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Note

Quantitation of gangliosides by scanning densitometry of thin-layer chromatography plates*

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Gangliosides are a diversified family of glycolipids characterized by the presence of sialic acid. Although ubiquitous in vertebrate plasma membranes [1], their biological function remains elusive. The human central nervous system is especially rich in gangliosides and their separation by high-performance thin-layer chromatography (HPTLC) in one dimension reveals the presence of at least twelve ganglioside bands [2]. The five major ganglioside species of this tissue were chosen for study in this report (Table I).

The conventional method for determining the relative distribution of individual ganglioside species in tissues employs thin-layer chromatography to separate the ganglioside components of a mixture, resorcinol staining of the sialic acid moiety, and quantitation of the stained ganglioside bands by scanning densitometry [2—7]. The percentage of an individual ganglioside is generally calculated from the ratio of the densitometric detector response due to the ganglioside band relative to the total detector response of all ganglioside bands separated from the mixture. However, this method is valid only if all ganglioside species yield equal densitometric values per mole of lipid-bound sialic acid after chromatographic separation and staining on thin-layer plates.

We report here that gangliosides G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} after migration and resorcinol staining on silica gel thin-layer plates produce different densitometric detector responses per mole of sialic acid. This finding must be taken into account when assessing the content of individual ganglioside species in tissues by scanning densitometry of thin-layer plates.

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TABLE I

CHEMICAL STRUCTURES OF GANGLIOSIDES UNDER STUDY

Abbreviations: gal = galactose; glu = glucose; galNAc = N-acetylgalactosamine; NANA = N-acetylneuraminic acid or sialic acid.

Ganglioside*	
G_{M4}	Ceramide-gal NANA
G_{M1}	Ceramide-glu-gal-galNAc-gal NANA
G_{D1a}	Ceramide-glu-gal-galNAc-gal NANA NANA
G_{D1b}	Ceramide-glu-gal-galNAc-gal NANA NANA
G_{T1b}	Ceramide-glu-gal-galNAc-gal NANA NANA NANA

*Ganglioside nomenclature is that of Svennerholm [11].

EXPERIMENTAL

Materials

Chloroform and methanol were analytical grade. Silica gel 60, extra pure, particle size 0.063—0.200 mm, and HPTLC plates (10 × 10 cm and 10 × 20 cm) precoated with a 0.2-mm layer of silica gel 60 of 5-μm particle size were from E. Merck (Darmstadt, F.R.G.). N-acetylneuraminic acid · $1H_2O$ used as sialic acid standard was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). Resorcinol was from Sigma (St. Louis, MO, U.S.A.).

Ganglioside purification

Gangliosides G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} were extracted from adult human central nervous system white matter [8] and purified by the classical procedure of Suzuki [9]. In G_{M4} purification Folch partitioning was omitted. As a final purification step, each ganglioside was chromatographed in a 1.3-m silica gel column using chloroform—methanol—water (60:35:8 or 55:45:10).

Thin-layer chromatography of gangliosides

HPTLC plates were prewashed by migration in chloroform—methanol (2:1) and activated at 140°C for 10 min. Ten samples were applied to each plate in 3 × 1 mm bands with a 1- or 5-μl positive-displacement microsyringe (Hamilton, 7000 series). Bands were placed 10 mm from the bottom of plates and dried under a constant flow of warm air (30—40°C). Two different developing solvents were studied: solvent A, chloroform—methanol—water—1% $CaCl_2$ (55:45:8:2) and solvent B, chloroform—methanol—water—1% $CaCl_2$ (60:35:7:1). Plates were suspended above solvent A for 60 or solvent B for

30 min to insure a saturated atmosphere in the developing tank. The plates were then lowered into the solvent. The height to which the solvent front was allowed to rise above the origin was varied. Following development, plates were heated at 140°C for 10 min to remove traces of solvent prior to spraying with resorcinol—HCl reagent [10]. Sprayed plates were covered with clean glass covers and again heated at 140°C for 10 min to visualize the blue ganglioside bands.

Scanning densitometry

Ganglioside bands were scanned with a Shimadzu Model CS-910 dual-wavelength TLC scanning densitometer (Kyoto, Japan) at 580 nm (sample wavelength) in the transmission mode at 6 mm/min. The slit length was adjusted to be 10% greater than the longest band after development (G_{M4}) and the slit width was 0.2 mm. The peak area of each band was measured after subtraction of the background absorbance at 720 nm (reference wavelength) by means of a Shimadzu data processor (C-R1B). The linearizer was not used.

