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Journal of Chromatography B, 864 (2008) 22–28
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative determination of lysophosphatidic acid by LC/ESI/MS/MS employing a reversed phase HPLC column

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Received 17 October 2007; accepted 19 January 2008
Available online 2 February 2008

Abstract

Lysophosphatidic acid (LPA) is a class of lipids that play multiple biological functions. Several reports show that they are potential biomarkers for diagnosing ovarian cancer. Therefore, it is necessary to accurately quantify their levels in biological samples. Here we report a high throughput LC/ESI/MS/MS (liquid chromatography electrospray tandem mass spectrometry) method employing a reversed phase C18 column to quantify LPA. In this method, a [$^{13}C_{16}$] labeled 16:0 LPA is used as the internal standard and the lipids are extracted out from biological samples using Bligh–Dyer method under highly acidic condition. The total run time is 8 min. The detection limits of the assay reach fmol level and the CV% of the assay are within 10%. Using this method, we quantify the levels of six LPA species (16:0, 18:2, 18:1, 18:0, 20:4, and 22:6 LPA) in plasma samples. We find that some unknown compounds present in plasma can interfere with the quantification of LPA if they are not well separated from LPA. These unknown compounds are more hydrophobic than LPA and can be removed by thin-layer chromatography (TLC). We also find that the levels of LPA species in human plasma generally follow the order: 18:2 LPA > 16:0 LPA, 20:4 LPA > 18:1 LPA, 22:6 LPA, and 18:0 LPA.

Keywords: LPA; Lysophosphatidic acid; LC/ESI/MS/MS; LPA quantification

1. Introduction

Lysophosphatidic acid (LPA) is a class of phospholipids containing a glycerol backbone attached by a phosphate group, an aliphatic chain, and a hydroxyl group, with the phosphate group attached to *sn*-3 position, the aliphatic chain attached to either *sn*-1 or *sn*-2 position, and the hydroxyl group attached to the remaining *sn*-1 or *sn*-2 position [1]. Up to date, six possible pathways have been found to produce LPA *in vivo*. LPA can be generated from glycerol-3-phosphate and acyl-CoA by glycerophosphate acyl transferase [2] or from monoacylglycerol by monoacylglycerol kinase in mitochondria and microsomes [3]. The reduction of acyl dihydroxy acetone phosphate in peroxysomes is another pathway for LPA formation [4]. LPA can also be formed through the hydrolysis of phosphatidic acid by Phospholipase A_1 or A_2 [5,6] or through the hydrolysis of lysophospholipids by lysophospholipase D [7]. In addition, it is found that oxidation of LDL (low-density lipoprotein) can lead to the formation of LPA [8].

LPA shows multiple biological activities *in vivo* through a family of G protein-coupled receptors (GPCR) [9]. It has been found that LPA induces smooth muscle contraction [10], platelet aggregation [11], cytoskeleton rearrangement [12], Ca^{2+} mobilization [13], chemotaxis [14], neurotransmitter release [15], and cell proliferation [16]. High levels of LPA in human plasma have been found to be associated with ovarian cancer [17] and atherosclerosis [18]. Due to its important physiological role and possibility to serve as a biomarker for diagnosing ovarian cancer, it is significant to develop a sensitive, accurate and high-throughput analytical method to quantify the levels of LPA in biological samples.

Up to date, several analytical methods have been employed to quantify the levels of LPA in biological samples, including: radioenzymatic assay [19], capillary electrophoresis (CE) [21], gas chromatography (GC) [17], high-performance liquid chromatography (HPLC) [20], liquid chromatography

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doi:10.1016/j.jchromb.2008.01.031

electrospray mass spectrometry (LC/ESI/MS) [22], and flow injection liquid chromatography electrospray tandem mass spectrometry (LC/ESI/MS/MS) [23,24]. However, radioenzymatic assay, CE, GC, and HPLC can only quantify the levels of total LPA, not the levels of individual LPA species, compromising their usefulness. Also, in the methods using radioenzymatic assay, CE, and GC, LPA species must be purified by thin-layer chromatography (TLC) prior to analysis, which greatly reduces the throughput of the assay. Although TLC is not utilized in the HPLC method [20], the throughput is still low because each run takes about 60 min. In addition, the assay lacks precision due to the poor peak shape of the HPLC chromatogram.

