# Thin-layer chromatography of ceramides

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ABSTRACT Ceramides with mono-, di-, and trihydroxy long-chain bases, and normal (saturated and unsaturated), branched-chain, and 2-hydroxy fatty acids have been analyzed by thin-layer chromatography. In most cases the compounds were also run as acetates. Borate, arsenite, and silver ions were used as complexing agents, and the effects of number, position, and stereochemistry of hydroxy groups, and of unsaturation, were studied. The results are discussed in view of analysis of natural ceramide species.

SUPPLEMENTARY KEY WORDS sphingosine · phytosphingosine · dienic long-chain base · normal fatty acids · 2-hydroxy fatty acids · acetyl derivatives · borate chromatography · arsenite chromatography · silver-ion chromatography

Ceramides (*N*-acyl derivatives of long-chain bases) are the lipophilic parts of all natural sphingolipids. They may exist free in animal (1) and plant (2) tissues or may be prepared from more complex compounds by enzymatic (3–8) or chemical (9, 10) degradation. Due to the existence in nature of about 60 bases (for a review, see Ref. 11) and at least as many sphingolipid fatty acids (12), a great number of ceramide species may exist. Compared with analyses of molecular species of glycerides (e.g., 13), relatively few studies of fatty acid–base combinations have been reported. Ceramide diacetates, derived from human plasma sphingomyelins, have been separated on silver ion-containing thin layers (4, 14), followed by hydrolysis and GLC analysis of bases and fatty acids. Free ceramides of human brain have been separated according to fatty acid chain length by TLC (15), and trihydroxy base-containing ceramide of bovine kidney sphingomyelin was prepared by TLC (16). Recently, GLC–MS was used to study ceramide species, as trimethylsilyl ethers, of human blood plasma and bovine brain (8, 17–20). In this way, direct information of both base and fatty acid structures was obtained. However, ceramide species differing only in unsaturation do not separate on GLC (18, 19), and homologous series differing in the number of hydroxy groups may overlap (19, 21, 22). Therefore, a group separation by TLC is needed before the analysis (8, 19). In the present paper, results from the analysis of a number of homogeneous ceramide species (see Table 1) by TLC are given, in order to allow selection of conditions for the study of natural mixtures. Some of the results have been presented before (6). Paper chromatography (23) and TLC (8, 10, 14–16, 24, 25) have been used to analyze or prepare a limited number of natural ceramide species.

## MATERIALS AND METHODS

The preparation of the long-chain base derivatives listed in Table 1 will be published eslewhere.[1] Acetylation was done with acetic anhydride and pyridine (26). Natural ceramide, shown in Fig. 7, sample 7, was prepared from bovine brain cerebroside (9), and contains d18:1 (95%) and d18:0 (5%) bases, and mostly long-chain (24:1) normal and 2-D-hydroxy fatty acids.

For TLC, Silica Gel G (Fluka A. G., Buchs, Switzerland, batch 117736 K) was used; the spreader (C. Desaga, G.m.b.H., Heidelberg, Germany) was adjusted to 0.25 mm, and the glass plates measured 20 × 20 cm. The slurry was prepared by shaking 30 g of adsorbent with 55 ml of water for 90 sec. Plates containing glycol-complexing agents were prepared by replacing water with an equal amount of 1% aqueous solutions of sodium

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, combined gas–liquid chromatography and mass spectrometry; Ac, acetyl; diAc, diacetyl; triAc, triacetyl; tetAc, tetraacetyl; AcO, 2-acetoxy; h, 2-hydroxy; m, monohydroxy; d, dihydroxy; t, trihydroxy; carbon chains are designated by chain length: number of double bonds. Shorthand formulas and systematic names of different ceramides are given in Table 1.

[1] Karlsson, K.-A., and I. Pascher. Unpublished data.

