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Improved separation of isomeric gangliosides by anion-exchange high-performance liquid chromatography

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Abstract

A new anion-exchange HPLC method for separation of gangliosides has been developed using TMAE (trimethylaminoethyl)-Fractogel as the stationary phase and a gradient system of ammonium acetate in methanol for elution. Chromatography of mouse brain gangliosides resulted in separation according not only to their degree of sialylation into mono-, di-, tri- and tetrasialogangliosides, but also in separation of positional isomers within the four elution domains, *e.g.* G_{D1a} ($IV^3Neu5Ac,II^3Neu5Ac$-$GgOse_4Cer$) and G_{D1b} ($II^3(Neu5Ac)_2$-$GgOse_4Cer$) of the disialoganglioside fraction. Furthermore, base-line separation of α2–3 and α2–6 sialylated neolacto-series gangliosides *e.g.* $IV^3Neu5Ac$-$nLcOse_4Cer$ and $IV^6Neu5Ac$-$nLcOse_4Cer$, isolated from human granulocytes, was achieved and pure fractions of each ganglioside were obtained. Thus, the high-resolution power of the strong anion-exchanger TMAE-Fractogel allowed the isolation of pure gangliosides on a preparative scale by one-step column chromatography.

1. Introduction

Gangliosides are distinguished from other glycosphingolipids (GSLs)[1] by the presence of sialic acid, a characteristic carbohydrate constituent [1]. Structures and functions of gangliosides have been widely reviewed [2–4]. They act as receptors for toxins and bacteria [5], viruses [6] and other ligands and are believed to be involved in cell–cell recognition phenomena and in the development of anti-tumor immunity [7].

Due to the biological importance of gangliosides much effort has been spent on their

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[1] Abbreviations

GSL(s), glycosphingolipid(s); HGG, human granulocytes gangliosides; MBG, mouse brain gangliosides; Neu5Ac,N-acetylneuraminic acid [22]; TMAE, trimethylaminoethyl. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [23] and the ganglioside nomenclature system of Svennerholm [24] was used. LacCer or lactosylceramide, Galß1-4Glcß1-1Cer; gangliotriaosylceramide or $GgOse_3Cer$, GalNAcß1-4Galß1-4Glcß1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer; lacto-N-*neo*tetraosylceramide or $nLcOse_4Cer$, Galß1-4GlcNAcß1-3Galß1-4Glcß1-1Cer; lacto-N-*nor*hexaosylceramide or $nLcOse_6Cer$, Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Galß1-4Glcß1-1Cer; G_{M3}, $II^3Neu5Ac$-LacCer; G_{M2}, $II^3Neu5Ac$-$GgOse_3Cer$; G_{M1}, $II^3Neu5Ac$-$GgOse_4Cer$; G_{D1a}, $IV^3Neu5Ac,II^3Neu5Ac$-$GgOse_4Cer$; G_{D1b}, $II^3(Neu5Ac)_2$-$GgOse_4Cer$; G_{T1b}, $IV^3Neu5Ac,II^3(Neu5Ac)_2$-$GgOse_4Cer$; G_{Q1b}, $IV^3(Neu5Ac)_2,II^3(Neu5Ac)_2$-$GgOse_4Cer$.)

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isolation and structural characterization. Anion-exchange chromatography is convenient as a first purification step of crude GSL extracts because of the complete separation of neutral GSLs and gangliosides. Since the first report by Momoi *et al.* [8], the separation of gangliosides using DEAE-resins has become one of the most popular methods for the preparation and purification of gangliosides. To achieve fractionation of gangliosides according to their degree of sialylation, the use of various anion-exchange DEAE-coupled matrices has been reported [9–12]. Further recent improvements in separation of gangliosides were the application of DEAE-Fractogel [13] as well as the strong anion-exchanger Q-Sepharose [14]. In this report, we describe an improved HPLC anion-exchange column chromatographic method using TMAE (trimethylaminoethyl)-Fractogel, which allows the separation of ganglioside positional isomers on a preparative scale. A preliminary report of some of the results has already been published [15].

2. Experimental

2.1. Gangliosides

Chloroform and methanol of analytical grade (Merck, Darmstadt, Germany) were distilled before use. Gangliosides from female CBA/J mouse brains were isolated according to standard procedures [2]. Two pools of brain were prepared (MBG1 and MBG2) which showed some differences in their relative amounts of gangliosides. Structures of mouse brain gangliosides are listed in Table 1.

