Thin-layer chromatographic separation of sphingosine and related bases*

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» Methods for the separation and identification of sphingosine and related bases are essential to the study of the chemistry and metabolism of sphingolipids. Previous reports on the separation of sphingosine bases and their derivatives include a paper chromatographic separation of sphingosine and dihydrosphingosine (1), reverse phase column chromatography of the *N*-succinyl derivatives (2), paper chromatography of the 2,4-dinitrophenyl derivatives (3), and a gas-liquid chromatographic method for the separation of aldehydes derived from the bases (4). A number of closely related bases of sphingosine were separated on paper impregnated with silicic acid (5). Recently two papers appeared that included methods for the separation of sphingosine bases by thin-layer chromatography (6, 7). Reported here is a rapid thin-layer chromatographic method useful for the qualitative separation and identification of the free bases of *erythro*-sphingosine, *threo*-sphingosine, dihydrosphingosine, phytosphingosine, and 3-O-methylsphingosine.

A commercial apparatus (Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.) was used to prepare thin layers of silicic acid (250 μ) on glass plates with Silica Gel G (E. Merck, A.G., Darmstadt, Germany). The plates were activated by heating at 105° for $^1/_2$ hr and stored over silica gel. About 50 μg of the sample was spotted from a chloroform–methanol 1:1 solution[1] and developed at 25° in glass chambers containing chloroform–methanol–2 N ammonia 40:10:1 for 50 min. The plates were allowed to dry at room temperature for 5–10 min and the spots visualized with ninhydrin spray (0.2 g ninhydrin in 95 ml *n*-butanol and 5 ml pyridine). The pink spots which were visible within $^1/_2$ hr disappeared within 24 hr. A more permanent record can be obtained by over-spraying the plates with 50% sulfuric acid and charring. About 5 μg of sphingosine can be easily seen using either spray.

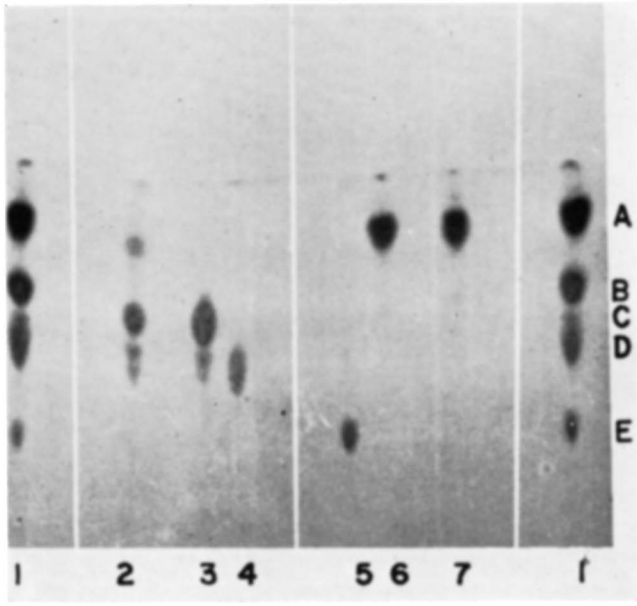

Fig. 1. Separation of sphingosine and related bases by thin-layer chromatography. 1, mixture of sphingosine, dihydrosphingosine, 3-O-methylsphingosine, and phytosphingosine; 2, sphingosine from triacetylsphingosine; 3, purified sphingosine from human grey matter sphingolipids; 4, dihydrosphingosine; 5, phytosphingosine; 6 and 7, stereoisomers of 3-O-methylsphingosine. Spot identification: A, 3-O-methylsphingosine; B, *erythro*-sphingosine; C, *threo*-sphingosine; D, dihydrosphingosine; E, phytosphingosine.

Samples of phytosphingosine and triacetylsphingosine were kindly supplied by Dr. H. E. Carter of the University of Illinois. Sphingosine was obtained from triacetylsphingosine by hydrolyzing for 6 hr with a mixture of methanol and concentrated hydrochloric acid 80:16 (v/v). This reagent minimizes the formation of 3-O-methylsphingosine (4). Sphingosine was also prepared from human brain grey matter sphingolipids by hydrolysis with methanol–sulfuric acid 95:5 and re-

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[1] All solvent ratios, v/v.

