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RAPID SEPARATION OF GANGLIOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a high-performance liquid chromatographic (HPLC) procedure for the rapid separation of individual ganglioside components on a 5-μm porous silica gel column using a mixture of isopropanol—hexane—water with increasing water content and decreasing hexane content. Total ganglioside mixtures were first fractionated on DEAE-silica gel according to the number of sialic acid residues. Each fraction was then separated into individual ganglioside components by HPLC. Total elution time was less than 2 h. This procedure has been applied for the separation of major ganglioside components of human erythrocytes and beef brain and is highly reproducible.

INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) has been used as an analytical tool for the quantitation of glycolipids from animal tissues or cells [1—11]. All these procedures involved derivatization to introduce an aromatic chromophore for detection in the standard ultraviolet detector system. Unfortunately, it has not been possible to regenerate the native glycolipids from derivatized components. Thus the available methods could not be utilized for isolation of intact glycolipids. Gangliosides have been separated without derivatization using HPLC with a chloroform–methanol–hydrochloric acid system, equipped with moving-wire flame ionization detector [12]. Due to the non-availability of this type of the detector system and the danger of using hydrochloric acid to isolate labile gangliosides, this solvent system does not seem to be appropriate. Recently, Watanabe and Arao [13] have used a mixture of isopropanol—hexane—water as a solvent system and were able to separate underivatized neutral glycosphingolipids (GSLs) from

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human erythrocytes. In this present paper, we report a HPLC procedure for purification of underivatized ganglioside components from human erhythrocytes and beef brain using isopropanol—hexane—water as a solvent system as described [13]. The procedure is highly reproducible and the separation can be achieved in a much shorter time in comparison to the conventional column chromatographic procedures [14—20].

EXPERIMENTAL

The gangliosides from human erythrocytes and beef brain were isolated as described previously [21, 22]. The erythrocyte ganglioside mixtures were further fractionated on DEAE-silica gel into mono and disialo species [19]. The beef brain ganglioside mixtures were similarly separated into mono-, di-, tri-

