Analytical Biochemistry **302,** 191–198 (2002)
doi:10.1006/abio.2001.5552, available online at http://www.idealibrary.com on IDEAL®

Phospholipid Composition of Cell-Derived Microparticles Determined by One-Dimensional High-Performance Thin-Layer Chromatography

A. M. Weerheim,* A. M. Kolb,† A. Sturk,†,‡ and R. Nieuwland†,‡

**Department of Dermatology and †Department of Clinical Chemistry, Leiden University Medical Center, Leiden; and ‡Clinical Chemistry, Academic Medical Center, 1100 DD Amsterdam, The Netherlands*

Received August 3, 2001; published online February 11, 2002

Microparticles in the circulation activate the coagulation system and may activate the complement system via C-reactive protein upon conversion of membrane phospholipids by phospholipases. We developed a sensitive and reproducible method to determine the phospholipid composition of microparticles. Samples were applied to horizontal, one-dimensional high-performance thin-layer chromatography (HPTLC). Phospholipids were separated on HPTLC by chloroform: ethyl acetate:acetone:isopropanol:ethanol:methanol: water:acetic acid (30:6:6:6:16:28:6:2); visualized by charring with 7.5% Cu-acetate (w/v), 2.5% $CuSO_4$ (w/v), and 8% H_3PO_4 (v/v) in water; and quantified by photodensitometric scanning. Erythrocyte membranes were used to validate the HPTLC system. Microparticles were isolated from plasma of healthy individuals (n = 10). On HPTLC, mixtures of (purified) phospholipids, i.e., lysophosphatidylcholine, phosphatidylcholine (PC), sphingomyelin (SM), lysophosphatidylserine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidylethanolamine (PE), and phosphatidylinositol, could be separated and quantified. All phospholipids were detectable in erythrocyte ghosts, and their quantities fell within ranges reported earlier. Quantitation of phospholipids, including extraction, was highly reproducible (CV < 10%). Microparticles contained PC (59%), SM (20.6%), and PE (9.4%), with relatively minor (<5%) quantities of other phospholipids. HPTLC can be used to study the phospholipid composition of cell-derived microparticles and may also be a useful technique for the analysis of other samples that are available only in minor quantities.

Key Words: **membrane phospholipid; HPTLC; microparticle.**

Low numbers of cell-derived microparticles are found in the systemic circulation of healthy individuals, and increased numbers are present in the circulation of patients at risk for thromboembolic events (1–3). These microparticles originate predominantly from blood platelets and, to a lesser extent, from white blood cells, erythrocytes, and—possibly—endothelial cells (3, 4). Elevated numbers of microparticles are found in a variety of diseases and there is accumulating evidence that these vesicles play a role in the development of atherosclerosis and thromboembolic events (5). The microparticles bind to and activate target cells to produce inflammatory mediators such as interleukin-6 and, more directly, they promote coagulation by exposing negatively charged phospholipids and—under certain pathological conditions—tissue factor, the initiator of coagulation *in vivo* (6). The phospholipid composition of circulating microparticles, however, is still unknown.

Although a number of laboratory techniques are already available that can be used to determine the phospholipid composition of biological membranes, most of these techniques lack overall sensitivity and are unable to detect relatively rare membrane phospholipids such as lysophosphatidylserine (L-PS)[1] and lysophosphatidylethanolamine (L-PE) (7–13). Previously, Ponec and Weerheim used one-dimensional high-performance thin-layer chromatography (HPTLC) to separate and identify PE, phosphatidylinositol (PI), PS, phosphatidylcholine (PC), sphingomyelin (SM), and lyso-PC (L-PC) in lipid extracts

[1] Abbreviations used: L-PS, lysophosphatidylserine; L-PE, lysophosphatidylethanolamine; HPTLC, high-performance thin-layer chromatography; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; L-PC, lysophosphatidylcholine; RBC, red blood cells; PBS, phosphate-buffered saline.

0003-2697/02 $35.00

from keratinocytes (13). With that method the membrane phospholipids L-PS and L-PE could not be detected, but other phospholipids could be identified and quantified. The aim of the present study was to improve this technique to assess the phospholipid composition of biological samples, not only of cells but in particular of *in vivo* circulating microparticles which are available only in minor quantities.

MATERIALS AND METHODS

Materials

Phospholipid standards were obtained from Sigma (St. Louis, MO): *l*-α-L-PC (L-4129), SM (S-7004). *l*-α-PC (P-7318), *l*-α-L-PS (L-5772), *l*-α-L-PE (L-4754), *l*-α-PS (P-7769), *l*-α-PI (P-0639), and *l*-α-PE (P-7693). Chloroform, ethyl acetate, acetone, methanol, ethanol, dichloromethane, isopropanol, acetic acid (all analytical grade), and HPTLC plates (Cat. No. 1.05641; 20 × 10 cm, Silicagel 60Å pore size, particle size 5–17 μm, 0.2 mm layer thickness) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical quality.

