Enhanced thin-layer chromatographic separation of G_{M1b}-type gangliosides by automated multiple development

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Abstract

Enhancement in separation of gangliosides on silica gel precoated high-performance TLC plates has been obtained by automated multiple development chromatography. A less polar mixture of the standard solvent chloroform–methanol–20 mM aqueous $CaCl_2$ (120:85:20, v/v) was used. Lowering the water content achieved separation of two complex monosialoganglioside fractions, isolated from murine YAC-1 T lymphoma and MDAY-D2 lymphoreticular cells. Three-fold chromatography in the solvent chloroform–methanol–20 mM aqueous $CaCl_2$ (120:85:14, v/v) resulted in TLC separation of G_{M1b}-type gangliosides, substituted with C_{24} and C_{16} fatty acids and with Neu5Ac and Neu5Gc as well, which could not be achieved by unidirectional standard chromatography. Compared to conventional single chromatography, the technique described allows high-resolution separation of extremely heterogenous ganglioside mixtures and offers a convenient tool for both analytical and preparative TLC.

Keywords: Automated multiple development; Gangliosides

1. Introduction

Gangliosides are a diverse group of glycosphingolipids (GSLs)[1] composed of long-chain aminoalcohol (sphingosine) and fatty acid, which together form the ceramide portion, and a carbohydrate moiety [1,2]. They 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 play crucial roles in various biological functions [3]. Gangliosides act as receptors for toxins and bacteria [4], viruses [5] and other ligands and are involved in e.g. signal transduction processes [6,7] and immunomodulation [8–10].

One-dimensional TLC is an easy and still commonly used method for the analysis of ganglioside mixtures [11,12]. This method has been continuously optimized with emphasis on the minor components of the total ganglioside mixtures. Further enhancement of GSL chromatography has been achieved by

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[1] Abbreviations used: AMD, automated multiple development; HPTLC, high-performance thin-layer chromatography; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid [30]. The designation of the following glycosphingolipids follows the IUPAC–IUB recommendations [31] and the ganglioside nomenclature system of Svennerholm [32] for G_{M1a}-type gangliosides. Gangliotriaosylceramide or GgOse$_3$Cer, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or GgOse$_4$Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; G_{M2}, II^{3}Neu5Ac-GgOse$_3$Cer; G_{M1} or G_{M1a}, II^{3}Neu5Ac-GgOse$_4$Cer; G_{M1b}, IV^{3}Neu5Ac-GgOse$_4$Cer; GalNAc-G_{M1b}, IV^{3}Neu5Ac-GgOse$_5$Cer; G_{D1a}, IV^{3}Neu5Ac,II^{3}Neu5Ac-GgOse$_4$Cer; G_{D1b}, II^3(Neu5Ac)$_2$-GgOse$_4$Cer; G_{T1b}, IV^{3}Neu5Ac,II^3(Neu5Ac)$_2$-GgOse$_4$Cer. Only Neu5Ac-substituted gangliosides are listed in this list of abbreviations.

the techniques of short-bed continuous [13] and automated multiple development [14].

In this study improved separation of G_{M1b}-type gangliosides on HPTLC plates was obtained by automated multiple development (AMD), that permits high-resolution separation of complex monosialoganglioside mixtures. The theoretical goal of increasing separation of bands by decreasing solvent strength [15] was achieved by three repeated runs in the same solvent with a polarity which is lower than that used in conventional single TLC.

2. Experimental

2.1. Reference gangliosides

Gangliosides from murine T lymphoma YAC-1 and lymphoreticular tumor cell line MDAY-D2 were isolated according to our established procedures [16,17]. Whole gangliosides were separated into mono- and disialoganglioside fractions by gradient anion-exchange chromatography as described.

A mixture of reference gangliosides from human brain composed of G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} was purchased from Supelco (Bellefonte, PA, USA). Their structures are listed in Table 1.

