Quantitative Analysis of Lysophosphatidic Acid in Human Plasma by Tandem Mass Spectrometry

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Analysis of lysophosphatidic acids (LPAs) is of clinical importance as they can serve a potential marker for ovarian and other gynecological cancers and obesity. It is critically important to develop a highly sensitive and specific method for the early detection of gynecological cancers to improve the overall outcome of this disease. We have established a novel quantification method of LPAs in human plasma by negative ionization tandem mass spectrometry (MS-MS) using multiple reaction monitoring (MRM) mode without the conventional TLC step. Protein-bound lipids, LPAs in plasma were extracted with methanol : chloroform (2:1) containing LPA C14:0 as an internal standard under acidic condition. Following back extraction with chloroform and water, the centrifuged lower phase was evaporated and reconstituted in methanol. The reconstituted solution was directly injected into electrospray source of MS/MS. For MRM mode, Q1 ions selected were m/z 409, 433, 435, 437 and 457 which corresponds to molecular mass $[M-H]^-$ of C16:0, C18:2, C18:1, C18:0 and C20:4 LPA, respectively. Q2 ions selected for MRM were m/z 79, phosphoryl product. Using MS/MS with MRM mode, all the species of LPAs were completely separated from plasma matrix without severe interferences. This method allowed simultaneous detection and quantification of different species of LPAs in a plasma over a linear dynamic range of 0.01-25 $\mu molL^{-1}$. The detection limit of the method was 0.3 pmol/mL, with a correlation coefficient of 0.9983 in most LPAs analyzed. When applied to the plasmas of normal and gynecological cancer patients, this new method differentiated two different groups by way of total LPA level.

Key words : Tandem mass spectrometry, Negative ionization, Multiple reaction monitoring, Lysophosphatidic acid, Gynecological cancer

Introduction

In South Korea, ovarian cancer, which ranks first in incidence among gynecological cancers, caused an estimated 2,584 deaths in 2000.[1] More than 75% of women with ovarian cancer were diagnosed in an advanced stage, and the survival rate for these women remains very poor. If the disease were would be detected in stage I, the long-term survival rate would be approximately 90%. However, ovarian cancer develops silently. Symptoms usually occur only in advanced stages when tumor dissemination within the peritoneal cavity induces ascites, with the resultant increase in abdominal girth. Therefore, it is critically important to develop a highly sensitive and specific method for the early detection of gynecological cancer to improve the overall outcome of this disease.

The search for a marker for ovarian cancer has been ongoing in many research laboratories over the last 20 years. More than 20 markers have been examined, including CA 125.[2-8] Unfortunately, none of these markers effectively detect early stage ovarian cancer, although some of them are good prognostic markers and are very useful for ovarian cancer patient management. Transvaginal sonography can detect the early stage of the disease, but lacks specificity. In addition, such methods are too expensive to be widely used for screening.[4] The present study was conducted to determine whether lysophosphatidic acid (LPA) is important clinically as a potential marker for ovarian and other gynecological cancers and obesity.[9]

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA),[10] once thought of only as an intermediate in the biosynthesis of phospholipids, has since been shown to be an important multifunctional biological mediator. LPA is the most widely studied example of a family of phospholipid growth factors whose members elicit their cellular effects through specific G-protein-coupled receptors. LPA elicits numerous cellular responses, including mitogenic[11] and antimitogenic[12] effects, on the cell cycle; regulation of the actin cytoskeleton,[13] cellular motility,[14] and cancer cell invasiveness;[15] and mobilization of intracellular calcium.[16,17] These pleiotropic growth factor-like effects have suggested roles for LPA as a factor in cellular homeostasis,[18] a mediator of wound healing,[19] and a modulator of carcinogenesis.[20]

Previous methods have utilized an indirect procedure to quantify the LPA level. Bioassays, such as voltage clamped Xenopus oocytes,[21,22] have been used to generate titers of LPA-like activity. Although sensitive, this approach is cannot distinguish compound classes or molecular structures. Other investigators have analyzed LPA derived fatty acid methyl esters in an effort to determine LPA concentration.[23]

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This methodology includes partial purification of LPA by thin-layer chromatography (TLC), followed by hydrolysis to generate fatty acid methyl esters for analysis by gas chromatography. Also, recent studies used TLC for sample preparation and analyzed the resulted by electrospray mass spectrometry.[24] This protocol has two significant problems. First, appropriate standards for the control of recovery are lacking throughout the procedure. Second, various LPA salts (free acid, sodium and calcium salts) differ in mobility when chromatographed by TLC. This is true for acidic, neutral and basic TLC analysis. Both of these difficulties could lead to underestimation of LPA levels.

