A two-dimensional thin-layer chromatography procedure for simultaneous separation of ceramide and diacylglycerol species

Jacques Bodennec, Gerard Brichon,[1] Omanand Koul,* Mohammed El Babili, and Georges Zwingelstein

Institut Michel Pacha, Universite Claude Bernard, Lyon I, 1337 Corniche Michel Pacha, F 83500 La Seyne sur Mer, France, and Shriver Center for Mental Retardation,* 200 Trapelo Road, Waltham, MA 02254 and Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02215

Abstract We have developed a novel, simple, and rapid, two-dimensional thin-layer chromatography method to separate 1,2-, 1,3-diacylglycerols and ceramides containing α-hydroxy and normal fatty acids from other neutral lipids on one 10 × 10 cm precoated silica gel plate. The three solvent systems used in succession leave the phospholipids at the origin and separate neutral lipids of interest into component species. We have applied this method to incorporation of 9,10-[^{3}H]myristic acid into lipids of gills from sea bass and obtained results that are similar and comparable to those obtained by described methods.—**Bodennec, J., G. Brichon, O. Koul, M. El Babili, and G. Zwingelstein.** A two-dimensional thin-layer chromatography procedure for simultaneous separation of ceramide and diacylglycerol species. *J. Lipid Res.* 1997. **38:** 1702–1706.

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Lipid second messengers including ceramides and diacylglycerols (DAG) play an important role in signal transduction (1, 2). DAGs activate defined isotypes of protein kinase C in many cellular types (1) and induce differentiation or cell growth. Ceramides have been implicated in various cellular events including cell growth, differentiation, apoptosis (2), and in events induced by osmotic stress (3, 4). The two second messengers (DAGs and ceramides) are produced by enzymatic hydrolysis of glycerophospholipids and sphingolipids, respectively (5–7). Because DAGs, once produced, also activate the endosomal sphingomyelinase and release ceramides (8–10) it is important to have a method to properly separate the two second messenger lipids at the same time from a single extract. Although there are radioactive methods for quantification of DAG and ceramides (11) there is no method that resolves the two second messengers lipid species simultaneously for radioactivity measurements. We have therefore developed a simple two-dimensional TLC method to separate and quantitate 1,2- and 1,3-DAG, and ceramides containing α-hydroxy- and normal fatty acids on the same plate. We have used this procedure and a previously described method (4, 12) to study lipid alterations in gills from euryhaline sea bass during an osmotic stress. The data obtained by the two methods are similar and comparable, and additionally our method allows us to obtain data on DAG species as well.

MATERIALS AND METHODS

Chemicals

Solvents, all of analytical grade, were purchased from SDS (Peypin, France) or Carlo Erba (Milano, Italy). The following standard lipids: cholesterol, cholesteryl oleate, oleic acid methyl ester (esterified fatty acids), oleic acid (FFA), oleyl alcohol, triolein (TAG), diolein (18:1,*cis*-9) containing 85% 1,3-DAG and 15% 1,2-DAG isomers, 1(2)-monoolein, ceramides types III and IV from bovine brain were purchased from Sigma Chemi-

Abbreviations: DAG, diacylglycerol; TLC, thin-layer chromatography; BSA, bovine serum albumin; SW, sea water; 2-D, two-dimensional.

[1]To whom correspondence should be addressed.

cal Co. (St Quentin Fallavier, France). The plates were pre-coated silica gel 60 without fluorescence indicator from Merck (Darmstadt, Germany). Radiolabeled myristic acid, [9,10-^{3}H(N)] (24,0 Ci/mmol) was purchased from Amersham (Les Ulis, France). Fraction V bovine serum albumin (fatty acid free) (BSA) was from Sigma Chemical Co.

Procedure for two-dimensional lipid separations

Standard lipid mixture (20 µl) was applied to the plate alone or mixed with lipid from tissues. The standard mixture (20 µl) contained 20 µg each of soybean phospholipids, cholesterol, cholesteryl oleate, methyl oleate, oleic acid, triolein, diolein, monoolein, ceramides (Type IV with α-hydroxy fatty acids and Type III with normal fatty acids) and oleyl alcohol, 1,3- and 1,2-DAG at 30 µg, 8.5 µg and 1.5 µg, respectively.