Preparation of Erythrocyte Ghosts

Freshly collected venous EDTA blood (10 ml) was centrifuged for 10 min at 180*g* and 20°C to separate red blood cells (RBC) from plasma. RBC were separated from the buffy coat by centrifugation for 20 min at 1560*g* and 20°C. Subsequently, 28 ml phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L sodium phosphate, pH 7.5) containing 0.1 mM EDTA (pH 7.4) was added to 2 ml RBC. This suspension was mixed and centrifuged for 20 min at 1560*g* and 20°C to pellet the erythrocytes. Erythrocytes (0.9 ml) were lysed in ice-cold lysis buffer (16.2 ml; 155 mM NH_4Cl, 1 mM EDTA, and 10 mM $KHCO_3$, pH 7.4) on melting ice for 10 min. The lysate was centrifuged for 20 min at 150*g* and 20°C. The supernatant, approximately 16.3 ml, was collected from which 0.8-ml portions were used for phospholipid extraction.

Preparation of Microparticles

Venous blood, anticoagulated with 0.32% sodium citrate, was obtained with informed consent from 10 healthy individuals. The blood was centrifuged for 20 min at 1550*g* to obtain cell-free plasma. This plasma was frozen as 250-μl aliquots in liquid nitrogen and stored at −80°C until use. After thawing, the plasma was centrifuged for 30 min at 17,590*g* and 20°C. The supernatant (225 μl) was removed and PBS containing 0.32% trisodium citrate (225 μl; pH 7.4) was added to the pellet. After being mixed, the microparticles were again centrifuged for 30 min at 17,590*g* and 20°C. Again, supernatant (225 μl) was removed and the pellet was washed once more. When microparticle preparations prepared this way are analyzed by flow cytometry, 90–95% of all events bind both annexin V, a protein that binds with high specificity and affinity to negatively charged phospholipids, and antibodies directed against cell-specific antigens such as CD61 (glycoprotein IIIa) for platelets (approximately 75% of the annexin V-positive events), glycophorin A for erythrocytes (10%), or CD66e for granulocytes (15%) (1, 2).To the washed microparticle pellet, in the remaining 25 μl, 3 ml methanol:chloroform (2:1) and 775 μl 0.5% acetic acid were added. The samples were thoroughly mixed for 30 s. Then, chloroform (1 ml) and 0.5% acetic acid (800 μl) were added. The samples were mixed for another 30 s and centrifuged for 10 min at 1560*g* (4°C). The chloroform fraction was isolated, and the acetic acid fraction (water phase) was washed twice with chloroform (1 ml). The three chloroform fractions were pooled and subsequently dried under nitrogen at 40°C. Finally, the microparticle-derived phospholipids were redissolved in methanol:chloroform (25 μl; 2:1) and applied to HPTLC. The quantity of organic phosphate for microparticles isolated from 250 μl plasma, determined as described previously (14, 15), was (mean ± SD) 276 ng ± 24 (n = 4). For phosphorus determination the microparticles were isolated as described, but PBS was replaced by saline. After drying at 40°C, 0.5 M HCl (200 μl) was added and the mixture was heated for 15 min at 100°C. Samples were applied to a 96-well microtiter plate (75 μl/well) and 10% ascorbine:0.42% ammonium molybdate (1:6; 175 μl) was added. The mixture was incubated for 20 min at 45°C. The optical density was determined at 750 nm using a Spectramax 250 (Sopachem; Wageningen, The Netherlands).

Phospholipid Extraction

Phospholipids were extracted according to Bligh and Dyer (16). Because the recoveries of especially PS and PI are only about 50% (17), the water phase was replaced by acetic acid (0.5% v/v; Prof. Dr. H. van der Bosch, personal communication). Recoveries of phospholipid standards with this modified Bligh and Dyer procedure are discussed under Results. On HPTLC, the recovery itself was determined by comparing the spot density (by photodensitometric scanning) of directly applied phospholipid standard mixtures (400 ng per standard) to phospholipid standards first extracted by the Bligh and Dyer procedure before being subjected to HPTLC.

HPTLC

TLC was performed on HPTLC plates, which were pretreated (full height) in a Camag horizontal developing chamber (Merck) to remove impurities by methanol:

ethyl acetate (6:4). The plates were activated for 10 min at 130°C. Samples, dissolved in methanol:chloroform (2:1), were applied as narrow bands (3 mm) to the HPTLC plate at 5 mm from the edge using the Camag Linomat IV (Merck) under N_2. A total of 25 samples could be applied on one HPTLC plate. The plate was then developed in a Camag horizontal developing chamber containing dichloromethane:ethyl acetate:acetone (80:16:4) for 70 mm to separate cholesterol, free fatty acids, and triglycerides from phospholipids to prevent overloading of the silica particles at the "start" position. The plate was dried under an air stream (40°C) on a heating plate (DESAGA, Heidelberg, Germany) for 10 min at 40°C. Subsequently, phospholipids were separated by chloroform:ethyl acetate:acetone: isopropanol:ethanol:methanol:water:acetic acid (30:6: 6:6:16:28:6:2) elution for 55 mm. The plate was dried for 5 min at 130°C. After the plate had cooled down, 10 ml charring reagent was carefully applied using a Gilson (Middleton, WI) P-5000 (5 ml) pipette. Subsequently, the plate was incubated with the charring reagent for approximately 60 s by swerving the plate gently from side to side. Finally, the excess of charring reagent was removed by decanting. The back of the HPTLC plate was cleaned with a dry tissue to remove the excess of charring reagent before being dried on the heating plate for 15 min at 60°C. Subsequently, the temperature was increased to 160°C (approximately 15 min) and then left at 160°C for 15 min to complete charring. The charring reagent was a mixture of 7.5% Cu-acetate (w/v), 2.5% $CuSO_4$ (w/v), and 8% H_3PO_4 (v/v) in water (18). The density of the spots was analyzed by photodensitometric scanning (Linotype Hell, Saphir Ultra 2, 16 bit; Digital Image, Sassenheim, The Netherlands), quantified using Quantity One software version 4 (Bio-Rad, Veenendaal, The Netherlands), and expressed as arbitrary units.

Statistics

Data were analyzed with SPSS for Windows, release 9. Differences were considered statistically significant at $P < 0.05$ using the Mann–Whitney U test.

RESULTS

Separation and Quantification of Purified Phospholipids

Phospholipid standards were applied separately and in combination. As shown in Fig. 1A, mixtures of PC, L-PC, PI, PE, L-PE, PS, L-PS, and SM could be separated by HPTLC, using a solvent containing chloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid (30:6:6:6:16:28:6:2). Figure 1B shows that by charring and subsequent spot density image analysis, concentrations of all phospholipids were quantified. The individual phospholipid concentration curves, analyzed by photodensitometric scanning and expressed as arbitrary units, were mathematically analyzed using Prism (release 3.0; GraphPad Software, Inc., San Diego, CA)—in the equation $y = a \times [x/(b + x)]$. Unknown quantities of individual phospholipid spots were, after scanning, calculated using the appropriate phospholipid concentration curve. Thus, R^2 values (as an indicator of the extent of the fit of the data to the best-fit nonlinear curve) of 0.983, 0.992, 0.989, 0.976, 0.990, 0.982, 0.990, and 0.991 were obtained for L-PC, SM, PC, L-PS, L-PE, PS, PI, and PE standard curves up to 1600 ng, respectively. Such standard curves were used to determine the *within-day* and *day-to-day* variations for individual phospholipids. Table 1 shows both the within-day and the day-to-day variation when phospholipid standards were directly applied to HPTLC, i.e., without phospholipid extraction.

For the within-day variation, one mixture of phospholipid standards (400 ng/phospholipid) was directly applied to one HPTLC-plate. The individual phospholipid spots were identified and quantified by photodensitometric scanning by using the standard curves of individual phospholipids, of which a representative example is shown in Fig. 1B. Within-day CVs of less than 1% were obtained for all phospholipids.

The day-to-day variation was determined by preparing one mixture of phospholipid standards that was subjected to HPTLC on 7 different days and the resulting spots of each phospholipid were quantified on standard curves run that day. Day-to-day CV of less than 5% were obtained for all phospholipids.

Table 2 shows the within-day variation when phospholipid standards were first, i.e., before being applied to one HPTLC plate, extracted by the (modified) Bligh and Dyer procedure. Although the within-day variation slightly increased compared to the within-day variation without phospholipid extraction (Table 1), the variation for all phospholipid standards was only 7% or less.

Phospholipid Composition of Erythrocyte Ghosts

The phospholipid composition of erythrocyte membranes ("ghosts") is well documented. To test whether the novel HPTLC assay was also suitable for determining the phospholipid composition of biological membranes, we prepared erythrocyte ghosts and extracted and analyzed their phospholipid composition. Figure 2 shows a representative experiment in which phospholipid standards (lane A), microparticle-derived phospholipids from two individuals (lanes B1 and B2), and erythrocyte ghost-derived phospholipids (lane C) were separated. The data on the phospholipid composition of erythrocyte ghosts are summarized in Table 3. Again,

