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Note

Separation of monoacetyldiglycerides by argentation thin—layer chromatography

ARUN KUMAR DAS, RAMA GHOSH and JYOTIRMOY DATTA*
Department of Chemistry, Bose Institute, Calcutta 700 009 (India)
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Interest has recently grown in the different molecular species involved in the major phospholipid classes present in cellular membranes, in order to determine the influence of these components on the functional aspects of such membranes[1–3].

One of the essential steps involved in the determination of phospholipids is the production of the corresponding diglycerides and their resolution, preferably as monoacetyl derivatives, according to the number of double bonds in the molecule by argentation thin-layer chromatography ($AgNO_3$-TLC). The composition of the different monoacetyldiglycerides (MADGs) in each fraction is obtained either by direct gas chromatography–mass spectrometry[4] or by computation from gas–liquid chromatographic (GLC) data on the component MADGs and the distribution of fatty acids at positions 1 and 2 of these molecules[5]. Thus, the efficiency of this procedure depends to a large extent on the degree of resolution of MADGs on the TLC plates.

A number of solvents, mostly mixtures of chloroform–methanol[5,6], benzene–chloroform–methanol[7] and benzene–diethyl ether[8], has been used for the TLC separation of MADGs but none of these could completely separate the components. In particular, the compounds containing more than four double bonds tend to segregate in one or two bands.

In the present communication we report the use of a new solvent to separate these highly unsaturated MADGs on $AgNO_3$-TLC plates.

MATERIALS AND METHODS

Reference compounds and reagents

Synthetic L-α-distearoylphosphatidylcholine, methyl pentadecanoate and reference mixtures of fatty acids, all >99% pure, were purchased from Applied Science Labs. (State College, PA, U.S.A.). Phospholipase C (E.C. 3.1.4.3.) from *Clostridium perfringens*, type I, was a product of Sigma (St. Louis, MO, U.S.A.). Silica gel G for TLC and neutral Al_2O_3 for adsorption column chromatography were obtained from E. Merck (Darmstadt, G.F.R.). Most of the other reagents and solvents used were of A.R. grade. The solvents were in general redistilled. Diethyl ether was freed from peroxides, washed, dried and redistilled before use.

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Preparation of hen egg phosphatidylcholine (PC)

This was extracted[9] from fresh yolk and partially purified by precipitation from cold acetone[10]. Pure PC was isolated chromatographically using a neutral Al_2O_3 column[11], the purity being checked by TLC against reference PC.

Preparation of MADGs from egg PC

sn-1,2-Diglycerides from egg PC were prepared by a method recently developed in this laboratory. The PC was enzymatically (phospholipase C) hydrolysed to the corresponding *sn*-1,2-diglycerides on a preparative TLC plate. The diglycerides were separated from other products by development of the same plate and extracted from the adsorbents using diethyl ether[12]. The diglycerides were acetylated to the corresponding MADGs using acetic anhydride and pyridine, the products being purified by preparative TLC[13].

Subfractionation of MADGs on $AgNO_3$-TLC plates

Glass plates (28 × 14 cm) were coated with 0.5 mm of silica gel G, impregnated with 25% (w/w) $AgNO_3$, dried in air for about 30 min, activated at 120°C for 2 h, cooled and stored in desiccator under silica gel before use. MADGs (10–15 mg) in chloroform were applied on the plate as a narrow band 2 cm from one of the shorter edges. The plate was developed twice up to 22 and 24 cm in light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (75:35:1, v/v/v) in a saturated chamber at 25°C. The bands were located under UV light after spraying the developed chromatogram with a 1% methanolic solution of 2′,7′-dichlorofluorescein.

The content of each band was isolated from the adsorbents by repeated extraction with diethyl ether. The pooled extract was freed from $AgNO_3$ by washing with water, dried under anhydrous Na_2SO_4 and adjusted to an appropriate concentration.

