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Improved thin-layer chromatographic separation of gangliosides by automated multiple development

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Abstract

Automated multiple development chromatography has been utilized to enhance separation of gangliosides on silica-gel precoated high-performance TLC plates. Three-fold chromatography of a complex mixture of neolacto-series monosialogangliosides in the solvent chloroform–methanol–water (120:85:14, v/v, 2 m*M* $CaCl_2$) resulted in a *ca.* three-fold increase in separation distance of *e.g.* α2-3 and α2-6 sialylated ganglioside isomers compared to conventional single chromatography in the standard solvent chloroform–methanol–water (120:85:20, v/v, 2 m*M* $CaCl_2$). An extremely heterogenous murine disialoganglioside mixture was developed three times in chloroform–methanol–water (120:85:16, v/v, 2 m*M* $CaCl_2$) leading to a more than two-fold increase in separation distance. Chloroform–methanol–water (120:85:22, v/v, 2 m*M* $CaCl_2$) was the solvent of choice for multiple chromatography of ganglio-series polysialogangliosides from embryonic chicken brain.

1. Introduction

Gangliosides are membrane-bound glycosphingolipids (GSLs)[1], containing one or more sialic acid residues. Their structures and functions have been widely reviewed [1–3]. Due to their localization in the outer leaflet of the cell membrane they act as receptors for toxins, bacteria [4], viruses [5] and other ligands and are assumed to modulate a variety of biological functions [6,7] including cell–cell recognition phenomena and the development of antitumor immunity [8].

Analytical and preparative high-performance thin-layer chromatography (HPTLC) are widely used for separation and identification as well as for isolation and purification of individual GSLs [9]. One-dimensional TLC is the simplest and still commonly used method for analysis of ganglioside mixtures. This method has been continuously optimized with emphasis on the minor components of the total ganglioside mix-

[1] Abbreviations used: AMD, automated multiple development; Neu5Ac, N-acetylneuraminic acid [33]. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations [34] and the nomenclature of Svennerholm [35]. Lactosylceramide or LacCer, Galß1-4Glcß1-1Cer; gangliotetraosylceramide or $GgOse_4Cer$, Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer; lacto-N-neotetraosylceramide or nLcOse$_4$Cer, Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Galß1-4Glcß1-1Cer; G_{M3}, II^3Neu5Ac-LacCer; G_{M1} or G_{M1a}, II^3Neu5Ac-$GgOse_4Cer$; G_{D1a}, IV^3Neu5Ac, II^3Neu5Ac-$GgOse_4Cer$; G_{D1b}, $II^3(Neu5Ac)_2$-$GgOse_4Cer$; G_{T1b}, IV^3Neu5Ac, $II^3(Neu5Ac)_2GgOse_4Cer$; G_{Q1b}, $IV^3(Neu5Ac)_2$, $II^3(Neu5Ac)_2$-$GgOse_4Cer$; G_{Q1c}, IV^3Neu5Ac, $II^3(Neu5Ac)_3$-$GgOse_4Cer$; G_{P1c}, $IV^3(Neu5Ac)_2$, $II^3(Neu5Ac)_3$-$GgOse_4Cer$; G_{H1c}, $IV^3(Neu5Ac)_3$, $II^3(Neu5Ac)_3$-$GgOse_4Cer$.

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tures. The combination of two consecutive unidirectional runs in different solvent systems led to improved separation of multisialogangliosides [10]. Two-dimensional TLC has been developed specifically for the recognition of alkali-labile gangliosides [11]. Furthermore, the technique of short-bed continuous development thin layer chromatography of GSLs has been utilized to enhance GSL separation [12]. Increased separation by TLC can be achieved by decreasing solvent strength, the basic theory for short-bed continuous [13] and programmed multiple development TLC [14].

In this study an improved method for the separation of gangliosides on HPTLC plates by automated multiple development is described, that permits high-resolution separation of complex ganglioside mixtures. The theoretical goal of increasing the separation of the different bands by decreasing the solvent strength was achieved by three consecutive runs in the same solvent but with lower polarity than that which is optimal for separation by conventional single HPTLC. A preliminary report of some results has been published [15].