Ganglioside purity

Ganglioside purity was evaluated by HPTLC of 1000 pmol ganglioside sialic acid in two different solvent systems, solvents A and B. Ganglioside bands were visualized with resorcinol reagent and quantitated by scanning densitometry. Each ganglioside species showed <1% contamination.

Preparation of ganglioside standards

The sialic acid content of ganglioside solutions was determined by a modification of the colorimetric assay of Svennerholm [10] in which the purple color product was solubilized into a single phase with tertiary butanol. A mixed ganglioside standard containing an equimolar sialic acid content of each of the five ganglioside species was prepared in chloroform—methanol (2:1).

Variability in densitometric quantitation of gangliosides

Intra-assay (intra-plate) variability of 5% was obtained when replicate ganglioside bands present on a single HPTLC plate were scanned.

Inter-assay (plate-to-plate) variability of 6% was determined by scanning three HPTLC plates each containing replicate applications of an unknown ganglioside mixture and mixed ganglioside standards (25, 50, 75 and 100 pmol). Amounts of individual ganglioside components within the unknown mixture were determined from densitometric standard curves run on the same plate.

RESULTS AND DISCUSSION

In order to define chromatographic conditions for the effective quantitation of gangliosides by scanning densitometry, TLC of a standard mixture of gangliosides containing equal sialic acid amounts of G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} was studied in two popular solvent systems in the range between 10 and 1000 pmol sialic acid for each ganglioside species. When gangliosides

