An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples

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Abstract Lipids, lysophospholipids and phospholipids in particular, have been shown to be biomarkers and potential therapeutic targets for human diseases. While many extraction and analytical methods have been developed for quantitative analyses of these molecules, most of them are laborious and time-consuming, with associated issues of poor reproducibility. This becomes one of the critical bottle-necks to move lipid markers to clinics. In the current work, we have developed an extremely simple method for lysophospholipids and phospholipids extraction from human plasma or serum samples, which only utilizes a single methanol (MeOH) solvent and involves a single step of centrifugation. This method has been subjected to strict validation by comparing it with classical lipid extraction methods.▮▮ This simple method will be extremely useful for the lipidomic, diseases marker, and lipid biochemistry fields not only for its potential wide applications associated with its simplicity and reproducibility, but also for its impact in moving lipid markers into clinics through high-throughput processing.—Zhao, Z., and Y. Xu. **An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples.** *J Lipid Res.* 2010. 51: 652–659.

Supplementary key words HPLC-ESI-MS/MS • MeOH method • cell pellet lipid • tissue lipid

Lipids play important roles in regulating and controlling cellular functions and are involved in human physical and pathological conditions (1–6). Lipidomics, the large-scale study of pathways and networks of cellular lipids in biological systems (7, 8), is an emerging field of basic and translational research. In the past decades, lysophospholipids, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) in particular, have been recognized to be important signaling molecules (9–17) and potential markers for ovarian cancers and other human diseases (18–21). In addition,

lysophosphatidylcholine (LPC) levels in plasma may be used as potential markers of colorectal cancer (22).

Extensive "omics" studies (including proteomics, genomics, and metabolomics) have been conducted in the past few years that have generated overwhelming amounts of data. However, almost none of the identified markers have been moved into clinics. One of the major problems is that very few of these markers have been confirmed and cross-examined in multiple labs. This is at least in part due to different methodologies used, which are critical for detecting the changes that occur during perturbation of the biological systems. We have discussed these issues in a recent review paper (23). One of the most critical factors clearly affecting the quantitative analysis of lipids in blood samples is extraction. Many different lipid extraction methods have been developed in the past decades and most of them are based on the original method developed by Blight and Dyer (24). In general, two organic solvents [methanol (MeOH) and chloroform] are used, and phase separation is involved. Lipids are dissolved in these solvents, and proteins and other hydrophilic materials are removed after phase separation. The original Bligh and Dyer method (BD method) is suitable for extracting major membrane phospholipids (PLs), including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and certain membrane sphingolipids. However, it is very poor in extracting lysophospholipids (LPLs), which are more hydrophilic. Modifications have been made to increase the efficiency of extracting these signaling molecules. We have improved LPA extraction by

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Abbreviations: BD method, Bligh and Dyer method; Cers, ceramides; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPLs, lysophospholipids; lyso-PAF, lyso-platelet activating factor; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLs, phospholipids; PS, phosphatidylserine; S1P, sphingosine-1-phosphate; SM, sphingomyelin.

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including HCl, which neutralizes the charge of LPA and forces it to be retained in the organic phase (23, 25, 26). Yatomi et al. (27) have included KCl, HCl, and NH_4OH in their extraction method to optimize extraction for both positively and negatively charged LPLs. However, there is concern that the acidic or alkaline conditions would induce hydrolysis of endogenous lipids, resulting in artificial generation of LPLs (28). Milder acid, such as citric acid, has been used to replace HCl (28, 29). In addition, butanol instead of MeOH and chloroform has been used in several labs as the optimized method for extraction of LPA and S1P (28–30). We have systematically compared these lipid extraction methods and found that none of them can efficiently extract all major classes of phospholipids with an acceptable efficiency (>80% recovery), and the yields vary 20–98%, dependent on the type of lipids to be extracted (24, 27, 29; data not shown). Furthermore, all of these methods involve several steps, including phase separation, centrifugation, and solvent evaporation. In particular, butanol is very hard to evaporate, which makes the process very time consuming.

The stability of blood lipids during sample processing is another major issue for using them as markers. EDTA-containing tubes have been used for plasma sample collection, which not only prevents the platelets from activation and blood from clotting but also inhibits enzymatic activities involved in lipid metabolism. EDTA plasma LPA levels were not significantly changed after 24 h of incubation at 37°C (29). In contrast, neither heparin nor calcium-trapping anticoagulants (including EDTA and acid citrate dextrose tube) block production of LPC (29, 31). In samples stored 1 day at room temperature, the LPC concentration increased 54% (31), and at 37°C incubation, LPC increased nearly 4-fold (29). Therefore, identifying conditions when plasma samples can be stored without affecting lipid levels is another critical factor for reproducibility.