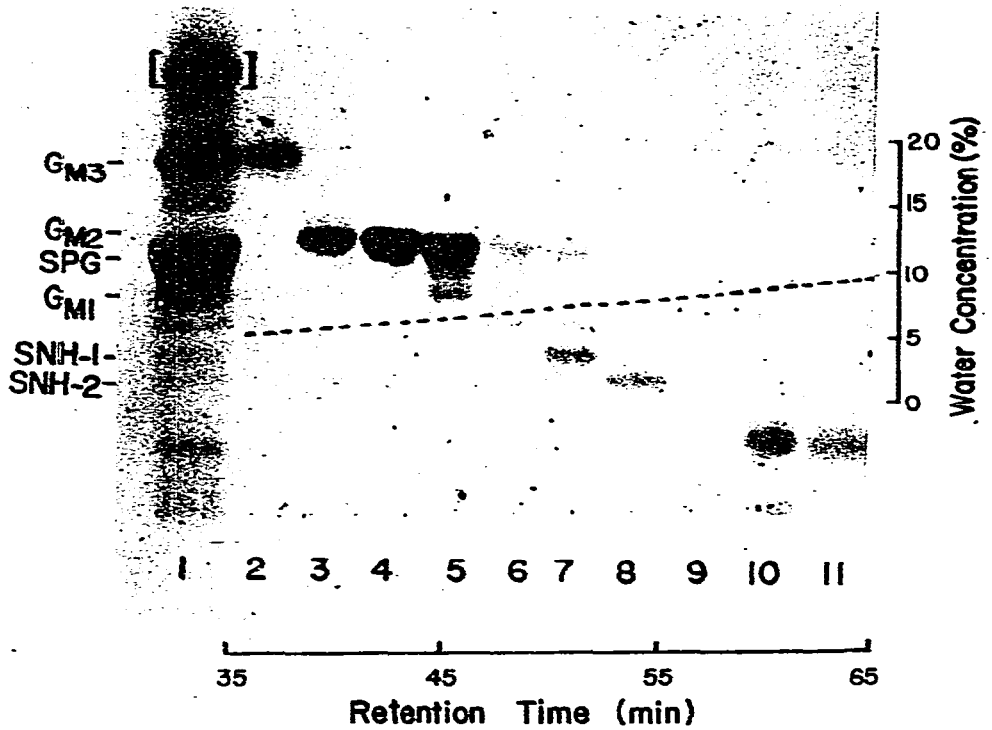

Fig. 1. Elution profile of the monosialoganglioside fraction of human erythrocytes separated by HPLC as monitored by TLC. HPLC column: Zorbax SIL (5 μm, 25 cm × 4.6 mm); sample applied, 500 μg. The column was eluted with a linear gradient of a mixture of isopropanol—hexane—water from a ratio of 55:42:3 to 55:25:20 during a period of 2 h. Fractions of 1 ml of effluent were collected and aliquots of 200 μl were spotted after evaporation and redissolving in 20 μl of chloroform—methanol—2.5 *N* ammonium hydroxide (50:50:10) on a precoated silica gel 60 plate. Lane 1 contains total monosialoganglioside fraction and lanes 2—11 contain eluted fractions. Solvent system: chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$. All bands were purple after detection by resorcinol spray [26]. The faint band in lane 1 above G_{M3} contained non-ganglioside impurities indicated by a bracket and was yellow in color. The dotted line indicates the concentration of water in the elution mixture. Faint ganglioside bands eluting in water concentrations between 10—15% were visible (data not shown).

and tetrasialo species [19]. For purification of individual ganglioside components of human erythrocytes by HPLC, mono- and disialoganglioside fractions were separately used. Similarly, for purification of individual ganglioside components of beef brain, mono-, di-, and trisialoganglioside fractions were separately employed. Total beef brain ganglioside mixtures were also used for purification of individual ganglioside components by HPLC. Standard gangliosides used in this study have been described previously [23, 24].

High-performance liquid chromatography

The apparatus included Altex Model 100 A pumps and a Model 420 microprocessor (Altex Scientific, Berkeley, CA, U.S.A.). A Zorbax Sil column (5 μm, 25 cm × 4.6 mm I.D., DuPont, Wilmington, DE, U.S.A.) was used and separation was carried out by programmed gradient elution. A linear gradient of isopropanol—hexane—water from 55:42:3 (solvent A) to 55:25:20 (solvent B) was used during a period of 2 h. The column was equilibrated with solvent A prior to injection of sample and after use the column was regenerated with the initial solvent for 15 min. The flow-rate was adjusted to 0.5 ml/min and the