Liquid chromatography–mass spectrometry is a widely used method to quantify small molecules in complex biological matrix due to its high sensitivity, specificity and throughput, making it an ideal method to quantify the levels of LPA species in biological samples. Up to date, several methods using liquid chromatography–mass spectrometry for LPA analysis have been reported. However, none of them are perfect for LPA quantification. Baker et al. [22] developed an LC/ESI/MS method employing a normal phase HPLC column to quantify individual LPA species. One disadvantage of this method is it is less specific than an LC/ESI/MS/MS method because it only detects LPA species according to their molecular mass ions, not the parent-to-daughter ion transitions.

Yoon et al. [24] developed a flow injection LC/ESI/MS/MS method to analyze LPA in human plasma samples. The limitation of this method for LPA quantification is due to flow injection presenting all the lipids in the samples injected to reach the mass spectrometer at the same time and all the lipids having the same parent-to-daughter ion transitions as those of LPA species will be counted in the quantification. We find that some unknown compounds present in plasma can produce the same parent-to-daughter ion transitions as LPA and interfere with the quantification of LPA in a flow injection LC/ESI/MS/MS method. This greatly reduces the accuracy, which has been demonstrated by our experiment shown in Section 3.3. In order to overcome this problem, Xiao et al. [23] used TLC to purify LPA and then run flow injection to quantify LPA. This definitely eliminates the unwanted lipids and increases the accuracy of quantification. However, the sample processing is too labor-intensive. Here we report a new high throughput LC/ESI/MS/MS method employing a reversed phase C18 column to quantify the levels of individual LPA species in biological samples. The run time is only 8 min and individual LPA species are well separated from each other. The interference from the unknown compounds is eliminated because LPA species are well separated with these unknown compounds. The detection limits of the assay reach fmol level and CV% of the assay are within 10%.

2. Materials and methods

2.1. Materials

16:0 LPA (Cat. No.: 857123), 18:2 LPA (Cat. No.: 790395), 18:1 LPA (Cat. No.: 857130), 18:0 LPA (Cat. No.: 857128), 20:4 LPA (Cat. No.: 790683), 22:6 LPA (Cat. No.: 790684), and heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA (Cat. No.: 790661) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Ammonium formate (Cat. No.: F-2004), ammonium acetate (Cat. No.: A-8920), monobasic ammonium phosphate (Cat. No.: A-1292), dibasic ammonium phosphate (Cat. No.: A-1167), ammonium hydroxide (Cat. No.: A-6899), hydrochloric acid (Cat. No.: H-1758), BSA (bovine serum albumin, essentially fatty acid free, Cat. No.: A7511), PBS buffer (Phosphate buffered saline, pH 7.4, Cat. No.: P-3813) and 8-anilino-1-naphthalene-sulfonic acid (Cat. No.: A-01028) were purchased from Sigma (St. Louis, MO, USA). Chloroform (Cat. No.: C607-4, 99.9%) was purchased from Fisher (Pittsburgh, PA, USA). DPBS buffer (Dulbecco's Phosphate-Buffered Saline, w/o calcium & magnesium) was purchased from Mediatech (Herndon, VA, USA). Methanol (Cat. No.: HP702, HPLC grade) and acetonitrile (Cat. No.: HP412, HPLC grade) were purchased from Spectrum Chemical Mfg. (Gardena, CA, USA). Silica Gel 60 TLC plate (Cat. No.: 5721-7) was purchased from EM Science (Gibbstown, NJ, USA).

2.2. Plasma sample

Twenty EDTA-anticoagulated plasma samples were obtained from 10 fasting women diagnosed with ovarian cancer and 10 fasting healthy women. The plasma samples were obtained by centrifuging whole blood drawn at $1600 \times g$ at 4 °C for 10 min. Then 100 μM (after mixing with plasma samples) 2,6-di-tert-butyl-4-methylphenol (BHT) was added to prevent oxidation. The samples were frozen and stored at −80 °C until used. Informed consents were obtained from all participants.