In the present study, we established a novel quantification method of LPAs in human plasma by turbo electrospray ionization tandem mass spectrometry (ESI-MS-MS), using multiple reaction monitoring (MRM) mode and precursor ion scan (PS) mode without the TLC step.

Experimental Section

Materials. LPAs (LPA C14:0, LPA C16:0, LPA C18:2, LPA C18:1, LPA C18:0 and LPA C20:4) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Chloroform, acetic acid and hydrochloric acid were purchased from Sigma-Aldrich Korea (Seoul, Korea). HPLC grade methanol and distilled water were purchased from Fisher Scientific Korea (Seoul, Korea). Figure 1 presents the structures of the various LPA species analyzed.

ESI-MS-MS Conditions. MS-MS was performed on an API 2000 triple quadrupole mass spectrometry equipped with turbo electrospray ion source (PerkinElmer Life Science Inc. Boston, MA). Twenty microliters of sample were delivered into the ESI source, using a micro-LC equipped with autosampler (PerkinElmer Series 200) without an LC column. The mobile phase used after degassing was 0.05% acetic acid in 95% methanol. Gradient elution of the mobile phase was from 200 to 400 μLmin^{-1} with a total running time of 1.5 min.

The instrument settings were as follow: the turbo ion-spray interface was maintained at 300 °C with a nitrogen nebulization. The nitrogen was kept at a pressure of 40 psi.

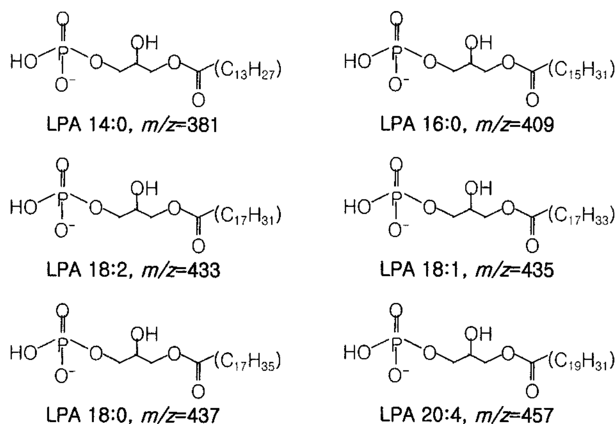

Figure 1. Structures of some lysophosphatidic acids (LPAs).

The turbo ion-spray drying gas (N_2) was kept at a pressure of 80 psi. For MRM scan mode with negative ion detection, the parameter setting was as follows -: The collision-activated dissociation gas (CAD) pressure was 5 psi and curtain gas (CUR) pressure was 20 psi; turbo ion-spray voltage, -4500 V; declustering potentials (DP), -27 V to -56 V; focusing potential (FP), -390 V; entrance potentials (EP), 10 V to 11 V; collision cell entrance potentials (CEP), -55 to -57 V; collision energies (CE), -61 V to -67 V; collision cell exit potentials (CXP), -11 V to -12 V; deflector (DF), -150 V and channel electron multiplier (CEM), 2300 V. The parameter setting for the for the PS scan mode with negative ion detection was as follows: DP, -56 V; FP, -390 V; EP, 10 V; CEP, -57 V; CE, -67 V; and CXP, -12 V.

Sample Preparation. The blood samples of three patients and five healthy controls were centrifuged at 3,000 rpm for 15 min at 4 °C. The plasma was transferred into coated microcentrifuge tubes (Supelco/Sigma, St. Louis, MO) and frozen at -50 °C or used immediately. All extraction procedures were performed in 1.5 mL microcentrifuge tubes. To 200 μL of plasma sample, 40 μL of 6 N hydrochloric acid and 800 μL of methanol : chloroform (2 : 1) containing LPA C14:0 as internal standard were added. The plasma was vortexed for 1 min and incubated for 20 min at -10 °C. The upper phase was transferred to another tube to which were added 200 μL of chloroform and 250 μL of distilled water for liquid-liquid extraction. The lower phase taken was vortexed for 1 min and incubated for 5 min at -10 °C. After centrifugation (13,000 rpm for 10 min at 4 °C), the lower phase was transferred to a new microcentrifuge tube and incubated for 30 min at -50 °C for the removal of lipid residue. The lower phase (100 μL) was transferred to a 96 well-microplate (Costar, Cambridge, MA), evaporated under a gentle nitrogen stream at 40 °C and redissolved in 100 μL of methanol. The solution was directly injected into the turbo electrospray ion source of the ESI-MS-MS.