In this work, we describe an extremely simple method for extracting all major classes of lysophospholipids (LPLs) and phospholipids (PLs) in blood samples. Because most of these lipids are composed of many subspecies (e.g., there are 10 different naturally occurring PCs), this method could efficiently (>80% yields) extract over 100 lipids from human blood samples. This method requires only a minimal amount of blood (≤10 µl) to extract/detect essentially all of these lipids and it is characterized by simplicity, universality, high recovery rate, and reproducibility.

EXPERIMENTAL PROCEDURES

Materials and reagents

Standard lipids, including PAs, PCs, PEs, PI (18:0/20:4), PS (18:0/18:1), LPAs, LPCs, lysophosphatidylethanolamines (LPEs), lysophosphatidylglycerol (18:1 LPG), lysophosphatidylinositol (LPIs), lysophosphatidylserine (18:1 LPS), lyso-platelet activating factor (lyso-PAF), sphingosine-1-phosphate (S1P), ceramides (Cers), and sphingomyelin (18:0 SM) were purchased from Avanti Polar lipids (Birmingham, AL) or Sigma (St. Louis, MO). HPLC-grade MeOH, chloroform, HCl, 1-butanol, citric acid, and disodium hydrogen phosphate were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Blood samples were collected from healthy adult volunteers who gave their informed consent. The Institutional Review Board of the Indiana University-Purdue University Indianapolis approved the study. Serum samples were obtained by allowing them to clot at room temperatures for 15 min and then centrifugating at 1,500 *g* for 5 min. Plasma samples were obtained by collecting whole blood in EDTA-containing tubes and centrifugating at 1,750 *g* for 15 min at room temperature. Serum or plasma samples were aliquoted into siliconized Eppendorf tubes (PGC Scientifics, Frederick, Maryland) and frozen at −80°C until use.

Ovarian cancer SKOV3 cells were cultured in RPMI 1640 supplemented with 10% FBS (Hyclone, South Logan, UT). Cells with approximately 85% confluence were collected by centrifugation and washed three times using PBS (1×). The cell pellets were stored at −80°C until use.

Extraction of lipids

All extractions were performed either in siliconized tubes or in glass tubes. We developed a simple phospholipid extraction method and call it the MeOH method. In brief, when it is used for extraction of choline-containing lipids, 2 µl of plasma or serum were added into 1 ml of MeOH with 100 pmol of 12:0 LPC as the internal standard. After vortexing and incubation on ice for 10 min, the mixture was centrifuged (10,000 *g*, 5 min, room temperature), and 120 µl of supernatant was directly used for MS analysis of lyso-PAF, LPCs, SMs, and PCs. For natively charged lipids, 10 µl of plasma or serum was added into 150 µl of MeOH with 10 pmol of 14:0 LPA and 5 pmol of 17:0 Cer as internal standards. After vortexing and incubation on ice for 10 min, the mixture was centrifuged (10,000 *g*, 5 min, room temperature), and 120 µl of supernatant was used for MS analysis of S1P, Cers, LPAs, LPEs, LPIs, LPG, LPS, PAs, PEs, PI, and PS.

When the BD method (24) was applied, the corresponding volume of plasma or serum (e.g., 2 µl for extraction of LPCs and PCs, 10 µl for extraction of S1P, LPAs, LPEs, PE, etc.) was diluted to 500 µl using PBS (1×) to which 3 ml of a 1:2 (v/v) mixture of $CHCl_3$: MeOH with or without HCl (10 µl, 6N) was added. The samples were vortexed and incubated on ice for 10 min. $CHCl_3$ (1 ml) and H_2O (1.3 ml) were then added and the samples were revortexed. The phases were separated by centrifugation (1,750 *g* for 10 min at 10°C) and the bottom phase was recovered. After solvent evaporation, the dried lipids were resuspended using corresponding volume of MeOH (e.g., 1000 µl of MeOH was used to dissolve dried lipids for MS analysis of LPCs, and 160 µl of MeOH was used to dissolve dried lipids for MS analysis of S1P, LPAs, LPEs, PEs, etc.) for MS analysis. The volumes used were determined based on our experience with endogenous concentrations of these lipids, the sensitivities of MS detection, and the best detection ranges for these lipids.