TABLE 1 LONG-CHAIN BASE DERIVATIVES

| Shorthand Formula (acid–base) | Systematic Name |
|---|---|
| | Derivatives of saturated monohydroxy base |
| Ac–m18:0 | 1-hydroxy-2-D-acetamidooctadecane |
| diAc–m18:0 | 1-acetoxy-2-D-acetamidooctadecane |
| 18:0–m18:0* | 1-hydroxy-2-D-octadecanoylamidooctadecane |
| DL h18:0–m18:0* | 1-hydroxy-2-D-(2-DL-hydroxyoctadecanoylamido)octadecane |
| DL AcO18:0–m18:0 | 1-hydroxy-2-D-(2-DL-acetoxyoctadecanoylamido)octadecane |
| | Derivatives of saturated dihydroxy base |
| Ac–d18:0 | D-*erythro*-1,3-dihydroxy-2-acetamidooctadecane |
| triAc–d18:0 | D-*erythro*-1,3-diacetoxy-2-acetamidooctadecane |
| 18:0–d18:0* | D-*erythro*-1,3-dihydroxy-2-octadecanoylamidooctadecane |
| 18:1–d18:0* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9-octadecenoylamido)octadecane |
| 18:2–d18:0* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9-*cis*-12-octadecadienoylamido)octadecane |
| DL h18:0–d18:0* | D-*erythro*-1,3-dihydroxy-2-(2-DL-hydroxyoctadecanoylamido)octadecane |
| DL AcO18:0–d18:0 | D-*erythro*-1,3-dihydroxy-2-(2-DL-acetoxyoctadecanoylamido)octadecane |
| | Derivatives of monounsaturated dihydroxy base |
| HCl–d18:1 | D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene hydrochloride |
| Ac–d18:1 | D-*erythro*-1,3-dihydroxy-2-acetamido-*trans*-4-octadecene |
| triAc–d18:1 | D-*erythro*-1,3-diacetoxy-2-acetamido-*trans*-4-octadecene |
| 18:0–d18:1* | D-*erythro*-1,3-dihydroxy-2-octadecanoylamido-*trans*-4-octadecene |
| 24:0–d18:1* | D-*erythro*-1,3-dihydroxy-2-tetracosanoylamido-*trans*-4-octadecene |
| 32:0–d18:1 | D-*erythro*-1,3-dihydroxy-2-dotriacontanoylamido-*trans*-4-octadecene |
| 18:1–d18:1* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9-octadecenoylamido)-*trans*-4-octadecene |
| 24:1–d18:1* | D-*erythro*-1,3-dihydroxy-2-(*cis*-15-tetracosenoylamido)-*trans*-4-octadecene |
| 18:2–d18:1* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9,*cis*-12-octadecadienoylamido)-*trans*-4-octadecene |
| D h18:0–d18:1 | D-*erythro*-1,3-dihydroxy-2-(2-D-hydroxyoctadecanoylamido)-*trans*-4-octadecene |
| DL h18:0–d18:1* | D-*erythro*-1,3-dihydroxy-2-(2-DL-hydroxyoctadecanoylamido)-*trans*-4-octadecene |
| D AcO18:0–d18:1 | D-*erythro*-1,3-dihydroxy-2-(2-D-acetoxyoctadecanoylamido)-*trans*-4-octadecene |
| phytanic–d18:1 | D-*erythro*-1,3-dihydroxy-2-(3-DL,7,11,15-tetramethylhexadecanoylamido)-*trans*-4-octadecene |
| | Derivatives of diunsaturated dihydroxy base |
| 18:0–d18:2* | D-*erythro*-1,3-dihydroxy-2-octadecanoylamido-*trans*-4-*trans*-8-octadecadiene |
| 18:1–d18:2* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9-octadecenoylamido)-*trans*-4-*trans*-8-octadecadiene |
| 18:2–d18:2* | D-*erythro*-1,3-dihydroxy-2-(*cis*-9,*cis*-12-octadecadienoylamido)-*trans*-4-*trans*-8-octadecadiene |
| | Derivatives of saturated trihydroxy base |
| Ac–t18:0 | D-*ribo*-1,3,4-trihydroxy-2-acetamidooctadecane |
| tetAc–t18:0 | D-*ribo*-1,3,4-triacetoxy-2-acetamidooctadecane |
| 18:0–t18:0* | D-*ribo*-1,3,4-trihydroxy-2-octadecanoylamidooctadecane |
| 24:0–t18:0 | D-*ribo*-1,3,4-trihydroxy-2-tetracosanoylamidooctadecane |
| DL h18:0–t18:0* | D-*ribo*-1,3,4-trihydroxy-2-(2-DL-hydroxyoctadecanoylamido)octadecane |
| DL AcO18:0–t18:0 | D-*ribo*-1,3,4-trihydroxy-2-(2-DL-acetoxyoctadecanoylamido)octadecane |

* The fully acetylated derivative was also analyzed.

