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Note

Improved one-dimensional thin-layer chromatographic technique for polar lipids

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The number of one-dimensional, thin-layer chromatographic (TLC) techniques employed for the resolution of lipids has increased rapidly over the past few years. The improvements brought about have made it possible to separate nearly all of the major polar lipid classes in a single migration step and have led to numerous methods for the quantitative, densitometric analysis of lipids, *in situ*, on the chromatographic plates[1–8]. These methods are less time-consuming and more sensitive than the more classical ones employing multi-migrational, two-dimensional TLC and subsequent determinations of phosphorus and galactose. However, one of the major problems inherent to one-dimensional TLC techniques is the high risk of contamination of one or more lipid bands by other lipids, leading to important errors in the interpretation of experimental observations. This risk will remain as long as there are lipids which have not yet been localized on the chromatographic plates and which exist in the biological material studied. Among the lipids which are frequently overlooked when devising TLC techniques are the lysophospholipids. It is particularly important that these lipids be localized on the chromatographic plates when metabolic studies, employing radiolabelled substrates that can be incorporated into lysophospholipids, are realized using radioautographic techniques following the resolution of the lipids by TLC.

Vitiello and Zanetta[9] devised a one-dimensional TLC technique which allows the resolution of sphingomyelin (Sph), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL) with phosphatidic acid (PA), phosphatidylethanolamine (PE), hydroxy- (H-S) and non-hydroxysulphatides (NH-S) and hydroxy- (H-CB) and non-hydroxycerebrosides (NH-CB). Cholesterol, cholesterol esters and neutral lipids migrate to the solvent front and are not resolved. As far as the polar lipids are concerned, the resolution is sufficiently good to permit the quantitative, densitometric analysis of all of them, after visualization by copper acetate/phosphoric acid charring[6], except for PS and PI when one of these two lipids, usually PS, is relatively abundant in the sample. The latter two lipids have similar R_F values, leading to overlap of the corresponding peaks obtained by densitometric scanning. The lysophospholipids were not localized.

We propose a variation of the solvent system described by Vitiello and Zanetta[9] that allows resolution of all the lipids cited by these authors, as well as of

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Lyso-PC, Lyso-PE, PA, CL, phosphatidylglycerol (PG), PE plasmalogen (pPE) and of the plant lipids digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG).

EXPERIMENTAL

Materials

All standard lipids, except for PI, MGDG and DGDG, were from animal sources and were obtained from Sigma Chemical Co. All reagents and solvents were of analytical reagent grade. 10 × 10 cm HPTLC plates (Silica gel 60 F-254) were from Merck.

Radiolabelled animal PI was obtained by culturing glioblastoma cells from the C-6 strain[10] in the presence of [^{14}C]inositol for 24 h. The total lipids were extracted three times from the pelleted cells with chloroform–methanol (2:1, v/v), washed with distilled water and then dried with Na_2SO_4. The radiolabelled PI was identified after HPTLC and radioautography. The mouse sciatic nerve lipid extracts were obtained by treating the excised nerves with chloroform–methanol (1:1, v/v) for 24 h at room temperature. Sonicating these delipidated nerves in the presence of fresh solvent did not ameliorate the extraction. The lipid extracts were stored at −20°C until use.

HPTLC and densitometry

The standard lipids and the sciatic nerve lipid extracts were spotted onto the HPTLC plates, using a 10-μl Hamilton syringe, 1.5 cm from the bottom edge. Each of the six lanes on each plate was 8 mm wide and separated from the neighbouring lanes by 7 mm. The chromatographic tanks were pre-saturated with the solvent systems for 1 h (18–20°C) before use, without lining them with filter-paper. The lipids were chromatographed with one of the following solvent systems: A, methyl acetate–*n*-propanol–chloroform–methanol–0.25% aqueous potassium chloride (25:25:25:10:9, v/v)[9]; B, methyl acetate–*n*-propanol–chloroform–methanol–0.25% aqueous potassium chloride (25:25:28:10:7, v/v).

Several other variations of the solvent system A containing different proportions of chloroform and KCl were tested. Increasing the proportion of chloroform resulted in a decrease of the R_F values of nearly all the polar lipids, while decreasing the proportion of KCl led to the decrease in the R_F values of the phospholipids, without any apparent effect on the migration of the glycolipids and the neutral lipids. We shall only discuss results with the solvent systems A and B detailed above.