2. Experimental

2.1. Gangliosides

Human leukocytes enriched with granulocytes were prepared from buffy coats as described previously [16] and were kindly provided by Prof. Dr. H. Tschesche (Institute of Biochemistry, University of Bielefeld, Bielefeld, Germany). Gangliosides were isolated and purified by standard procedures [2] as described in refs. 17 and 18.

Gangliosides from murine lymphoma YAC-1 cells were isolated as recently published [19]. Whole gangliosides were separated into mono- and disialoganglioside fractions by gradient anion-exchange chromatography as described previously [20].

Brains from 16 days old chicken embryos were disintegrated and extracted with chloroform (C)/methanol (M) (2:1), (1:1) and (1:2) (v/v). The combined extracts were evaporated, dissolved in water and dialysed against water. The dialysate was freeze-dried and neutral GSLs and gangliosides were separated by anion-exchange chromatography on a DEAE-Sepharose CL6B column in the acetate form [20]. Finally, gangliosides were taken up in C/M (85:15), applied to an Iatrobeads RS8060 (Macherey-Nagel, Düren, Germany) column and purified by stepwise elution with C/M (4:1), (3:1), (2:1), (1:1), (2:3) and (1:2) (v/v).

A mixture of reference gangliosides from human brain was purchased from Supelco (Bellefonte, PA, USA). Purified bovine brain G_{T1b} and G_{Q1b} were from Dr. Pallmann GmbH (Munich, Germany).

2.2. Analytical and preparative thin layer chromatography

Silica-gel precoated high-performance thin layer chromatography plates (HPTLC plates, 10×10 cm, thickness 0.24 mm, Merck, Darmstadt, Germany) were used for analytical and preparative purposes. Conventional single chromatography of gangliosides was performed for 35 min in the solvent C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) and visualized by resorcinol [21].

Preparative GSL amounts were applied to the HPTLC plates with an automated sample applier Linomat IV (CAMAG, Muttenz, Switzerland). GSLs were visualized with primulin (Aldrich-Chemie, Steinheim, Germany) 0.001% (w/v) in acetone–H_2O (8:2) [22]. Zones containing GSLs were localized under UV light, scraped off and the silica gel was transferred to small columns with sintered glass plugs. Gangliosides were freed from primulin by stepwise gradient elution with isopropanol–hexane–water from 55:42:3 to 55:10:35 (v/v) according to Levery *et al.* [23].

2.3. The automated multiple development (AMD) system

The AMD equipment (CAMAG, Muttenz, Switzerland) consisted of a development unit, a control unit and a vacuum pump. Details of the development unit are described in Fig. 1.

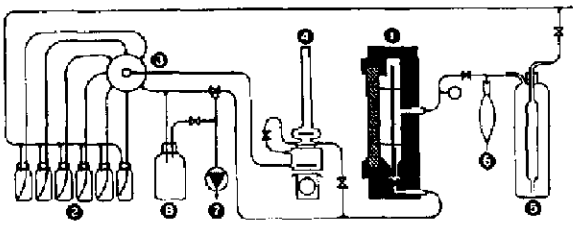

Fig. 1. Flow diagram of the AMD developing unit. Development chamber (1), solvent reservoir bottles (2), motor driven valve (3), gradient mixer (4), wash bottle (5), gas phase reservoir (6), vacuum pump (7), waste collection bottle (8).

2.4. AMD chromatography of gangliosides

Gangliosides were separated on HPTLC plates by three-fold automated multiple development. Monosialogangliosides of human granulocytes were chromatographed in C–M–W (120:85:14, v/v, 2 m*M* $CaCl_2$). Disialogangliosides from murine YAC-1 lymphoma cells were separated in C–M–W (120:85:16, v/v, 2 m*M* $CaCl_2$) and polysialogangliosides of embryonic chicken brain were developed in C–M–W (120:85:22, v/v, 2 m*M* $CaCl_2$), each three times in the same solvent mixture. All runs were performed for 55 min each with intermediate vacuum drying periods of 10 min.

3. Results and discussion

3.1. AMD chromatography of gangliosides

High-performance thin layer chromatography is one of the major techniques for analysis of GSLs and its ease and reproducibility make it an ideal method for analytical and preparative purposes [24]. However, several complex ganglioside mixtures have been described, that could not be separated by conventional one-step chromatography on HPTLC plates. The aim of this work was to improve in some cases the unsatisfactory separations of individual gangliosides in complex mixtures considering analytical as well as preparative HPTLC.