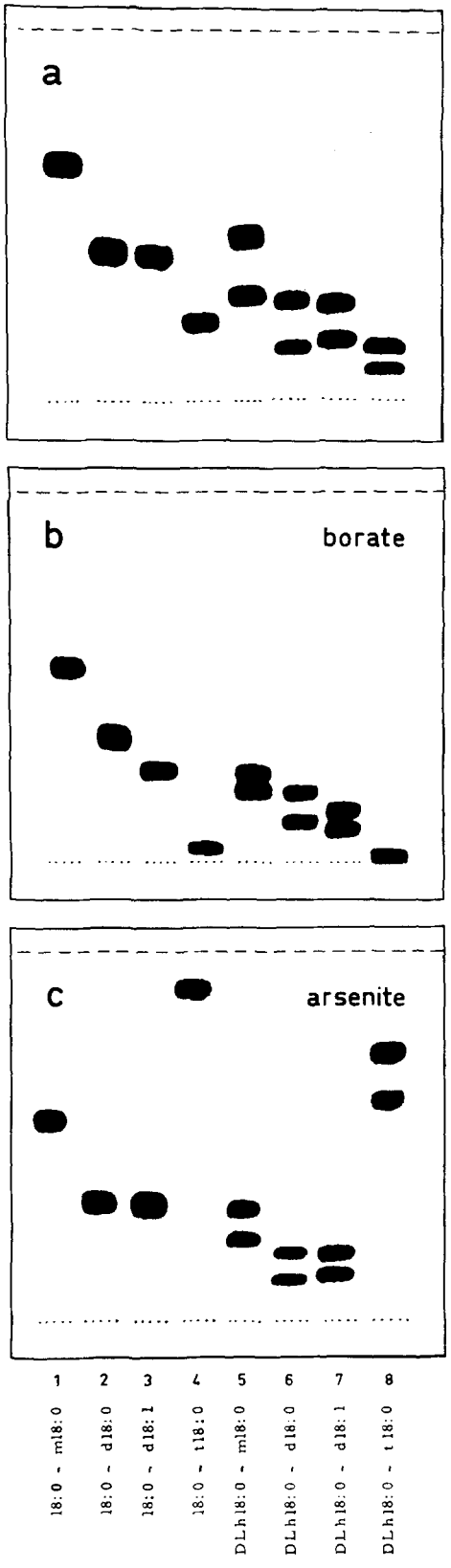
borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) and sodium *meta* arsenite ($NaAsO_2$), respectively. Silver ion-containing layers were prepared by mixing 1.6 g of $AgNO_3$ with the adsorbent prior to the addition of water. The slurry was applied with a stainless steel spreader. All plates were dried for 30 min at 120°C immediately before use, and were run in tanks lined with filter paper. For comparison of separation properties, all plates with free ceramides were run in chloroform–methanol 95:5 (v/v). For ceramide acetates, chloroform–benzene–acetone 80:20:5 (v/v/v) (on ordinary silica gel) or 80:20:10 (v/v/v) (on silver ion layers) was used. 30–40-μg samples (7 μl of a 0.5% solution) were applied as an elongated spot with a micropipette. To make spots visible the plates were sprayed with a 3% cupric acetate solution in 8% aqueous $H_3PO_4$ (27) until uniformly transparent, and then they were heated for 25 min at 200°C. Unsaturated compounds appear faster than saturated ones. After spraying, silver ion-containing plates do not darken.