Lipids were applied at the lower right-hand corner of the 10 × 10 cm (or 5 × 10 cm) TLC plate. The plate was developed in the first dimension (10 or 5 cm) in solvent system I [chloroform–methanol 50:5 (v/v)] and solvent was allowed to run up to 1 cm from the top. After drying the plate in air, it was developed in the second direction (see Fig. 1) in solvent system II [hexane–ethyl ether–acetic acid 80:20:1 (v/v/v)]. The solvent was allowed to run to the top of the plate, and after air-drying the plate was developed again in the same direction with solvent system III [heptane–diisopropyl ether–acetic acid 60:40:4 (v/v/v)]. The solvent was allowed to run to one-third of the length of plate. The plate was then removed, air dried, and sprayed with Dittmer and Lester reagent (13) and heated at 180°C for 1 min to visualize the lipid spots. However, iodine was used for visualization when radioactive lipids were chromatographed.

[9,10-^{3}H]myristic acid incorporation into gills

Gills from SW-adapted sea bass (*Dicentrarchus labrax*) were cut off at their bases, and each pair was transferred to ultrafiltrated Mediterranean sea water (SW) (salinity 3.7%) or diluted SW (salinity 1%), rinsed, and blotted on paper. Then, one gill of each pair was incubated, at 17°C for 4 h, in either SW (salinity 3.7%) or in diluted SW (salinity 1%) containing 5 µM [9,10-^{3}H]myristic acid (30 Ci/mmol, Amersham, UK) complexed to BSA (14). Lipids were extracted by the method of Folch, Lees, and Sloane Stanley (15) as modified by Chapelle et al. (16). Radioactivity in the lipid extracts was determined. An aliquot of radioactive lipids was mixed with 20 µl of standard lipid mixture described above and spotted for 2-D TLC. The separated spots were visualized by exposure to iodine vapor, and the spots were scraped into counting vials. Three ml of water–ethanol 1:1 (v/v) was mixed with the gel and 8 ml of Pico-fluor 30 scintillation fluid (Packard, Downers Grove, IL) was added and the mixture was mixed thoroughly (17). Radioactivity was determined in a Packard Tricarb 460 β radio spectrometer. Counting efficiency was determined by the external standard channels ratio method. Results are expressed in dpm.

Analysis of radioactivity in free ceramides of gill lipids after alkaline methanolysis (13) [previously described method]

Radioactive lipid extracts were subjected to mild alkaline methanolysis in 0.4 M methanolic NaOH at 37°C for 1 h. Concentrated HCl was added to obtain a final concentration of 0.4 M acid, and hydrolysis was continued for 1 h at the same temperature (4). Chloroform extracts were made and carrier lipids (pure ceramides containing both normal and α-hydroxy fatty acids) were added. The samples, dissolved in minimal amounts of chloroform, were applied to aminopropyl bonded silica cartridge columns (100 mg LC-NH_2 from Supelco, Bellefonte, PA) that were preconditioned with hexane. Neutral lipids containing free ceramides were eluted with 3 ml of chloroform–isopropanol 2:1 (v/v) and free fatty acids with 1.4 ml of isopropyl ether–acetic acid 98:2 (v/v). Sphingomyelin and residual phospholipids were eluted with 3 ml of 0.3 N methanolic HCl. Radioactivity was determined in aliquots of each fraction.

Free ceramides in neutral lipid fraction were separated by one-dimensional TLC on 5 × 10 cm or 10 × 10 cm precoated silica-gel plates in chloroform–methanol 50:3 (v/v). The two ceramide lipid spots containing normal and α-hydroxy fatty acids and neutral lipids spots, visualized with iodine, were scraped into scintillation-counting vials and radioactivity was determined as described above.

Statistics

Mean values for radioactivity were compared using one-way analysis of variance. Percentages were arcsine-transformed. All values are presented as means ± SEM.

RESULTS AND DISCUSSION

Figure 1 shows a typical 2-D chromatogram of lipids from gills mixed with standard lipid and run according to the protocole given in the Methods above. All major classes of neutral lipids are well resolved. Additionally, 1,3-diacylglycerols and 1,2-diacylglycerols are well separated from cholesterol and fatty alcohols.

