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Note

Method for determination of different phospholipids by circular high-performance thin-layer chromatography

RUDI KRAUS, JÜRGEN WUTHE and RODERICH RÜFER*

Institut für Pharmakologie und Toxikologie der Fakultät für Klinische Medizin Mannheim der Universität Heidelberg, Maybachstrasse 14–16, D-6800 Mannheim 1 (F.R.G.)

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Surface-active phospholipids have been reported to have an important influence on the mechanical behaviour of the lungs [1,2]. In pathological conditions, quantitative as well as qualitative alterations of this fraction are believed to be responsible for respiratory distress syndrome (RDS) [3,4]. Therefore, sensitive and specific methods for phospholipid analysis are needed.

In recent years numerous methods for quantitative determination of phospholipids by high-performance thin-layer chromatography (HPTLC) [5–11] have been developed. HPTLC plates combined with a detection reagent, such as 8-anilino-1-naphthalene sulphonate (8-ANS) [12], allow quantitative determination of nanogram amounts of phosphatidylcholine (PC) and sphingomyelin (SM).

In this paper the use of 2,5-bis-[5-*tert.*-butylbenzoxazolyl(2′)] thiophene (BBOT) as detection reagent is described for the first time. In combination with the Camag Circular U-chamber system, this reagent allowed the measurement of nanogram amounts of lysophosphatidylcholine (LPC) even in biological material. The main advantages of our method are the fast and reproducible separation of phospholipids and the quantitative determination of LPC due to the sensitive detection reagent BBOT. Up to 24 samples can be accommodated on one plate, since the geometry of the circular technique with an increasing lateral distance towards the periphery prevents cross-contamination of two neighbouring samples during development of the chromatogram.

EXPERIMENTAL

Reagents

All chemicals and the HPTLC plates were purchased from Merck (Darmstadt,

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F.R.G.). We used spectroscopic-grade chloroform and methanol, chromatographic-grade ethanol, analytic-reagent-grade acetic acid and silica gel 60 HPTLC plates (10×10 cm), as well as L-α-dipalmitoylphosphatidylcholine (DPPC), SM, L-α-LPC (γ-palmitoyl) and BBOT as detection reagent (phospholipids and BBOT from Sigma, München, F.R.G.).

Chromatographic equipment

For sample application we used a Nanoapplicator in combination with a Nanomat. For development we applied a U-chamber. Quantitative determination was carried out in a TLC scanner (all chromatographic equipment from Camag, Berlin, F.R.G.) combined with a basic programmable integrator (Spectra-Physics, Darmstadt, F.R.G.).

Chromatographic procedure

Plates were activated at 110°C for 2 h, and 200-nl samples were applied to the plates by the Nanoapplicator in combination with the Nanomat [5]. Application of the samples was started at 0° with a radius of 5 mm from the centre of the plate and then continued with one sample every 15°. Standard solutions with three different concentrations and four samples were applied according to the data-pair procedure. After application, the plates were kept for 1 h at 50°C and then stored in a desiccator until room temperature was reached. With this procedure the reproducibility was considerably improved.

The plates were developed in the U-chamber with 900 μl of solvent system I [chloroform—methanol—acetic acid—water (70:30:4:3)] or II [chloroform—methanol—acetic acid—ethanol—water (60:30:4:10:3)] (solvent flow-rate, 0.5 $\mu l\,s^{-1}$) (Fig. 1). BBOT was dissolved in the methanol part of the solvent system at a concentration of 20 mg/l. After development the plates were dried with a hair dryer, and 20 min before scanning they were put into the TLC scanner in order to reach the temperature of the scanning chamber (37°C) at the beginning of the scanning. Plates in the TLC scanner were peripherally evaluated by means of a 366-nm mercury lamp with a scanning velocity of 0.15 s^{-1}. The analogue signal of the scanner was recorded and integrated by an integrator. By means of a basic program, chromatograms were evaluated according to a double logarithmic or linear regression [13]. Therefore, only 5 min after termination of the test procedure, a complete evaluation with calibration curve and all quantitative values was available.