When the butanol method (28) was applied for extraction of lipids, the corresponding volume of plasma or serum was diluted to 500 µl using a buffer containing 30 mM citric acid and 40 mM disodium hydrogen phosphate (pH 4.0) as described (28). After it was mixed with 1 ml of 1-butanol and vortexed, phases were separated by centrifugation (1,750 *g*, 10 min, and 10°C). The upper phase was transferred to a new glass tube and the extraction was reperformed with 0.5 ml of water-saturated 1-butanol. The upper phases were combined and evaporated. After evaporation, the dried lipids were resuspended using corresponding volumes of MeOH for MS analysis. We also investigated the difference in LPL extraction by adding the blood sample after the buffer (containing citric acid) was mixed with the organic solvent (butanol).

For cell pellet lipid assays, a pellet from 1×10^6 cells was resuspended in 50 µl of water before it was transferred into 450 µL of methanol (10 pmol of 14:0 LPA, 100 pmol 12:0 LPC, and 50 pmol of 17:0 Cer as internal standards). After vortexing and centrifugation (10,000 g, 5 min, room temperature), 100 µl of the supernatant was used for analysis of PCs, SMs, lyso-PAF, and LPCs, and the left MeOH extract was removed to a new glass tube. After evaporation by nitrogen, the dried lipids were resuspended into 100 µl of MeOH for negatively charged lipid MS analysis.

For tissue lipid analyses, we used frozen tissues from brains of mice, which were crushed and transferred into glass tubes, followed by adding 2 ml water. Tissue powders were further homogenized using a Brinkmann POLYTRON PT 10/35 Homogenizer. From the homogenate, 50 µl (~5.0 mg of the tissue) from each sample was used for lipids extraction and analyses. The same procedures as described above for cell pellets were used.

HPLC-ESI-MS/MS

MS analyses were performed using the API-4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Forster City, CA) with the Analyst data acquisition system. The instrument was equipped with a Z-spray ionization source. Both the nebulizer and desolvation gases were nitrogen and the collision gas was argon. Typical operating parameters were as follows: collision gas (CAD) 8, curtain gas (CUR) 10, ion source gas 1 (GS1) 15, ion source gas 2 (GS2) 35, electrospray voltage 5000 with positive ion MRM mode or −4,200 with negative ion MRM mode, and a temperature of heater at 500°C.

Negative ion MRM mode was used for the quantitative analysis of S1P, LPAs, LPEs, 18:1 LPG, LPIs, 18:1 LPS, PAs, PEs, PI, and PS. HPLC conditions for the separation of LPCs from LPAs, and PCs from PAs were established to avoid the interference of LPCs and PCs with the quantification of LPA and PAs. To achieve the shortest time for separation, a short C18 HPLC column (TARGA C18 5 µM, 2.1 mm ID × 10 mm TR-0121-C185, Higgins Analytical, Southborough, MA) was used. Samples (10 µl) were loaded through a LC system (Agilent 1100) with an auto sampler. The mobile phase was MeOH/water/NH₄OH (90:10:0.1, v/v/v) and the HPLC separations were 15 min/sample using the following scheme: 1) constant rate of 0.2 ml/min for 2 min; 2) the flow rate increased from 0.2 to 0.8 ml/min in 2 min; 3) constant rate of 0.8 ml/min for 9 min; and 4) decreased flow rate from 0.8 to 0.2 ml/min in 2 min. Cers were also detected by negative ion MRM mode, and lyso-PAF, LPCs, SMs, and PCs were determined by positive ion MRM mode for the quantitative analysis. While HPLC separation was not necessary for these lipids, samples (10 µl) were directly injected into the MS ion source, and the mobile phase was MeOH/water/NH₄OH (90:10:0.1, v/v/v) and the flow rate was 0.2 ml/min, with a duration time 1.5 min/sample. The parent-to-daughter transition ion pairs we used for MRM mode are listed in Tables 2–4.

Quantitative analysis of lipids

Standard curves were established for quantitative analyses of all lipids. In brief, different amounts of lipids standards were spiked into plasma or serum in the presence of internal standard and then extracted by the methanol method and analyzed by MS analyses. The peak area ratios (a lipid/internal standard) versus the molar ratios (a lipid/internal standard) were plotted and fitted to a linear regression. For commercially unavailable lipids, the slopes of the closest structures were used. For example, we used the slope of 18:1 LPC for 18:2 LPC and the slope of 20:0 LPC for 20:4 LPC, respectively.

The efficiency of extraction was calculated by the formula: $R\,(\%) = A_{extraction}/\,(A_{extraction} + A_{left}) * 100$, where R represents the extraction recovery, $A_{extraction}$ represents the amount of analyte in samples extracted by the MeOH method we used, and A_{left} represents the amount of analyte left in samples where we extracted them by BD or butanol method.

