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Quantitative analysis of acyl-lysophosphatidic acid in plasma using negative ionization tandem mass spectrometry

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Abstract

Analysis of acyl-lysophosphatidic acids (LPAs) has clinical importance as a potential biomarker for ovarian and other gynecological cancers or obesity from the point of view of prevention. Here we report a simple sample preparation and analytical method with high sensitivity and specificity for the early detection of gynecological cancers to improve the overall outcome of this disease. We established a novel quantification method for acyl-LPAs in plasma by electrospray negative ionization tandem mass spectrometry (MS–MS) using multiple reaction monitoring mode without conventional TLC step. Protein-bound lipids, acyl-LPAs in plasma were extracted with methanol/chloroform (2:1) containing LPA $C_{14:0}$ as internal standard under acidic conditions. Following back-extraction with chloroform and water, the centrifuged lower phase was evaporated and reconstituted in methanol and then analyzed. Using ESI-MS–MS with negative ionization MRM mode, all the species of LPAs were completely separated from plasma matrix without severe interference. For MRM mode, Q1 ions selected were *m*/*z* 409, 433, 435, 437 and 457 which corresponds to molecular mass $[M\text{-}H]^-$ of $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, $C_{18:0}$ and $C_{20:4}$ LPA, respectively. Q2 ions selected for MRM was *m*/*z* 79, phosphoryl product. Using MS–MS with MRM mode, all the species of LPAs were completely separated from plasma matrix without severe interference. This method allowed simultaneous detection and quantification of different species of LPAs in plasma over a linear dynamic range of 0.01–25 μmol/l. The method detection limit was 0.3 pmol/ml with correlation coefficient of 0.9983 in most LPAs analyzed. When applied to plasma from normal and gynecological cancer patients, this new method differentiated two different groups by way of total LPA level.

Keywords: Acyl-lysophosphatidic acid

1. Introduction

In South Korea, ovarian cancer ranks first in incidence among gynecological cancers and will cause an estimated 2584 deaths [1]. More than 75% of women diagnosed with ovarian cancer present at an advanced stage, and survival rate for these women remains very poor. If the disease is detected at stage I, the long-term (more than 5 years survival) survival rate is approximately 90%. However, ovarian cancer develops silently. Symptoms usually occur only in advanced stages when tumor dissemination within

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the peritoneal cavity induces ascites with a 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 past 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 are good prognostic markers and are very useful in the management of ovarian cancer patients. Transvaginal sonography can detect early stage disease, but lacks specificity. In addition, such methods are too expensive to be widely used for screening [4]. The purpose of this study was to determine whether lysophosphatidic acid (LPA) is of clinical importance as a potential marker for ovarian and other gynecological cancers or obesity [9].

Lysophosphatidic acid (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA) [10], was once thought to be only an intermediate in the biosynthesis of phospholipids. However, it has 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 used an indirect method to quantify 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 unable to distinguish compound classes or molecular structures. Other investigators have analyzed LPA derived fatty acid methyl esters in an effort to determine LPA concentration [23]. This methodology included partial purification of LPA by thin-layer chromatography (TLC), followed by hydrolysis to generate fatty acid methyl esters for analysis by gas chromatography. Also, a recent report used TLC for sample preparation with analysis 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 mobilities when chromatographed by TLC. This is true for acidic, neutral and basic TLC analysis. Both of these difficulties could lead to underestimation of LPA level.

In this study, we have 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.

2. Experimental

2.1. Chemicals and standard solution

Acyl-lysophosphatidic acids (LPAs; LPA $C_{14:0}$, LPA $C_{16:0}$, LPA $C_{18:2}$, LPA $C_{18:1}$, LPA $C_{18:0}$ and LPA $C_{20:4}$) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Fig. 1 presents the structures of acyl-LPAs analyzed.

Guaranteed or analytical grade reagents and solvents (e.g. methanol, chloroform, acetic acid, hydrochloric acid) were purchased from Sigma–Aldrich

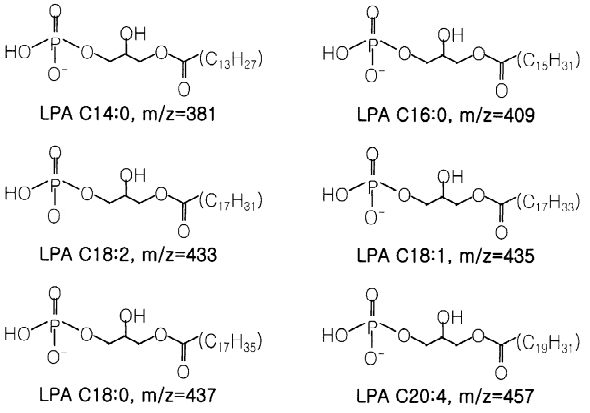

Fig. 1. Structures of lysophosphatidic acid (LPA).