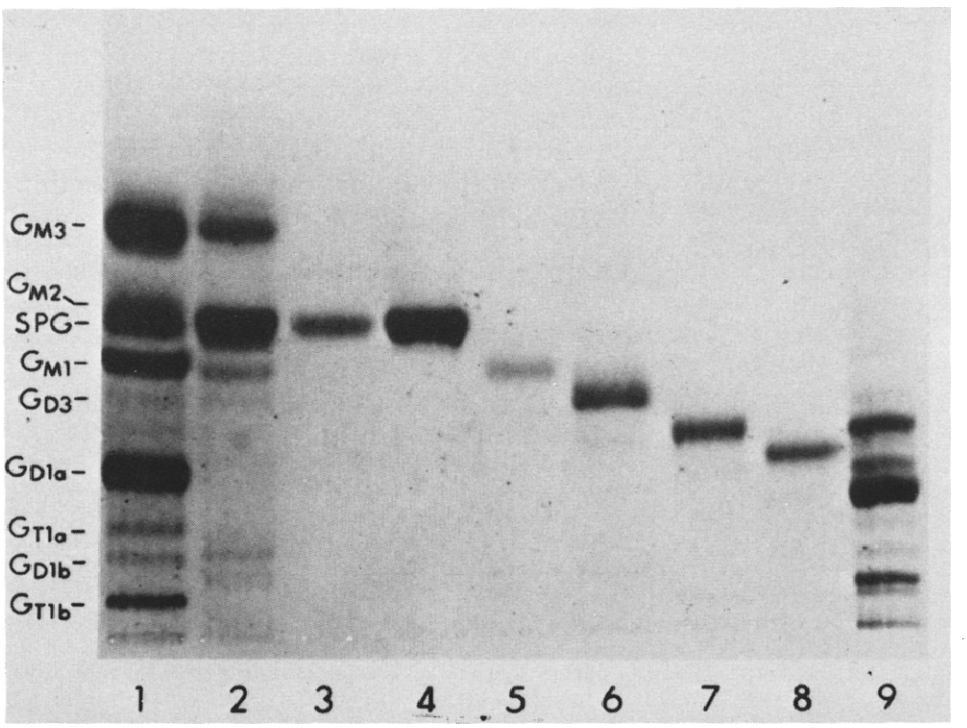

Fig. 2. TLC of purified monosialogangliosides of human erythrocytes separated by HPLC. Lane 1 contains standard mixtures of G_{M3}, G_{M2}, SPG and beef brain gangliosides. Lane 2 contains total monosialoganglioside fraction. Lane 9 contains total disialoganglioside fraction of human erythrocytes. Lanes 3—8 are purified monosialogangliosides of human erythrocytes. Lane 3 was identified as G_{M2}; lane 4 as SPG; lane 5 as G_{M1}; lane 7 as SNH-1 containing C_{22} and C_{24} fatty acid and lane 8 as SNH-2 containing C_{16} fatty acid [27]; lane 6 was not identified. Solvent system and spray reagent as in Fig. 1.

ganglioside sample (up to 500 μg) dissolved in 50 μl of isopropanol—hexane—water (55:30:15) by mild sonication was injected and gradient elution started. Fractions of 1 ml were collected by a fraction collector. The elution profile of gangliosides was monitored by thin-layer chromatography (TLC).

Thin-layer chromatography

TLC was performed on precoated silica gel 60 plates (E. Merck, Darmstadt, G.F.R.) with chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$ (solvent 1) or chloroform—methanol—2.5 *N* ammonium hydroxide (60:40:9) (solvent 2) as solvent systems [25]. Gangliosides were revealed by spraying the plate with resorcinol reagent [26] and heating the plate at 100° C for 10 min. Fractions were pooled after TLC examination.

RESULTS*

The elution profile of the monosialoganglioside fraction from human erythrocytes by the HPLC procedure is presented in Fig. 1. The elution was complete in approximately 1 h and gangliosides were eluted in a water concentration of between 5 and 9%. The three gangliosides viz. G_{M3} (lane 2), G_{M2} (lane 3) and sialosylparagloboside (SPG, lane 4) that comprise more than 90% of the monosialoganglioside fraction were well separated. For the isolation of other minor ganglioside components, the partially purified fractions in lanes 5, 6, 7 and 8 were separately reloaded on the HPLC column and eluted exactly under identical conditions as described for the total mixture but fractions of 250 μl of eluate were collected. After TLC examination, the pure fractions were pooled. Fig. 2 shows a thin-layer chromatogram of purified monosialogangliosides from human erhythrocytes (G_{M3} not shown). The purity of each ganglioside component was established by running duplicate TLC plates in solvent systems 1 and 2.