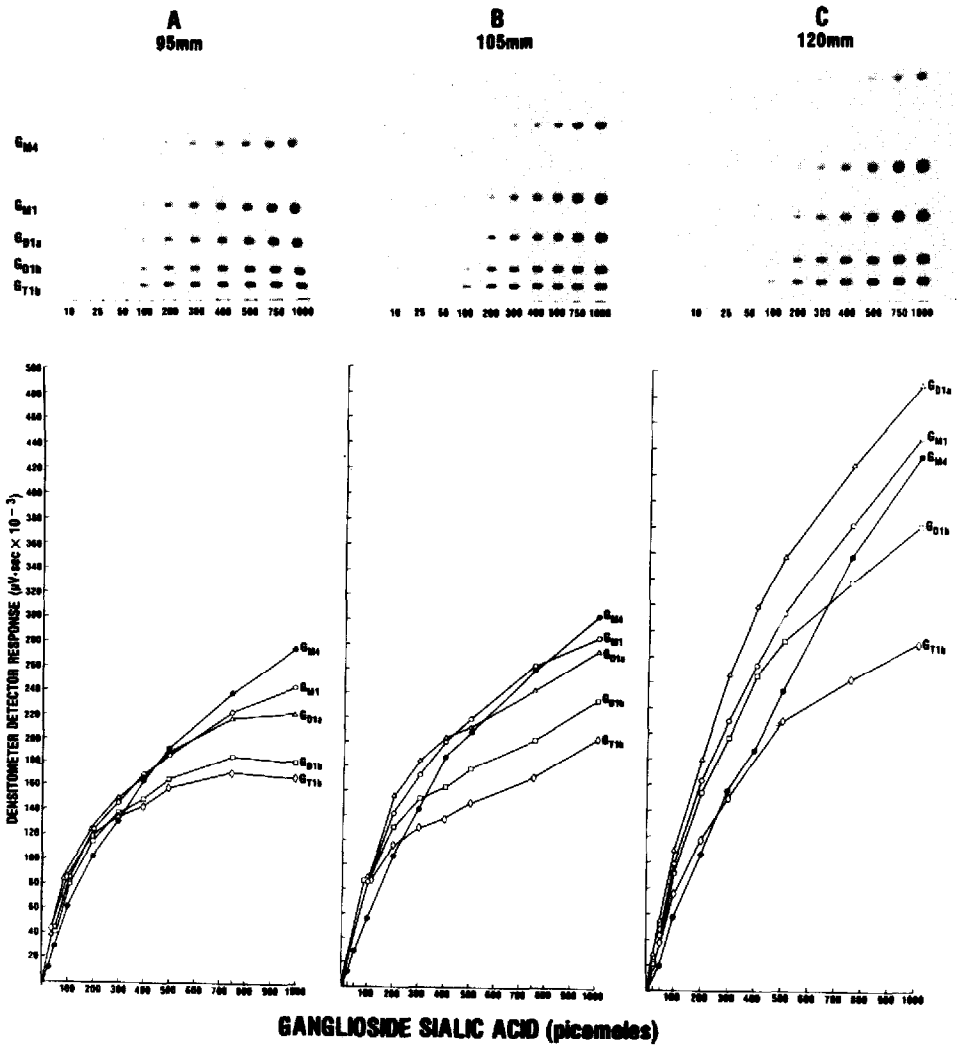

Fig. 1. TLC with corresponding densitometric standard curves of the mixed ganglioside standard in solvent A: chloroform—methanol—water—1% $CaCl_2$ (55:45:8:2). Gangliosides were separated on HPTLC plates and stained with resorcinol reagent. Each lane of the chromatogram contains the indicated pmol sialic acid of each ganglioside species. Each band was scanned at 580 nm in the transmission mode. The densitometer detector response is plotted as a function of amount of ganglioside sialic acid present. Detector response is the peak area due to band absorbance at 580 nm after subtraction of background at 720 nm. Heights of solvent fronts above origin: A, 95 mm; B, 105 mm; C, 120 mm.