Determination of the fatty acid compositions of MADG subfractions

Mixed methyl esters corresponding to the MADGs in each band were prepared by alkaline methanolysis[14], and the fatty acid compositions were determined by GLC of the mixed methyl esters, isothermally at 180°C on stainless-steel columns (6 ft. × 1/8 in.) packed with 15% DEGS coated on Gas-Chrom Z (100–120 mesh) (Applied Science Labs.). A dual-column Pye Unicam gas chromatograph, Model GCD, equipped with a flame ionization detector was used. The peaks were identified by comparing their retention parameters with those of the reference methyl esters. Compositions were calculated from peak areas, obtained from the peak height × width at half height and applying suitable correction factors determined from the chromatograms of the reference mixtures of methyl esters. The relative molar percentages of MADGs in the bands were computed from the chromatograms of fatty acid methyl esters with methyl pentadecanoate as internal standard[15].

RESULTS

Fig. 1 shows that MADGs from egg PC can be resolved into ten well separated bands on a 25% $AgNO_3$ treated silica gel G plate developed in the present solvent system. The absence of any fully saturated species in egg PC is evident from the fact that the top band appeared in the chromatogram only when fully saturated MADG

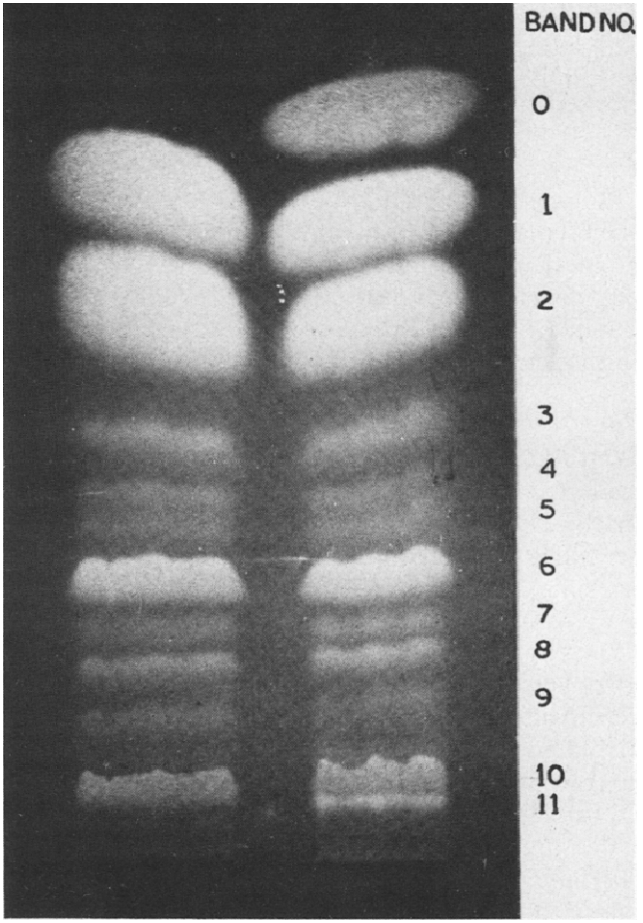

Fig. 1. Separation of monoacetyldiglycerides (MADGs) on an $AgNO_3$-TLC plate. Conditions: 28 × 14 cm TLC glass plate coated with 0.5 mm of silica gel G impregnated with 25% (w/w) $AgNO_3$. MADGs (10 mg) derived from hen egg PC were applied as a small band on the left-hand side of the plate; on the right-hand side, the same mixture together with 1 mg MADGs from authentic 1,2-distearoylphosphatidylcholine was similarly applied. The plate was developed twice up to 22 and 24 cm respectively in light petroleum (b.p. 60 – 80 C) – diethyl ether–acetic acid (75:35:1, v/v/v). Bands were visualized under UV-light after spraying with a 1% methanolic solution of 2',7'-dichlorofluorescein. Bands: O = 3-acetyl-1,2-distearoylglycerol (OOAC); 1–10 = MAGDs of egg PC; 11 = traces of 2',7'-dichlorofluorescein not removed during processing.

prepared from reference distearoylphosphatidylcholine was mixed with MADGs of egg PC. From the chromatogram it appears that bands 1, 2, 3, 6 and 10 are major subfractions in the MADGs of egg PC. This was confirmed from the relative proportions of the subfractions estimated by GLC analysis. The very faint band between bands 9 and 10 was taken with band 9 for extraction of the content.

MADGs prepared from rat liver PC, when run side by side with MADGs from egg PC, produced almost identical chromatograms with respect to the number of bands and their R_F values, but the former contained an additional saturated band (the chromatogram is not shown but is available on request).