In the two dimensional TLC system described here,

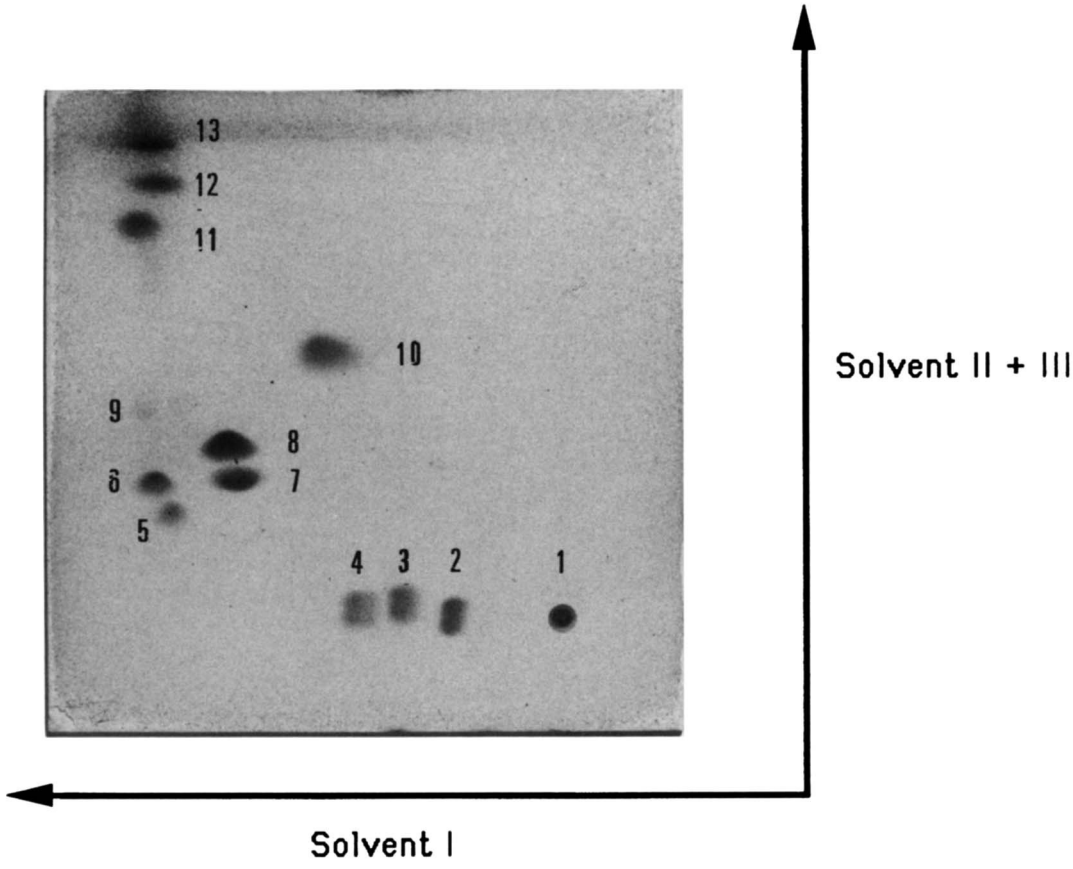

Fig. 1. Representative example of a two-dimensional thin-layer chromatography of standard lipids diluted with radioactive lipids from gills. 1: Origin (phospholipids plus cerebrosides and sphingosine bases); 2: ceramide 1 (ceramide type IV Sigma with α-hydroxy fatty acids); 3: MG; 4: ceramide 2 (ceramide Type III Sigma with normal fatty acids); 5: 1,2-DAG; 6: 1,3-DAG; 7: cholesterol; 8: oleyl alcohol; 9: unknown; 10: FFA; 11: TG; 12: esterified fatty acids; 13: cholesteryl esters (plus hydrocarbons).

solvent II gave a better resolution between triglycerides, esterified fatty acids, and cholesterol esters. Solvent III separates free fatty acids from fatty alcohols, 1,2-diacylglycerols, 1,3-diacyglycerols, and cholesterol, but the resolution between the latter two compounds is not possible. The separation between 1,3-diacylglycerols and cholesterol is achieved by using solvent I.

Solvents used for developing TLC plates for separation of neutral lipids are nearly always mixtures of polar and nonpolar solvents of varying degrees. Increasing the polarity of the solvents increases the R_f values of all components and improves resolution between compounds of low R_f and decreases the resolution between the compounds of higher R_f values (18). Nevertheless, cholesterol and 1,3 DAG are particularly difficult to separate as they often migrate together in the conventional solvent mixtures. Similarly, monoglycerides and ceramides often overlap phospholipids. In fact, it is often impossible to obtain complete separation among various classes of compounds on the same plate because most of the lipid mixtures contain compounds of widely different polarities (19).