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*Abbreviations of the ganglio series gangliosides are according to the nomenclature of Svennerholm [29]. G_{M3} = NeuAc(α2-3)Gal(β1-4)Glc-Cer; G_{M2} = GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{M1} = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{D3} = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc-Cer; G_{D1a} = NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{D1b} = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(1-4)Glc-Cer; G_{T1a} = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer; G_{T1b} = NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer; SPG (sialosylparagloboside) = NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)-Gal(β1-4)Glc-Cer; DPG (disialosylparagloboside) = NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc-NAc(β1-3)Gal(β1-4)Glc-Cer; SNH (sialosylnorhexaosylceramide) = NeuAc(α2-3)Gal(β1-4)Glc-NAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer; DPGC (disialosylpentaglycosylceramide) = $\begin{matrix}\text{NeuAc}(\alpha 2\text{—}6)\\ \text{NeuAc}(\alpha 2\text{—}3)\end{matrix}$Gal(β1-3)GalNAc(1-3)Gal(1-4)Gal(β1-4)Glc-Cer; DL10C (disialosyllacto-N-isooctaosylceramide) = $\begin{matrix}\text{NeuAc}(\alpha 2\text{-}3)\text{Gal}(\beta 1\text{-}4)\text{GlcNAc}(\beta 1\text{—}6)\\ \text{NeuAc}(\alpha 2\text{-}3)\text{Gal}(\beta 1\text{-}4)\text{GlcNAc}(\beta 1\text{—}3)\end{matrix}$Gal(β1-4)GlcNAc(β1-3)-Gal(β1-4)Glc-Cer; where Gal = D-galactose; Glc = D-glucose; GalNAc = N-acetyl-D-galactosamine; GlcNac = N-Acetyl-D-glucosamine; NeuAc = N-acetylneuraminic acid; Cer(ceramide) = N-acylsphingosine.

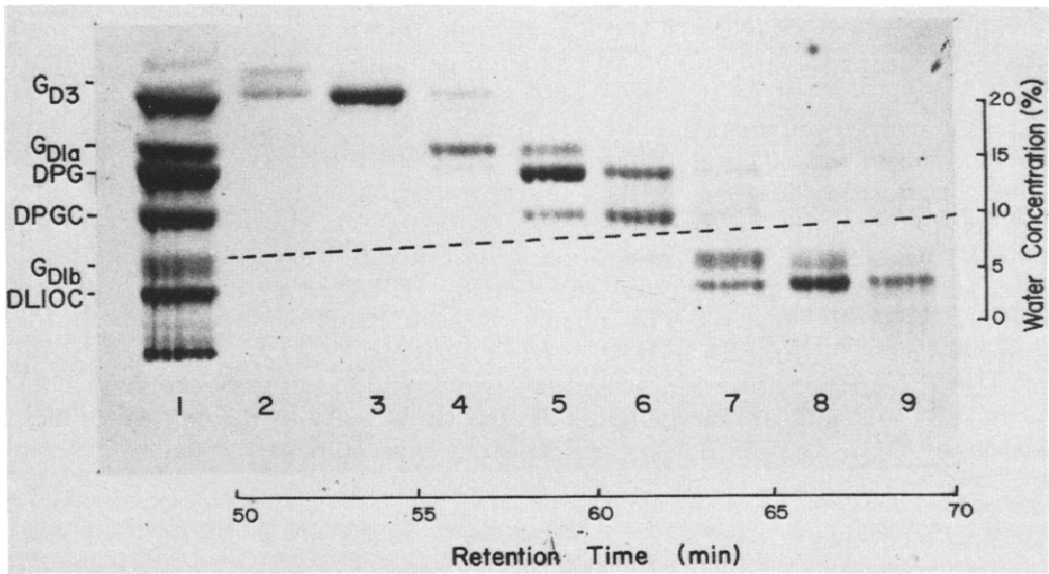

Fig. 3. Elution profile of the disialoganglioside fraction of human erythrocytes separated by HPLC as monitored by TLC. Solvent system: chloroform—methanol—2.5 *N* ammonium hydroxide (60:40:9). All bands were purple after detection by resorcinol spray. Lane 1 contains total disialoganglioside fraction and lanes 2—9 contain eluted fractions. Faint ganglioside bands eluting in water concentrations between 10—16% were visible (data not shown). Other details as in Fig. 1.