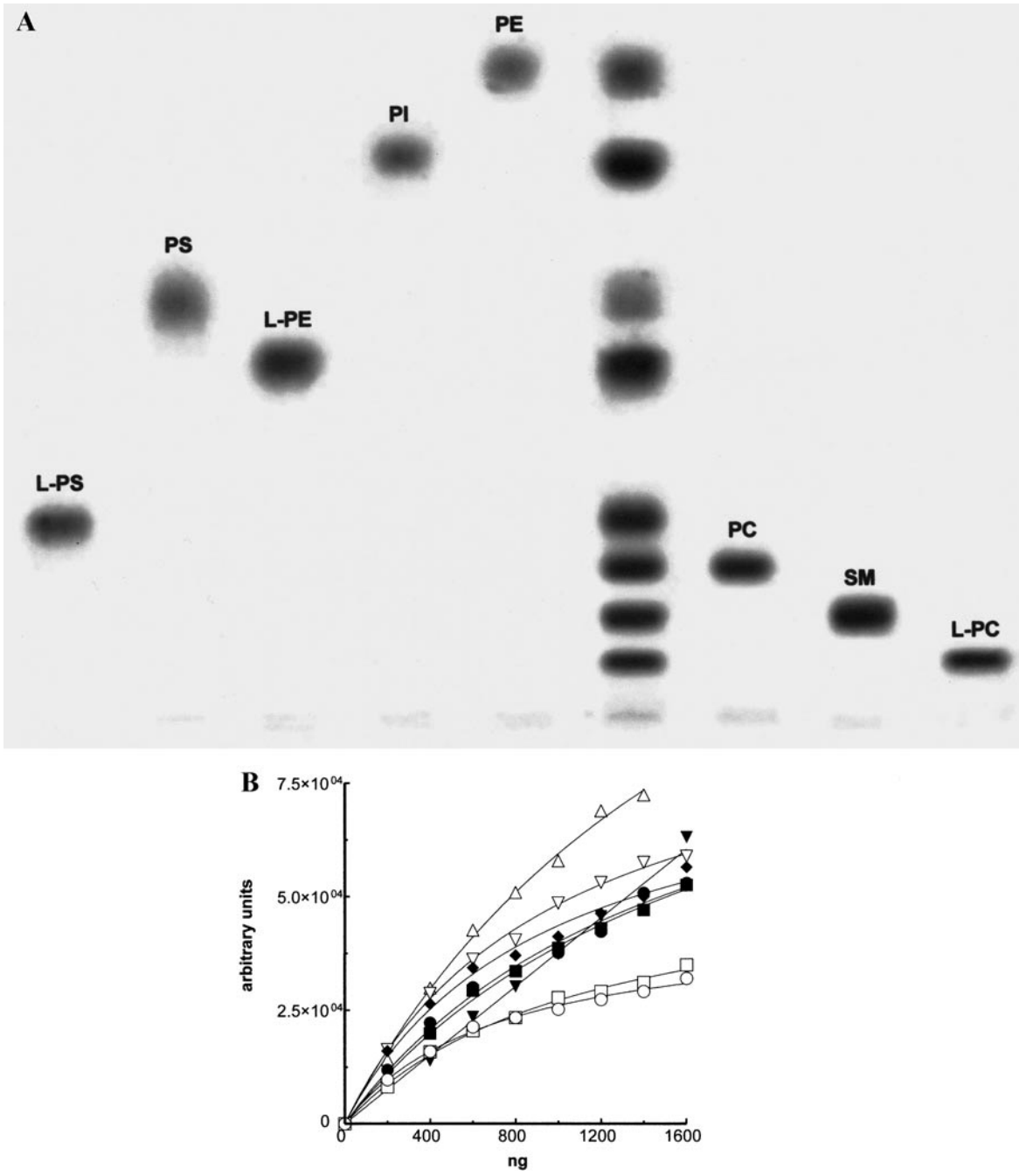

FIG. 1. Separation of phospholipids by one-dimensional HPTLC. (A) A representative example of a one-dimensional HPTLC plate on which a mixture of phospholipids (L-PC, SM, PC, L-PS, L-PE, PS, PI, PE; 500 ng per standard) was separated. Individual phospholipids were applied as 500 ng in this particular experiment (SM, L-PC, PC, PS, PE), 250 ng (L-PE, L-PS), and 100 ng (PI). (B) Standard curves of lipid standards determined after charring and photodensitometric analysis are shown for L-PC (○), SM (◆), PC (●), L-PS (□), L-PE (▽), PS(■), PI (△), and PE (▼).

both the within-day and the day-to-day variation were determined, and, for comparison, data from literature were included. Clearly, the phospholipid composition of the erythrocyte ghost membrane analyses was within the range of previous reports. Also, the ranges of the phospholipid compositions found between days were considered acceptable. Finally, the erythrocyte ghosts were spiked with individual phospholipid standards (100, 200, and 400 ng of each standard) before phospholipid extraction and their recovery was determined

TABLE 1

Direct HPTLC Analysis of Phospholipid Standards (*without* Phospholipid Extraction)

	Within-day variation			Day-to-day variation		
Standard	*N*	Mean (%)	CV (%)	*N*	Mean (%)	CV (%)
L-PC	10	100.0	0.48	7	100.2	1.94
SM	10	100.4	0.92	7	100.3	3.54
PC	10	98.5	0.95	7	94.3	3.78
L-PS	10	100.0	0.42	7	100.8	2.06
L-PE	10	100.0	0.55	7	97.4	4.19
PS	10	101.4	0.43	7	99.9	3.88
PI	10	99.2	0.64	7	99.6	4.74
PE	10	100.9	0.83	7	97.7	4.20

Note. The *within-day* variation was determined by directly applying a mixture of phospholipid standards (400 ng per phospholipid standard), i.e., without phospholipid extraction, to one HPTLC plate ($n = 10$). A standard curve was prepared for each individual phospholipid standard. The phospholipid spots of interest were quantitated by photodensitometric scanning by using the standard curve for that particular phospholipid. For the *day-to-day* variation one mixture of phospholipid standards, again without phospholipid extraction, was subjected to HPTLC on 7 different days and the resulting spot of each phospholipid quantified on a standard curve run with that analysis.

