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Large scale purification of gangliosides G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) by trimethylaminoethyl-Fractogel high-performance liquid chromatography

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Abstract

A preparative anion-exchange high-performance liquid chromatographic method for the separation of the closely allied monosialogangliosides G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) has been developed. Hybridoma cells, readily available material derived from industrial monoclonal antibody production, were used as ganglioside source and led to fractions with pure G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) in high milligram quantities. The crude ganglioside extract was loaded onto columns filled with the strong anion-exchanger trimethylaminoethyl (TMAE)-Fractogel. Gangliosides were eluted from the stationary phase with a gradient system of ammonium acetate in methanol. The scaled-up approach ranged over more than one order of magnitude from 20 to 500 mg batches of G_{M3} gangliosides. Thus, the high-resolution power of the strong anion-exchanger TMAE-Fractogel allowed the preparative isolation by one-step column chromatography of two G_{M3} specimens which only differ in one hydroxyl group at position 5 of the neuraminic acid (N-acetyl- versus N-glycolylneuraminic acid).

Keywords: Gangliosides

1. Introduction

Gangliosides are characterized by the presence of one or more sialic acid units in the oligosaccharide chain. The parent compounds are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), which are known to play crucial roles in various biological events [1]. Structures, functions, and metabolism of gangliosides have been widely reviewed [2–5]. Ganglioside G_{M3}(Neu5Ac), the main ganglioside in non-neuronal human tissue, acts, for instance, as a modulator of cell growth [6–8] and shows immunosuppressive activity [9]. N-Glycolylated sialyllactosylceramide, G_{M3}(Neu5Gc), also known as Hanganutziu–Deicher antigen 3, represents an important tumor-associated carbohydrate antigen in man [10].

Various anion-exchange DEAE-coupled matrices are in use for separation of neutral GSLs and gangliosides, for instance Sephadex [11], Sepharose [12], controlled porous glass [13–15] and Fractogel [16]. Further improvements in separation of gan-

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gliosides were achieved with the strong anion-exchanger Q-Sepharose [17] and trimethylaminoethyl (TMAE)-Fractogel [18]. The preparative HPLC purification of G_{M3} employing a Zorbax-NH_2 column has been recently published [19].

G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) serve as starting material for various G_{M3} derivatives, e.g. lyso-G_{M3}, de-N-acetyl-G_{M3}, G_{M3}-amides and other compounds [20–22] representing bioactive modulators of many cell functions in vitro [23]. An excellent source of G_{M3}(Neu5Ac) as well as G_{M3}(Neu5Gc) are hybridoma cells from murine, rat, and human origin [24]. In this study we report on the rapid and efficient separation of bulk quantities of mixtures of closely allied G_{M3}-specimens, leading to pure G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) fractions. For this purpose, we developed a one-step high-performance liquid anion-exchange chromatography procedure, using the strong anion exchanger TMAE-Fractogel instead of widely used diethylaminoethyl (DEAE)-coupled matrices.

2. Experimental

2.1. Ganglioside extraction

Mouse–mouse hybridoma cells (15.6 kg wet mass) were homogenized, lyophilized and 3-fold extracted with 25 l methylisobutylketone–methanol–water (40:80:30, v/v/v) in total according to a procedure recently published [25]. After evaporation, the crude extract was resuspended in water, dialyzed and freeze dried. The extract was divided into four aliquots, each taken up in 1.5 l chloroform–methanol–water (30:60:8, v/v/v) and applied to a column packed with 300 ml DEAE-Sepharose CL-6B (Pharmacia, Freiburg, Germany) in the acetate form [26]. Neutral lipids were removed by washing the column with 0.75 l of the same solvent and 0.75 l methanol. Gangliosides were eluted with 1.5 l of 0.45 *M* ammonium acetate in methanol. The eluates were pooled, evaporated and salts were removed by dialysis. To saponify contaminating phospholipids, the freeze dried samples were incubated with 500 ml aqueous 1 *M* NaOH for 1 h at 37°C. After neutralization with 50 ml 10 *M* acetic acid, the material was pooled, dialyzed, freeze dried and purified by adsorption chromatography on Iatrobeads 6RS-8060 (Macherey and Nagel, Düren, Germany) according to Ueno et al. [27]. The chloroform–methanol (1:2, v/v) eluate contained purified whole gangliosides. Yields of lipid extracts (see Section 3) were determined gravimetrically.