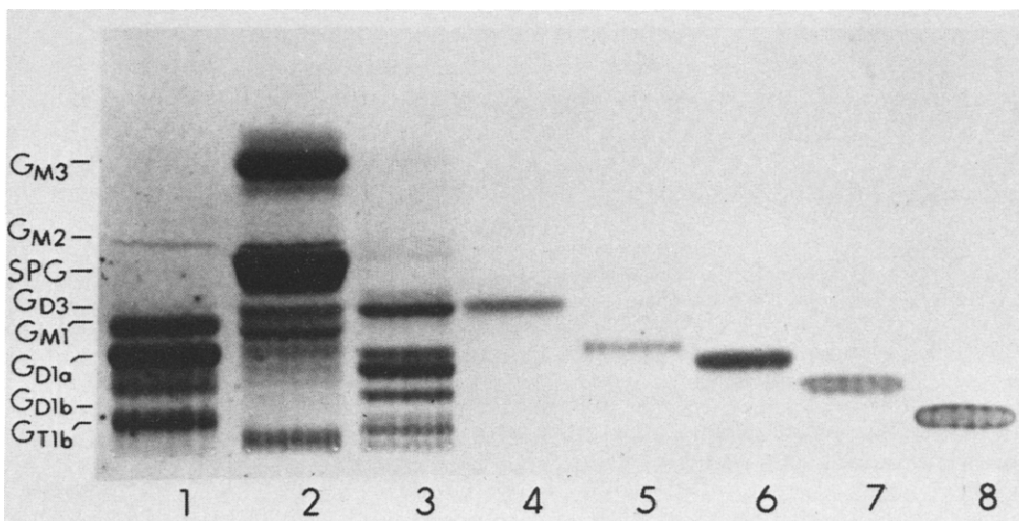

Fig. 4. TLC of purified disialogangliosides of human erythrocytes separated by HPLC. Lane 1 contain beef brain ganglioside mixture. Lane 2 contains monosialoganglioside fraction of human erythrocytes. Lane 3 contains disialoganglioside fraction of human erythrocytes. Lane 4—8 are purified disialogangliosides of human erythrocytes. Lane 4 was identified as G_{D_3}; lane 5 as $G_{D_{1a}}$; lane 6 as disialosylparagloboside; lane 7 was identified as a new ganglioside that has disialosylpentaglycosylceramide structure [28]. Ganglioside $G_{D_{1b}}$ which runs in between bands 7 and 8 is not shown. Solvent system and spray reagent as in Fig. 3.

The elution pattern of the disialoganglioside fraction from human erythrocytes which comprises about 15% of the total ganglioside [19, 22] is presented in Fig. 3. The majority of the gangliosides were eluted off the column in 1 h 15 min in a water concentration of between 7 and 10%. Ganglioside G_{D3} (lane 2, two bands and lane 3) was obtained in sufficient purity but for purification of other gangliosides, lanes 4, 5, 6, 7 and 8 were pooled together and reloaded on the HPLC column as described for monosialoganglioside fraction. The ganglioside fraction in lane 9 was separately reloaded as above. The disialogangliosides of human erythrocytes purified by HPLC procedure are presented in Fig. 4 (G_{D1b} not shown). The purity of each ganglioside was checked by running duplicate TLC plates in both solvent systems.

For HPLC purification of individual ganglioside components from beef brain, mono-, di- and trisialoganglioside fractions were employed. Excellent separation of G_{M3}, G_{M2} and G_{M1} gangliosides was achieved from the mono-