Results and Discussion

Xu *et al*. used two-dimensional TLC to separate LPA from other lipids.[9,25] All major lysophospholipids (LPLs) species, including lysophosphatidylchloline (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), lyso-platelet activating factor (LPAF) and PAF were separated in plasma with the solvent system (chloroform : methanol : ammonium hydroxide = 65 : 35 : 5.5). Although this TLC step showed the advantage of decreased viscosity of the extracts during the plasma preparation, it did not appear that the partial TLC purification step properly improved sensitivity and specificity for the quantification of LPA due to the background fluctuation in the spectrum of product scan mode.[24] Therefore, we developed a novel quantification method for LPAs by negative ion ESI-MS-MS, especially using turbo ion electrospray without tedious, time consuming and labor-intensive TLC clean-up.

The linear dynamic range of LPA C16:0 and C18:0

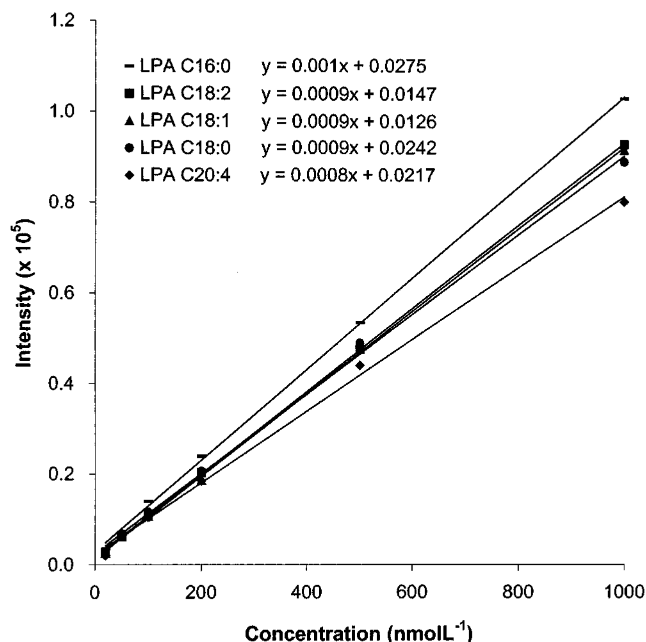

Figure 2. Calibration curve of LPA species.

Table 1. Linearity and detection limit of LPA species

LPAs	Regression line[a]		Correlation coefficient (r)	Detection limit ($nmolL^{-1}$)
	m	b		
LPA C16:0	0.0010	0.0275	0.9983	1.0
LPA C18:2	0.0009	0.0147	0.9987	0.5
LPA C18:1	0.0009	0.0126	0.9984	0.3
LPA C18:0	0.0009	0.0242	0.9986	0.5
LPA C20:4	0.0008	0.0217	0.9991	0.5

[a]y = mx + b

species were between 0.01 $\mu molL^{-1}$ and 25 $\mu molL^{-1}$, which is adequate for the detection of biologically excreted low concentration of LPA in plasma. The concentration of LPAs was calculated by measuring the height of each LPA relative to that of internal standards following a calibration curve of each LPA (Figure 2). The regression lines for all LPAs show excellent linearity with a correlation coefficient of higher than 0.9983 in the range of 0.01-25 $\mu molL^{-1}$ (Table 1). The detection limit for this method was 0.3-1.0 $pmolmL^{-1}$ for the quantification of LPAs in plasma.