## RESULTS AND DISCUSSION

### *Effects of Number, Position, and Stereochemistry of Hydroxy Groups*

As far as known, ceramides contain from two to four hydroxy groups (two or three of the base, and none or one of the fatty acid). In addition to these, ceramides containing the monohydroxy base (sphingine, not found in nature) were studied for comparison. The configura-

tion at asymmetric centers of all natural bases analyzed thus far (11) is D, that is, D-*erythro* for dihydroxy and D-*ribo* for trihydroxy bases; in the present paper only



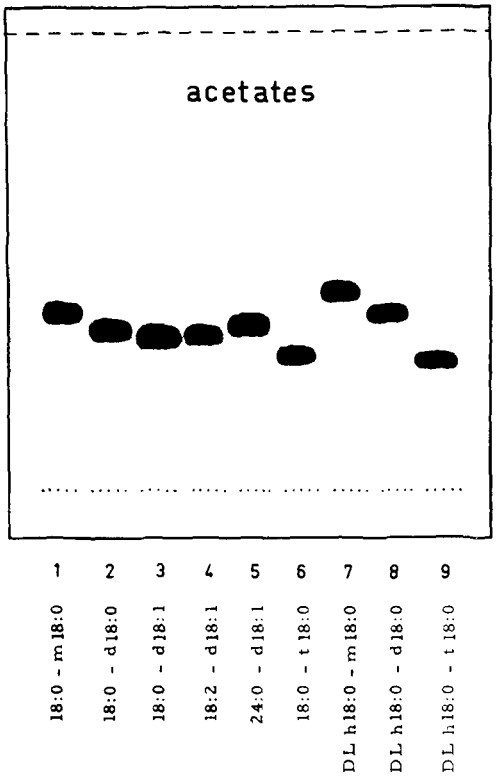




FIG. 2. Thin-layer chromatogram showing the separation of ceramide acetates on ordinary silica gel. Solvent: chloroform–benzene–acetone 80:20:5 (v/v/v). Detection as given for Fig. 1.

bases with these configurations have been analyzed. For 2-hydroxy fatty acids of natural glycosphingolipids (28, 29) and phosphosphingolipids (30), a D configuration has been assigned. In the present study, ceramides containing D- or L-hydroxy acid, or a racemate, were used. As shown in Fig. 1 *a–c* (samples *5–8*), ceramides differing only in configuration at this center do separate well in different systems; those containing the L isomer always move faster. A similar separation is achieved with 2-acetoxy isomers (Fig. 4, samples *7* and *8*), except for trihydroxy base-containing ceramides (sample *9*). However, no separation is obtained for the corresponding fully acetylated derivatives (Fig. 2, samples *7–9*). For comparison, a ceramide containing phytanic acid with a 3-DL methyl group is shown (Fig. 5, sample *6*), with a tendency to separate. This separation is complete for the corresponding 1-β-D-galactosylceramide derivative.[1]

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FIG. 1. *Thin-layer chromatograms of ceramides demonstrating* the effect of number, position, and stereochemistry of hydroxy groups on separations. Ordinary silica gel (*a*), borate-impregnated gel (*b*) and arsenite-impregnated gel (*c*) were used as adsorbents, and chloroform–methanol 95:5 (v/v) as developing solvent. Spots (30–40 μg) were made visible with cupric acetate–$H_3PO_4$ reagent (27). For ceramides containing racemic hydroxy acid, the L forms always move faster (samples *5–8*).