peated crystallization of the sphingosine bases from petroleum ether. The filtrates were used for the preparation of *erythro*- and *threo*-3-O-methylsphingosine following the procedure of Carter *et al.* (8). Dihydrosphingosine was obtained by hydrogenation of sphingosine in ethyl ether solution at room temperature for 2 hr at 10 lb/in^2 using 5% palladium on charcoal as catalyst. Chemically synthesized DL-*erythro-trans*-sphingosine and DL-*threo-trans*-sphingosine were generously supplied by Dr. P. W. O'Connell of the Upjohn Company, Kalamazoo, Mich. *Erythro*-sphingosine contained a faster moving contaminant (1,3-dihydroxy-2-amino-4-octadecyne), which is the immediate precursor in the chemical synthesis. Gangliosides were prepared from human grey matter (9) and from calf brain by a similar procedure. Beef gangliosides (purified, type II) were purchased from Sigma Chemical Co., St. Louis, Missouri.

The separation of the different sphingosine bases is shown in Fig. 1. The R_f values generally varied slightly from plate to plate due to the variable loss of ammonia from the solvent mixture. The relative mobilities of the different bases compared to 3-O-methylsphingosine were as follows: 3-O-methylsphingosine, 1; *erythro*-sphingosine, 0.80; *threo*-sphingosine, 0.66; dihydrosphingosine, 0.61; phytosphingosine, 0.37. All the bases tested separated from one another except the stereoisomers of 3-O-methylsphingosine.[2]

Both samples of sphingosine in Fig. 1 (Samples 2 and 3) contained a small amount of dihydrosphingosine and a spot that moved between sphingosine and dihydrosphingosine. This spot had the same mobility as *threo*-sphingosine, as seen in Fig. 2. The addition of *threo*-sphingosine to the sphingosine (Sample 7) intensified the spot between sphingosine and dihydrosphingosine and no other new spot was found. Upon hydrogenation, both sphingosine and this spot disappeared, forming dihydrosphingosine (Samples 3 and 4, Fig. 1). When a preparation of sphingosine (Sample 3) was refluxed with methanol–sulfuric acid, this spot appeared to intensify. *Threo*-sphingosine has been previously isolated from aqueous methanolic hydrolysates of cerebrosides (10). Sweeley and Moscatelli (4) analyzed the aldehydes derived from a sample of triacetylsphingosine obtained from Dr. Carter by gas-liquid chromatography and found only sphingosine,

[2] We have been able to separate these stereoisomers by spotting small quantities and developing the plates until the solvent moved to the top. The 3-O-methylsphingosine I moves faster, and 3-O-methylsphingosine II is contaminated by some 3-O-methylsphingosine I. The 3-O-methylsphingosine I may have the *erythro* configuration by analogy to *erythro*-sphingosine, which moves faster than the *threo* isomer. Carter *et al.* (8) did not determine the configuration of these isomers.

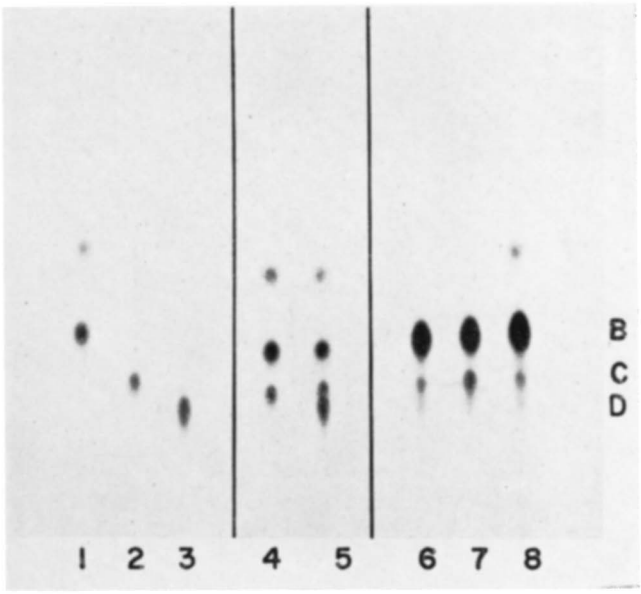

FIG. 2. Separation of the stereoisomers of sphingosine. 1, DL-*erythro-trans*-sphingosine with a faster moving contaminant; 2, DL-*threo-trans*-sphingosine; 3, dihydrosphingosine; 4, mixture of 1 + 2; 5, mixture of 1 + 2 + 3; 6, purified sphingosine from human grey matter sphingolipids; 7, mixture of 6 + 2; 8, mixture of 6 + 1. Spot identification: same as in Fig 1.