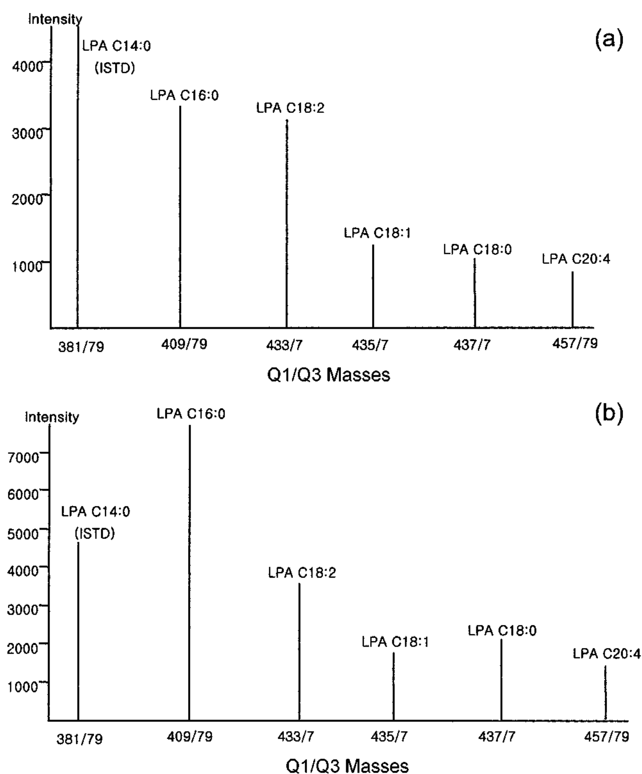

Figure 3. The MRM spectrum of LPAs from plasma. (a) healthy control, (b) gynecological cancer patient.

Recovery of LPA C16:0 and C18:0 was between 100 and 110%, with RSD of less than 7% from the plasma fortified with three different concentrations (0.1, 0.5 and 1 $nmolmL^{-1}$) of LPAs (Table 2). This excellent recovery result proved the superiority of our method in terms of reproducibility compared with that of Xu *et al.*'s method,[20] which showed 70% of recovery with TLC procedure.

For achieving high sensitivity and specificity, we used both MRM and PS scan mode for the purpose of quantification and identification of LPAs, respectively. MRM spectrum (Figure 3) and PS spectrum (Figure 4) were presented from the plasmas of healthy control and gynecological

Table 2. Recovery, precision and accuracy data for quantification of LPA C16:0 and LPA C18:0

Conc. added ($\mu molL^{-1}$)		Inter-day assay[a]				Intra-day assay[a]			
		LPA C16:0	Recovery (%)	LPA C18:0	Recovery (%)	LPA C16:0	Recovery (%)	LPA C18:0	Recovery (%)
0.10	Mean	0.10	100	0.11	110	0.10	100	0.11	110
	SD	0.01		0.01		0.01		0.01	
	%RSD	6.8		4.7		5.9		5.7	
0.50	Mean	0.50	100	0.52	104	0.50	100	0.52	104
	SD	0.01		0.01		0.01		0.01	
	%RSD	2.4		2.5		2.4		2.5	
1.00	Mean	1.01	101	1.03	103	1.01	101	1.03	103
	SD	0.03		0.02		0.03		0.02	
	%RSD	2.6		2.3		2.5		1.8	

[a]n = 5; number of independent replicate.

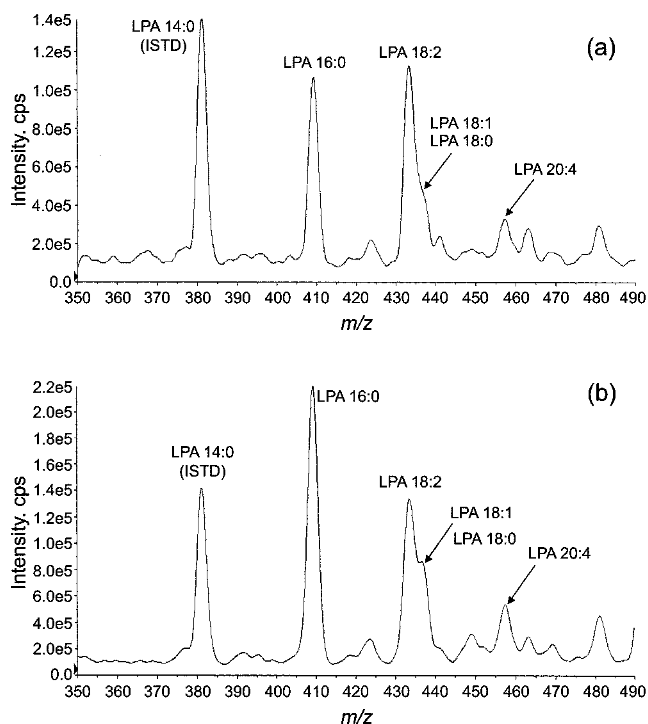

Figure 4. The PS spectrum of LPAs from plasma. (a) healthy control, (b) gynecological cancer patient.