An increased resolution of bands was achieved by three consecutive runs in a solvent with reduced polarity compared with the optimal separation of the compounds in question by conventional single-step HPTLC. Three-fold chromatography was performed by automated multiple development as described in the Experimental section. The three compound solvent C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) was the basic solvent due to its high resolution power and its wide applicability in ganglioside separation. A lower polarity, obtained by decreasing the water content, led to improved separation of complex mono- and disialoganglioside mixtures whereas increased polarity gave excellent separation of polysialogangliosides as demonstrated in the following sections.

3.2. AMD chromatography of human granulocytes gangliosides

Monosialogangliosides II^3Neu5Ac-LacCer (G_{M3}), IV^3Neu5Ac-nLcOse$_4$Cer, IV^6Neu5Ac-nLcOse$_4$Cer, and VI^3Neu5Ac-nLcOse$_6$Cer are the main gangliosides in human granulocytes. The fractions with higher R_f values of ganglioside pairs are characterized by the presence of $C_{24:1}$ and to some extent by $C_{22:0}$ fatty acids in their ceramide portions, whereas $C_{16:0}$ fatty acids are characteristic for the fractions showing lower R_f values [17,25]. The structures of the main gangliosides from human granulocytes are listed in Table 1. As shown in Fig. 2A, single chromatography in the conventional solvent C–M–W (120:85:20, v/v, with 2 m*M* $CaCl_2$) led to a set of eight tightly spaced bands. Three-fold development in C–M–W (120:85:14, v/v, 2 m*M*

Table 1
Structures of gangliosides from human granulocytes

No.[a]	Fatty acid	Structure
1	24:1, 22:0	II^3Neu5Ac-LacCer (G_{M3})
2	16:0	II^3Neu5Ac-LacCer (G_{M3})
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] According to Fig. 2.

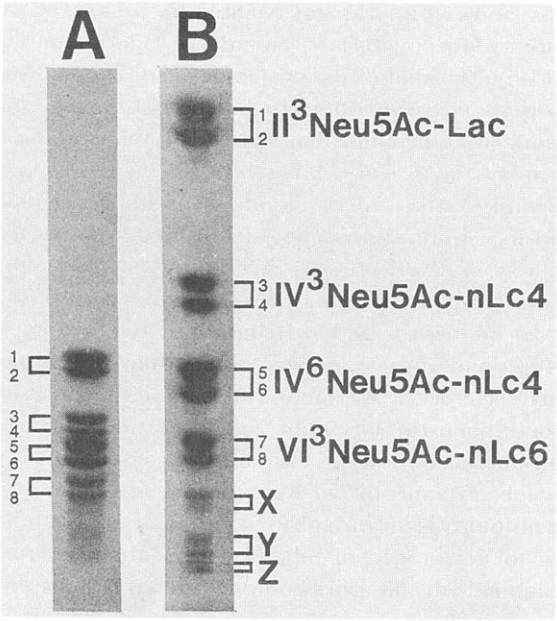

Fig. 2. AMD chromatography of human granulocytes gangliosides. Gangliosides (15 μg) were chromatographed in C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) (A) and separated by three-fold automated multiple development in C–M–W (120:85:14, v/v, 2 m*M* $CaCl_2$) (B). Gangliosides were visualized by resorcinol. Structures of gangliosides are listed in Table 1. Lac, lactosylceramide; nLc4, $nLcOse_4Cer$; nLc6, $nLcOse_6Cer$.

$CaCl_2$) resulted in a *ca.* three-fold increase in separation distance of the four GSL pairs (Fig. 2B). Enhanced resolution of *e.g.* bands 4 and 5, corresponding to $IV^3Neu5Ac\text{-}nLcOse_4Cer$ (C_{16} fatty acid) and $IV^6Neu5Ac\text{-}nLcOse_4Cer$ (C_{24} fatty acid), now permits feasible and more efficient preparative HPTLC of these former closely spaced GSLs [24]. Moreover, the unidentified gangliosides designated X, Y and Z, will now become accessible for structural characterization by combination of AMD separation and preparative HPTLC. Since neolacto-series gangliosides from human granulocytes (and from cells of other species) were found to bind *e.g.* to influenza and Sendai virus [17,26] as well as to a variety of microorganisms [4] the described improved high-resolution separation would *e.g.* facilitate the specific detection of microorganism–ganglioside interactions on the HPTLC plate and furthermore enables the isolation of these important receptor molecules by preparative HPTLC in an effective, easy and convenient way [24].