2.2. Analytical thin-layer chromatography

Silica gel precoated high-performance thin-layer chromatography plates (HPTLC plates, 10 cm×10 cm, thickness 0.2 mm; Merck, Darmstadt, Germany) were used for conventional single and automated multiple development chromatography. Standard chromatography was performed for 35 min in the solvent chloroform–methanol–20 mM aqueous CaCl$_2$ (120:85:20, v/v) and gangliosides were visualized by resorcinol [18].

Table 1
Structures of brain gangliosides

Symbol[a]	Structure
G_{M1}	II^{3}Neu5Ac-GgOse$_4$Cer
G_{D1a}	IV^{3}Neu5Ac,II^{3}Neu5Ac-GgOse$_4$Cer
G_{D1b}	II^3(Neu5Ac)$_2$-GgOse$_4$Cer
G_{T1b}	IV^{3}Neu5Ac,II^3(Neu5Ac)$_2$-GgOse$_4$Cer

[a] According to Svennerholm [32].

2.3. AMD thin-layer chromatography of gangliosides

The AMD equipment (CAMAG, Muttenz, Switzerland) consisted of a development unit, a control unit and a vacuum pump. Details of this technique have been described in a previous paper [14]. Gangliosides were chromatographed in chloroform–methanol–20 mM aqueous CaCl$_2$ (120:85:14, v/v). For each sample the procedure was repeated twice and each run was performed for 55 min followed by a vacuum drying period of 10 min [14].

2.4. TLC-immunostaining (overlay-technique)

After chromatography and evaporation of the solvent, the silica gel of the HPTLC plate was fixed with polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany), followed by a specially amended overlay-assay based on the procedure reported by Bethke et al. [19]. The plates were soaked for 15 min in solution A (phosphate-buffered saline (PBS) supplemented with 1% (w/v) bovine serum albumin) for blocking of nonspecifc protein binding and then overlayed for 1 h with polyclonal rabbit anti-GgOse$_4$Cer [20] or chicken anti-GalNAc-G_{M1b} antibodies (see below), diluted 1:2000 in solution A. Unbound antibodies were removed by washing the plate five times with solution B (PBS, 0.05% Tween 21).

Affinity chromatography-purified goat anti-rabbit IgG and rabbit anti-chicken IgG, labeled with alkaline phosphatase (Dianova, Hamburg, Germany) were diluted 1:2000 in solution A. After 1 h incubation, the plates were washed as described above, followed by two-fold rinsing with 0.1 M glycine buffer, pH 10.4, supplemented with 1 mM ZnCl$_2$ and 1 mM MgCl$_2$, for the removal of phosphate. Bound antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) dissolved in the same buffer.

2.5. Detection of G_{M1b}-type gangliosides

After chromatography and silica gel fixation, the HPTLC plate was incubated with 5 mU/ml *V. cholerae* neuraminidase (Behring Werke, Marburg, Germany; EC 3.2.1.18) for 2 h at 37°C according to

Müthing and Mühlradt [20]. This enzyme treatment leads to removal of terminally bound neuraminic acid from G_{M1b} and thus results in generation of $GgOse_4Cer$, which can be detected with specific anti-$GgOse_4Cer$ antibody. G_{M1a}, which carries internally bound neuraminic acid, remains intact [12] and does not bind to the specific anti-$GgOse_4Cer$ antibody [20]. Then the plate was overlayed with the rabbit anti-$GgOse_4Cer$ and alkaline phosphatase-conjugated secondary goat anti-rabbit antibody, followed by visualization as described above.

GalNAc-G_{M1b} gangliosides were identified with a specific chicken antiserum according to previous reports [16,17].

In the case of automated multiple development, the evaporated HPTLC plate was thoroughly sprayed with methanol before and after the silica gel fixation step.

3. Results

3.1. AMD thin-layer chromatography of gangliosides

Due to its high resolving power [12], the three compound solvent mixture chloroform–methanol–20 mM aqueous $CaCl_2$ (120:85:20, v/v) is the most commonly used solvent for standard ganglioside separation. However, several complex ganglioside mixtures have not been separated by conventional single-step chromatography on HPTLC plates. The aim of this work was to achieve complete separation of individual G_{M1b}-type gangliosides within complex mixtures. An increased resolution of distinct bands was achieved by three consecutive runs in the solvent chloroform–methanol–20 mM aqueous $CaCl_2$ (120:85:14, v/v) with reduced polarity compared to that which is optimal for separation using conventional single-step chromatography.