(Seoul, South Korea) or Fisher (Seoul, South Korea). Distilled water was free from interference, purified by a standard water purification system (Millipore–Milli-Q™ system, Tokyo, Japan). A Millipore membrane filter (pore size 0.2 μm) was used for filtration in preparation of distilled water or solvent.

Stock standard solutions of each acyl-LPAs and internal standards (LPA $C_{14:0}$) (4000 μg ml^{-1}) were prepared in HPLC grade methanol. We did not find endogenous excretion of $C_{14:0}$ LPA. Working standard solutions were obtained by diluting the stock solutions with HPLC grade methanol. All solutions were stored at 4 °C until the time of analysis. Calibration standard solutions were prepared at concentrations between 25 and 200 μg ml^{-1}.

2.2. ESI-MS–MS conditions

ESI-MS–MS was carried out on a Perkin-Elmer Wallac MS2 triple quadrupole tandem mass spectrometer equipped with a turbo electrospray ion source, Harvard pump, micro-LC, and autosampler (Series 200, Perkin-Elmer Life Science, Boston, MA, USA). Twenty microliters of compound (or sample) was directly delivered into the ESI source through a PTFE line (0.22 μm I.D.) using the PE micro-LC system and autosampler where LC column was not installed. Compounds (or samples) were eluted with a mobile phase of 95% methanol (including 0.05% acetic acid). Gradient elution of mobile phase was carried out from 200 to 400 μl min^{-1} with a total run time of 1.5 min. The ESI-MS–MS settings used were as follows. Turbo electrospray ionisation source was maintained at 300 °C with a drying gas flow of 10 l h^{-1} and data were collected in the negative ion mode from 100 to 1000 m/z at 13 000 a.m.u. s^{-1}. The pressures of nitrogen nebulization and drying gas (nitrogen) were 40 and 80 p.s.i., respectively.

Multiple reaction monitoring and precursor scan of m/z 79 were carried out in negative ionization mode. Parameter settings for multiple reaction monitoring were as follows. Selected ions for Q1 were m/z 409, m/z 433, m/z 435, m/z 437 and m/z 457 which correspond to molecular masses $[M\text{-}H]^-$ of $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, $C_{18:0}$ and $C_{20:4}$ acyl-LPA, respectively. Selected ion for Q2 was m/z 79, which corresponds to the fragment of phosphoryl moiety.

The instrument settings used are as follows: the turbo ion-spray interface was maintained at 300 °C with nitrogen nebulization. The nitrogen was at a pressure of 40 p.s.i. The turbo ion-spray drying gas (N_2) was at a pressure of 80 p.s.i. For MRM scan mode with negative ion detection, parameter settings were as follows: the collision activated dissociation gas (CAD) was at a pressure of 5 p.s.i. and curtain gas (CUR) was at a pressure of 20 p.s.i.; turbo ion-spray voltage, −4500 V; declustering potentials (DP), −27 to −56 V; focusing potential (FP), −390 V; entrance potentials (EP), 10 to 11 V; collision cell entrance potentials (CEP), −55 to −57 V; collision energies (CE), −61 to −67 V; collision cell exit potentials (CXP), −11 to −12 V; deflector (DF), −150 V and channel electron multiplier (CEM), 2300 V. For the PS scan mode with negative ion detection, the parameter settings were as follows: DP, −56 V; FP, −390 V; EP, 10 V; CEP, −57 V; CE, −67 V; and CXP, −12 V.

2.3. Sample preparation

Blood samples were collected from patients admitted at Obstetrics and Gynecology and from healthy controls and then were centrifuged at 2000 *g* for 15 min at 4 °C. The plasma was transferred into coated microcentrifuge tubes (Supelco/Sigma, St Louis, MO, USA) and kept frozen at −50 °C or used immediately. All extraction procedures were carried out in 1.5-ml microcentrifuge tubes. To 200 μl of plasma sample, 40 μl of 6 *M* hydrochloric acid and 800 μl of methanol/chloroform (2:1) containing LPA $C_{14:0}$ 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, and then 200 μl of chloroform and 250 μl of distilled water were added for liquid–liquid extraction. The lower phase was vortexed for 1 min and incubated for 5 min at −10 °C. After centrifugation (25 000 *g* 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, USA) 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 ESI-MS–MS.