(Table 3). The recoveries of phospholipid standards, all more than 90% except for L-PE (83.5%), were independent of the concentrations added.

TABLE 2

Phospholipid Extraction *and* HPTLC Analysis of Phospholipid Standards

	Within-day variation, including Bligh and Dyer extraction		
Standard	*N*	Mean (%)	CV (%)
L-PC	12	100.9	3.78
SM	12	93.2	3.83
PC	12	101.5	3.75
L-PS	12	92.2	6.62
L-PE	12	97.9	3.17
PS	12	90.3	5.94
PI	12	95.0	6.58
PE	12	96.5	7.05

Note. The within-day variation *including* the phospholipid extraction was determined by preparing a mixture of phospholipid standards (400 ng each). This mixture was divided over 12 different tubes, which all were separately subjected to phospholipid extraction. Subsequently, these 12 samples were all applied to one HPTLC plate. For each individual phospholipid standard a standard curve was prepared. The phospholipid spots of interest were then analyzed and quantified by using the standard curve for that particular phospholipid. Data are shown as the percentages of each phospholipid within the total amount of phospholipids.

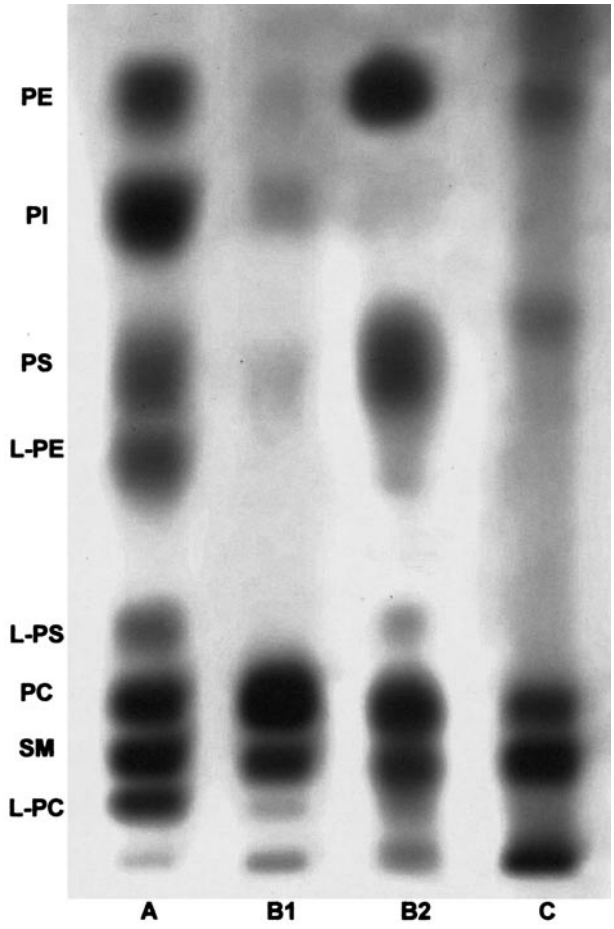

FIG. 2. Separation of phospholipids from erythrocyte ghosts and microparticles. Erythrocytes and microparticles were prepared as described under Materials and Methods. Phospholipids were extracted and applied to one-dimensional, horizontal HPTLC. Representative separations are shown of a mixture of purified phospholipids (lane A), cell-derived microparticles isolated from plasma of two healthy individuals (lanes B1 and B2), and erythrocyte ghosts (lane C).

Phospholipid Composition of in Vivo Cell-Derived Microparticles

As shown in Fig. 2, by HPTLC even minor quantities of membrane phospholipids from *in vivo* microparticles could be separated, identified, and quantified. Microparticles from healthy individuals contained predominantly PC (59.2%) and to a lesser extent SM (20.6%) and PE (9.40%). All other phospholipids present, i.e., L-PC, L-PS, L-PE, PS, and PI, were relatively rare at quantities generally below 5% (Table 4). However, results between individuals varied considerably, as evident from the two examples shown in Fig. 2 and the indicated ranges in Table 4. Remarkable differences between the phospholipid compositions of the microparticles, predominantly of platelet origin, and the platelet membranes were observed (Table 4).