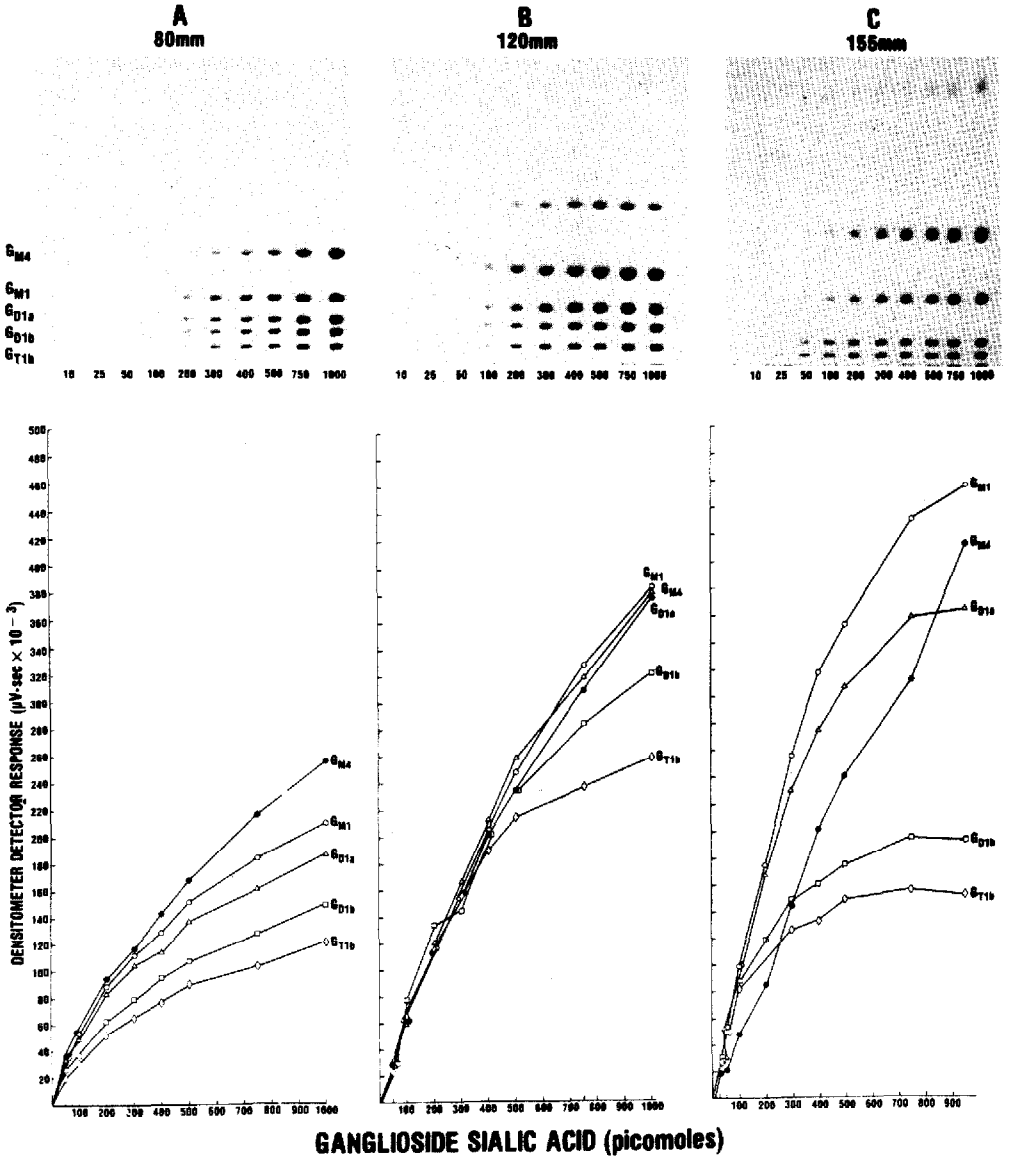

Fig. 2. TLC with corresponding densitometric standard curves of the mixed ganglioside standard in solvent B: chloroform—methanol—water—1% $CaCl_2$ (60:35:7:1). Gangliosides were separated on HPTLC plates and stained with resorcinol reagent. Each lane contains the indicated pmol sialic acid for each ganglioside species. Each band was scanned at 580 nm in the transmission mode. The densitometer detector response is plotted as a function of amount of ganglioside sialic acid present. Detector response is the peak area due to band absorbance at 580 nm after subtraction of background at 720 nm. Heights of solvent fronts above origin: A, 80 mm; B, 120 mm; C, 155 mm.

were chromatographed in solvent A, individual ganglioside species yielded divergent densitometric standard curves (Fig. 1). Standard curves for all gangliosides were essentially non-linear until the solvent front was allowed to reach a height of 105 mm (Fig. 1B). When the front reached 120 mm, all gangliosides had standard curves with an enhanced range of linearity (Fig. 1C). G_{M4} curves were consistently the most linear, while G_{T1b} curves were the least linear. Dense thin ganglioside bands, such as seen with high amounts of G_{T1b} and G_{D1b}, caused the linearity of standard curves to fall off. In contrast, gangliosides of higher R_F which formed larger less dense bands, such as G_{M4} and G_{M1}, had standard curves which were linear throughout a longer range of sialic acid values.

When the ganglioside standard was chromatographed in a less polar system (solvent B), more linear densitometric standard curves were obtained at lower migration heights (Fig. 2A). When the front reached 120 mm above the origin (Fig. 2B), all ganglioside species had overlapping curves which were linear to 500 pmol ganglioside sialic acid. Since the latter chromatographic conditions yielded standard curves with the longest range of linearity, these conditions were chosen when determining the relative distribution of gangliosides within an unknown mixture.

In either solvent system, as little as 10 pmol ganglioside sialic acid could be reproducibly detected, a sensitivity ten-fold greater than that previously reported by Ando et al. [6].

We sought to determine whether differences noted in the densitometric standard curves of individual gangliosides resulted from a differing ability of the gangliosides to form a color product with resorcinol reagent, i.e., had differing molar extinction values. Pure ganglioside solutions were applied to HPTLC plates in 3-mm bands and stained with resorcinol reagent without solvent migration. This resulted in ganglioside bands of nearly identical size and shape, thus negating any influence these factors might have in densitometric measurements. No significant difference in the molar extinction values of gangliosides was detected. This indicated that the individual ganglioside species adsorbed on silica gel were equally reactive with resorcinol reagent. Thus differences in the densitometric standard curves of individual ganglioside species resulted from differences in band size or geometry following migration in solvent. This necessitates the use of standard curves when quantitating individual gangliosides within a mixture.

In summary, when determining the relative distribution of ganglioside species within a mixture by scanning densitometry of thin-layer chromatograms, it is necessary to reference experimental densitometric values to densitometric standard curves of gangliosides present in the mixture. Since the solvent system and distance of solvent front migration can strongly influence band geometry and subsequently densitometric values, unknown mixtures and ganglioside standards must be run on the same plate. TLC conditions which both effectively separate gangliosides and yield linear standard curves are migration in solvent B to a height of 120 mm above the origin.

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