3.2. Conventional single-step chromatography of YAC-1 and MDAY-D2 monosialogangliosides

The murine lymphoma YAC-1 expresses a complex mixture of G_{M1b}-type gangliosides (G_{M1b} and GalNAc-G_{M1b}). Due to the substitution with Neu5Ac and Neu5Gc as well as C_{24}- and C_{16}- fatty acids, four types of G_{M1b} and GalNAc-G_{M1b} are present in YAC-1 cells [16,21]. The same gangliosides (G_{M1b} and GalNAc-G_{M1b}) are expressed by the murine lymphoreticular cell line MDAY-D2, but in contrast to YAC-1 only Neu5Ac was found in MDAY-D2 gangliosides [17] according to Laferté et al. [22].

Table 2
Main monosialogangliosides from murine lymphoma YAC-1 and lymphoreticular MDAY-D2 cells

Ganglioside fraction[a]	No.[b]	Fatty acid	Symbol[b]	Sialic acid	YAC-1	MDAY-D2
-II	1	24:0,24:1	G_{M2}	Neu5Ac	−	+
-I	2	16:0	G_{M2}	Neu5Ac	−	+
0	3	24:0,24:1	G_{M1a}	Neu5Ac	−	+
I	4	16:0	G_{M1a}	Neu5Ac	−	+
	5*	24:0,24:1	G_{M1b} *	Neu5Ac	+	+
II	6*	16:0	G_{M1b} *	Neu5Ac	+	+
	7	24:0,24:1	GalNAc-G_{M1b}	Neu5Ac	+	+
	8*	24:0,24:1	G_{M1b} *	Neu5Gc	+	−
III	9*	16:0	G_{M1b} *	Neu5Gc	+	−
	10	16:0	GalNAc-G_{M1b}	Neu5Ac	+	+
	11	24:0,24:1	GalNAc-G_{M1b}	Neu5Gc	+	−
IV	12	16:0	GalNAc-G_{M1b}	Neu5Gc	+	−

[a] According to Figs. 1 and 2.
[b] Variations of ganglioside G_{M1b}, detectable by immunostaining with anti-$GgOse_4Cer$ antibody after V. cholerae neuraminidase treatment, are marked with asterisks; structural data drawn from Müthing et al. [16] and Müthing et al. [17].

The main monosialoganglioside of MDAY-D2 cells is G_{M1a}(Neu5Ac), carrying C_{24}- and C_{16}-fatty acids, whereas YAC-1 cells express negligible amounts of G_{M1a} type. The predominant monosialogangliosides of both cell lines are listed in Table 2.

The resorcinol stained thin-layer chromatogram of YAC-1 and MDAY-D2 cells is shown in Fig. 1A (lanes b and c, respectively) together with reference gangliosides from human brain, lane a. G_{M1a}(Neu5Ac, C_{24}- and C_{16}-fatty acids) dominates in MDAY-D2 cells (lane c, fraction 0 and I), whereas G_{M1b} is the main monosialoganglioside in YAC-1 cells (lane b, fractions I, II and III), which appear in three bands due to additional substitution with Neu5Gc (see Table 2).

G_{M1b} was detected in YAC-1 and MDAY-D2 monosialogangliosides by incubation of gangliosides with *V. cholerae* neuraminidase followed by specific anti-GgOse$_4$Cer antibody staining on the HPTLC plate (Fig. 1B). The above mentioned variability in the GSL composition of YAC-1 cells leads to ganglioside mixtures, in which different gangliosides e.g. G_{M1b}(Neu5Ac, $C_{16:0}$) and G_{M1b}(Neu5Gc, $C_{24:0,24:1}$) co-chromatograph in one band, i.e. compounds 6 and 8 in band II (see Table 2 and Fig. 1B, lane b). Regardless of solvents used, until now these components still could not be separated within whole ganglioside mixtures by conventional single chromatography.
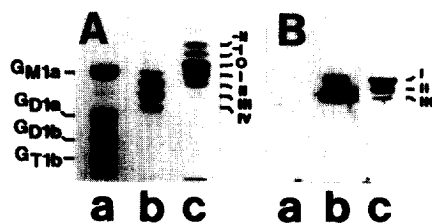
3.3. AMD thin-layer chromatography of YAC-1 and MDAY-D2 monosialogangliosides