The solvent system of Bowyer and King (20) that is used to separate neutral lipids in petroleum ether–diethyl ether–acetic acid 40:60:0.1 (v/v/v) in the first dimension followed by petroleum ether–ethyl acetate–acetic acid 95:2:2 (v/v/v) in the second dimension resolves 1,2- and 1,3-diacylglycerols, but ceramides, monoacylglycerols, and phospholipids overlap. In contrast, our 2-D TLC system that uses three solvents in succession allows the separation of all these compounds on one plate.

We recommend small plates (10 × 10 cm or 5 × 10 cm) as the development time is shorter and the resolution is not compromised. Shorter time also keeps the spots from diffusing and improves resolution (20). In addition the condensed spot allows better sensitivity of detection.

TABLE 1. Incorporation of [9,10-^{3}H]myristic acid into lipids of gills after incubation in sea water (SW) or diluted sea water (Diluted SW)

Lipid	Diluted SW	SW
	% of total lipid radioactivity	
PL	54.4 ± 3.3	32.9 ± 0.9[a]
α-Hydroxy-FA ceramide	3.6 ± 0.5	4.1 ± 0.9
MG	2.1 ± 0.4	1.9 ± 0.8
Normal-FA ceramide	3.2 ± 0.5	7.0 ± 0.6[a]
Cholesterol + fatty alcohol	9.0 ± 0.6	1.0 ± 0.2[a]
1,2-DAG	1.4 ± 0.3	2.7 ± 0.1
1,3-DAG	1.6 ± 0.3	4.0 ± 0.2[a]
FFA	4.7 ± 0.6	8.2 ± 0.3[a]
Unknown	1.9 ± 0.6	4.7 ± 1.3
TG	15.3 ± 1.8	31.3 ± 1.3[a]
Cholesteryl esters	2.7 ± 1.2	1.9 ± 0.9

Results are expressed as the mean ± SEM of four fish. Each determination was done in duplicate.

[a]After arcsine transformation, the SW and Diluted SW values were statistically different, $P < 0.001$.

We have used this double-dimensional TLC method to obtain data on incorporation of labeled myristic acid into DAG and free ceramides from gills of sea bass incubated in either sea water (SW) or diluted SW. The data are summarized in **Table 1.** A decrease in salt concentration decreased the incorporation of radioactivity into ceramides with normal fatty acids and in 1,3-DAG. There was no difference in incorporation into 1,2-DAG. The results on the incorporation into ceramides are similar and comparable to those obtained after processing the sample by classical methodology of alkaline methanolysis and single dimensional TLC (4, 12) to obtain ceramide species (**Table 2**). The ratio of radioactivity incorporated into ceramides containing α-hydroxy fatty acids (C1) and normal (C2) fatty acids determined by the two methods is shown in **Table 3.** Both methods gave similar data for ceramides so that there is no statistically significant difference. In addition, our 2-D method described here generates simultaneous data on two DAG species not possible with the classical methodology.

In conclusion, we have developed a novel 2-D TLC method to resolve 1,2- and 1,3-DAG species and ceramides containing α-hydroxy and normal fatty acids and other neutral lipids on one plate at the same time. The method is simple and allows a better resolution of species of ceramides and diacylglycerols than conventional TLC methods. As not many steps are involved between sample preparation and TLC, losses are minimal and the data on mass or radioactivity can be obtained quantitatively and reproducibily in minimum time. Further data obtained are similar and comparable to that obtained with classical methods.

TABLE 2. Radioactivity incorporated into molecular species of free ceramide as determined after alkaline methanolysis (12)

Incubation Medium	Diluted SW	SW
	% of lipid radioactivity	
With normal fatty acids	3.7 ± 0.2	6.6 ± 0.5[a]
With α-hydroxy fatty acids	2.5 ± 0.1	2.7 ± 0.1

[a]SW and Diluted SW values are statistically different after arcsine transformation, $P < 0.001$.

TABLE 3. Comparison of the radioactivity incorporated into α-hydroxy (C1)- and normal fatty acid (C2)-containing free ceramides determined by the two methods described in this report

	2D-TLC	Alkaline Hydrolysis	
Diluted sea water	0.92 ± 0.05	0.69 ± 0.06	NS
Sea water	0.63 ± 0.11	0.43 ± 0.10	NS

Results are expressed as ratio: α-hydroxy/normal fatty acids containing ceramides. NS, 2D-TLC and alkaline hydrolysis values are not statistically different after arcsine transformation.

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