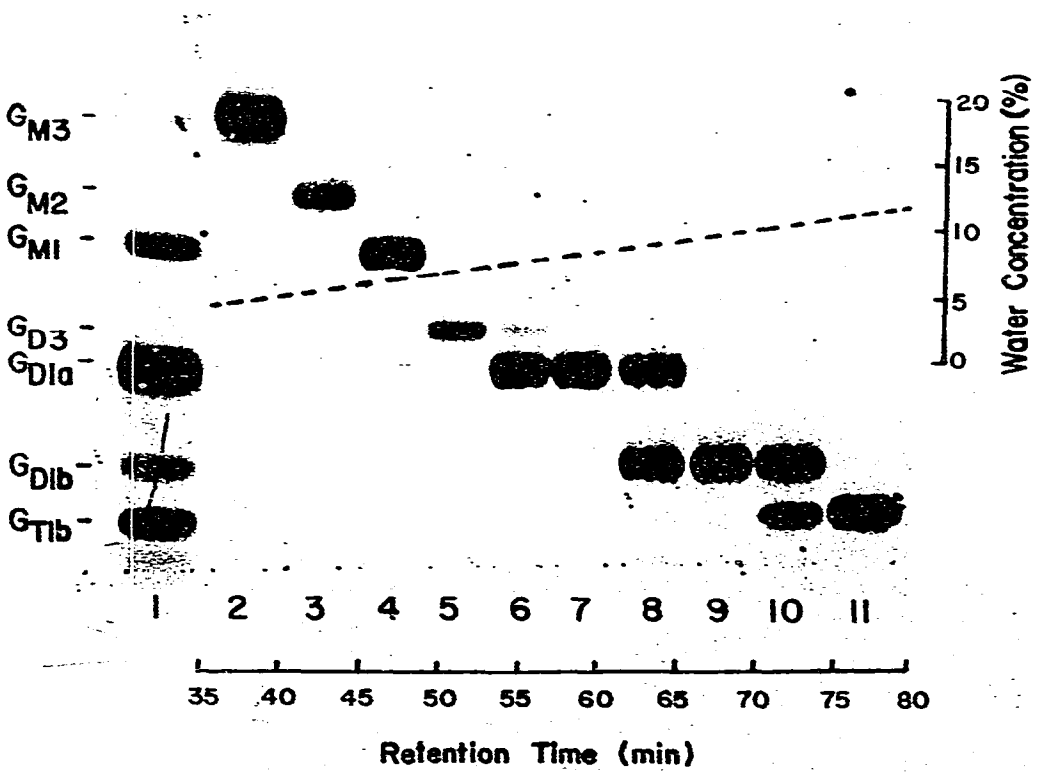

Fig. 5. Elution profile of the total beef brain ganglioside mixture separated by HPLC as monitored by TLC. Solvent system: chloroform—methanol—water (55:45:10) containing 0.02% (w/v) $CaCl_2 \cdot 2H_2O$. All bands were purple by resorcinol spray. Lane 1 contains total beef brain ganglioside mixture and lanes 2—11 contain eluted fractions. Faint ganglioside bands eluting in water concentrations of between 14—20% were visible (data not shown). Other details as in Fig. 1.