Threefold AMD separation was performed in chloroform–methanol–20 mM aqueous CaCl$_2$ (120:85:14, v/v). The TLC resorcinol stain is shown in Fig. 2A, indicating splitting of ganglioside bands II and III. As demonstrated in Fig. 2B, lane (b), four clearly resolved G_{M1b}-bands (compounds 5, 6, 8 and 9, see Table 2) are detectable in YAC-1 gangliosides by the immunostaining procedure. G_{M1b}(Neu5Gc, $C_{24:0,24:1}$; No. 8) and G_{M1b}(Neu5Gc, $C_{16:0}$; No. 9) are not expressed by MDAY-D2 cells (Fig. 2B, lane c), whereas G_{M1b}(Neu5Ac, $C_{24:0,24:1}$; No. 5) and G_{M1b}(Neu5Ac, $C_{16:0}$; No. 6) are present in both cell lines. The main monosialogangliosides G_{M1a}(Neu5Ac, $C_{24:0,24:1}$; No. 3, band 0) and G_{M1a}(Neu5Ac, $C_{16:0}$; No. 4 of band I) of MDAY-D2 (see Table 2), which interfere with corresponding G_{M1b} species during single chromatography (see Fig. 1, lanes c), showed higher R_F values after AMD separation, resulting in distinct immunostaining of G_{M1b} free from any interference (Fig. 2B, lane c).

GalNAc-G_{M1b} variants (see Table 2) showed chromatographical behaviour comparable to their G_{M1b} counterparts. According to sialylation and fatty acid substitution, four individual bands were detected

Fig. 1. Detection of G_{M1b} in monosialoganglioside fractions of murine lymphomas YAC-1 and MDAY-D2. (A) Resorcinol stain and (B) immnunostain with specific anti-GgOse$_4$Cer antibodies after neuraminidase treatment. Gangliosides (10 µg) from human brain (a) and 5 µg each of monosialogangliosides from YAC-1 (b) and MDAY-D2 (c) were chromatographed for 35 min in chloroform–methanol–20 mM aqueous CaCl$_2$ (120:85:20, v/v). The positions of human brain gangliosides (see Table 1) are marked in the margin. Gangliosides of fractions -II to IV are listed in Table 2.
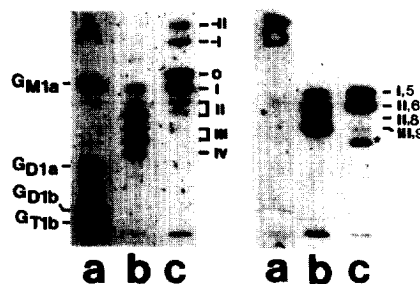
Fig. 2. Detection of G_{M1b} in monosialoganglioside fractions of murine lymphomas YAC-1 and MDAY-D2 after automated multiple development. (A) Resorcinol stain and (B) immunostain with specific anti-GgOse$_4$Cer antibodies after neuraminidase treatment. Gangliosides (10 µg) from human brain (a) and 5 µg each of monosialogangliosides from YAC-1 (b) and MDAY-D2 (c) were chromatographed three-fold for 55 min, each, in chloroform–methanol–20 mM aqueous CaCl$_2$ (120:85:14, v/v). The positions of human brain gangliosides (see Table 1) are marked in the margin. Gangliosides of fractions -II to IV are listed in Table 2. *=unknown compound.