Gangliosides from human granulocytes were isolated as recently described by Müthing *et al.* [16]. Briefly, cell lipids were extracted with chloroform–methanol (2:1, v/v) and (1:2, v/v). The combined extracts were evaporated and partitioned according to Folch *et al.* [17]. Gangliosides of Folch's upper phase were separated from neutral glycosphingolipids by anion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) as described by Müthing *et al.* [18]. Gangliosides were eluted with 0.45 *M* ammonium acetate in methanol. After evaporation and desalting by dialysis, the ganglioside fraction was incubated for 1 h at 37°C in 1 *M* NaOH to hydrolyse phospholipids, followed by neutralization with acetic acid and desalting by dialysis. Gangliosides were then further purified by Iatrobeads 6RS-8060 chromatography (Macherey-Nagel, Düren, Germany) according to Ueno *et al.* [19]. The total ganglioside fraction composed of gangliosides numbered 1 to 8 (see Table 2) and designated HGG1, was eluted with chloroform–methanol (1:2, v/v). Alternatively, stepwise elution was performed with chloroform–methanol (85:15, 3:1, 2:1, 1:2, v/v), and finally with pure methanol. A neolacto-series ganglioside fraction without G_{M3}, composed of gangliosides 3 to 8 (G_{M3} depleted, see Table 2) and designated HGG2, was obtained in the chloroform–methanol (1:2) eluate.

2.2. High-performance thin layer chromatography

Gangliosides were separated on glass-backed silica gel 60 precoated HPTLC plates (size 10 cm × 10 cm, thickness 0.24 mm; E. Merck, Darmstadt, Germany) in the solvent chloroform–methanol–water (120:85:20, v/v) with 2 m*M* $CaCl_2$ in standard separating chambers equipped with filter paper. Gangliosides were applied to the HPTLC plates with the automatic Desaga TLC-applicator AS 30 (Desaga, Heidelberg, Germany). Volumes of 5 μl were applied with a high-precision 10-μl syringe. Gangliosides were visualized by resorcinol as described by Svennerholm [20] and lipid bound sialic acid was estimated by densitometry. Resorcinol stained ganglioside chromatograms were scanned with a Desaga CD 60 scanner equipped with an IBM compatible personal computer and densitometric software. Intensities of bands were measured in the reflectance mode at 580 nm with a light beam slit of 0.1 mm × 2 mm.

2.3. Anion-exchange HPLC of gangliosides

The Superformance universal glass-cartridge system of Merck was used for HPLC. Glass cartridges (150 × 10 mm) either filled with Frac-

togel EMD DEAE-650(S) (Merck, No. 20282) or with Fractogel EMD TMAE-650(S) (Merck, No. 20286) 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 anion-exchanger was converted into the acetate form by successive rinsing with 100 ml of NaCl/Tris buffer (0.5 *M* NaCl, 0.25 *M* Tris), 150 ml Milli Q-water (Millipore, Bedford, MA, USA; Milli-Q water purification system), 100 ml of 1 *M* acetic acid and 150 ml Milli-Q water. Finally, the column was equilibrated with chloroform–methanol–water (30:60:8, 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. After rinsing with 220 ml of chloroform–methanol–water (30:60:8, v/v) and 40 ml of methanol, DEAE-Fractogel bound gangliosides were eluted with an NH_4OAc gradient in methanol as follows: flow-rate 0.5 ml/min; linear gradient from methanol to 0.03 *M* NH_4OAc (280 min), followed by a linear gradient from 0.03 *M* to 0.1 *M* NH_4OAc (100 min) and 0.1 *M* NH_4OAc (60 min). TMAE-Fractogel bound gangliosides were eluted with an NH_4OAc gradient in methanol as follows: flow-rate 0.5 ml/min; linear gradient from methanol to 0.1 *M* NH_4OAc (20 min) followed by a linear gradient from 0.1 *M* to 1 *M* NH_4OAc (220 min) and 1 *M* NH_4OAc (60 min). The details on the chromatographic parameters are given in the respective figures (see below). The relative elution volume was calculated as the quotient of the elution volume to the bed volume of the column.