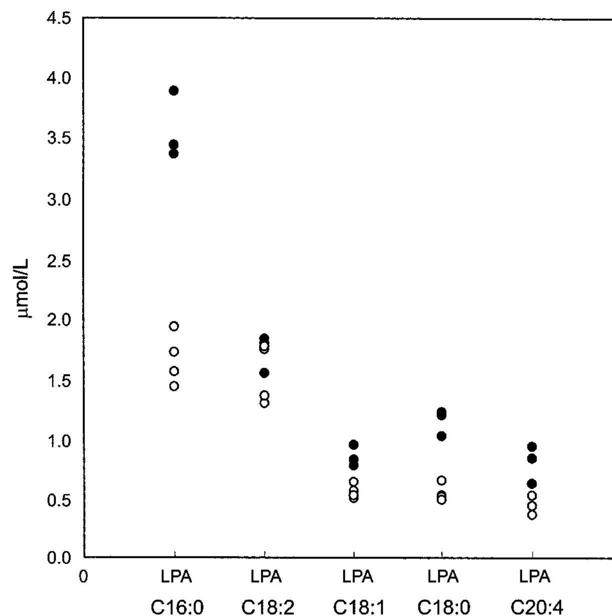

Figure 5. Concentration of LPAs in plasmas from healthy control (○) and gynecological cancer patient (●).

cancer patients. Figure 3 and 4 show an excellent mass spectrum, which minimized any interferences that could be derived from plasma.

We applied the new method to the plasmas from with normal and gynecological cancer patients. Total LPAs from plasma from gynecological cancer patients were 1.5 times higher than that from plasma from normal control (Table 3), showing that gynecological cancer patients are clearly differentiated from normal control. This result implies the future possibility of the availability of LPAs as a biological marker for the early medical intervention for a variety of gynecological cancer patients. To support the clinical use of this marker, a more intensive study should be performed with an extended large-scale population.

In Table 3, we used total LPAs level for differentiating the patient from the normal. However, if the levels of the palmitoyl LPA (C16:0), oleyl LPA (C18:1), and stearoyl LPA (C18:1) are used as a biological marker, the difference between a healthy individual and a patient would be quite prominent as shown in Figure 5. It seems that this result may support and have some connection with the report by Xu *et al.*[9] The ovarian cancer activating factor (OCAF) is composed of various species of LPA, including LPAs, with polyunsaturated fatty acyl chains. However, OCAF is more potent than *sn*-1 of palmitoyl, oleyl, and steroyl LPA in increased $[Ca^{+}]_i$ in ovarian cancer cells.[25] Therefore this interesting finding will be an important consideration when we develop a strategy for further studies.

The importance of this study is a new method development and its tentative evaluation for clinical use for the diagnosis of gynecological cancers compared with a control in obstetric patients. Plasma LPA may represent a more sensitive maker for gynecological cancers. The plasma LPA assay offers the possibility of the diagnosis of gynecological cancers, a disease that is associated with a poor outcome mainly because it is rarely detected at early stages.

We need to address the fact our results are preliminary and are based on a limited population. Further studies will be able to verify the general usefulness of LPA as a biomarker

Table 3. Concentration of LPAs in plasmas from patients and healthy controls
unit : μmolL^{-1}

Sample	LPA C16:0	LPA C18:2	LPA C18:1	LPA C18:0	LPA C20:4	Total
Patient 1[a]	3.897	1.826	0.943	1.223	0.827	8.716
Patient 2[b]	3.442	1.536	0.774	1.199	0.621	7.573
Patient 3[c]	3.370	1.790	0.818	1.018	0.932	7.927
Control 1	1.716	1.740	0.637	0.522	0.431	5.046
Control 2	1.925	1.292	0.496	0.648	0.362	4.722
Control 3	1.555	1.765	0.555	0.510	0.430	4.817
Control 4	1.435	1.360	0.525	0.487	0.520	4.327

[a]Female, 53Y, Primary peritoneal carcinoma. [b]Female, 42Y, Kruckenberg tumor in both ovaries. [c]Female, 47Y, cervical carcinoma.

for gynecological cancers and whether a combination of LPA and other assays, such as CA125, are even more useful for cancer detection.

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