In Table I the fatty acid compositions (mole%) are expressed in terms of the number of double bonds in the molecule by adding together the percentages of the fatty acids containing identical numbers of double bonds. The average number of double bonds per mole of the MADGs in a band, A, is calculated from:

$$A = \frac{\Sigma \text{ (mole\% of each fatty type} \times \text{number of double bonds in the type} \times 2)}{100}$$

From the acid compositions of the last five bands and the corresponding A values, it is concluded that the present chromatographic method can effectively separate highly unsaturated MADGs containing four or more double bonds. The predominance (over 45%) of each of the two types of fatty acids, one saturated and the other a tetra-, penta- or hexanoic, in bands 6, 8 and 10 respectively indicates that these bands might contain about 90% of the 04AC, 05AC and 06AC types of MADGs. Again the tetra-, penta- and hexaenoic acids are almost entirely distributed in the above three bands respectively, which indicates that the polyunsaturated acids in egg PC remain mainly in combination with saturated fatty acids.

From the fatty acid compositions, applying similar arguments, the 01AC, 02AC and 03AC types of MADGs are considered to be the probable major components of bands 1, 2 and 5 respectively. In the case of bands 3, 4, 7 and 9, where the fatty acid compositions are more complex, additional help was needed from the established elution order of MADGs in $AgNO_3$-adsorption chromatography[16]. For example, the major component of band 4 was considered to be 22AC from the facts that 65% of the fatty acids were of the dienoic type and that the 22AC type of MADG is eluted between 12AC and 03AC.

Considering the fatty acid compositions and the molar proportions of the components of the bands, we conclude that about 50 mole% of egg PC is of the 01 type, the other major types being 02 (32%), 11 (6%), 04 (5.6%), 12 (1.4%) and 06 (1.2%).

DISCUSSION

Resolution of MADGs is a very important step in the determination of the distributions of phosphoglycerides in different tissues; the accuracy of such determinations depends to a large extent on the degree of resolution. The present method is more efficient than the previous ones[4–8]; in the latter, good resolution of highly unsaturated MADGs containing four or more double bonds could not be achieved, whereas these were resolved into four distinct bands by our method.

Ambient humidity is a factor known to have a profound influence on the degree of resolution in $AgNO_3$-TLC. During the development of the present method we found that this effect becomes very prominent when the developing solvent contains hydrophilic components like methanol or ethanol. At high ambient humidity the resolution was poor with these solvent mixtures. With the present solvent system, which does not contain any highly hydrophilic component, well reproducible chromatograms could be obtained under widely varying ambient humidities. High resolution and good reproducibility are the two major advantages of the present method over the others. A third advantage lies in the use of a single solvent for the two

TABLE I

MOLE PERCENTAGES, FATTY ACID COMPOSITIONS, AVERAGE NUMBERS OF DOUBLE BONDS AND THE PROBABLE MAJOR COMPONENTS OF THE DIFFERENT MADG BANDS SEPARATED ON $AgNO_3$-TLC

Band no.	*Mole%*	*Fatty acid composition*							*Average no. double bonds*	*Probable major component**
		Saturated	*Monoenoic*	*Dienoic*	*Trienoic*	*Tetraenoic*	*Pentaenoic*	*Hexaenoic*		
1	49.7	50.1	49.9	–	–	–	–	–	1.0	01AC
2	37.9	42.2	15.9	41.9	–	–	–	–	2.0	02AC
3	1.9	8.2	46.1	41.6	4.1	–	–	–	2.8	12AC
4	0.6	12.2	14.5	65.4	7.9	–	–	–	3.4	22AC
5	0.6	40.8	3.0	6.2	45.4	4.6	–	–	3.4	03AC
6	6.2	46.2	3.5	1.3	1.6	47.4	–	–	4.0	04AC
7	0.4	8.9	31.8	10.4	12.5	36.4	–	–	4.7	14AC
8	0.8	45.1	4.6	–	–	3.4	46.9	–	5.0	05AC
9	0.6	1.8	8.6	2.9	75.1	4.3	7.3	–	5.9	33AC
10	1.3	46.1	1.8	1.0	2.0	–	1.0	48.1	6.1	06AC

* The numerals denote the number of double bonds in the two acyl groups in MADG.

consecutive developments, whereas in most of the previous methods[5,7,8] two different solvents were used.

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