Short bed–continuous development thin-layer chromatography of glycosphingolipids

William W. Young, Jr.[1] and Cheryl A. Borgman

Department of Pathology, University of Virginia Medical Center, Charlottesville, VA 22908

Summary The technique of short bed–continuous development chromatography has been utilized to increase the separation of glycosphingolipids on high performance thin-layer chromatography plates. The theoretical goal of increasing separation of bands by decreasing solvent strength was achieved within a practical time span as a result of the high solvent velocities in the short bed tank. Examples are given for increased separation of short and long chain neutral glycolipids, acetylated neutral glycolipids, and gangliosides. —**Young, W. W., Jr., and C. A. Borgman.** Short bed–continuous development thin-layer chromatography of glycosphingolipids. *J. Lipid Res.* 1986. **27:** 120–124.

Supplementary key words gangliosides • neutral glycolipids • TLC-immunoblotting

Abbreviations: ceramide dihexoside (CDH), Gal β 1 → 4 GlcCer; gangliotriaosylceramide ($GgOse_3Cer$; asialo GM2), GalNAc β 1 → 4 Gal β 1 → 4 GlcCer; globoside, GalNAc β 1 → 3 Gal α 1 → 4 Gal β 1 → 4 GlcCer; Forssman glycolipid, GalNAc α 1 → 3 GalNAc β 1 → 3 Gal α 1 → 4 Gal β 1 → 4 GlcCer; N-glycolylhematoside (GM3), NeuNGc α 2 → 3 Gal β 1 → 4 GlcCer; GM1, Gal β 1 → 3 GalNAc β 1 → 4 [NeuNAc α 2 → 3] Gal β 1 → 4 GlcCer; GM1b, NeuNAc α 2 → 3 Gal β 1 → 3 GalNAc β 1 → 4 Gal β 1 → 4 GlcCer; GT1, NeuNAc α 2 → 3 Gal β 1 → 3 GalNAc β 1 → 4 [NeuNAc α 2 → 8 NeuNAc α 2 → 3] Gal β 1 → 4 GlcCer. TLC, thin-layer chromatography; CD-HPTLC, continuous development high performance thin-layer chromatography.

[1] Address for reprint requests: Dr. William W. Young, Jr., Department of Pathology, Box 214, University of Virginia Medical Center, Charlottesville, VA 22908.

Cell surface glycosphingolipids have been implicated as antigens (1), mediators of cell growth control (2, 3), and as receptors for toxins (4, 5) and microbes (6–8). Thin-layer chromatography is one of the major techniques used for the analysis and purification of glycosphingolipids. In recent years high performance thin-layer chromatography (HPTLC) plates have been widely used because of increased band resolution as compared to conventional plates. However, HPTLC plates have the disadvantage that the bands, although clearly resolved, may be very closely spaced. Thus, scraping of individual bands from HPTLC plates may be difficult.

As described in detail by Perry (9), the separation by TLC of any two components can be increased in almost all cases by decreasing the solvent strength, which for practical purposes is equivalent to decreasing solvent polarity. The limitation of this theory is that a decrease of solvent strength is accompanied by an exponential decrease in R_f (9). This difficulty can be overcome by the high solvent velocities achieved by continuous development in a short bed chamber. The inverse relationship between solvent velocity and bed length is illustrated elsewhere (10). The present report described the use of continuous development (CD-HPTLC) for increasing the separation of several types of glycosphingolipids.

MATERIALS AND METHODS

Materials

The short bed–continuous development chamber was obtained from Regis Chemical Co. (Morton Grove, IL). Glass-backed and aluminum-backed HPTLC plates were

obtained from E. Merck (Federal Republic of Germany). Enhance spray was obtained from New England Nuclear (Boston, MA).

Glycolipid standards were purified from the following sources using established procedures (11): ceramide dihexoside (CDH) and globoside from human erythrocytes, N-glycolyl hematoside (GM3) from horse erythrocytes, gangliotriaosylceramide ($GgOse_3Cer$) from guinea pig erythrocytes, gangliosides GM1 and GT from bovine brain, and Forssman ceramide pentasaccharide from sheep erythrocytes. The neutral glycolipids from human meconium were partially purified by Iatrobead chromatography as previously described (12). Mouse L5178Y lymphoma cells were metabolically labeled with [^{3}H]galactose, and the glycolipids were purified as previously described (13).