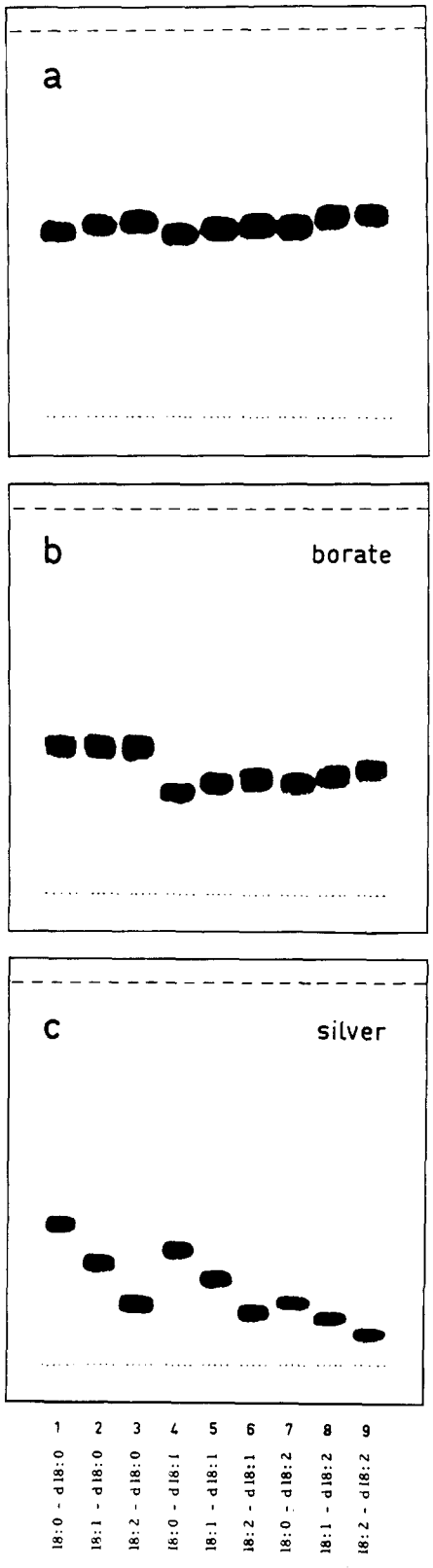


FIG. 3. Thin-layer chromatograms showing the effects of borate (*b*) and silver ions (*c*) compared with ordinary conditions (*a*) on saturated to tetraunsaturated dihydroxy ceramides. Solvent and detection as given for Fig. 1.

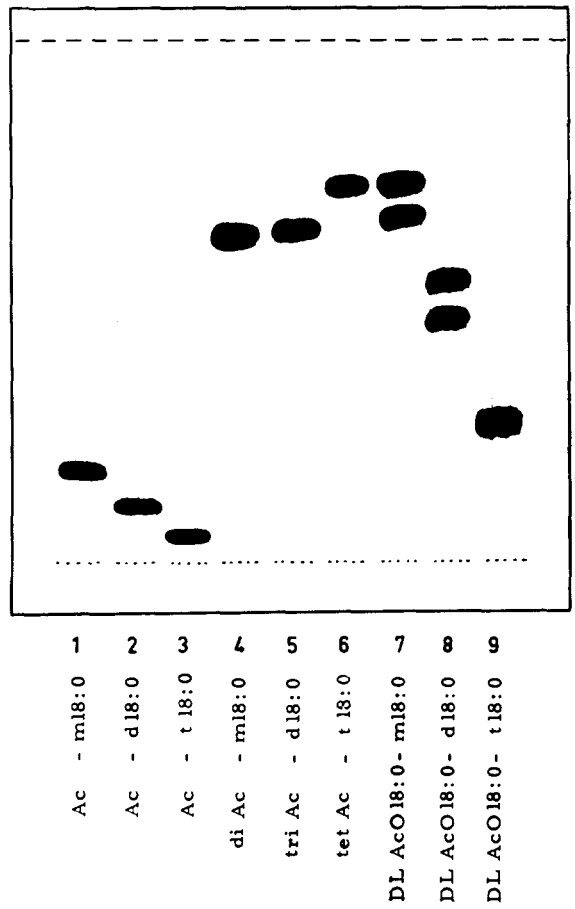


FIG. 4. Thin-layer chromatogram with partially and fully acetylated derivatives of monohydroxy base (samples *1*, *4*, and *7*), dihydroxy base (samples *2*, *5*, and *8*), and trihydroxy base (samples *3*, *6*, and *9*) on ordinary silica gel. Solvent and detection as given for Fig. 1. For ceramides containing racemic acetoxy acid, the L forms always move faster (samples *7* and *8*).