2.2. Anion-exchange HPLC of gangliosides

The Superformance universal glass cartridge system of Merck was used for anion-exchange HPLC of gangliosides. Two glass cartridges of different size (150×10 mm, Merck No. 20286, and 110×30 mm, Merck No. 16887, with column volumes of 12 ml and 78 ml, respectively) filled with Fractogel EMD TMAE-650(S) were fitted into a HPLC system (Gilson Abimed, Langenberg, Germany) consisting of three M303 HPLC pumps, a high-pressure mixer M811 and a fraction collector M202.

The following washing, regeneration and equilibration steps are described for the small column (150×10 mm) using a flow-rate of 0.5 ml/min. The procedure for the large column (110×30 mm) is identical, but the flow-rate was set to 3 ml/min resulting in a 6-fold volume increase.

The anion-exchanger was converted into the acetate form by successive rinsing with Milli-Q water (Millipore, Bedford, MA, USA; Milli-Q water purification system), NaCl–Tris buffer (0.5 *M* NaCl, 0.25 *M* Tris, pH 8.5), Milli-Q water, 1 *M* acetic acid, Milli-Q water and methanol, each for 200 min. Finally, the column was equilibrated with chloroform–methanol–water (30:60:8, v/v/v) and gangliosides were applied in this solvent onto the column via a Rheodyne 7125 manual injector connected with a 5-ml sample loop. The HPLC program for gradient elution with increasing concentrations of ammonium acetate is outlined in Table 1. The details on the chromatographic parameters are given in the figure legends. After the HPLC run, the column was regenerated by successive rinsing with the solvents described above for converting the anion-exchanger into the acetate form.

Gangliosides containing samples were pooled (see Section 3), evaporated and resuspended in water for subsequent dialysis. Alternatively, the residues were taken up in 0.88% KCl and desalted by reversed-

Table 1
HPLC program for ganglioside isolation on TMAE fractogel[a]

Time[b] (min)	Flow[c] (ml/min)	A[d] (%)	B (%)	C (%)
0	0.0	100	0	0
3	0.5	100	0	0
210	0.5	100	0	0
220	0.5	0	100	0
247	0.5	0	100	0
370	0.5	0	90	10
450	0.5	0	75	25
490	0.5	0	0	100
546	0.5	0	0	100
547	0.0	0	0	100

[a] 150×10 mm column.
[b] Fractionation was started at $t=247$ min.
[c] Using the 110×30 mm column, the flow-rate was 3.0 ml/min.
[d] A: chloroform–methanol–water (30:60:8, v/v), B: methanol, C: 1 *M* ammonium acetate in methanol.

phase chromatography on octadecyl columns (0.5 g; Burdick and Jackson, Inert SPE System; Muskegon, MI, USA) according to Williams and McCluer [28].

2.3. High-performance thin-layer chromatography

High-performance thin-layer chromatography plates (HPTLC plates, size 10×10 cm, glass backed and precoated with 0.2 mm silica gel 60, No. 5633, Merck, Darmstadt, Germany) were used. Glycosphingolipids were delivered with the automatic sample applicator AS30 (Desaga, Heidelberg, Germany). HPTLC separation of gangliosides was performed in the neutral solvent 1, chloroform–methanol–water (120:85:20, v/v/v) and alkaline solvent 2, chloroform–methanol–2.5 *M* NH_4OH (120:85:20, v/v/v), each with 2 m*M* $CaCl_2$ [29]. Lipid bound sialic acid was visualized with resorcinol according to Svennerholm [30].

Resorcinol stained ganglioside chromatograms were scanned with the CD60 scanner (Desaga) equipped with an IBM compatible personal computer and densitometric software. Band intensities were measured in reflectance mode at 580 nm with a light beam slit of 2×0.1 mm. Reference gangliosides were prepared from human granulocytes as described elsewhere [31].

3. Results

3.1. Ganglioside isolation from hybridoma cells

The extraction of 15.6 kg wet mass of hybridoma cells, corresponding to 730 g dry mass, resulted in a crude extract of 267.9 g. After dialysis and freeze drying, 58.8 g were recovered. DEAE chromatography, dialysis and freeze drying gave 10.5 g, and the final ganglioside amount obtained after alkaline treatment, dialysis, freeze drying and Iatrobeads chromatography was 1.36 g.