Sample preparation

According to the expected concentrations of our samples we chose the following standard concentrations: (1) 20 ng of LPC, 500 ng of SM, 1200 ng of DPPC; (2) 30 ng of LPC, 750 ng of SM, 1800 ng of DPPC; and (3) 40 ng of LPC, 1000 ng of SM, 2400 ng of DPPC per 200 nl of chloroform—methanol (2:1) in all cases.

To demonstrate the efficiency of our method we prepared a synthetic sample with the following concentrations: 22 ng of LPC, 812 ng of SM, 2395 ng of DPPC. These concentrations of phospholipids are of the same order of those in our biological material. This sample was analysed on six HPTLC plates with solvent system I. A natural sample was obtained by lipid extraction from rat lungs by the

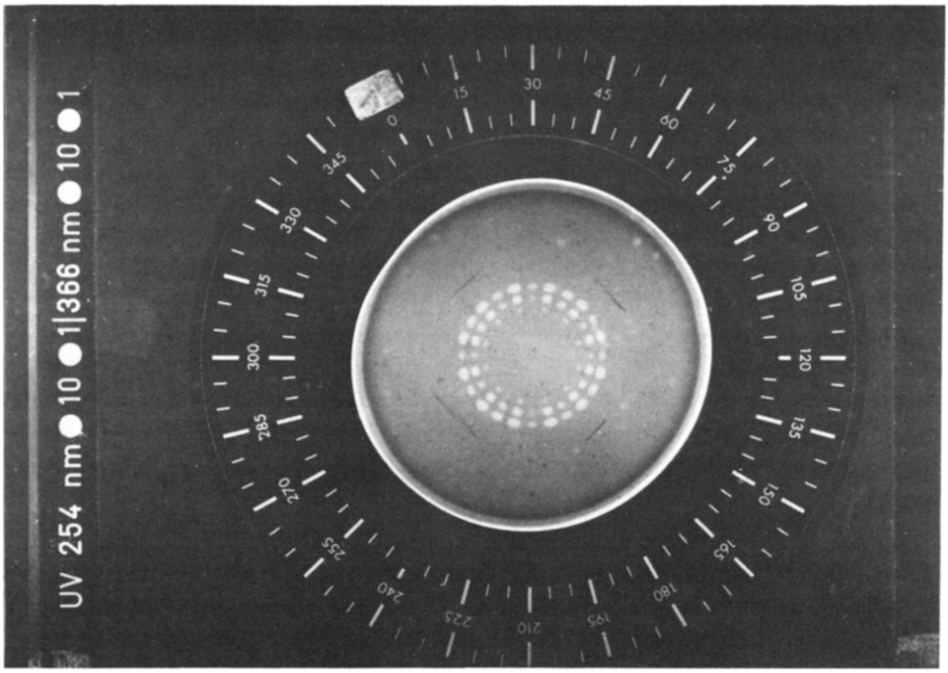

Fig. 1. HPTLC plate after application of standards and a synthetic sample. Developed with chloroform—methanol—acetic acid—water (70:30:4:3), visualized in UV light of 366 nm.

method of Folch et al. [14]. We determined PC on two plates with solvent system II and SM and LPC on another two plates with solvent system I. In this sample we determined the lipid phosphate content by the method of Martin and Doty [15] and the lecithin content with an enzymic test kit (Boehringer Mannheim, Mannheim, F.R.G.).

RESULTS AND DISCUSSION

Fig. 2 shows as an example the phospholipid separation of a synthetic sample on a HPTLC plate after development with solvent system I. Bright fluorescing spots of lipids can be seen against a dark and only weakly fluorescing background in UV light at 366 nm. The best contrast was achieved by completely removing the solvent from the plate. We further observed that better reproducibility was obtained when plates were kept for 1 h at 50°C after application.