As shown in Fig. 1*a*, ceramides differing in number of hydroxy groups are well separated (if ceramides containing unnatural 2-L-hydroxy acid are neglected). Isomers that differ in hydroxy group position are also separated, e.g., 18:0–d18:0 (sample *2*) and 18:0–d18:1 (sample *3*) from h18:0–m18:0 (sample *5*), or 18:0–t18:0 (sample *4*) from h18:0–d18:0 (sample *6*) and h18:0–d18:1 (sample *7*). Fig. 1, *b* and *c*, demonstrates the effect obtained by glycol-complexing agents, borate and arsenite, respectively. These have been used before to improve separations of polyhydroxy compounds (31). An earlier study on *N*-dinitrophenyl derivatives of isomeric long-chain bases had shown that borate decreases the mobility of trihydroxy derivatives, while arsenite increases the mobility, compared with normal conditions.[2] A similar effect is obtained for trihydroxy base-containing ceramides. On borate plates (Fig. 1 *b*), 18:0–t18:0 (sample *4*) and h18:0–t18:0 (sample *8*) are retarded, while there is a strong opposite effect on arsenite.

[2] Karlsson, K.-A. Unpublished data.

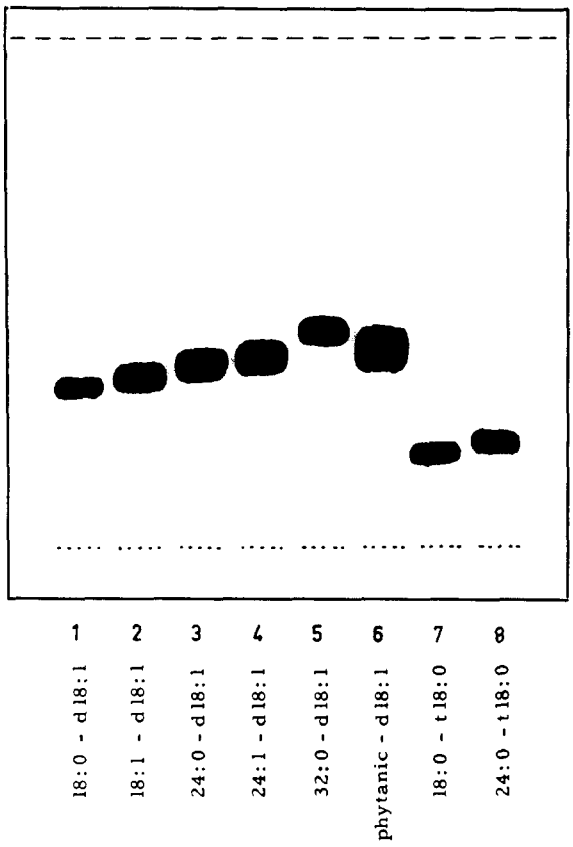


FIG. 5. The effect of chain length on the separations of ceramides on thin layers of ordinary silica gel. Solvent and detection as given for Fig. 1.

A borate effect is also observed for ceramides containing a 4-5 *trans* double bond (compare 18:0–d18:0, sample *2*, with 18:0–d18:1, sample *3;* and h18:0–d18:0, sample *6*, with h18:0–d18:1, sample *7*, in Fig. 1 *a* and *b*). This is also seen in Fig. 3 *a* and *b*, and has been observed before (24, 32). The effect of complexing agents on fatty acid hydroxyls is not significant.

Concerning fully acetylated derivatives, ceramides with trihydroxy base run slower than ceramides with dihydroxy base (Fig. 2). However, a reversal of migration is observed for fully acetylated trihydroxy base and dihydroxy base (Fig. 4).

### *Carbon Chain Length*

The effect of carbon chain length on TLC separation is shown in Fig. 5. With increasing chain length the compounds travel faster. Acetylation does not improve the separation (Figs. 2 and 6). As shown in Fig. 6, samples *4* and *5*, there is an unexpected improvement in separation of homologues of ceramides with *cis*-unsaturated fatty acids (18:1 and 24:1) on silver-ion plates (compare Fig. 5, samples *1–4*), which has been observed before (8). This effect is probably due to double bond position (9 and 15 positions, respectively).