3.3. AMD chromatography of disialogangliosides from murine lymphoma YAC-1 cells

The resorcinol stained chromatogram of the disialoganglioside fraction from YAC-1 cells showed a continuum of poorly separated bands after conventional HPTLC as demonstrated in

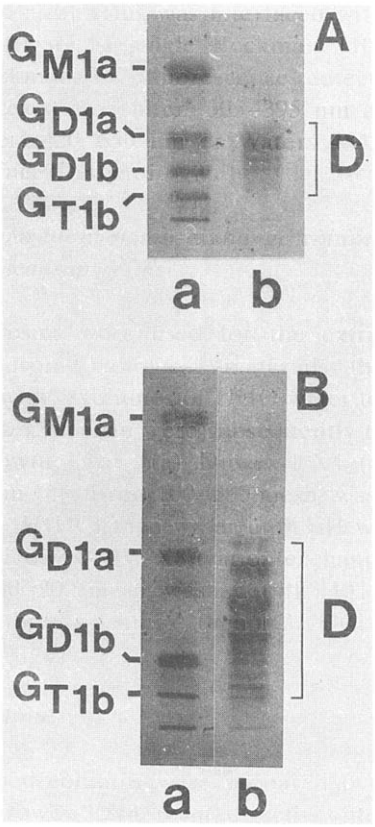

Fig. 3. AMD chromatography of disialogangliosides from murine lymphoma YAC-1 cells. Human brain gangliosides (10 μg) (lane a, references) and 5 μg disialogangliosides from YAC-1 (lane b) were chromatographed in C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) (A) and separated by threefold automated multiple development in C–M–W (120:85:16, v/v, 2 m*M* $CaCl_2$) (B). Gangliosides were visualized by resorcinol. The positions of human brain gangliosides are marked in the margin. D, disialoganglioside fraction.

Fig. 3A (lane b). Improved separation of this highly complex mixture, consisting of several different yet unknown components, which chromatograph between reference G_{D1a} and G_{T1b}, was obtained by three-fold chromatography in C–M–W (120:85:16, v/v, 2 m*M* $CaCl_2$) as demonstrated in Fig. 3B (lane b). This mixture will now also become accessible for structural characterization by combined AMD separation and preparative HPTLC.

3.4. *AMD chromatography of polysialogangliosides from embryonic chicken brain*

Conventional chromatography of gangliosides with more than three sialic acids results in poor resolution due to their very slow migration rates. For example, single HPTLC of polysialogangliosides, isolated from embryonic chicken brains, gave insufficient resolution in C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) as shown in Fig. 4. A special advantage of the AMD technique is the excellent separation of highly polar gangliosides, moving more slowly than G_{T1b}, which was achieved by three-fold chromatography in C–M–W (120:85:22, v/v, 2 m*M* $CaCl_2$) as demonstrated in Fig. 5 (lane c). Five fractions of individual polysialogangliosides, corresponding to G_{T1b}(I), G_{Q1b}(II), G_{Q1c}(III), G_{P1c}(IV) and G_{H1c}(V) (Fig. 5, lanes d–h), were isolated by preparative HPTLC after AMD separation. Their structures are listed in Table 2. The presented data show that the combination of AMD chromatography and preparative HPTLC is successful for the isolation of individual polysialogangliosides from complex GSL mixtures like those of *e.g.* fishes [27,28] and embryonic brains [29,30]. The technique described offers a