RESULTS

The development and optimization of the MeOH method for efficient and reproducible extraction of PLs and LPLs

In our search for a simple and universal method for extraction of PLs and LPLs in blood samples, we found that by using a single MeOH solvent without any other additions (such as acid or alkali), we could replace most if not all "classical" methods for PL and LPL extraction. This method extracted PLs and LPLs from blood samples by precipitating blood proteins in MeOH (32). Moreover, MeOH is compatible for ESI-MS analysis. Thus, lipids extracted in MeOH could be directly used for analyses and the solvent evaporation step could be eliminated.

We optimized the volume of blood samples and MeOH for extraction of negatively charged LPLs, including S1P and LPAs. We found that 10 µl of a serum sample was enough for LC-ESI-MS-based quantitative analyses of these LPLs. While 150 µl of MeOH extracted 8.5% more S1P and 3.8–11.6% more LPAs when compared with 50 µL, further increase in the volume of MeOH used, e.g., to 500 µL, did not increase the yield of extraction. For choline-containing PLs and LPLs, 2 µl of a serum sample was sufficient, because their natural concentrations are higher than those of negatively charged LPLs. Choline-containing LPLs and PLs in 2-µl blood samples could be extracted with equal efficiency by 100 µl or 1,000 µl of MeOH. Based on these results, we have chosen to use 150 µl MeOH for extracting negatively charged LPLs and PLs from 10 µl of blood samples and 1,000 µl MeOH for extracting choline-containing PLs and LPLs from 2 µl of blood samples, because the extracted lipids in these volumes of MeOH can be directly used in MS analyses without further concentration or dilution. Theoretically, 0.2 µl of blood samples would be enough for MS analyses of choline-containing PLs and LPLs. Practically, however, it would be difficult to accurately pipet samples in 0.2 µl. Nevertheless, lower volumes of blood (0.2–10 µl) were needed than in most previous publications (18, 22, 28–30) to accurately analyze PLs and LPLs.

Validate the efficiency and reproducibility in extracting S1P, LPAs, and LPCs using the MeOH method

To critically evaluate the MeOH method, we first applied this method to extract S1P, LPAs, and LPCs due to the importance of these lipids in biological systems. We tested the efficiency of the extraction by determining the recovery rates of the MeOH method for these LPLs. Standard curves were established using commercially available standards, as described previously (26). The recovery rates were obtained by MS analyses and calculation of the concentrations of spiked standard lipids in four different concentrations into a human plasma sample. The endogenous

LPL concentrations were subtracted. The concentration ranges of each LPL used were chosen based on the ranges of their concentrations in human blood samples (22, 28) (**Table 1**). The simple MeOH method had an average recovery rate of approximately 90% (ranging from 84.1 to 114.8%) for most LPLs in most concentrations ranges.

To further confirm the efficiency of the MeOH method in extracting these LPLs, we tested whether additional lipids could be extracted from the precipitates after the one-step MeOH extraction using the butanol method, the BD method, or the new MeOH method again. The butanol method has been considered to be the optimal method for extraction of S1P and LPA (28, 29), and it was found that approximately 11.2% more S1P and 14.8–17.7% more LPAs could be further extracted from the precipitates, suggesting that the simple MeOH method extracted ~85% S1P and LPAs. However, the butanol method may also generate some artifacts, as discussed below. For LPCs, <1% could be extracted from the precipitates using either the BD or the new MeOH method, suggesting that the recovery rate of the one-step MeOH method was >99% for these lipids.

The new MeOH method involved only one solvent and one step of centrifugation, which saved solvents and time, and it was extremely simple to significantly improve repro-ducibility. To test the reproducibility of this new MeOH method in biological sample lipid analyses, we extracted lipids from a plasma sample four times. The high repro-ducibility was proven by the small variations with the peak areas of lipids obtained by MS from 0.9% to 2%.

Comparison of the MeOH method for extraction of S1P and LPAs with established methods

To examine the MeOH method further, we compared it with other well-established lipid extraction methods currently used, namely, the BD, the modified BD (BD/HCl), and the butanol method (24, 26, 28, 29). The MS peak areas of lipids extracted (represented the relative yields) from serum samples using different methods were compared. The peak areas obtained using the MeOH method were taken as 100%. The MS peak areas of S1P and LPAs extracted by the BD method were only 3.2–25.1% of those extracted by the MeOH method, indicating that the BD method is not suitable for extraction of these lipids as shown in our previous studies (25, 26) (**Table 2**).