The fluorescence intensity of the spots was found to be temperature-dependent. Higher temperature caused lower intensity. Therefore, it was necessary to reach a stable temperature on the plate and in the scanner during the measurement in order to exclude a temperature drift. Fluorescence was nearly stable for a period of 24 h. During this time we observed a decrease in fluorescence by ca. 10–15% only. Owing to this relatively long fluorescence stability, it was possible to repeat the scanning several times.

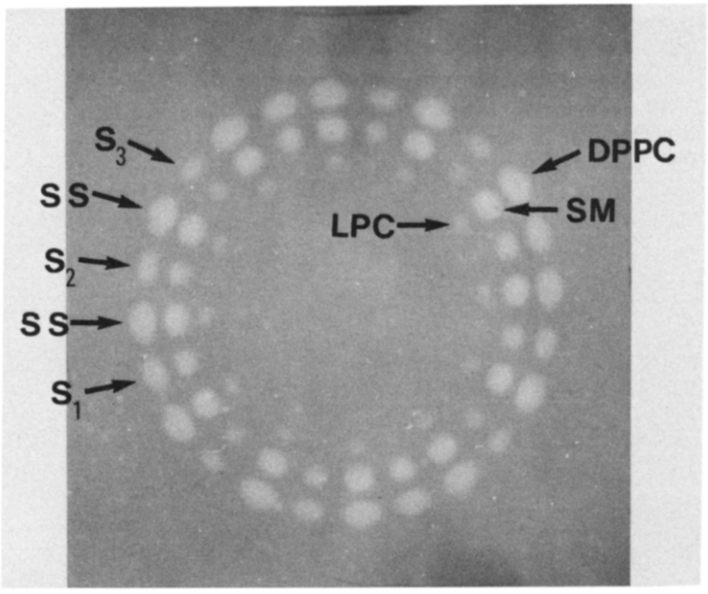

Fig. 2. Separation of three different standards (S_1—S_3) and a synthetic sample (SS) with chloroform—methanol—acetic acid—water (70:30:4:3).

Analysis of a synthetic sample

Each chromatogram of the synthetic sample was evaluated with reference to four calibration curves: both peak height and area were therefore evaluated in a linear and double logarithmic fashion. The results are shown in Table I.

Analysis of biological material

Table II shows the results of the phospholipid determination in a sample of rat lung: the lipid phosphate content was 2913 μg phosphate per g dry weight of lung. With regard to PC we compared our chromatographic results with those measured by means of an enzymic test kit. The PC content so found (38 487 ± 1087 μg/g dry weight) was in agreement with the value obtained by HPTLC.

With this method the three types of lipid can be quantified over a broad range of concentrations. Evaluation by double logarithmic regression should be preferred as the relative standard deviation is smaller. Results of peak-height and peak-area evaluation were almost identical. A deviation of the two values by more than 10% made a repetition of the separation necessary and could be avoided by strictly controlling experimental conditions. The detection limit for LPC was below 10 ng, and that for SM and DPPC below 20 ng, i.e. 25 μg/g of sample for LPC and 50 μg/g of sample for SM and DPPC. The linearity range for LPC was from 10 to 300 ng, for SM from 20 to 1600 ng and for PC from 20 to 4000 ng. For multiple determination on one plate the coefficient of variation was better than 10%. The correlation coefficients (r) for the calibration curves were 0.99 and better.