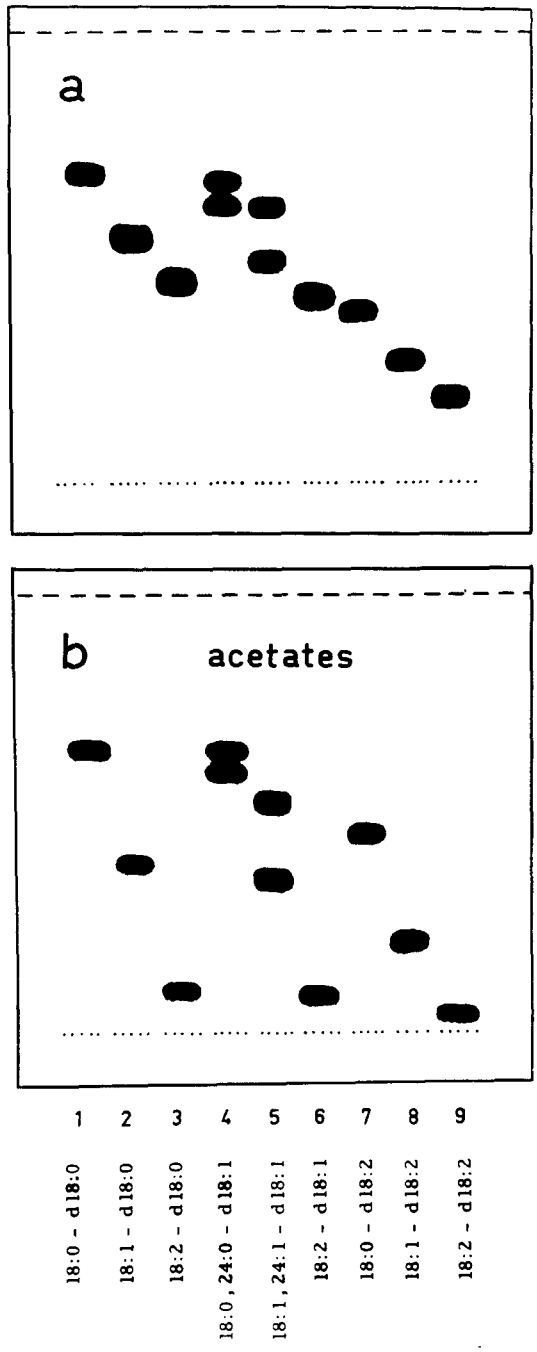


FIG. 6. Thin-layer chromatograms showing the separation of free ceramides (*a*) and ceramide acetates (*b*) on silver ion-containing layers. Plate *a* was developed three times with chloroform–methanol 95:5 (v/v), and plate *b* once with chloroform–benzene–acetone 80:20:10 (v/v/v). Detection as given for Fig. 1. Ceramides with $C_{24}$ acid (samples *4* and *5*) move faster than ceramides with $C_{18}$ acid.

### *Unsaturation*

The effect of unsaturation is demonstrated in Figs. 3 *a–c*, 5, and 6, where saturated and mono-, di-, tri-, and tetra-unsaturated ceramides are shown. On ordinary gel (Fig. 3 *a*) a slight increase in mobility is observed with increas-