Including HCl in the BD method was very effective in extracting S1P and LPAs, as shown previously (23, 25, 26); however, approximately 20–70% more LPAs were observed than the amounts obtained by the MeOH method (Table 2).

TABLE 1. Standard curves and recovery percentages of MeOH method for extraction of additions of standard LPLs to human serum or plasma samples

Analyte	Concentration Added, µM	Concentration Measured (n = 3), µM[c]	Recovery of Added Analyte, %[c]	Linear Regression Equation
S1P[a]	0	0.83 (0.03)		$y = 0.5237x + 0.0087$ ($r^2 = 0.9978$)
	0.2	1.03 (0.04)	98.6 (1.3)	
	0.5	1.33 (0.11)	100.6 (1.3)	
	1	1.83 (0.04)	100.2 (2.3)	
	2	2.95 (0.16)	106.0 (2.8)	
16:0 LPA[a]	0	0.72 (0.08)		$y = 1.5188x - 0.0053$ ($r^2 = 0.9989$)
	0.5	1.21 (0.20)	98.3 (3.4)	
	1	1.74 (0.12)	102.4 (2.6)	
	2	2.66 (0.10)	97.2 (2.8)	
	3	3.74 (0.23)	100.6 (0.9)	
18:1 LPA[a]	0	0.81 (0.06)		$y = 1.300x - 0.021$ ($r^2 = 0.9902$)
	0.5	1.24 (0.06)	86.2 (4.5)	
	1	1.65 (0.10)	84.1 (4.1)	
	2	2.85 (0.11)	102.0 (4.9)	
	3	4.06 (0.20)	108.5 (4.8)	
18: 0 LPA[a]	0	0.20 (0.04)		$y = 0.7924x + 0.0232$ ($R^2 = 0.9938$)
	0.2	0.37 (0.08)	86.7 (2.3)	
	0.5	0.68 (0.03)	95.0 (2.5)	
	1	1.20 (0.10)	99.4 (3.6)	
	2	2.39 (0.12)	109.3 (4.5)	
16:0 LPC[b]	0	67.7 (1.3)		$y = 0.6796x + 0.0123$ ($r^2 = 0.9981$)
	10	78.0 (2.4)	103.4 (2.5)	
	25	94.9 (3.1)	108.8 (3.3)	
	50	118.1 (2.5)	100.8 (1.2)	
	100	153.8 (4.3)	86.2 (1.2)	
18:1 LPC[b]	0	14.3 (0.6)		$y = 0.6011x + 0.0104$ ($r^2 = 0.9966$)
	5	20.1 (1.1)	114.8 (4.6)	
	10	24.5 (0.5)	101.8 (1.3)	
	25	41.0 (0.2)	106.5 (2.3)	
	50	68.5 (1.4)	108.2 (1.2)	
18:0 LPC[b]	0	22.9 (0.8)		$y = 0.8153x + 0.0058$ ($r^2 = 0.9974$)
	5	27.9 (1.3)	100.3 (0.3)	
	10	32.9 (1.5)	99.8 (1.2)	
	25	51.6 (2.1)	114.8 (3.3)	
	50	72.4 (1.6)	99.0 (2.5)	

[a] Extracted lipids from 10 µl of serum samples, and the internal standard was 14:0 LPA (10 pmol);
[b] Extracted lipids from 2 µl of plasma samples, and the internal standard was 12:0 LPC (100 pmol);
[c] Data are presented as the mean (SD).

TABLE 2. Comparisons of the recoveries of different methods for extraction of S1P and LPAs

Compound	MRM Transition Pair		BD Method[c]		Modified BD Method[c]		Butanol Method 1[c]		Butanol Method 2[c]	
	Parent Ion	Daughter Ion	Mean[a]	SD[b]	Mean[a]	SD[b]	Mean[a]	SD[b]	Mean[a]	SD[b]
S1P	378.0	79	3.2	0.7	37.0	0.2	64.8	2.4	97.1	15.3
LPAs										
16:0	409.0	153	6.6	1.9	128.5	9.2	165.6	11.2	113.1	0.2
18:2	433.0	153	8.6	2.1	134.3	1.3	185.1	10.1	89.1	2.0
18:1	435.0	153	11.3	2.7	119.6	3.6	166.4	20.9	111.0	2.4
18:0	437.0	153	25.1	0.6	138.5	4.5	248.5	116.0	109.2	5.8
20:4	457.0	153	14.6	4.3	161.4	6.3	208.1	12.3	78.7	0.9
22:6	481.0	153	18.9	6.9	168.5	26.5	199.5	27.7	47.6	5.8

[a] Peak area obtained by MS analysis of lipids extracted by Butanol method, BD method and modified BD method, respectively, as percent of that obtained by MS analysis of lipids extracted by MeOH method.
[b] SD.
[c] 10 μl of serum were used for extraction of lipids.