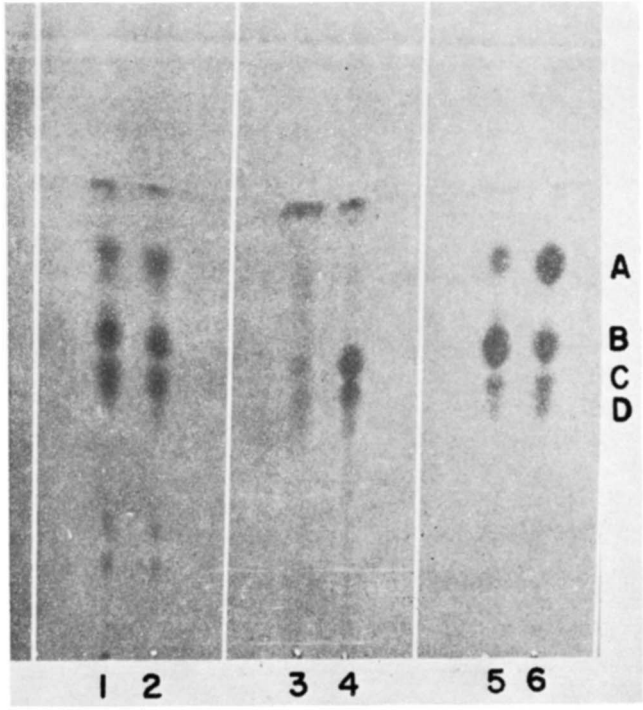

FIG. 3. Separation of sphingosine bases obtained from various lipid sources. 1, human grey matter gangliosides; 2, calf brain gangliosides; 3, beef brain gangliosides; 4, commercial sphingosine sulfate (General Biochemicals, Chagrin Falls, Ohio); 5, human grey matter sphingolipids (once crystallized from petroleum ether); 6, human grey matter sphingolipids. Spot identification: same as in Fig. 1.

dihydrosphingosine, and 3-O-methylsphingosine. This method does not distinguish between *erythro* and *threo* isomers of sphingosine. The foregoing observations strongly suggest that the spot between sphingosine and dihydrosphingosine is the *threo* isomer of sphingosine. It seems likely that some *threo* isomer is formed by inversion during acid hydrolysis (11). However, this does not rule out the possibility that some *threo* isomer occurs naturally.

When a sphingosine preparation (Sample 3, Fig. 1) was refluxed with methanol–sulfuric acid, a new spot corresponding to that of 3-O-methylsphingosine was formed. However, when dihydrosphingosine was treated in a similar manner, no new spot could be found. The allylic double bond on the sphingosine molecule is apparently essential for the formation of the ether linkage under these conditions (12). We could not conclude that any *threo*-dihydrosphingosine is formed under these conditions, since both *erythro*- and *threo*-dihydrosphingosine have the same mobility in this system. The R_f values of *threo*-sphingosine and dihydrosphingosine are very similar, and it is advisable to spot small quantities and allow the solvent front to run to the top of the plate in order to effect better separation.

The separation of sphingosine bases obtained from various sources is shown in Fig. 3. In general, 10–20 mg of the lipid was hydrolyzed with 1–2 ml of 2 N methanolic HCl for 6–8 hr. After removal of methyl esters with petroleum ether, methanol was removed under vacuum and the hydrolysate made alkaline with 1 N sodium hydroxide. Sphingosine bases were then extracted from the alkaline solution with ethyl ether. The ether solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. As seen from Fig. 3, the sphingosine bases from the gangliosides do not contain any appreciable amount of dihydrosphingosine. However, dihydrosphingosine is readily observed in the samples derived from human grey matter sphingolipids and commercial sphingosine sulfate. All the lipids tested contained the spot between sphingosine and dihydrosphingosine. Two unidentified ninhydrin-positive spots were found in the case of human grey matter gangliosides and calf brain gangliosides with R_f values slightly lower than that of phytosphingosine. These spots may not necessarily be sphingosine bases or related compounds. When the sulfuric acid spray was used, two additional faint spots appeared, one above and one below the 3-O-methylsphingosine. These also were not identified. The spots at the solvent front gave a yellow color with ninhydrin spray instead of the usual purple color.

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