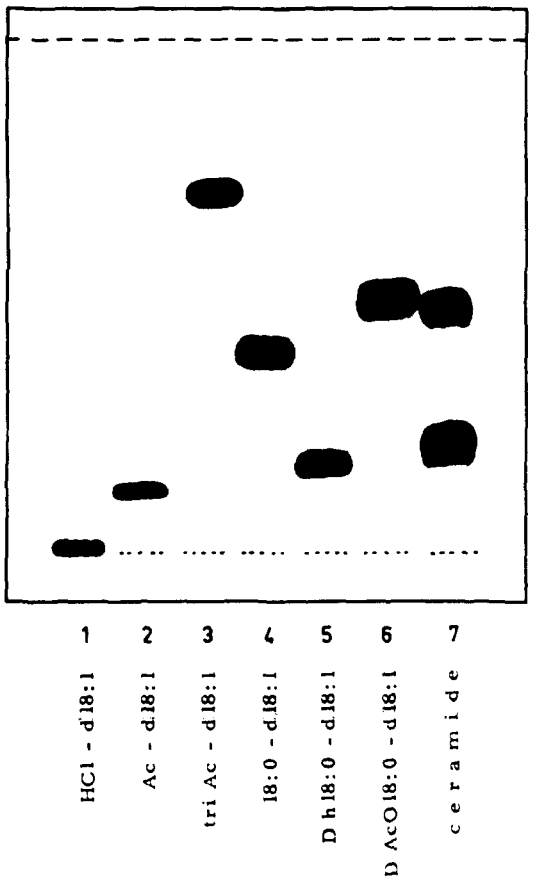


FIG. 7. Thin-layer chromatogram showing mobilities of characteristic sphingosine (d18:1) derivatives on ordinary silica gel. Solvent and detection as given for Fig. 1. The natural ceramide (sample 7) was prepared from bovine brain cerebroside (9), and contains d18:1 (95%) and d18:0 (5%) bases, and mostly long-chain (24:1) normal and 2-D-hydroxy fatty acids.

ing *cis* unsaturation of the fatty acid and also by addition of an isolated *trans* double bond to the base (compare 18:0–d18:1, sample *4*, with 18:0–d18:2, sample *7*). The 4-5 *trans* double bond of the base has no effect unless borate complexes are used (Figs. 1 *b*, and 3 *b*).

As shown in Fig. 3 *c* and Fig. 6, silver ion complexing retards compounds according to number of double bonds. The separation of ceramides with and without 4-5 *trans* unsaturation of the base (compare, e.g., samples *1* and *4* of Figs. 3 *c* and 6) is less pronounced than for ceramides with and without 8-9 *trans* unsaturation of the base (compare, e.g., samples *4* and *7* of Figs. 3 *c* and 6). The effect of increasing *cis* unsaturation of the fatty acid is similar for ceramides containing the three types of bases (d18:0, d18:1, and d18:2). As demonstrated earlier, compounds with *cis* double bonds are more retarded than compounds with *trans* double bonds (33); this should be considered when geometrical isomers may be present. Fig. 6 demonstrates a better separation of acetylated than nonacetylated ceramides, and also several reversals of migration between the two systems (e.g., samples *3* and *7*, *6* and *7*, and *6* and *8*).

### *Separation into Groups for Analysis of Natural Fatty Acid–Base Combinations*

When a total natural ceramide fraction (most possible species present) is considered for preparative TLC, e.g., preceding GLC–MS analysis, an initial separation with respect to the number of hydroxy groups is recommended, preferably on arsenite-containing layers (Fig. 1 *c*). The complex formation does not seem to interfere with subsequent analysis (34). In this way (Fig. 1 *c*) four completely separated main ceramide groups are obtained: trihydroxy base–normal acid (sample *4*), trihydroxy base–hydroxy acid (sample *8*, lower spot), dihydroxy base–normal acid (samples *2* and *3*), and dihydroxy base–hydroxy acid (samples *6* and *7*). A heterogeneity in chain length or unsaturation (which may elongate the spots) of natural compounds is not expected to cause overlapping of these groups. Only in extreme cases may a dihydroxy ceramide travel as a trihydroxy ceramide (see Ac–d18:1, sample *2*, and h18:0–d18:1, sample *5*, in Fig. 7). Isolated di- to tetrahydroxy ceramides may then be separated into subgroups according to degree of unsaturation by use of silver ions, possibly as acetates (Fig. 6). The poor effect on the 4-5 *trans* double bond of the base (compare, e.g., 18:1–d18:0, sample *2*, and 18:1–d18:1, sample *5*, lower spot, in Fig. 6) may cause problems in separating ceramides containing saturated and monosaturated dihydroxy bases. This may be overcome by using borate complexing (Figs. 1 *b*, and 3 *b*). The ceramide subgroups obtained may finally be separated according to carbon numbers by GLC (8, 19), and establishment of fatty acid–base combinations is possible by use of GLC–MS (8, 17–22).

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