Because we have shown that the MeOH method had ∼85% yields, the increase in LPAs (when it was >20%) was likely artificial generation of LPAs due to acid hydrolysis, as previously reported (28). The butanol method has been considered the optimal method for LPA extraction (28–30). This method uses a weak acid (citric acid). However, we have found that these modifications did not prevent the detection of artificially increased LPAs (Table 2), suggesting that a change from a strong acid HCl to a weak acid is unlikely to prevent an artificial increase in LPAs. However, we found that if the buffer (containing citric acid) was first mixed with the organic solvent (butanol) prior to adding blood samples, essentially no extra LPAs were detected (Table 2, last columns). These data suggest that acid-catalyzed hydrolysis may be prevented when it is copresented with an organic solvent. Under this condition, however, the extraction efficiencies of the butanol method for unsaturated LPAs were relatively low (47.6-89.1%). Moreover, the butanol method suffered two additional major weaknesses. First, butanol is incompatible with MS detection and has to be evaporated before MS analyses, but it is much harder to evaporate than MeOH. Second, butanol coextracts other materials, which contaminate the MS ion source.

The application of the MeOH method for extraction of PLs, lyso-PAF, LPCs, Cers, and SMs

It will be a great advantage when a simple method can universally extract all major classes of PLs and LPLs in biological samples. Currently, different methods are used for optimally extracting different PLs and LPLs. The BD method was originally developed for effectively extracting major cell membrane PLs, including PCs and PEs, as well PIs, PSs, and PAs. Many groups, including ours, also used it to extract lyso-PAF, LPCs, Cers, and SMs (3, 22). We compared the extracting efficiencies of the MeOH method and the BD method for these lipids. For major PLs, the MeOH method was either as good as or out-performed the BD method with higher efficiencies (**Table 3**). The MS peak areas of lyso-PAF, LPCs, Cers, and SMs extracted by the BD method possess 63.3–111.2% (Table 3), indicating that the MeOH method extracts more or similar lipids as those by other classical methods. To further confirm the efficiency of the MeOH method in extracting these lipids, we reextracted the pellets after MeOH extraction by the BD method or the MeOH method again. In either case, <1% of these lipids could be reextracted, suggesting that the efficiency of the one-step MeOH method was > 99% for these lipids.

The application of the MeOH method for extraction of LPIs, LPEs, LPG, and LPS

We further investigated the potential of the MeOH method for extraction of other LPLs, such as LPIs, LPEs, LPG, and LPS. For LPIs, the MeOH method was superior, because the recoveries (assessed by comparing MS peak areas of these lipids extracted by different methods) by either butanol or the BD methods were low (**Table 4**). Again, HCl could increase the yields, but it may also induce artifact evidence by ≥2-fold increase in lipid levels.

For LPEs, although the butanol method and BD method showed higher yields for certain forms, these improvements were marginal (Table 4). It is worthwhile to note that inclusion of HCl resulted in very high levels of certain forms of LPEs, which were likely due to artificial acid-induced hydrolysis of endogenous lipids in the blood samples (such as from PE) and thus strong acid should be avoided for endogenous LPE analyses. In extracting LPG and LPS (using one of their standard forms in each of these lipids for proof-of-principle studies), the MeOH method out-performed other two methods (Table 4).

MeOH stabilized most lipids in plasma samples

The MeOH method was used to extract PL and LPL in healthy control plasma samples. S1P (0.5–1 μM), 6 LPAs (0.2–0.5 μM), 3 LPIs (4–6 μM), 5 LPEs (3–5 μM), 18:1 LPG (0.4–0.7 μM), 7 Cers (1–4 μM), 9 PEs (60–100 μM), lyso-PAF (0.4–0.6 μM), 7 LPCs (100–150 μM), 3 SMs (200–300 μM), and 10 PCs (1.5–2 mM) were detected by HPLC-ESI-MS/MS.