TABLE 3

HPTLC Analysis of Erythrocyte Ghosts and Spike Recovery

Ghost phospholipid	Within-day variation			Day-to-day variation			Spike recovery			Literature range
	Mean (%)[a]	SD	Range	Mean (%)[a]	SD	Range	Mean (%)	SD	Range	
L-PC	0.42	0.28	0.0–0.9	0.51	0.78	0.0–1.7	94.3	3.67	90.6–98.0	1.0–2.4[b]
SM	27.8	2.57	26.0–34.1	31.3	2.95	27.6–35.4	105.6	4.28	101–110	22.0–30.1[b]
PC	23.6	0.83	22.4–24.7	18.0	3.38	14.1–23.2	93.9	2.95	91.0–96.9	21.0–34.1[b]
L-PS	0.5	0.51	0.0–1.8	0.49	0.61	0.0–1.3	89.6	5.24	84.4–94.8	1.0[c]
L-PE	0.01	0.02	0.0–0.05	0.6	0.61	0.0–1.4	83.5	5.60	77.9–89.1	4.0[c]
PS	15.8	3.03	11.4–21.0	25.9	4.52	20.4–31.8	97.9	4.14	93.8–102	8.0–17.5
PI	0.28	0.08	0.2–0.4	1.26	0.96	0.0–2.2	90.5	5.14	85.4–95.6	0.6–2.3[b]
PE	31.6	2.35	27.8–34.4	21.8	4.18	15.7–26.5	93.2	10.9	82.3–104	17.2–32.8[b]

Note. Erythrocyte ghosts were prepared as described under Materials and Methods and divided over 10 different tubes. Then, phospholipids were extracted and applied to one HPTLC plate (within-day variation *including* phospholipid extraction). For day-to-day variation the ghosts were divided over seven different tubes, and phospholipids were extracted and stored at −20°C and finally applied to HPTLC on 7 different days. For the spike experiments ($n = 3$), individual phospholipid standards (100, 200, and 400 ng of each standard) were added to the erythrocyte ghosts.

[a] The percentage of each phospholipid within the total amount of phospholipids is presented.

[b] Refs. (8, 23, 25–36).

[c] Ref. (28).

DISCUSSION

This study shows that by one-dimensional HPTLC it is possible to separate, identify, and quantify minor quantities of (membrane) phospholipids. Previous studies showed that the phospholipid composition of biological membranes can be determined by both HPLC and (HP)TLC (7–13). However, previous HPLC studies either did not address the separation of all phospholipid classes or did not pay attention to the simultaneous separation of phospholipids and lysophospholipids (19–21). Not until very recently was a one-step normal-phase HPLC procedure described by Lesnefsky and co-workers to separate and quantify all major (membrane) phospholipids and lysophospholipids (22). By one-dimensional TLC phospholipids such as L-PC, SM, PC, PS, PE, and PI can be separated but relatively high quantities (μg range) have to applied (23, 24), but again hardly any attention was paid to the detection of lysophospholipids (11, 23–25). With two-dimensional TLC, L-PC, SM, PC, L-PE, PS, PI, and PE can be separated and identified, but relatively high quantities (2 μg) of (total) phospholipid are required, only one sample can be applied per plate, and L-PS is not detectable (8).

Previously, one-dimensional horizontal HPTLC was used to separate and identify L-PC, SM, PC, PS, PI, and PE in membranes from keratinocytes (13). For this separation two elution solvents were used, (i) chloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid (36:6:6:6:16:28:2:1; 20 mm) and (ii) chloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water (48:6:6:6:6:24:4; 25 mm). In the present study, these two elution solvents were combined by changing the percentages of chloroform, ethanol, methanol, water, and acetic acid (chloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid; 30:6:6:6:16:28:6:2; 55 mm) in such a way that both L-PS and L-PE became detectable. In the previous work of Ponec and Weerheim the L-PS comigrated with PC, and L-PE comigrated with PS (A. Weerheim, personal communication). Thus, the improvements as presented in the present study are evident since now it has become possible to separate, identify, and quantify also

TABLE 4

Phospholipid Composition of Cell-Derived Microparticles Isolated from Blood of Healthy Individuals

Phospholipid	Microparticle phospholipids			Platelet plasma membrane range[a]
	N	Mean (%)	Range	
L-PC	10	2.05	0.0–4.2	ND[b]
SM	10	20.6	14.0–24.3	19.0–22.7
PC	10	59.2	50.9–65.5	31.8–35.5
L-PS	10	1.05	0.0–3.3	ND
L-PE	10	0.83	0.0–3.8	ND
PS	10	3.63	0.8–7.7	12.0–13.7
PI	10	3.21	1.5–7.8	0.4–0.9
PE	10	9.40	5.2–20.5	31.9–35.0

Note. Cell-derived microparticles were isolated from human plasma as described under Materials and Methods. Phospholipids were extracted and applied to HPTLC. Because approximately 75% of these microparticles are of platelet origin (1), for comparison data on the phospholipid composition of purified platelet plasma membranes are presented.

[a] Ref. (37).

[b] ND, not detected.

the lysoforms of the major classes of membrane phospholipids. Compared to the previously reported HPTLC technique, the sensitivity of phospholipid detection was further improved by optimizing the phospholipid extraction procedure and by replacing the charring reagent of 0.5% $CuSO_4$ in acetic acid:H_2SO_4:H_3PO_4:water (5:1:1:95) by 7.5% Cu-acetate, 2.5% $CuSO_4$, and 8% H_3PO_4 in water (18). The detection range of the HPTLC system was 5–15 ng for individual phospholipids, which is on average a fivefold improvement (13).