3. Results and discussion

The resorcinol stained thin layer chromatograms of mouse brain gangliosides (MBG1, see Experimental) and human granulocytes gangliosides (HGG1, see Experimental) are shown in Fig. 1. The major gangliosides from mouse brain are the ganglio-series gangliosides G_{M1}, G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}. Their structures are shown in Table 1. In a previous paper [16] we

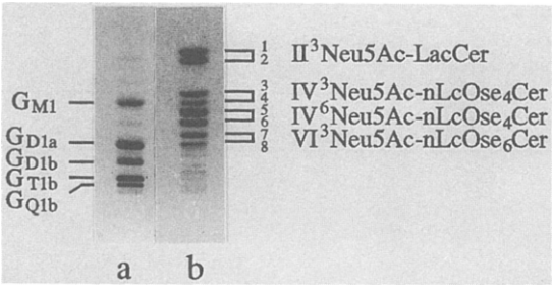

Fig. 1. Thin layer chromatogram of 10 μg gangliosides, each, of mouse brain (a, MBG1) and human granulocytes (b, HGG1). For abbreviations see Experimental. After chromatography, gangliosides were visualized by spraying the plate with resorcinol-HCl reagent. The structures of gangliosides from mouse brain and human granulocytes are listed in Tables 1 and 2, respectively.

showed that, besides G_{M3}, the neolacto-series gangliosides $IV^3Neu5Ac\text{-}nLcOse_4Cer$, $IV^6Neu5Ac\text{-}nLcOse_4Cer$ and $VI^3Neu5Ac\text{-}nLcOse_6Cer$ were the major gangliosides isolated from a pool of human granulocytes, according to results of Fukuda *et al.* [21]. The characteristic pattern of four ganglioside double bands (Fig. 1, lane b) is caused by substitution of fatty acids with different chain length (mainly

Table 1
Structures of mouse brain gangliosides

Symbol[a]	Structure
G_{M1}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer $^{3}_{2}\vert\alpha$ Neu5Ac
G_{D1a}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer $^{3}_{2}\vert\alpha$ $^{3}_{2}\vert\alpha$ Neu5Ac Neu5Ac
G_{D1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer $^{3}_{2}\vert\alpha$ Neu5Ac8-2αNeu5Ac
G_{T1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer $^{3}_{2}\vert\alpha$ $^{3}_{2}\vert\alpha$ Neu5Ac Neu5Ac8-2αNeu5Ac
G_{Q1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer $^{3}_{2}\vert\alpha$ $^{3}_{2}\vert\alpha$ Neu5Ac8-2αNeu5Ac Neu5Ac8-2αNeu5Ac

[a] According to Svennerholm [24].

Table 2
Structures of monosialogangliosides from human granulocytes[a]

No.	Fatty acid	Structure
1	24:1, 22:0	II^3Neu5Ac-LacCer
2	16:0	II^3Neu5Ac-LacCer
3	24:1, 22:0	IV^3Neu5Ac-nLcOse$_4$Cer
4	16:0	IV^3Neu5Ac-nLcOse$_4$Cer
5	24:1	IV^6Neu5Ac-nLcOse$_4$Cer
6	16:0	IV^6Neu5Ac-nLcOse$_4$Cer
7	24:1	VI^3Neu5Ac-nLcOse$_6$Cer
8	16:0	VI^3Neu5Ac-nLcOse$_6$Cer

[a] Data drawn from Müthing *et al.* [16].

C_{24}- and C_{16}-fatty acids) in the ceramide portions (see Table 2).

3.1. HPLC of ganglio-series gangliosides on DEAE-Fractogel

To ensure binding of all gangliosides to the weak anion-exchange resin, ganglioside mixtures with a relatively low concentration of 30 nmol/ml (*ca.* 0.05 mg/ml) were applied to the column. Increasing of the ganglioside concentration prevented binding of monosialogangliosides, which passed through the column without binding to the DEAE-groups. The maximum loading capacity of the column was *ca.* 0.5 μmol per ml of resin, as calculated for the mouse brain ganglioside mixture MBG1 (see Experimental). The HPLC elution profile of MBG1 and the corresponding TLC pattern of the single fractions are shown in Fig. 2. Gangliosides were separated according to their degree of sialylation into mono-, di-, tri- and tetrasialogangliosides within the relative elution volume of twelve column volumes. Complete elution of all gangliosides is ensured with 0.1 *M* NH_4OAc. Within the mono-, di- and trisialoganglioside fractions, respectively, all individual gangliosides co-eluted, *e.g.* G_{D1a} and G_{D1b} of the disialoganglioside fraction. Only in the tetrasialoganglioside elution peak the two less polar compounds eluted prior to G_{Q1b}.