We consider the quantitative determination of LPC, which has not yet been described for the HPTLC circular technique, as one of the main novel achieve-

TABLE I

RECOVERY AND REPRODUCIBILITY

Separation on six HPTLC plates of 72 aliquots of one sample of known phospholipid concentrations in the U-chamber with chloroform—methanol—acetic acid—water (70:30:4:3), scanned in UV light at 366 nm. Amounts found represent mean ± standard error of the mean. C.V. = coefficient of variation

Lipid	Amount added (ng)	Evaluation by area		Evaluation by height	
		Amount found (ng)	C.V. (%)	Amount found (ng)	C.V. (%)
Double logarithmic regression					
LPC	22	23 ± 0.7	3.1	21 ± 0.2	0.9
SM	812	809 ± 2.4	0.3	808 ± 4.0	0.5
DPPC	2395	2432 ± 38.9	1.6	2388 ± 7.2	0.3
Linear regression					
LPC	22	20 ± 1.8	8.9	20 ± 1.8	8.9
SM	812	822 ± 12.3	1.5	824 ± 10.7	1.3
DPPC	2395	2439 ± 46.3	1.9	2400 ± 7.2	0.3

ments of this method. An advantage is seen in the fact that we can apply 24 samples on one plate, thereby exceeding the average capacity of the linear HPTLC method by a factor of two. This offers the possibility of either measuring a large number of samples on one plate or measuring a smaller amount (up to four samples) several times simultaneously with the standard solutions on the same plate. Furthermore, the application of a detection reagent dissolved in the solvent system seems advantageous as this procedure makes simple and quick handling possible. The method is superior to the spraying and submersion technique commonly used [8–11,16]. In our opinion BBOT as a detection reagent is to be preferred to 8-ANS, which was applied by Blass and Ho [12], because of the better fluorescence stability over 24 h. Quick and reproducible evaluation by means of the basic programmable integrator is another advantage.

Problems arise when natural samples are investigated. A peripheral spreading

TABLE II

PHOSPHOLIPID CONCENTRATION IN RAT LUNG

Twenty-four aliquots of the sample were applied to two HPTLC plates. Separation in the U-chamber; LPC and SM with chloroform—methanol—acetic acid—water (70:30:4:3), PC with chloroform—methanol—acetic acid—ethanol—water (60:30:4:10:3)

Phospholipid	Concentration (mean ± standard error of the mean) (μg/g dry weight)	Percentage of whole phospholipid content
LPC	1241 ± 30	1.7
SM	10317 ± 250	13.5
PC	34279 ± 432	47.1

of the solvent front is inherent to the circular technique. This results in broader spots at greater R_F values. Low R_F values, e.g. LPC, result in sharply bordered spots, high R_F values, e.g. phosphatidyl glycerol (PG) and phosphatidyl ethanolamine (PE), lead to broader spots with diffuse fringes. When lung samples were measured, we encountered the following problem. PC with a medium R_F value was highly concentrated. Phospholipids with a higher R_F value, e.g. PG, yielded considerably lower intensities owing to spot spreading. In order to measure these phospholipids the sample had to be more concentrated to start with. Therefore, the plate was likely to be overloaded by PC and an acceptable separation would no longer be possible. This is the reason why we confined our analysis to the three types of phospholipid mentioned.

Another disadvantage of this method is that we did not succeed in quantifying PC, SM and LPC in natural samples by means of one single solvent system. This is due to the fact that in lung homogenate individual phospholipids do not represent homogeneous substances. Every head-group may have a variety of saturated or unsaturated fatty acids of different lengths attached to it. Such lipids, which differ only in their acyl chains, may display a different chromatographic behaviour [17,18]. When solvent system I is used, unsaturated PC has different R_F values in contrast to saturated PC. This leads to an extension of the PC spot (DPPC R_F value 0.59, diarachoidyl PC R_F value 0.63). By use of solvent system II such tailing could be avoided. This resulted in a difference of only 10% between the enzymic and HPTLC methods. Such a difference seems acceptable according to general standards.

Our method showed a higher sensitivity to unsaturated PC (diarachoidyl) compared with equimolar amounts of saturated PC (dipalmitoyl). However, the lipid phosphate content directly measured in lung tissue and the PC content found by means of the enzymic method were in good agreement and were well within the range of comparable values indicated in the literature [19]. Therefore, the effect described above can be ignored. No broadening of spots was observed with LPC and SM.

Despite the limitations described, our method for determination of the three phospholipids is quick and reproducible.

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