To validate the HPTLC system, the phospholipid composition of erythrocyte ghosts was determined and compared to the literature (8, 23, 25–36). The phospholipid composition (within-day variation) as determined by HPTLC was within the ranges reported previously. Some slight differences became apparent with regard to the day-to-day variation. Whereas especially PC tended to be somewhat lower, PS slightly increased. Please note that the within-day variation was performed immediately after preparation of erythrocyte ghosts, whereas the day-to-day variation involved a period of 3–4 weeks during which the ghosts were stored at −20°C. Most likely, these differences may explain the discrepancies in phospholipid composition. The spiking experiments showed that all individual phospholipid standards, including the lysophospholipids, have an acceptable (>80%) recovery.

The phospholipid composition of microparticles obtained from healthy individuals clearly differed from both erythrocyte ghosts and purified platelet plasma membranes (37). Especially the latter was not anticipated because in general more than 75% of the microparticles in healthy subjects originate from platelets (1). Compared to purified platelet plasma membranes, the microparticles contained about twofold more PC, three- to sixfold more PI, four- to fivefold less PS, and three- to fourfold less PE. Apart from differences in the methodology, these differences are most likely explained by (i) the presence of microparticles from nonplatelet origin and/or (ii) the selective shedding of (particular) membrane phospholipids. There were also considerable differences in the microparticle phospholipid fractions from the various donors. For instance, 5 of the 10 donors had ≤1.8% PS, two donors were intermediate (2.7 and 3.9%), and 3 donors ranged between 7.0 and 7.7%. Additional studies are required to determine the causes and possibly the functional relevance of these differences. Incubation of a microparticle fraction from one of the donors with pancreas phospholipase A_2 resulted in the hydrolysis of PC (untreated 61.9%, with enzyme 23.9%) and in a concurrent increase of L-PC from 0.9% (untreated) to 32.2% (treated), thus confirming the correct identification of (L-)PC in this preparation (data not shown).

In various diseases it has now been firmly established that the numbers and cellular origin of microparticles in blood differ between healthy individuals (1–3). Moreover, during inflammation high levels of enzymes that degrade phospholipids are present. The present HPTLC method therefore offers the opportunity to establish the phospholipid composition of microparticles in detail, not only during physiological but also under pathological conditions, and thus may be a first step to determine the influence of individual phospholipids on coagulation and inflammation, which is one of the final goals of our studies.

REFERENCES

1. Berckmans, R. J., Nieuwland, R., Böing, A. N., Romijn, F., Hack, C. E., and Sturk, A. (2001) Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb. Haemostasis* **85,** 639–646.
2. Nieuwland, R., Berckmans, R. J., Rotteveel-Eijkman, R. C., Maquelin, K. N., Roozendaal, K. J., Jansen, P. G. M., ten Have, K., Eijsman, L., Hack, C. E., and Sturk, A. (1997) Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation* **96,** 3534–3541.
3. Nieuwland, R., Berckmans, R. J., McGregor, S., Böing, A. N., Romijn, F. P. H. T. M., Westendorp, R. G. J., Hack, C. E., and Sturk, A. (2000) Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* **95,** 930–935.
4. Combes, V., Simon, A.-C., Grau, G.-E., Arnoux, D., Camoin, L., Sabatier, F., Mutin, M., Sanmarco, M., Sampol, J., and Dignat-George, F. (1999) In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J. Clin. Invest.* **104,** 93–102.
5. Mallat, Z., Hugel, B., Ohan, J., Lesèche, G., Freyssinet, J. M., and Tedgui, A. (1999) Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques. *Circulation* **99,** 348–353.
6. Nemerson, Y. (1988) Tissue factor and hemostasis. *Blood* **71,** 1–8.
7. Goppelt, M., and Resch, K. (1984) Densitometric quantitation of individual phospholipids from natural sources separated by one-dimensional thin-layer chromatography. *Anal. Biochem.* **140,** 152–156.
8. Mallinger, A. G., Yao, J. K., Brown, A. S., and Dippold, C. S. (1993) Analysis of complex mixtures of phospholipid classes from cell membranes using two-dimensional thin-layer chromatography and scanning laser densitometry. *J. Chromatogr.* **614,** 67–75.
9. Hiramatsu, K., Nozaki, H., and Arimori, S. (1980) Lipid content of human platelets quantitated by thin-layer chromatography in combination with flame ionization detection. *J. Chromatogr.* **182,** 301–309.
10. Samet, J. M., Friedman, M., and Henke, D. C. (1989) High-performance liquid chromatography separation of phospholipid classes and arachidonic acid on cyanopropyl columns. *Anal. Biochem.* **182,** 32–36.
11. Wang, W. Q., and Gustafson, E. J. (1992) One-dimensional thin-layer chromatographic separation of phospholipids and lysophospholipids from tissue lipid extracts. *J. Chromatogr.* **581,** 139–142.
12. Silversand, C., and Haux, C. (1997) Improved high-performance liquid chromatographic method for the separation and quantification of lipid classes: Application to fish lipids. *J. Chromatogr. B.* **703,** 7–14.

