of the separation process. The method used a column loading of 2.4 mg of the separated mixture per 1 ml of ion-exchanger; this, however, does not represent maximum capacity of the ion-exchanger employed.

This type of ion-exchange chromatography may serve as a method of prefractionation for the subsequent rapid column chromatography on silica gel of the constituent oligosialic fractions, or for subsequent TLC. The purpose of the preparative method is to isolate the particular gangliosides for structural study, or as substrates for metabolic studies and for the detection of metabolic disorders in Tay-Sachs disease and in G_{M1} gangliosidosis, or for the production

of monoclonal antibodies. Important in terms of analysis is the combination of Spheron ion-exchange chromatography with the technique of ganglioside mapping on high-performance thin-layer chromatography plates of silica gel. In complex mixtures of gangliosides, it permits the distinction between minor gangliosides of the same polarity but different charges.

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