3.2. TLC separation of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc)

The resorcinol stained thin-layer chromatograms of the whole ganglioside fraction from hybridoma cells are shown in Fig. 1, chromatographed in neutral solvent 1 (Fig. 1A, lane a) and alkaline solvent 2 (Fig. 1B, lane a) compared to reference gangliosides from human granulocytes (Fig. 1, lanes b). The chromatography in the neutral solvent resulted in three G_{M3}-bands, whereas in alkaline solvent two double bands were revealed, the upper pair represented G_{M3}(Neu5Ac) and the lower pair G_{M3}(Neu5Gc), each substituted with C_{24}- (upper band) and C_{16}-fatty acid (lower band) [24].

3.3. TMAE-Fractogel HPLC on a 150×10 mm column

The small TMAE-Fractogel column (12-ml volume) was loaded with 20 mg whole gangliosides from hybridoma cells, and gangliosides were eluted by ammonium acetate gradient chromatography (Fig. 2A). G_{M3}(Neu5Ac) containing fractions (with G_{M3}(Neu5Gc) contaminants) and G_{M3}(Neu5Gc) harbouring fractions were combined in three pools (Fig. 2B). After desalting by reversed-phase chromatography, 6.5 mg, 10.5 mg, and 2.1 mg gangliosides were recovered in pools I, II, and III, respectively, corresponding to a yield of 95.5%. Pure G_{M3}(Neu5Gc) with differing ceramide portions was obtained in pools II and III, whereas the G_{M3}(Neu5Ac) enriched pool I contained minor quantities of G_{M3}(Neu5Gc). Therefore, pool I was reapplied to the column and repeatedly chromatographed

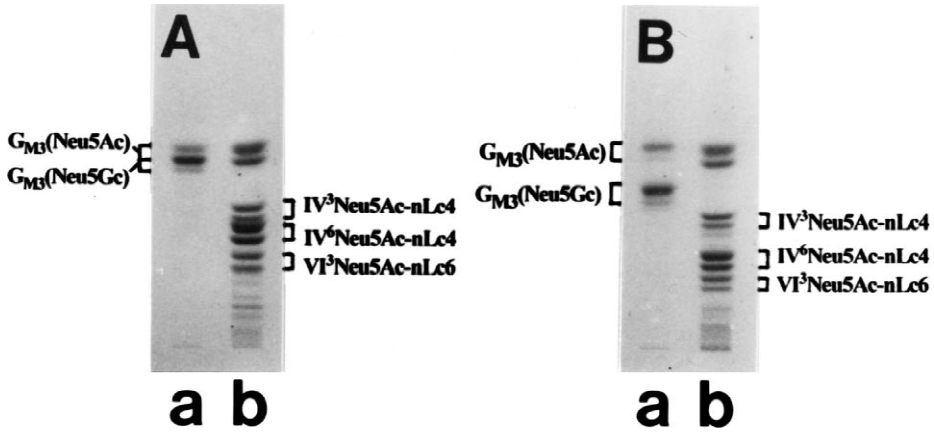

Fig. 1. Thin-layer chromatogram of gangliosides from hybridoma cells run in solvent 1 (A) and solvent 2 (B). Lanes (a) 5 μg gangliosides of hybridoma cells; (b) 15 μg reference gangliosides of human granulocytes. After chromatography, gangliosides were stained with resorcinol–HCl reagent.

under the same conditions described above. As shown in Fig. 3, this procedure results in pure G_{M3}(Neu5Ac) (pool IV) and G_{M3}(Neu5Gc) (pool V).

3.4. TMAE-Fractogel HPLC on a 110×30 mm column

The large TMAE-Fractogel column (78-ml volume) was loaded with 130 mg whole gangliosides of hybridoma cells, and gangliosides were eluted by ammonium acetate gradient chromatography (Fig. 4A). According to the elution profile, pure G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) fractions were obtained in pool I (G_{M3}(Neu5Ac), 20.0 mg) and pools III and IV (G_{M3}(Neu5Gc), 32.2+13.2 mg= 45.4 mg) (Fig. 4B). The intermediate collection (pool II) was found to be composed of almost equal amounts of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) (48.4 mg in total). Pools were desalted by dialysis with a total recovery of 113.8 mg (87.5%).