It is well known that lipid levels are unstable in blood samples. Differences in processing procedures and especially the differences in storage times of the blood samples have been recognized as major factors for inconsistencies of lipid results obtained (23). We tested whether MeOH could stabilize lipid levels in plasma samples, because

TABLE 3. Comparisons of the recoveries by the BD method and the MeOH method for extraction of lyso-PAF, LPCs, Cers, SMs, and other major membrane PLs

	MRM Transition Pair		BD Method	
Compound	Parent Ion	Daughter Ion	Mean[a]	SD[b]
PCs[c]				
16:0/18:3	756.6	184	98.9	4.0
16:0/18:2	758.6	184	96.5	6.4
16:0/18:1	760.6	184	99.5	6.0
16:0/18:0	762.6	184	99.1	6.9
16:0/20:4	782.6	184	91.8	4.1
16:0/22:6	806.5	184	96.5	7.9
18:0/18:3	784.9	184	100.5	7.7
18:0/18:2	786.8	184	91.9	8.2
18:0/18:1	788.8	184	94.8	5.8
18:0/20:4	810.6	184	97.1	9.8
PEs[d]				
16:0/18:2	714.5	279	78.0	1.3
16:0/18:1	716.5	281	65.9	0.8
16:0/18:0	718.5	283	66.3	1.2
16:0/20:4	738.5	303	74.3	6.7
16:0/22:6	762.5	327	71.6	5.2
18:0/18:2	742.5	279	47.3	5.9
18:0/18:1	744.5	281	33.8	4.7
18:0/20:4	766.6	303	47.4	2.5
18:0/22:6	790.6	327	36.9	4.4
PAs[d]				
16:0/18:2	671.6	279	83.1	1.2
16:0/18:1	673.5	281	50.5	2.9
18:0/ 20:4 PI[d]	885.5	283	82.6	14.3
18:0/18:1 PS[d]	788.4	283	78.7	3.5
Lyso-PAF[c]	482.0	104	73.6	7.7
LPCs[c]				
16:0	496.0	184	71.5	3.1
18:2	520.0	184	87.2	0.5
18:1	522.0	184	88.8	0.9
18:0	524.0	184	92.5	1.1
20:4	544.0	184	93.6	0.9
22:6	568.0	184	97.8	2.9
Cers[d]				
16:0	536.6	280	73.6	7.1
18:1	562.6	306	78.0	2.1
18:0	564.6	308	63.3	1.5
20:0	592.6	336	68.8	2.3
22:0	620.6	364	80.2	6.1
24:1	646.7	390	85.0	3.2
24:0	648.6	392	101.9	2.6
SMs[c]				
16:0	703.4	184	103.0	4.7
18:1	729.5	184	111.2	4.0
18:0	731.5	184	102.9	1.9

[a] Peak area obtained by MS analysis of lipids extracted by BD method as percent of that obtained by MS analysis of lipids extracted by methanol method.
[b] SD.
[c] 2 µl of plasma were used for extraction of lipids.
[d] 10 µl of plasma were used for extraction of lipids.

MeOH could denature and precipitate many proteins and possibly lipid metabolic enzymes. Plasma samples (10 µl) were added to 500 µl MeOH. The levels of some LPLs and PLs were analyzed after samples were incubated (4 h at 37°C or room temperature for 24 h) and compared with the samples without incubation. Parallel experiments showed that LPC levels did not change in the presence of MeOH (with variations from −1.5% to 4.5%). In addition, the levels of S1P, LPAs, LPIs, 18:1 LPG, Cers, PEs, lyso-PAF, SPC, SMs, and PCs were not significantly changed after incubation (<5%), suggesting that MeOH is a very

good reagent to stabilize LPL levels, and the plasma samples can be safely kept in MeOH. However, the stability of LPEs was an exception, which decreased approximately 5–20%, suggesting that either MeOH cannot block the enzyme(s) responsible for LPE metabolism or LPEs are unstable nonenzymatically in MeOH.

The application of the MeOH method for extracting LPLs and PLs from cell pellets and tissues

The suitability of the MeOH method for extracting LPLs and PLs from cell pellets and animal tissues were investigated. For PAs, PCs, PEs, PI, PS, SM, Cers, lyso-PAF, and LPCs, the MS peak areas extracted by the BD method were 70.5–112.0% of those extracted by the MeOH method (taken as 100%), suggesting that the MeOH method is suitable for extracting these lipids from either cell pellets or tissues. To further confirm this, we reextracted lipids from the MeOH precipitates using the classical BD method and found that <5.6% of lipids could be extracted, indicating that the recovery rate of the one- step MeOH method was >94% for these lipids. The levels of LPAs in cell pellets and tissues were very low and the efficiencies for extracting other LPLs were 85.5% for S1P, 84.1–99.1% for LPIs, 82.2–99.4 for LPEs, 95% for LPG, and 75.6–99.7% for LPS, suggesting that the simple MeOH method can also be used for extracting both PLs and LPLs from cell pellets and tissues.