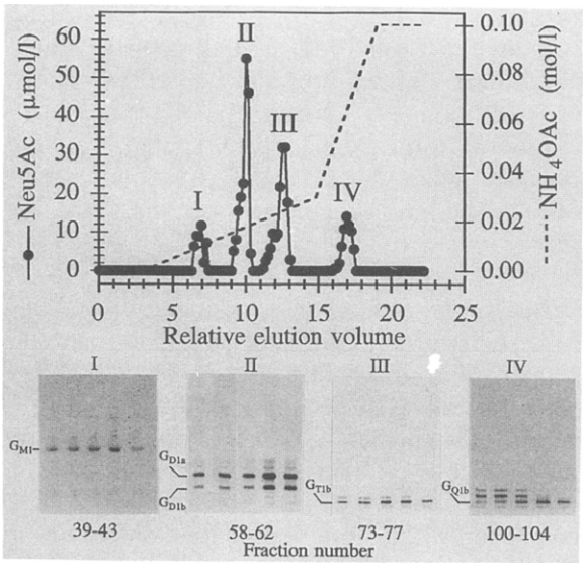

Fig. 2. HPLC of mouse brain gangliosides on DEAE-Fractogel. Gangliosides (10 mg MBG1, see Experimental) were chromatographed and collected in 2-ml fractions (= 4-min fractions). Aliquots (2%) of single fractions were analyzed by TLC. I = mono-, II = di-, III = tri- and IV = tetrasialogangliosides. The structures of mouse brain gangliosides are listed in Table 1.

3.2. HPLC of ganglio-series gangliosides on TMAE-Fractogel

The strong anion-exchanger enabled application of ganglioside mixtures with a higher concentration up to 300 nmol/ml (*ca.* 0.5 mg/ml) without any loss of monosialogangliosides. Compared to DEAE-Fractogel, ganglioside concentrations could be increased about ten-fold leading to drastically reduced duration of sample application. The maximum loading capacity of the TMAE-Fractogel tested was found to be at least *ca.* 1 μmol per ml resin as calculated for the mouse brain mixture MBG2 (see Experimental). The HPLC elution profile of MBG2 and the TLC patterns of single fractions are shown in Fig. 3. Due to the strong anion-exchanger TMAE, about a ten-fold higher concentration of NH_4OAc was required for ganglioside elution compared to the DEAE-Fractogel. Gangliosides eluted according to their degree of sialylation as described for DEAE-Fractogel, but ganglioside

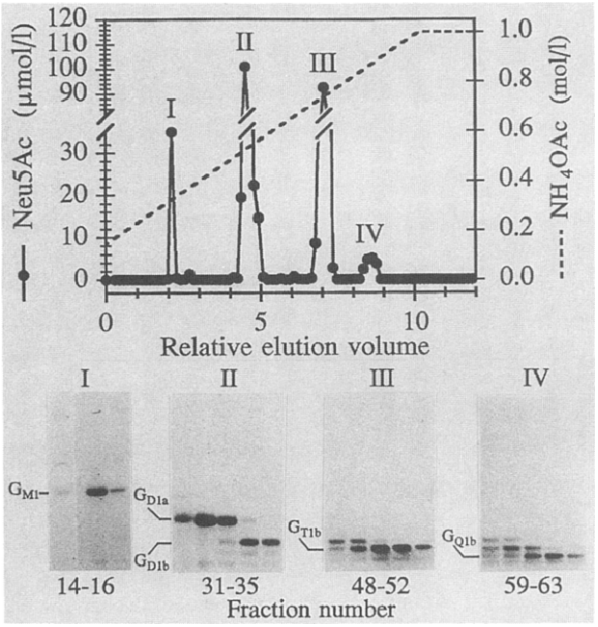

Fig. 3. HPLC of mouse brain gangliosides on TMAE-Fractogel. Gangliosides (20 mg MBG2, see Experimental) were chromatographed and collected in 1.5-ml fractions (= 3-min fractions). Aliquots (1%) of single fractions were analyzed by TLC. I = mono-, II = di-, III = tri- and IV = tetrasialogangliosides. The structures of mouse brain gangliosides are listed in Table 1.

separation was completed with seven relative column volumes, indicating a higher resolution capacity of the TMAE-Fractogel. In all four elution peaks less polar gangliosides eluted prior to the more polar gangliosides. Thus, fractions of pure gangliosides were obtained, and good separation of isomers, *e.g.* G_{D1a} and G_{D1b}, was achieved. The higher resolution capacity of strong anion-exchangers has also been reported by Hirabayashi *et al.* [14], who used Q-Sepharose for large-scale fractionation of bovine brain gangliosides.