Methods

Continuous development HPTLC was performed by applying samples at the origin, 1.5 cm from the bottom of the plate. The short bed-continuous development tank was filled with either 50 ml of solvent B or D or 30 ml of solvent F (see below). Plates were placed in position 4 (next to the most horizontal position) for solvents B and D and position 3 for solvent F; see Fig. 1 of ref. 10 which shows the plate positions. In CD-HPTLC the solvent reached the top of the tank in about 10–15 min, and development was allowed to continue for the times indicated below. For conventional HPTLC, plates were removed from tanks when the solvent reached the top of the plate (8.5 cm bed length). Solvents and development times: A, chloroform–methanol–water (C–M–W) 62:30:6 (v/v), 30 min; B, C–M–W 75:18:2.5, 2.5 hr; C, C–M–0.25% $CaCl_2$ in water 60:40:9, 37 min; D, C–M–0.25% $CaCl_2$ 62.5:30:6, 2 hr; E, C–M–W 55:40:11, 47 min; and F, C–M–W 60:35:8, 45 min.

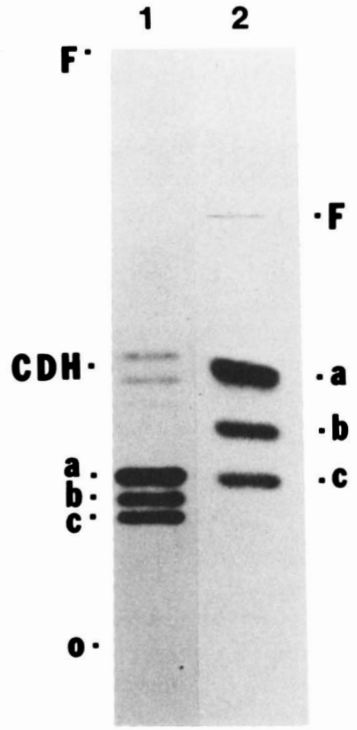

Fig. 1. Comparison of gangliotriaosylceramide bands separated by conventional versus CD-HPTLC. Lymphoma cells were metabolically labeled with [^{3}H]galactose and extracted, and the lower phase glycolipids were purified by the acetylation procedure. Aliquots (5,000 cpm) were chromatographed on glass-backed HPTLC plates as follows: lane 1, regular chamber using solvent A; lane 2, continuous development chamber (plate at position 4) using solvent B. After spraying the plates with Enhance spray, fluorography was performed by exposing the plates to X-ray film at −70°C for 2 days (lane 1) or 3 days (lane 2). F, solvent front; O, origin. CDH, ceramide dihexoside. R_f of glycolipid standards: lane 1, CDH 0.42, $GgOse_3Cer$ (from guinea pig erythrocytes) 0.26, and globoside 0.19; lane 2, CDH 1.0, $GgOse_3Cer$ 0.65, and globoside 0.29.

Antibody staining of TLC plates was performed as previously described (12) based on the method of Magnani et al. (14). Briefly, aliquots of human meconium neutral glycolipids were applied to aluminum-backed HPTLC plates and chromatographed as described in Fig. 3. After drying, the sheets were dipped in hexane containing 0.05% polyisobutylmethacrylate (Polysciences, Warrington, PA), blocked with 5% bovine serum albumin in phosphate-buffered saline, and then incubated in succession with anti-Lewis a antibody CF4C4 and iodinated staphylococcal protein A. After washing and drying, the sheets were exposed at −70°C for 1–2 hr to Kodak XAR-5 film utilizing an intensifying screen.

RESULTS

Neutral glycolipids

Gangliotriaosylceramide (asialo GM2), the major neutral glycolipid of mouse L5178Y lymphoma cells, is present in the cells in three forms separable by HPTLC (13). The fastest migrating form was found previously to contain mainly C24 fatty acids, the middle band C16 fatty acid, and the slowest band C16 alpha-hydroxy fatty acid (15, 16). Although the three bands were clearly resolved by conventional HPTLC (**Fig. 1**, lane 1), the bands were too closely spaced (5 mm between bands a and c) to allow them to be scraped separately in order to quantitate incorporation of radiolabeled precursors into individual bands. However, chromatography in a continuous development chamber resulted in a threefold increase in separation of the three bands (Fig. 1, lane 2), thus making scraping of separate bands a straightforward process. Even though chromatography proceeded for 2.5 hr, the bands were nearly as sharp as those obtained by conventional HPTLC.