Table 1
Linearity and detection limit of LPA species

LPAs	Regression line[a]		Correlation coefficient (r)	Detection limit (nmol/l)
	m	b		
LPA $C_{16:0}$	0.0010	0.0275	0.9983	1.0
LPA $C_{18:2}$	0.0009	0.0147	0.9987	0.5
LPA $C_{18:1}$	0.0009	0.0126	0.9984	0.3
LPA $C_{18:0}$	0.0009	0.0242	0.9986	0.5
LPA $C_{20:4}$	0.0008	0.0217	0.9991	0.5

[a] $y = mx + b$.

3. Results and discussion

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

The linear dynamic ranges of LPA $C_{16:0}$ and $C_{18:0}$ species were between 0.01 and 25 μmol/l, which could detect biologically excreted low concentration of LPA in plasma. Concentration of LPAs was calculated by measuring the height of each LPA relative to that of internal standards following the calibration curve of each LPA (Table 1). The regression lines for all LPAs showed excellent linearity with a correlation coefficient of higher than 0.9983 in the range of 0.01–25 μmol/l (Table 1). The method detection limit was 0.3–1.0 pmol/ml for the quantification of LPAs in plasma.

Recoveries of LPA $C_{16:0}$ and $C_{18:0}$ were between 100 and 110% with RSD of less than 7% from the plasma fortified with three different concentrations (0.1, 0.5 and 1 nmol/ml) of LPAs (Table 2). This excellent recovery result proved the superiority of our method in terms of reproducibility when compared with that of Xu et al. [20], which showed 70% recovery with the TLC procedure.

To achieve high sensitivity and specificity, we used both MRM and PS scan mode for the purpose of quantification and identification of LPAs, respectively. MRM spectrum (Fig. 2) and PS spectrum (Fig. 3) were presented from the plasmas of healthy control and gynecological cancer patients. Figs. 2 and 3 showed excellent mass spectra, which minimized any interference that could be derived from plasma.

We applied the new method to the plasmas from

Table 2
Recovery, precision and accuracy data for quantification of LPA $C_{16:0}$ and LPA $C_{18:0}$

Conc. added (μmol/l)		Inter-day assay[a]				Intra-day assay[a]			
		LPA $C_{16:0}$	Recovery (%)	LPA $C_{18:0}$	Recovery (%)	LPA $C_{16:0}$	Recovery (%)	LPA $C_{18:0}$	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 replicates.