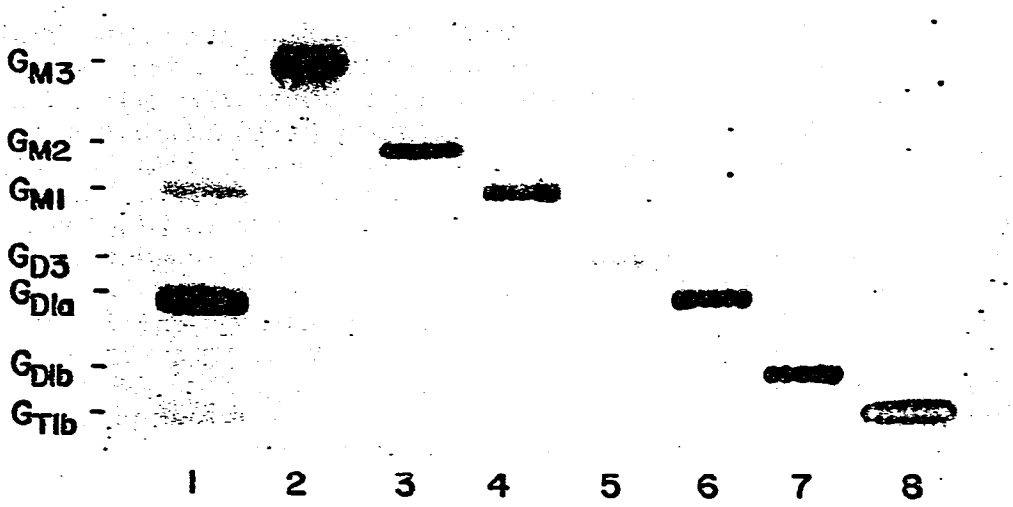

Fig. 6. TLC of purified gangliosides of beef brain separated by HPLC. Lane 1 contains total beef brain ganglioside mixture. Lanes 2—8 are purified gangliosides of beef brain. Lane 2 was identified as G_{M3}; lane 3 as G_{M2}; lane 4 as G_{M1}; lane 5 as G_{D3}; lane 6 as G_{D1a}; lane 7 as G_{D1b} and lane 8 as G_{T1b}. Attempts were not made to isolate other minor gangliosides. Solvent system and spray ragent as in Fig. 5.

sialoganglioside fraction. The elution was complete in 1 h and gangliosides were eluted in a water concentration of between 5 and 7.6%. From the disialoganglioside fraction, G_{D3}, G_{D1a} and G_{D1b} were well separated in a water concentration of between 10 and 11.5%. The major gangliosides of beef brain were also purified by the HPLC procedure with the total ganglioside mixture. The elution profile of the gangliosides from total beef brain ganglioside mixture is shown in Fig. 5. The elution was complete within 1 h 30 min and gangliosides were eluted in a water concentration of between 5 and 12%. Excellent separation of G_{M3} (lane 2), G_{M2} (lane 3), G_{M1} (lane 4) and G_{D3} (lane 5) gangliosides was achieved. The other major gangliosides viz. G_{D1a} (lane 7), G_{D1b} (lane 9) and G_{T1b} (lane 11) were also obtained in pure forms although the gangliosides in lanes 6, 8 and 10 were not homogeneous. These fractions were separated into pure components by reloading the partially purified fractions on to the HPLC column and eluting under identical conditions as described for the total mixture but fractions of 250 μl were collected. The gangliosides purified from total beef brain ganglioside mixture are shown in Fig. 6. The purity of each ganglioside component was established by running duplicate TLC plates in both solvent systems.

DISCUSSION

For the purification of gangliosides, silica gel column chromatographic procedures have been most frequently used [14—20]. Because of the complexity of these compounds, gradient elution and repeated chromatographic separation are often needed to purify the ganglioside components. Preparative TLC procedures commonly used in many cases [15], are not feasible for larger

amounts of gangliosides. A major disadvantage of the preparative TLC procedure is due to the poor recovery of the gangliosides from the silica gel scrapings. In recent years, HPLC procedures which utilize silica gel of controlled pore diameter have been found to be extremely useful for the quantitative separation of derivatized (aromatic chromophore) neutral GSLs [1—11]. More recently, Watanabe and Arao [13] have been able to separate underivatized neutral GSLs containing mono to dodecasaccharides on HPLC using a mixture of isopropanol—hexane—water as an eluting solvent. An advantage of this solvent system is its non-toxicity and better resolution power compared to the conventionally employed chloroform—methanol—water system. We have shown in this report that complex gangliosides from human erythrocytes and beef brain can be separated in pure forms using HPLC on a silica gel column and similar solvent mixtures as was employed by Watanabe and Arao [13]. The separation pattern of ganglioside components is highly reproducible because the gradient elution solvent could be strictly controlled with the HPLC system. Moreover, the gangliosides could be separated in a matter of hours compared to a few days in conventional column chromatography. We have used from 50—500 μg of gangliosides in our system and have been able to reproduce the results. We have been able to regenerate the same column without loss of any resolving power even after 50 applications. We plan to exploit this procedure on a much larger scale utilizing a preparative silica gel column. Therefore, this HPLC procedure provides a highly efficient method for purification of individual ganglioside components on micro or macro scale in many other tisues or cells.

ACKNOWLEGDEMENTS

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