Gangliosides

Mouse L5178Y lymphoma cells contain a major ganglio-

side that was previously identified as GM1b (13). Chromatography in a conventional tank produced a set of four bands (**Fig. 2**, lane 1), which were too tightly spaced to permit individual bands to be scraped separately. In contrast, continuous development increased band separation threefold (Fig. 2, lane 2) and made the scraping of each band feasible.

Acetylated neutral glycolipids

The following example was chosen to illustrate the improved separation of acetylated glycolipids that is possible using CD-HPTLC. Acetylated gangliotriaosylceramide and acetylated globoside co-migrate when chromatographed in a conventional tank using the solvent dichloroethane–acetone 55:45 (v/v). Initial attempts with CD-HPTLC utilized less polar mixtures of dichloroethane and acetone. The optimal conditions of dichloroethane–acetone 80:20 for 3 hr, bed length 6.2 cm produced a separation of bands of only 3 mm. However, diluting the solvent with hexane produced a dramatic increase in band separation. Continuous development in the solvent dichloroethane–acetone–hexane 20:30:50 for 1.5 hr separated the bands by 1.5 cm (bed length 5.8 cm, acetylated $GgOse_3Cer$ R_f 0.81, acetylated globoside R_f 0.55).

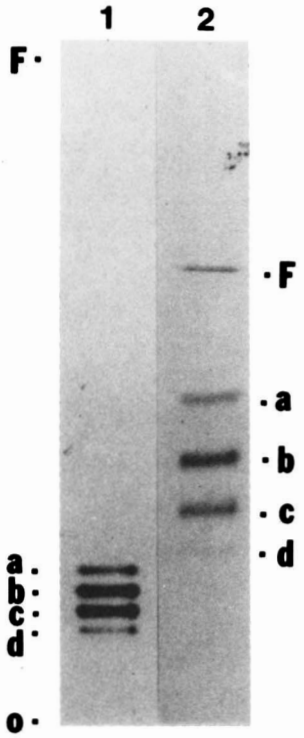

Fig. 2. Comparison of ganglioside GM1b bands separated by conventional versus CD-HPTLC. Lymphoma cells were metabolically labeled with [^{3}H]galactose and extracted, and upper phase lipids were purified on Bond Elut C18 reverse phase columns. Aliquots (10,000 cpm) were chromatographed on glass-backed HPTLC plates as follows: lane 1, regular chamber using solvent C; lane 2, continuous development chamber (plate at position 4) using solvent D. Fluorography was performed as described in Fig. 1, using an exposure time of 3 days at −70°C for both lanes 1 and 2. R_f of ganglioside standards: lane 1, N-glycolyl hematoside (GM3) 0.33, GM1 0.19, and GT 0.01; lane 2, GM3 1.0, GM1 0.77, and GT 0.04.

TLC–immunostaining

The technique developed by Magnani et al. (14) for staining glycolipids on TLC plates with monoclonal antibodies has been of great value in identifying new glycolipid species, many of which are highly complex structures (17). The following example was chosen to indicate the increased resolution of TLC–immunostaining that can be achieved using CD-HPTLC. Human meconium contains several glycolipids that bear the Lewis a human blood group determinant (Gal β 1 → 3[Fuc α 1 → 4] GlcNAc) as we described previously (12). By conventional HPTLC these reactive species appear as two bands, a faster band that co-migrates with authentic Lewis a ceramide pentasaccharide and a broad slower band (**Fig. 3**, lane 1). CD-HPTLC in solvent F not only increased the separation between the two main bands but also separated the slower main band into two components (Fig. 3, lane 2). An alternative solvent for separating complex glycolipids consists of n-propanol–water 80:20, which produced excellent separation in the GM1 to GT area when plates were run in position 3 for 1.5–2 hr (data not shown).