Finally, a large scale purification procedure of 500 mg whole gangliosides of hybridomas was performed as described above. Deducing from the

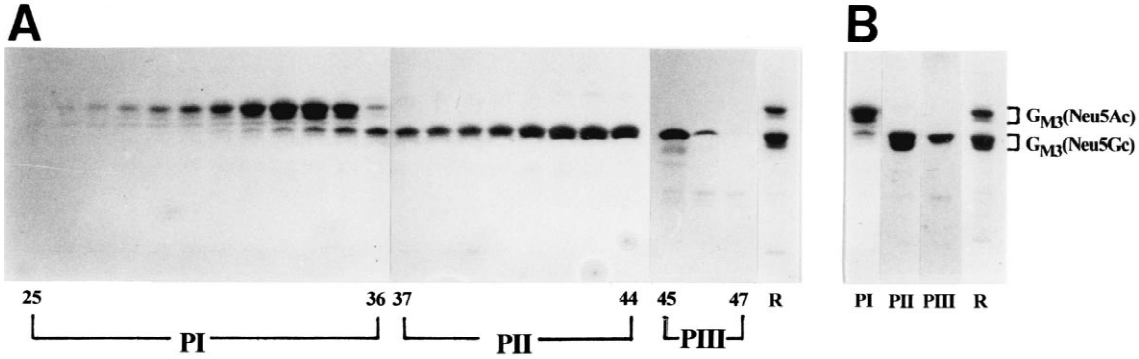

Fig. 2. TMAE-Fractogel HPLC of 20 mg hybridoma gangliosides (A). Gangliosides were dissolved in 40 ml chloroform–methanol–water (30:60:8, v/v/v), applied to a 150×10 mm column (for details of the gradient see Section 2), and collected in 1.5-ml fractions (3-min fractions). Aliquots of 10 μl (0.67%) of single fractions were analyzed by TLC. Fractions were pooled as indicated (PI to PIII) and separated by TLC (B). PI: 10 μg, PII: 10 μg, PIII: 4 μg, R (reference hybridoma whole gangliosides): 10 μg. Gangliosides were chromatographed in solvent 2 and stained with resorcinol–HCl reagent.

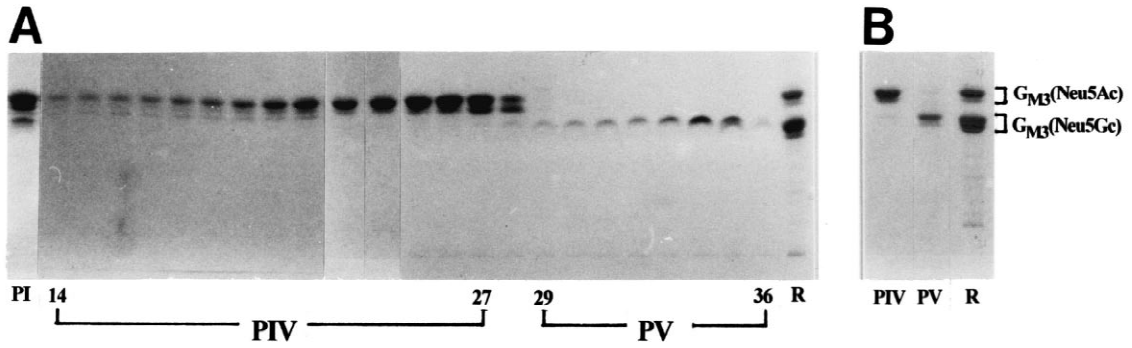

Fig. 3. TMAE-Fractogel HPLC of 6.5 mg gangliosides from pool I of Fig. 2 (G_{M3}(Neu5Ac)-enriched fraction) (A). Gangliosides were dissolved in 8 ml chloroform–methanol–water (30:60:8, v/v/v), applied to a 150×10 mm column (for details of the gradient see Section 2), and collected in 1.5-ml fractions (3-min fractions). Aliquots of 20 μl (1.33%) of single fractions were analyzed by TLC in comparison to 10 μg of PI (starting material for the HPLC run). Fractions were pooled as indicated (PIV and PV) and separated by TLC (B). PIV: 4 μg, PV: 2 μg, R (reference hybridoma whole gangliosides): 10 μg. Gangliosides were chromatographed in solvent 2 and stained with resorcinol–HCl reagent.

HPLC elution profile (Fig. 5A), pure G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) fractions were present in pool I (G_{M3}(Neu5Ac), 14.9 mg) and pools III and IV (G_{M3}(Neu5Gc), 135.8+64.4 mg=200.2 mg) (Fig. 5B). The intermediate collection (pool II) contained equal amounts of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) (184.4 mg in total) and a combined fraction of gangliosides with higher molecular masses than G_{M3} (not shown in Fig. 5) made up 27.1 mg. Again, pools were desalted by dialysis with a total recovery of 426.6 mg (85.3%).