Table 3
Synopsis of AMD-separation of G_{M1b} and GalNAc-G_{M1b} gangliosides from YAC-1 and MDAY-D2 cells

Standard single chromatography[a]	AMD chromatography	Ganglioside
I,5	I,5	G_{M1b}(Neu5Ac, $C_{24:0,24:1}$)
II,6,7,8	II,6	G_{M1b}(Neu5Ac, $C_{16:0}$)
	II,7	G_{M1b}(Neu5Gc,$C_{24:0,24:1}$)[b]
	II,8	GalNAc-G_{M1b}(Neu5Ac, $C_{24:0,24:1}$)[b,*]
III,9,10,11	III,9	G_{M1b}(Neu5Gc, $C_{16:0}$)[c]
	III,10	GalNAc-G_{M1b}(Neu5Ac, $C_{16:0}$)[c,*]
	III,11	GalNAc-G_{M1b}(Neu5Gc, $C_{24:0,24:1}$)[*]
IV,12	IV,12	GalNAc-G_{M1b}(Neu5Gc, $C_{16:0}$)[*]

[a] Abbreviations of gangliosides and designation of ganglioside bands according to Figs. 1 and 2, and Table 2.
[b] Co-chromatograph in one band.
[c] Co-chromatograph in one band.
Variations of ganglioside GalNAc-G_{M1b}, detectable by immunostaining with anti-GalNAc-G_{M1b} antibody, are marked with asterisks.

with the anti-GalNAc-G_{M1b} antibody, which chromatographed in the order (from top) GalNAc-G_{M1b}(Neu5Ac, $C_{24:0,24:1}$), GalNAc-G_{M1b}(Neu5Ac, $C_{16:0}$), GalNAc-G_{M1b}(Neu5Gc, $C_{24:0,24:1}$) and GalNAc-G_{M1b}(Neu5Gc, $C_{16:0}$) as listed in Table 3. Compounds II,7 and II,8 as well as gangliosides III,9 and III,10 do not separate as single substances even after AMD treatment. However, these four gangliosides could be clearly identified by specific TLC immunostaining (see Table 3).

It should be added, that immunostaining following AMD separation gave negative results in the beginning. However, this problem was solved by thoroughly spraying the silica gel layer with methanol after AMD separation, i.e. before and after silica gel fixation.

4. Discussion

G_{M1b}-type gangliosides are predominant in murine spleen [23], splenic T lymphoblasts, thymocytes [24,25] and in stimulated mouse macrophages [26] as well as in macrophage like WEHI-3 cells [27]. Furthermore, G_{M1b} was found to be strongly expressed in embryonic, but only in minute amounts in adult brains [28] suggesting embryonic stage specific appearance of this GSL. The technique described is helpful for the specific detection of this ganglioside species in complex GSL mixtures, in particular to

eliminate interference of G_{M1b}-type gangliosides with abundant monosialoganglioside G_{M1a}, which are both known to be typical compounds of e.g. murine spleen and thymus [29]. However, G_{M1a}(Neu5Ac, $C_{16:0}$) and G_{M1b}(Neu5Ac, $C_{24:0,24:1}$) still chromatograph together (fraction I, see Fig. 2), but spreading of the bulk of monosialogangliosides resulted in lowered interference of e.g. dominant G_{M1a}(Neu5Ac, $C_{24:0,24:1}$) with minor G_{M1b}(Neu5Ac, $C_{24:0,24:1}$) in the monosialoganglioside fraction of MDAY-D2 cells as shown in Figs. 1 and 2. In general this procedure enabled detection of minor gangliosides which were screened by major gangliosides in GSL mixtures.

5. Conclusion

In this study the applicability of the AMD thin-layer chromatography for analytical detection of individual gangliosides within complex mixtures is described. As a general rule, the chromatographic solvent chosen should be less polar than that which is optimal for separation of the compounds by standard single step TLC. This is generally achieved by reducing the most polar component, e.g. the aqueous content within the chloroform–methanol–20 mM aqueous $CaCl_2$ mixture. To achieve higher resolution, i.e. enhanced separation compared to conventional single chromatography, the HPTLC

plate can be developed multiple times with intermediate drying periods.

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