3.3. HPLC of neolacto-series gangliosides on DEAE-Fractogel

Due to the high content of monosialogangliosides in the ganglioside fraction of human granulocytes compared to that in the mouse brain mixture, ganglioside mixtures with up to 70 nmol/ml (*ca.* 0.1 mg/ml) were applied without any loss of material. The maximum loading capacity tested was 1.2 μmol per ml resin, as calculated for the ganglioside mixture HGG1 (see Experimental). This mixture mainly contains G_{M3}(Neu5Ac) and the neolacto-series gangliosides IV^{3}Neu5Ac-nLcOse$_4$Cer, IV^{6}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer (see Table 2). HPLC gave one strong double-peaked monosialo- and a small disialoganglioside fraction within a relative elution volume of five column volumes (Fig. 4), containing 95% monosialogangliosides (peaks Ia and Ib, Fig. 4) and 5% disialogangliosides (peak II, Fig. 4). Terminally α2–3 sialylated gangliosides G_{M3} (bands 1 and 2), IV^{3}Neu5Ac-nLcOse$_4$Cer (bands 3 and 4) and VI^{3}Neu5Ac-nLcOse$_6$Cer (bands 7 and 8) eluted in peak Ia prior to IV^{6}Neu5Ac-nLcOse$_4$Cer, which was the main compound of peak Ib (bands 5 and 6). The linkage type of

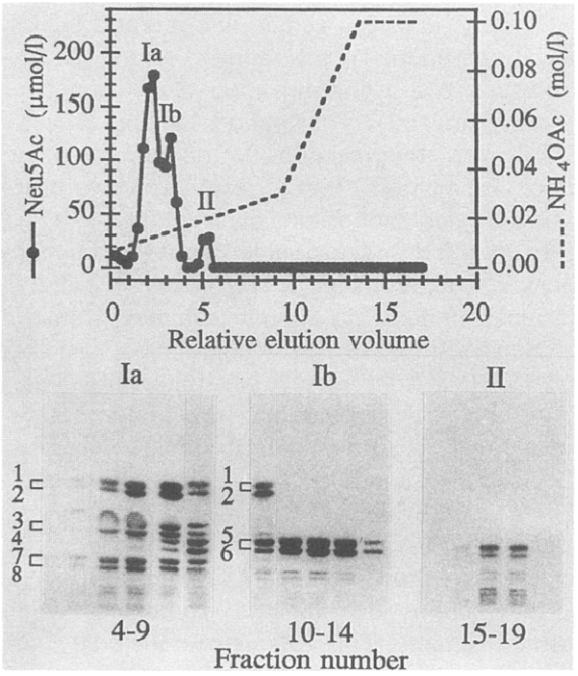

Fig. 4. HPLC of human granulocytes gangliosides on DEAE-Fractogel. A 20-mg amount of the total ganglioside mixture (HGG1, see Experimental) was applied and collected in 3.5-ml fractions (= 7-min fractions). Aliquots (2%) of single fractions were analyzed by TLC. I = mono- and II = disialogangliosides. The structures of human granulocytes gangliosides 1 to 8 are listed in Table 2.

Neu5Ac (α2–3, α2–6) hardly influenced binding, *i.e.* elution from DEAE-Fractogel. Isomeric IV^{3}Neu5Ac-nLcOse$_4$Cer (compounds 3 and 4) and IV^{6}Neu5Ac-nLcOse$_4$Cer (compounds 5 and 6) were separated to some extent under the conditions used. Terminally α2–3 sialylated IV^{3}Neu5Ac-nLcOse$_4$Cer (compounds 3 and 4) and α2–3 sialylated VI^{3}Neu5Ac-nLcOse$_6$Cer (compounds 7 and 8), differing in their oligosaccharide chain length by the disaccharide sequence Galß1-4GlcNAc, co-eluted in this system (see Fig. 4, fractions 6 to 9). The unknown disialogangliosides, which chromatographed in the range of the monosialoganglioside bands 5 and 6 and below bands 7 and 8, co-eluted in fractions 17 and 18 of peak II (see Fig. 4).