The chromatography was stopped when the solvent front was 1 cm from the top edge of the plate. The sciatic nerve lipids were identified by co-migration with standards in different lanes and by charging standard lipids onto the same lane as the sciatic nerve lipid extracts, checking that the nerve lipids migrated with the corresponding standard lipids. Sciatic nerve PI was identified by co-migration with the radiolabelled PI of the cell culture lipid extract. The lipid bands were visualized by copper acetate/phosphoric acid charring[6] and scanned, within 1 h, at 366 nm (mercury lamp), using a photodensitometer scanner (Camag) operating in the reflectance mode (beam dimensions, 4 × 0.1 mm; scan speed, 0.5 mm/sec), coupled to a SP4100 computing integrator (Spectra-Physics).

RESULTS AND DISCUSSION

We employed the original solvent system (A) proposed by Vitiello and Zanetta[9] to separate the lipids of a mouse sciatic nerve lipid extract containing Lyso-PE and various standard lipids. Lyso-PE co-migrated with PS (hR_F = 32.4 ± 0.6) (mean ± standard deviation). As expected[9], the same was true for PA and CL (hR_F = 42.4 ± 0.6). PG was not resolved and the plant lipid MGDG was insufficiently resolved from the neutral lipids to permit its densitometric analysis.

These observations led us to carry out a systematic study of the effects of variations in the solvent composition on the migration of the various lipids in order to try to resolve the co-migrating lipids (see Experimental). The best resolution was obtained with solvent system B.

We used the solvent system B to resolve mouse sciatic nerve lipids to which we added, separately or otherwise, standard lipids that are not usually observed in this biological material, because of their very low concentrations in freshly prepared lipid extracts, as well as standard lipids considered to be characteristic of plant tissues (MGDG and DGDG). The hR_F values for each of these lipids are given in Table I. In every case, each lipid added to the sciatic nerve lipid extract was resolved from the other lipids. Hence, even if the standard deviations observed for the hR_F values of two lipids suggest a possible co-migration, *e.g.* hR_F(PA) = 33.3 ± 1.3, hR_F(CL) = 35.5 ± 2.1, the two lipids were always resolved when they were both present.

TABLE I

hR_F VALUES OF MOUSE SCIATIC NERVE LIPIDS AND ADDED STANDARD LIPIDS RESOLVED BY THE SOLVENT SYSTEM B

Standard polar lipids (*) were added, separately or otherwise, to a mouse sciatic nerve lipid extract. HPTLC using the solvent system methyl acetate–*n*-propanol–chloroform–methanol–0.25% aqueous KCl (25:25:28:10:7, v/v) and densitometric scanning were carried out as described in Experimental. hR_F values were determined from the densitometric scan recordings. n = Number of different measurements; N = number of HPTLC plates; S.D. = standard deviation.

Lipid	hR_F *(mean ± S.D.)*	n	N
(*)Lyso-PC	3.1 ± 0.3	4	3
Sph	5.4 ± 0.4	15	6
PC + pPC	9.9 ± 0.6	15	6
PS	18.8 ± 1.4	15	6
(*)Lyso-PE	22.1 ± 0.7	8	4
(*)PI (soy bean)	24.7 ± 1.0	6	3
PI (sciatic nerve)	28.0 ± 1.5	15	6
(*)PA	33.3 ± 1.3	5	5
CL	35.5 ± 2.1	15	6
(*)PG	39.3 ± 1.1	2	2
PE	43.5 ± 2.9	14	5
pPE	45.8 ± 3.6	8	3
(*)DGDG	52.0 ± 2.8	3	3
H-S	55.1 ± 1.8	15	6
NH-S	59.3 ± 1.7	15	6
H-CB	69.3 ± 2.7	15	6
NH-CB	77.2 ± 2.5	15	6
(*)MGDG	88.1 ± 1.9	6	3

Lyso-PE and PS, which co-migrated with the previous solvent system, were resolved by solvent system B, hR_F(Lyso-PE) = 22.1 ± 0.7, hR_F(PS) = 18.8 ± 1.4. PE and pPE frequently give rise to a single peak on the densitometric scan recordings when they are present in unequal quantities. However, a double band is always observed on the plate when these two lipids are present. The lower band represents PF while pPE constitutes the upper band. The overlaps of the standard deviations observed for the above-mentioned lipids are simply due to the variations in the migrations, which were carried out independently on different days. Similar observations were made by Payne[11].