DISCUSSION

The short bed–continuous development HPTLC method should be of use for the analysis and preparative isolation of glycosphingolipids. One of the drawbacks to conventional HPTLC analysis is the difficulty of separating complex glycolipids in chloroform–methanol systems (reviewed in ref. 18). For example, type 3 chain blood group H and A reactive glycolipids were not recognized within mixtures of type 2 chain species until a monoclonal antibody reactive with type 3 chain structures became available (19). Several approaches have been taken to improve the separation of these complex glycolipids: *1*) some poorly resolved glycolipids can be well separated after acetylation; *2*) certain glycolipid mixtures can be separated on borate-impregnated plates (20); and *3*) a conventional plate can be developed multiple times with intermediate drying to achieve greater separation than that possible by a single development (21). Continuous development using a short bed tank accomplishes the same goal of the latter approach but in a much more efficient fashion. The high solvent velocities that can be achieved using the short bed system make it possible to achieve the theoretical goal of increasing separation of bands by decreasing solvent strength (9).

The several examples listed in this report should serve

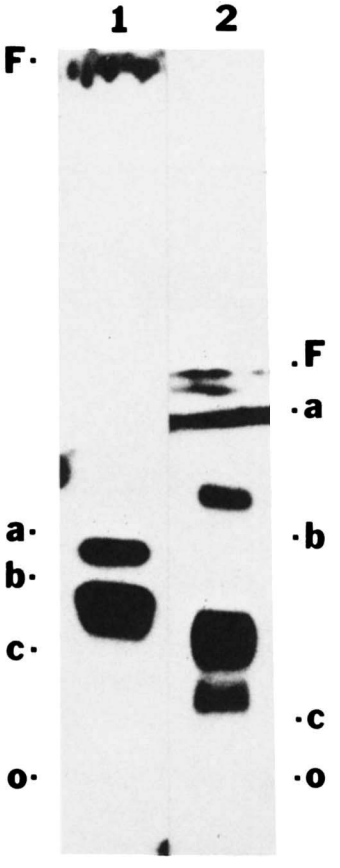

Fig. 3. Comparison of blood group Lewis a reactive glycolipids separated by conventional versus CD-HPTLC and visualized by TLC-immunostaining. Meconium neutral glycolipids were chromatographed on aluminum-backed HPTLC plates as follows: lane 1, regular chamber using solvent E; lane 2, continuous development chamber (plate at position 3) using solvent F. TLC-immunostaining was performed as described in Materials and Methods. Glycolipid standards: a, Forssman; b, GM1 ganglioside; and c, GT ganglioside. The dark area at the front of the chromatogram in lane 1 and the dark line below the front in lane 2 represent nonspecific staining.

as starting points for determining ideal CD-HPTLC conditions for a given application. The initial choice of solvent should be one that is less polar than that which is optimal for separation of the compounds in question by conventional HPTLC. Generally this means preparing a less polar mixture of the standard components (such as chloroform, methanol, and water); only with dichloroethane–acetone systems used for acetylated glycolipids did we find the technique of dilution with hexane to be of practical use (see Results).

For analytical purposes the shortest bed lengths (i.e., the most vertical plate positions) may be preferred because highest solvent velocities can be achieved with minimal diffusion of bands. However, for preparative purposes, a longer bed length will be required to obtain the necessary distance between bands (Fig. 1 and 2). In such preparative cases the radioautographic intensity of CD-HPTLC bands in general will be less than that obtained by conventional HPTLC, due in part to continuous development separation of species that are superimposed in conventional HPTLC.

We thank Dr. David Herold for suggesting the continuous development method, Ms. Jennifer Lovejoy for excellent technical assistance, and Ms. Cathy Murphy for typing the manuscript. This investigation was supported by grants IM-335/BC-489A from the American Cancer Society and grant AI-21916 from the NIH.

Manuscript received 22 July 1985.