3.4. HPLC of neolacto-series gangliosides on TMAE-Fractogel

The G_{M3} depleted ganglioside mixture HGG2 (see Experimental) was applied onto TMAE-Fractogel with a concentration of 470 nmol/ml (*ca.* 0.75 mg/ml). The highest loading capacity tested with this ganglioside mixture was 1.6 μmol per ml resin. HPLC resulted in two baseline separated monosialoganglioside peaks (Fig. 5, Ia and Ib) and a double-peaked disialoganglioside fraction (Fig. 5, II) within a relative elution volume of six column volumes. Isomeric IV^{3}Neu5Ac-nLcOse$_4$Cer (compounds 3 and 4) and IV^{6}Neu5Ac-nLcOse$_4$Cer (compounds 5 and 6) were clearly separated in this system. Separation of α2-3 sialylated IV^{3}Neu5Ac-nLcOse$_4$Cer (bands 3 and 4) and the Galß1-4GlcNAc elongated α2-3 sialylated VI^{3}Neu5Ac-nLcOse$_6$Cer (compounds 7 and 8) was partly achieved, as shown by the TLC control in Fig. 5. Both fractions 13 and 15 contained single purified isomers. Thus, all structurally well characterized major gangliosides of elution peak Ia contained a Neu5Acα2-3-terminus, whereas the Neu5Acα2-6-terminus of the neolacto-series was characteristic for the main components of elution peak Ib. From these data, the minor ganglioside of peak Ia (occurring in fractions 16 and 17 of the TLC insert in Fig. 5) is predicted to be

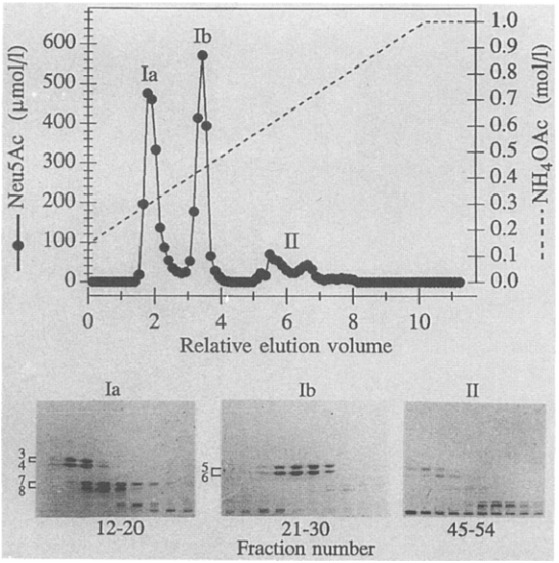

Fig. 5. HPLC of human granulocytes gangliosides on TMAE-Fractogel. A 30-mg amount of the G_{M3} depleted ganglioside mixture (HGG2, see Experimental) was applied and collected in 1.5 ml fractions (= 3 min fractions). Aliquots (1%) of single fractions were analyzed by TLC. I = mono- and II = disialogangliosides. The structures of human granulocytes gangliosides are listed in Table 2.

VIII^{3}Neu5Ac-nLcOse$_8$Cer, and the minor ganglioside of peak Ib (occurring in fractions 28 and 29) is speculated to represent VI^{6}Neu5Ac-nLcOse$_6$Cer. The latter assumption is supported by data of Fukuda *et al.*, who showed the expression of VI^{6}Neu5Ac-nLcOse$_6$Cer in human granulocytes [21].

In the separation of the disialogangliosides by TMAE-Fractogel chromatography (Fig. 5, peak II), two pairs of disialogangliosides of yet unknown structures were dissolved (Fig. 5, fractions 45 to 54), which were not separated before by DEAE-Fractogel chromatography (*cf.* to Fig. 4). To our knowledge, this is the first report on the expression of disialogangliosides in human granulocytes. Fukuda *et al.* [21] reported the putative presence of a disialoganglioside structure within a purified monosialoganglioside fraction, but no further structural data were available due to the low amounts present in granulocytes. Until now, the low concentrations present and the limited availability of material also prevented the isolation of sufficient amounts

of disialogangliosides, but their structural characterization is presently in progress.

4. Conclusions

In the case of the weak anion-exchanger DEAE-Fractogel, the number of sialic acid residues is the dominant parameter in the retention of gangliosides. In addition to separation according to the degree of sialylation, the strong anion-exchanger TMAE-Fractogel enabled the separation of isomeric compounds, which only differed in their type of sialylation (α2–3, α2–6). Gangliosides, identical in sialic acid substitution, but with differences in their respective neutral oligosaccharide moieties, could also only be separated by TMAE-Fractogel. Thus, besides improved separation of gangliosides, extended structural information about unknown gangliosides can be obtained by the strong anion-exchanger. Furthermore, the technique described offers the opportunity to accumulate minor components; this is beneficial for the isolation of minor gangliosides, that might be of relevance *e.g.* in cell–cell recognition, interaction with microorganisms or which might play a role as receptors for carbohydrate binding ligands.

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