DISCUSSION

Genomic and proteomic studies have identified genes that are either mutated or have altered expression (33, 34) and proteins that are down- or upregulated in cancers and other human diseases (35). Lipidomics and metabolomics are significant extensions of genomics and proteomics, which qualitatively and quantitatively analyze small molecules and investigate the relations among these small molecules, as well as with other biological molecules. Altered metabolite levels have been observed in many diseases, and certain lipids have recently been recognized as important cell signaling molecules.

However, almost none of the identified markers have been moved to clinics. One of the major problems is the lack of cross validation of these markers in different laboratories. For lipid markers, different sample collection and processing procedures, lipid extraction methods, and LC-MS conditions have been employed in different laboratories. A simple and efficient method for lipid extraction will have a significant impact on these issues. We presented the MeOH method, an extremely simple method with an across-the-board high efficiency for extracting major classes of LPLs and PLs in blood samples. Traditionally, several different extraction methods are needed to extract different classes of PLs and LPLs. This streamlined procedure is naturally associated with less error and thus high reproducibility.

The MeOH method has been critically evaluated and validated by several methods. First, we have shown that it extracts the most important LPLs (S1P, LPAs, and LPCs)

TABLE 4. Comparisons of the recoveries of different methods for extraction of LPIs, LPEs, LPG, and LPS

Compound	MRM Transition Pair		Butanol Method[c]		BD Method[c]		Modified BD Method[c]	
	Parent Ion	Daughter Ion	Mean[a]	SD[b]	Mean[a]	SD	Mean[a]	SD
LPIs								
16:0	571.0	153	33.6	7.2	28.6	8.2	114.6	2.2
18:0	599.0	153	79.2	4.7	16.8	3.7	217.9	12.2
20:4	619.0	153	28.4	3.7	18.5	6.4	112.8	8.3
LPEs								
16:0	452.2	255	70.9	1.2	90.9	26.0	124.7	5.7
18:1	478.3	281	73.4	5.1	89.7	19.1	142.9	0.9
18:0	480.5	283	59.9	5.9	78.1	14.4	92.4	1.1
20:4	500.5	303	131.4	13.9	110.7	20.9	886.4	4.3
22:6	524.5	311	128.9	17.7	107.0	31.0	876.0	8.8
18:1 LPG	509.4	327	78.7	42.8	54.8	1.7	137.5	20.7
18:1 LPS	522.2	339	62.0	1.5	18.0	3.1	164.7	1.0

[a] Peak area obtained by MS analysis of lipids extracted by the butanol method, the BD method, and the modified BD method, respectively, as percent of that obtained by MS analysis of lipids extracted by methanol method.
[b] SD.
[c] 10 μl of serum were used for extraction of lipids.

with >85% yields using spiked and known amounts of LPLs in human blood samples. Second, by comparison with other well-established methods, MeOH displays similar or superior extracting power for all major classes of blood PLs and LPLs. Third, reextraction of the precipitates suggests that a one-time MeOH extraction is highly effective and additional steps have little gain. Moreover, we have optimized the method such that the solvent evaporation step can be omitted to further simply the procedure.

Another important feature of the MeOH method is that because no acid or alkaline is involved, it is likely that this method extracts authentic and endogenous lipids and does not generate artifacts. Scherer et al. (28) have reported that strong acid could induce as much as 7-fold increases in the LPA concentration. Our study here suggests that 20–70% more LPA may be generated when HCl was used. The differences could be derived from the amounts of HCl used and the different incubation times during extraction. In addition, our data show that a weak acid (such as citric acid) cannot prevent this increase in LPA levels (**Table 2**), making the strong argument that any acid should be omitted if it is possible. Whereas the classical BD method is very poor in extracting LPLs, our newly developed MeOH method provides such a possibility, which is critically important for the lipid signaling and disease marker fields. In our view, the previous LPA marker studies (18–21, 36–38) are valid, because the same procedures have been applied to all the samples without any bias. Nevertheless, it is important to measure endogenous LPA levels without artifacts. We have also found that this method can be applied to extract lipid in tissues and cells. The only limitation is that when lipids are in very low concentrations (such as cell supernatants), a preconcentration step may be needed. Moreover, it is possible that this method can be used for extracting other classes of lipids, such as fatty acids, so that it may have an even broader application.

In summary, we presented a new and extremely simple method for the extraction of major classes of endogenous PLs and LPLs from biological samples with high efficiency and reproducibility without the generation of an artifact. This method can be easily applied in different laboratories, and such cross-examination of lipid analyses and lipid marker development can be feasibly conducted. This new method will be useful for both basic sciences and clinical applications.

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