All data of small and large scale preparative purification of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), i.e. total recoveries and yields of both types of gangliosides together with the relative column elution volumes, are summarized in Table 2.

4. Discussion

Anion-exchange chromatography is convenient as a first purification step for separation of neutral glycosphingolipids and gangliosides. Since the early reports of Winterbourn [32] and Momoi et al. [11], the separation of gangliosides using DEAE-resins has become one of the most popular methods, not only for the preparation of total ganglioside fractions, but also for the isolation of ganglioside

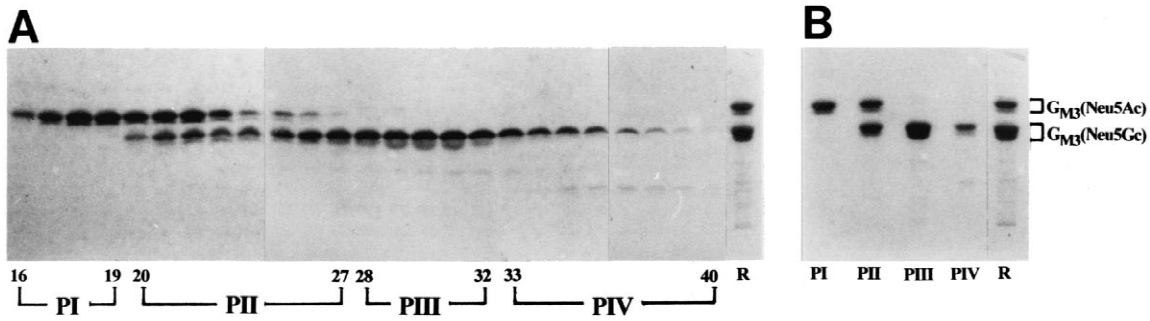

Fig. 4. TMAE-Fractogel HPLC of 130 mg hybridoma gangliosides (A). Gangliosides were dissolved in 160 ml chloroform–methanol–water (30:60:8, v/v/v), applied to a 110×30 mm column (for details of the gradient see Section 2), and collected in 9-ml fractions (3-min fractions). Aliquots of 10 μl (0.11%) of single fractions were analyzed by TLC. Fractions were pooled as indicated (PI to PIV) and separated by TLC (B). PI: 5 μg, PII: 10 μg, PIII: 10 μg, PIV: 2 μg, R (reference hybridoma whole gangliosides): 10 μg. Gangliosides were chromatographed in solvent 2 and stained with resorcinol–HCl reagent.

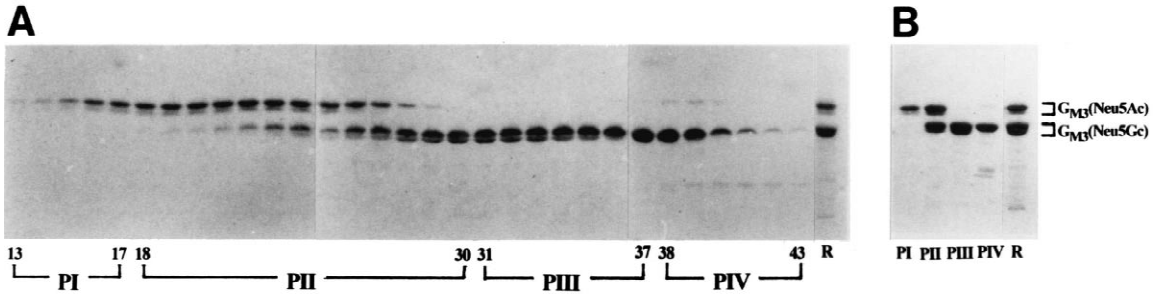

Fig. 5. TMAE-Fractogel HPLC of 500 mg hybridoma gangliosides (A). Gangliosides were dissolved in 260 ml chloroform–methanol–water (30:60:8, v/v/v), applied to a 110×30 mm column (for details of the gradient see Section 2), and collected in 9-ml fractions (3-min fractions). Aliquots of 2 μl (0.022%) of single fractions were analyzed by TLC. Fractions were pooled as indicated (PI to PIV) and separated by TLC (B). PI: 2 μg, PII: 10 μg, PIII: 6 μg, PIV: 4 μg, R (reference hybridoma whole gangliosides): 10 μg. Gangliosides were chromatographed in solvent 2 and stained with resorcinol–HCl reagent.