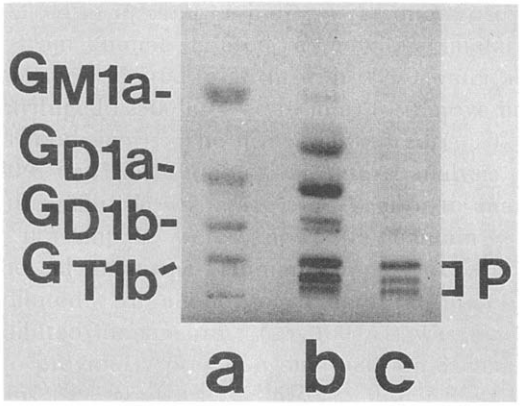

Fig. 4. Thin layer chromatography of embryonic chicken brain gangliosides. Human brain gangliosides (5 μg) (lane a), 10 μg gangliosides of Iatrobeads C–M (1:1) eluate (lane b) and 5 μg gangliosides of Iatrobeads C–M (2:3) eluate from embryonic chicken brains (lane c) were chromatographed in C–M–W (120:85:20, v/v, 2 m*M* $CaCl_2$) and stained with resorcinol. The positions of human brain gangliosides are marked in the margin. P, polysialogangliosides.

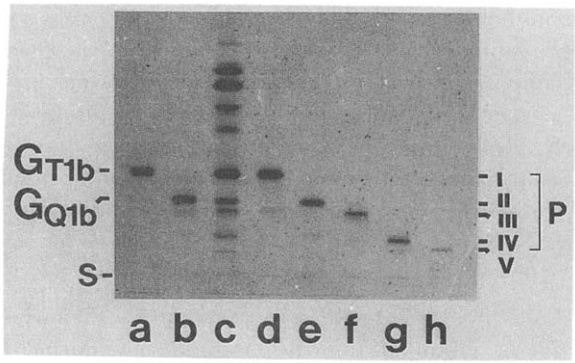

Fig. 5. AMD chromatography of individual polysialogangliosides from embryonic chicken brains. Gangliosides were chromatographed three-fold in C–M–W (120:85:22, v/v, with 2 m*M* $CaCl_2$) and stained with resorcinol: (lane a) 1 μg G_{T1b} from bovine brain (reference), (lane b) 1 μg G_{Q1b} from bovine brain (reference), (lane c) 10 μg of embryonic chicken brain gangliosides, C–M (2:3) Iatrobeads eluate. Individual polysialogangliosides from embryonic chicken brains isolated by preparative HPTLC were applied as follows: 1 μg G_{T1b} (lane d, I), 1 μg G_{Q1b} (lane e, II), 1 μg G_{Q1c} (lane f, III), 1 μg G_{P1c} (lane g, IV), 0.25 μg G_{H1c} (lane h, V). Structures of polysialogangliosides are listed in Table 2. P, polysialogangliosides; S, start.

Table 2
Structures of polysialogangliosides from embryonic chicken brain

No.[a]	Symbol	Structure
I	G_{T1b}	$IV^3Neu5Ac,II^3(Neu5Ac)_2$-GgOse$_4$Cer
II	G_{Q1b}	$IV^3(Neu5Ac)_2,II^3(Neu5Ac)_2$-GgOse$_4$Cer
III	G_{Q1c}	$IV^3Neu5Ac,II^3(Neu5Ac)_3$-GgOse$_4$Cer
IV	G_{P1c}	$IV^3(Neu5Ac)_2,II^3(Neu5Ac)_3$-GgOse$_4$Cer
V	G_{H1c}	$IV^3(Neu5Ac)_3,II^3(Neu5Ac)_3$-GgOse$_4$Cer

[a] According to Fig. 5.

convenient approach to analyze ganglioside expression with particular reference to the minor components within the total ganglioside mixture, components that might be relevant for cell proliferation and differentiation processes *e.g.* during brain development and aging [31] or in neuronal degeneration occurring for instance in Alzheimer's disease [32].

4. Conclusions

This study describes the applicability of AMD chromatography for the analytical detection and preparative isolation of individual gangliosides in complex mixtures. Examples are given for high-resolution separation of mono-, di- and polysialoganglioside fractions. The initial choice of chromatographic solvent should be one which is less polar than that which is optimal for the separation of the compounds in question by conventional single TLC. As demonstrated in this study, this generally implicates the preparation of a less polar solvent mixture by reducing the amount of the most polar component, *i.e.* water, in the chloroform–methanol–water mixture. The HPTLC plate can be developed multiple times, with intermediate drying periods, to achieve a higher resolution, *i.e.* enhanced separation, than can be obtained by single chromatography. The examples given should serve as a starting point for further applications.

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