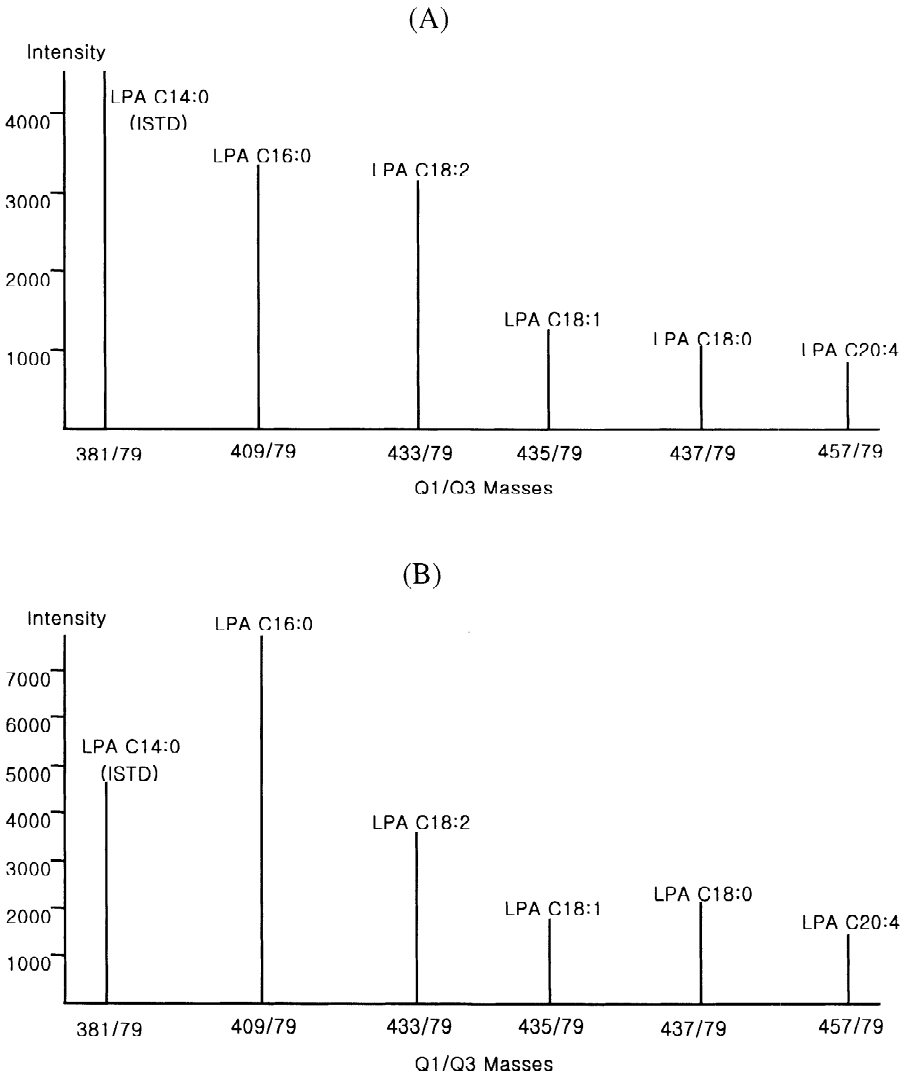

Fig. 2. The MRM spectrum of LPAs from plasma. (A) Healthy control, (B) gynecological cancer patient.

normal and gynecological cancer patients. Total LPAs in plasma from gynecological cancer patients were 1.5 times higher than that in plasma from normal control (Table 3), so gynecological cancer patients are clearly differentiated from normal controls. This result implies the future possibility of availability of LPAs as a biological marker for the early medical intervention in patients with gynecological cancer. For the clinical use of this marker, a more intensive study should be carried out with an extended large-scale population.

In Table 3, we used total LPAs level to differentiate cancer patients from normal controls. However, if the level of palmitoyl LPA ($C_{16:0}$), oleyl LPA ($C_{18:1}$), and stearoyl LPA ($C_{18:1}$) is used as a biological marker, the difference between normal and patient will be quite prominent as shown in Fig. 4. It seems that this result may support and have some connection with the report by Xu et al. [20]. The ovarian cancer activating factor (OCAF) is composed of various species of LPA, including LPAs with polyunsaturated fatty acyl chains. However,