subfractions, e.g. according to their degree of sialylation [26,33]. Various anion-exchange DEAE-coupled matrices are in use for ganglioside fractionation, for instance DEAE-Sephadex, DEAE-Sepharose, Spherosil-DEAE-Dextran, DEAE-derivatized controlled porous glass [12–15,34]. Further improvements in the separation of gangliosides were achieved with the strong anion-exchanger Mono Q [35], Q-Sepharose [17] and TMAE-Fractogel [18]. The latter was shown to enable the separation of ganglioside positional isomers on a preparative scale, e.g. IV^3Neu5Ac-nLcOse$_4$Cer and IV^6Neu5Ac-nLcOse$_4$Cer, and with Q-Sepharose the separation of Neu5Ac- and Neu5Gc-substituted gangliosides has been attained [17]. The preparative HPLC purification of G_{M3} (scale ≤20 mg per run) employing a Zorbax-NH_2 column has been recently published [19]. The aim of our study was to establish appropriate conditions for rapid and efficient scaled-up purification of the closely allied gangliosides G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc). Several practical advantages in using TMAE-Fractogel are the (1)

Table 2
Synopsis of ganglioside yields at small and large scale purification of gangliosides G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) by single chromatography on TMAE-Fractogel HPLC

	Dimension		
	Small scale[a]	Large scale[b]	
	(mg)	(mg)	
Starting material[c]	20.0	130.0	500.0
G_{M3}(Neu5Ac)	6.5[d]	20.0	14.9
G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc)[e]	–	48.4	184.4
G_{M3}(Neu5Gc)	12.6	45.4	200.2
Gangliosides with higher molecular mass than G_{M3}	–	–	27.1
Total recovery[f]	19.1 (95.5%)	113.8 (87.5%)	426.6 (85.3%)
Relative elution volume[g]	2.8	2.8	3.4

[a] 150×10 mm column with 12-ml bed volume.
[b] 110×30 mm column with 78-ml bed volume.
[c] Whole ganglioside fraction.
[d] Contains small G_{M3}(Neu5Gc) contamination.
[e] Mixed G_{M3}-fraction.
[f] Calculated after desalting of pooled samples.
[g] Calculated as the quotient of the elution volume to the column bed volume for G_{M3}-separation.

high loading capacity accounting for at least 6.4 mg G_{M3}-mixture per millilitre gel, (2) separation of gangliosides according to their small structural difference (Neu5Ac versus Neu5Gc), (3) achievement of high numbers of pure G_{M3}(Neu5Ac)- and G_{M3}(Neu5Gc)-fractions by one-step HPLC, (4) low relative column elution volumes (<4), and (5) online monitoring of the separation by short-wavelength UV detection due to elution with an ammonium acetate gradient in methanol (not shown). The total recoveries ranged from 85.3% to 95.5% and are lower than other previously published methods. However, since the ganglioside containing samples were pooled after HPLC and TLC analysis and quantified after desalting by reversed-phase chromatography or dialysis, the reduced yields might be due to some loss during the desalting procedures.

In view of the biological importance of G_{M3} in various tumor-associated phenomena and other biological events as well as the need for G_{M3} as starting material for various G_{M3}-derivatives [20–22], it appears likely that a demand for this ganglioside will arise in the future. Therefore, our improved method enables convenient scaled-up isolation of bulk quantities of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) ranging from 20 to 500 mg of G_{M3} gangliosides. Hybridoma cells from industrial monoclonal antibody production, a readily available source for sialylated lactosylceramide, were chosen as the starting material for preparative isolation of pure G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) specimens in high milligram quantities.

5. Abbreviations used

DEAE	Diethylaminoethyl
GSL(s)	Glycosphingolipid(s)
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid [36]
TMAE	Trimethylaminoethyl

The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [37] and the nomenclature of Svennerholm [38]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; Lacto-N-neotetraosylceramide or $nLcOse_4Cer$, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Lacto-N-norhexaosylceramide or $nLcOse_6Cer$, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; G_{M3}(Neu5Ac), II^3Neu5Ac-LacCer; G_{M3}(Neu5Gc), II^3Neu5Gc-LacCer.

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