13. Ponec, M., and Weerheim, A. (1990) Retinoids and lipid changes in keratinocytes. *Methods Enzymol.* **90,** 30–41.
14. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) Microdetermination of phosphorus. *Anal. Chem.* **28,** 1756–1758.
15. Ames, B. N., and Dubin, D. T. (1960) The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235,** 769–773.
16. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917.
17. Kolarovic, L., and Fournier, N. C. (1986) A comparison of extraction methods for the isolation of phospholipids from biological sources. *Anal. Biochem.* **156,** 244–250.
18. Weerheim, A., and Ponec, M. (2001) Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography. *Arch. Dermatol. Res.* **293,** 191–199.
19. Rehman, S. U. (1991) Rapid isocratic method for the separation and quantification of major phospholipid classes by high-performance liquid chromatography. *J. Chromatogr.* **567,** 29–37.
20. Grizard, G., Sion, B., Bauchart, D., and Boucher, D. (2000) Separation and quantification of cholesterol and major phospholipid classes in human semen by high-performance liquid chromatography and light-scattering detection. *J. Chromatogr. B* **740,** 101–107.
21. Bünger, H., and Pison, U. (1995) Quantitative analysis of pulmonary surfactant phospholipids by high-performance liquid chromatography and light-scattering detection. *J. Chromatogr. B* **672,** 25–31.
22. Lesnefsky, E. J., Stoll, M. S. K., Minkler, P. E., and Hoppel, C. L. (2000) Separation and quantitation of phospholipids and lysophospholipids by high-performance liquid chromatography. *Anal. Biochem.* **285,** 246–254.
23. Chapelle, S. M. (1979) Rapid quantitative analysis of phospholipid erythrocytes by densitometric thin-layer chromatography. *Clin. Chim. Acta* **92,** 11–18.
24. Touchstone, J. C. (1995) Thin-layer chromatographic procedures for lipid separation. *J. Chromatogr. B* **671,** 169–195.
25. Vaysse, J., Pilardeau, P., and Garnier, M. (1985) Rapid quantitative analysis of phospholipids in biological fluids after thin-layer chromatography. *Clin. Chim. Acta* **147,** 183–190.
26. Lautin, A., Cordasco, D. M., Segarnick, D. J., Wood, L., Mason, M. F., Wolkin, A., and Rotrosen, J. (1982) Red cell phospholipids in schizophrenia. *Life Sci.* **31,** 3051–3056.
27. Dougherty, R. M., Claudio Galli, B. A., Ferro-Luzzi, A., and Iacono, J. M. (1987) Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: A study of normal subjects from Italy, Finland, and the USA. *Am. J. Clin. Nutr.* **45,** 443–455.
28. Mitchell, K. T., Ferrell, J. E., Jr., and Huestis, W. H. (1986) Separation of phosphoinositides and other phospholipids by two-dimensional thin-layer chromatography. *Anal. Biochem.* **158,** 447–453.
29. Broekhuyse, R. M. (1974) Long-term storage of erythrocytes for quantitative analyses of lipids. *Clin. Chim. Acta* **52,** 53–58.
30. Turner, J. D. (1970) Precise quantitative determination of human blood lipids by thin-layer and triethylaminoethyl-cellulose column chromatography. *Anal. Biochem.* **38,** 423–436.
31. Iacono, J. M., Zellner, D. C., Paoletti, R., Ishikawa, T., Grigeni, V., and Fumagalli, R. (1973) Comparison of blood platelet and erythrocyte lipids in man in three age groups from three regions: Milan, Cincinnati and Sicily. *Haemostasis* **2,** 141–162.
32. Engelmann, B., Streich, S., Schönthier, U. M., Richter, W. O., and Duhm, J. (1992) Changes of membrane phospholipid composition of human erythrocytes in hyperlipidemias. *Biochim. Biophys. Acta* **1165,** 32–37.
33. Hägerstrand, H., and Isomaa, B. (1994) Lipid and protein composition of exovesicles released from human erythrocytes following treatment with amphiphiles. *Biochim. Biophys. Acta* **1190,** 409–415.
34. Iida, H., Takashima, Y., Maeda, S., Sekiya, T., Kawade, M., Kawamura, M., Okano, Y., and Nozawa, Y. (1984) Alterations in erythrocyte membrane lipids in abetalipoproteinemia: Phospholipid and fatty acyl composition. *Biochem. Med.* **32,** 79–87.
35. Broekhuyse, R. M. (1969) Quantitative two-dimensional thin-layer chromatography of blood phospholipids. *Clin. Chim. Acta* **23,** 457–461.
36. Broekhuyse, R. M. (1974) Improved lipid extraction of erythrocytes. *Clin. Chim. Acta* **51,** 341–343.
37. Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S., and Douste-Blazy, L. D. (1986) Biochemical characterization of plasma membranes and intracellular membranes isolated from human platelets using Percoll gradients. *Biochim. Biophys. Acta* **856,** 155–164.