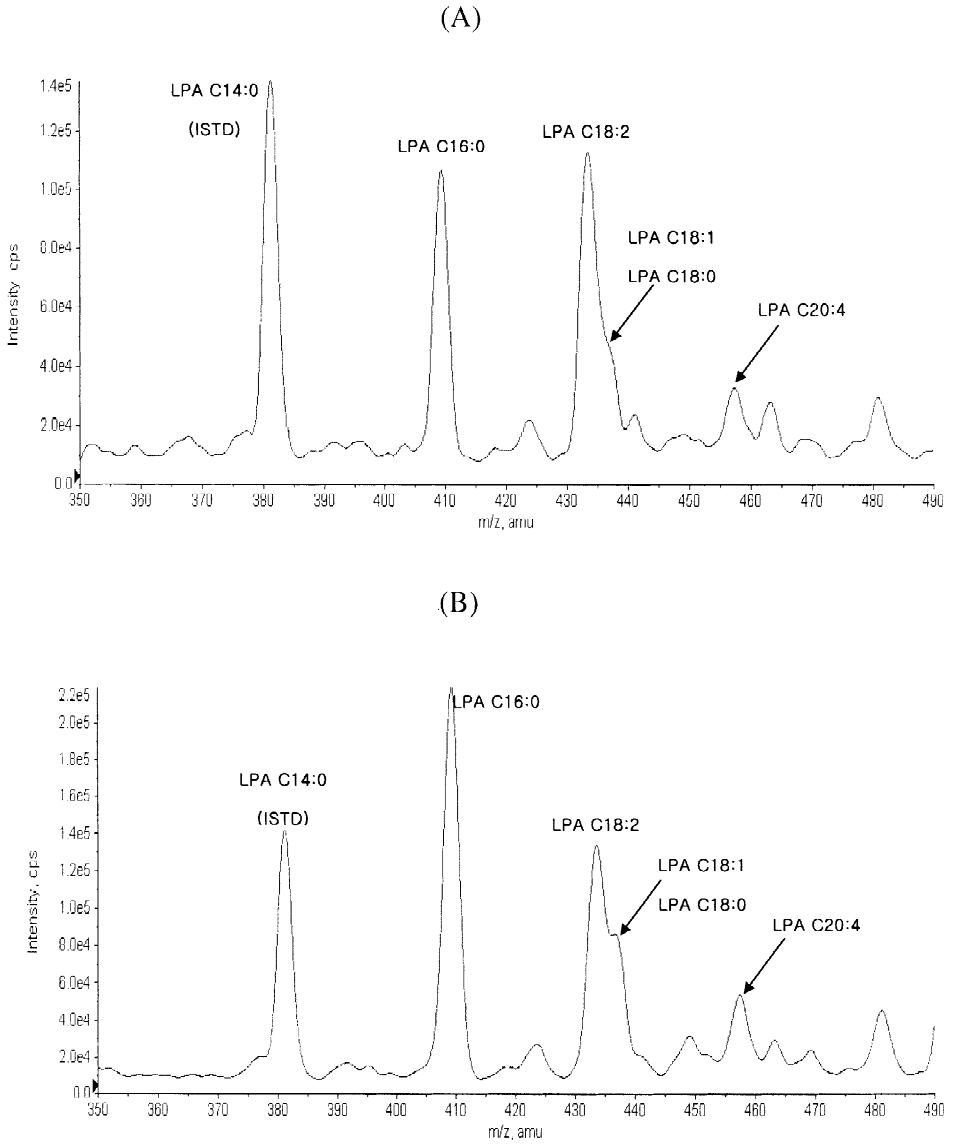

Fig. 3. The PS spectrum of LPAs from plasma. (A) Healthy control, (B) gynecological cancer patient.

OCAF is more potent than *sn*-1 of palmitoyl, oleyl, and stearoyl LPA in increased $[Ca^{+}]_i$ in ovarian cancer cells [25]. Therefore this interesting finding will be one of the considerations for extending the next study strategy.

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

Our results are preliminary and are based on a limited study population. Further studies will be required to determine the general usefulness of LPA as a biomarker for gynecological cancers and ex-

Table 3
Concentration (μmol/l) of LPAs in plasmas from patients and healthy controls

Sample	LPA $C_{16:0}$	LPA $C_{18:2}$	LPA $C_{18:1}$	LPA $C_{18:0}$	LPA $C_{20:4}$	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, 53 years, primary peritoneal carcinoma.
[b] Female, 42 years, Kruckenberg tumor in both ovaries.
[c] Female, 47 years, cervical carcinoma.

amine if a combination of LPA and other assays such as CA125 will prove even more useful for cancer detection.

Acknowledgements

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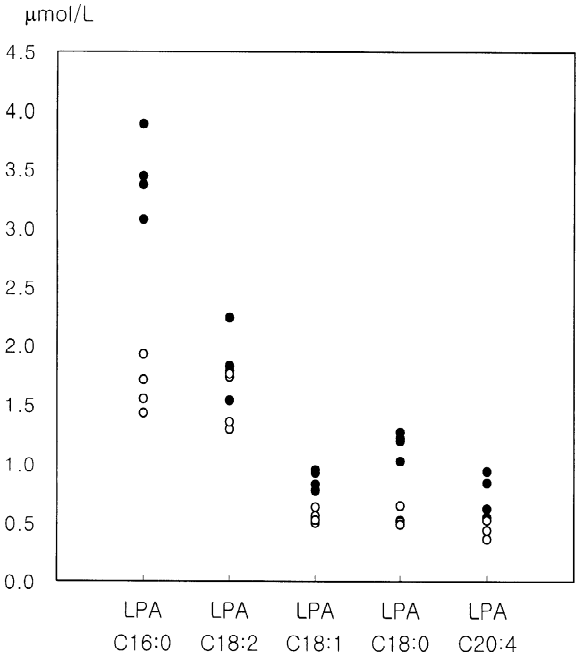

Fig. 4. Concentration of LPAs in plasmas from healthy control (○) and gynecological cancer patient (●).

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