In addition to the lipids resolved by the original solvent system, and the six lipids mentioned above (PS, Lyso-PE, PA, CL, PE and pPE), our solvent system also allows the resolution of Lyso-PC, PG, DGDG and MGDG. The resolution is sufficiently good to give well defined peaks after densitometric scanning of the plates. This is illustrated in Fig. 1, which represents a densitometric scan recording of a lane in which standard lipids (see legend to Fig. 1) were added to a mouse sciatic nerve lipid extract so as to show the relative positions of all of the polar lipids resolved by our solvent system. Plant PI was omitted for the sake of clarity. Indeed, PI from plants does not migrate to the same position as animal PI, $hR_F(PI_{plant})$ = 24.7 ± 1.0, $hR_F(PI_{animal})$ = 28.0 ± 1.5. This absence of co-migration of the two types of PI is also observed when employing other solvent systems[6,9,12] and visualization tech-

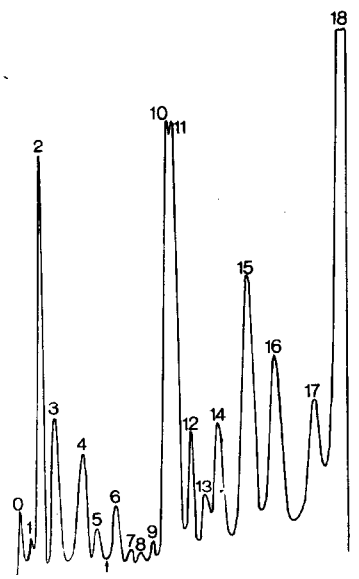

Fig. 1. Densitometric scan recording after HPTLC using the solvent system B, methyl acetate–*n*-propanol–chloroform–methanol–0.25% aqueous KCl (25:25:28:10:7). Standard lipids (Lyso-PC, Lyso-PE, PA, PG, DGDG and MGDG) were added to a mouse sciatic nerve lipid extract. The chromatography and densitometric scanning were carried out as described in Experimental. Peaks: O = origin; 1 = Lyso-PC; 2 = Sph; 3 = PC + pPC; 4 = PS; 5 = Lyso-PE; 6 = PI; 7 = PA; 8 = CL; 9 = PG; 10 = PE; 11 = pPE; 12 = DGDG; 13 = H-S; 14 = NH-S; 15 = H-CB; 16 = NH-CB; 17 = MGDG; 18 = neutral lipids and solvent front. The arrow shows the position of standard PI from the soy-bean (not added).

niques. Hence, the identification by co-migration of PI in animal lipid extracts with standard plant PI is highly hazardous. This phenomenon was not observed for any other phospholipid.

The well defined peaks obtained by densitometric scanning of the plates allow our solvent system to be employed for the quantitative analysis of animal and plant polar lipids by densitometry, *in situ*, on the chromatographic plates. Moreover, the contamination risk is very much reduced compared with existing one-dimensional HPTLC techniques, since we can separate up to seventeen identified lipids. To our knowledge, no other one-dimensional TLC technique allows the resolution of so many different types of polar lipids with a single migration and without preliminary fractionation of the lipids.

The neutral lipids, which migrate to the solvent front, can easily be resolved by a simple variation of this technique. The first elution, using the solvent system B, is allowed to continue for a distance of 6.5 cm (origin to solvent front). A second elution, using the solvent system hexane–diethyl ether–acetic acid (75:23:2, v/v), in the same direction as the first elution, is then carried out to the top of the chromatoplate. This second elution, which does not modify the positions of the polar lipids, allows the resolution of cholesterol, free fatty acids, diacylglycerols, triacylglycerols and cholesterol esters.

In our laboratory, we currently employ these solvent systems for quantitative analyses and metabolic studies of peripheral nerve lipids and plant lipids. For the quantitative analyses, we use a densitometric technique similar to that described by Macala *et al.*[6], except that we use the plant lipid MGDG as an external standard for peripheral nerve analyses, and Sph as the external standard for plant lipid analyses.

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