REFERENCES

1. Marcus, D. M. 1984. A review of the immunogenic and immunomodulatory properties of glycosphingolipids. *Mol. Immunol.* **21:** 1083–1091.
2. Hakomori, S. 1981. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu. Rev. Biochem.* **50:** 733–764.
3. Bremer, E. G., S. Hakomori, D. F. Bowen-Pope, E. Raines, and R. Ross. 1984. Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. *J. Biol. Chem.* **259:** 6818–6825.
4. Holmgren, J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. *Nature.* **292:** 413–418.
5. Critchley, D. R., P. G. Nelson, W. H. Habig, and P. H. Fishman. 1985. Fate of tetanus toxin bound to the surface of primary neurons in culture: evidence for rapid internalization. *J. Cell Biol.* **100:** 1499–1507.
6. Markwell, M. K., L. Svennerholm, and J. C. Paulson. 1981. Specific gangliosides function as host cell receptors for Sendai virus. *Proc. Natl. Acad. Sci. USA.* **78:** 5406–5410.
7. Kallenius, G., S. B. Svenson, H. Hultberg, R. Mollby, I. Helin, B. Cedergren, and J. Winberg. 1981. Occurrence of P-fimbriated *Escherichia coli* in urinary tract infections. *Lancet.* **ii:** 1369–1372.
8. Loomes, L. M., K. Uemura, and T. Feizi. 1985. Interaction of *Mycoplasma pneumoniae* with erythrocyte glycolipids of I and i antigen types. *Infect. Immun.* **47:** 15–20.
9. Perry, J. A. 1979. New look at solvent strength, selectivity, and continuous development. *J. Chromatogr.* **165:** 117–140.
10. Regis Technical Manual. 1979. Short bed/continuous development. Regis Chemical Co., Morton Grove, IL.
11. Laine, R. A., K. Stellner, and S. Hakomori. 1974. Isolation and characterization of membrane glycosphingolipids. *Methods Membr. Biol.* **2:** 205–247.
12. Young, W. W., Jr., H. S. Johnson, Y. Tamura, K. Karlsson, G. Larson, J. M. R. Parker, D. P. Khare, U. Spohr, D. A. Baker, O. Hindsgaul, and R. U. Lemieux. 1983. Characterization of monoclonal antibodies specific for the Lewis a human blood determinant. *J. Biol. Chem.* **258:** 4890–4894.
13. Kannagi, R., R. Stroup, N. A. Cochran, D. L. Urdal, W. W. Young, Jr., and S. Hakomori. 1983. Factors affecting expression of glycolipid tumor antigens: influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells. *Cancer Res.* **43:** 4997–5005.
14. Magnani, J. L., B. Nilsson, M. Brockhaus, D. Zopf, Z. Steplewski, H. Koprowski, and V. Ginsburg. 1982. A

monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. *J. Biol. Chem.* **257:** 14365–14369.
15. Hakomori, S., D. Urdal, M. Yokota, and W. W. Young, Jr. 1980. Mass spectrometric analysis of tumor-associated glycolipid antigens with particular reference to the incompatible blood group antigens in human cancer. *Proc. Jpn. Soc. Med. Mass. Spectrom.* **5:** 3–23.
16. Urdal, D. L., and S. Hakomori. 1983. Characterization of tumor-associated ganglio-N-triaosylceramide in mouse lymphoma and the dependency of its exposure and antigenicity on the sialosyl residues of a second glycoconjugate. *J. Biol. Chem.* **258:** 6869–6874.
17. Hakomori, S. 1984. Monoclonal antibodies directed to cell-surface carbohydrates. *In* Monoclonal Antibodies and Functional Cell Lines. R. Kennett, K. B. Bechtol, and T. J. McKearn, editors. Plenum Press, New York. 67–100.
18. Kanfer, J. N., and S. Hakomori. 1983. Handbook of Lipid Research. Vol. 3: Sphingolipid Biochemistry. Plenum Press, New York. 1–166.
19. Clausen, H., S. B. Levery, E. Nudelman, S. Tsuchiya, and S. Hakomori. 1985. Repetitive A epitope (type 3 chain A) defined by blood group A_1-specific monoclonal antibody TH-1: chemical basis of qualitative A_1 and A_2 distinction. *Proc. Natl. Acad. Sci. USA.* **82:** 1199–1203.
20. Gahmberg, C. G., and S. Hokomori. 1975. Surface carbohydrates of hamster fibroblasts. I. Chemical characterization of surface-labeled glycosphingolipids and a specific ceramide tetrasaccharide for transformants. *J. Biol. Chem.* **250:** 2438–2446.
21. Stellner, K., K. Watanabe, and S. Hakomori. 1973. Isolation and characterization of glycosphingolipids with blood group H specificity from membranes of human erythrocytes. *Biochemistry.* **12:** 656–661.