2.3. Extraction of LPA without using TLC

LPA in plasma was extracted using Bligh–Dyer method [28] under highly acidic condition (except for extraction recovery study, in which both neutral and acidic pH adjustment were employed) in the following procedure: first 200 pmol heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA was mixed with 500 μl plasma. One hundred microliters of 6 M HCl was then added to acidify the plasma, followed by addition of 2 ml 2:1 (v/v) methanol–chloroform. The mixture was vortexed and kept in ice for 10 min. One milliliter each of chloroform and water were then added. After vortexing, the mixture was centrifuged at $3000 \times g$ at 10 °C for 10 min. The bottom organic layer was transferred into another tube containing 100 μl 0.5 M ammonium acetate–ammonium hydroxide in methanol, pH 9.0 and dried under nitrogen. One milliliter chloroform was added to the remaining aqueous phase again for second extraction. The mixture was vortexed and centrifuged at $3000 \times g$ at 10 °C for 10 min. The bottom organic layer was transferred into the tube containing 100 μl 0.5 M ammonium acetate–ammonium hydroxide in methanol, pH 9.0 again and dried under nitrogen. The dried pellet was dissolved in 200 μl methanol and centrifuged at $6400 \times g$ for 5 min. The supernatant was injected and analyzed by LC/ESI/MS/MS.

Since a plasma matrix absence of LPA was not available, an artificial matrix made by dissolving 4% BSA in DPBS buffer was employed to set up the calibration curves. The calibrations curves (see Table 3) of this extraction method were established using varying amount of LPA and a fixed amount of [$^{13}C_{16}$] 16:0 LPA dissolved in 4% BSA in DPBS buffer, which were extracted following the procedure described above. The precision of the assay was assessed using a pooled plasma, which was utilized as a quality control for the assay. The Intra-day CV% of this extraction method were obtained using eight quality control samples in the same day while inter-day CV% of this extraction method were obtained using 24 quality control samples in 3 days (eight quality control samples for each day).

The extraction recovery of LPA at neutral pH and acidic pH was determined using the following procedure: 50 pmol of 22:6 LPA, 100 pmol 20:4 LPA, 50 pmol 18:0 LPA, 50 pmol 18:1 LPA, 400 pmol 18:2 LPA, and 100 pmol 16:0 LPA were added to 500 μl human plasma to make a spiked sample (pre-spiked sample). Then the pHs of a spiked human plasma samples and two unspiked human plasma samples (blank sample) were adjusted by adding either 100 μl pH 7.4 PBS buffer (containing 100 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) or 100 μl 6 M HCl. The samples were extracted following the extraction protocol described above. After extraction, the same amount of LPA, detailed above, was added into the extracted lipid pellet of an unspiked plasma sample to make a post-spiked sample. The lipid pellets of a pre-spiked sample, a post-spiked sample, and a blank sample were dissolved in methanol and injected into the LC/ESI/MS/MS instrument. The recovery was calculated using an equation listed below:

$$R(\%) = \frac{A_{pre} - A_b}{A_{post} - A_b} \times 100$$

where R represents the extraction recovery, A_{pre} represents the peak area of analyte in pre-spiked sample, A_{post} represents the peak area of analyte in post-spiked sample, and A_b represents the peak area of analyte in a blank sample.

2.4. Extraction of LPA using TLC

LPA in plasma was first extracted using the Bligh–Dyer method mentioned in Section 2.3, except that the organic layer obtained was not neutralized by 100 μl 0.5 M ammonium acetate–ammonium hydroxide in methanol, pH 9.0. The organic layer was dried under nitrogen and stored in a −20 °C freezer overnight. The following day, LPA in the extracted lipid was purified with TLC using the procedure described below: the dried lipids were first reconstituted in 50 μl 2:1 (v/v) methanol–chloroform and loaded onto Silica Gel 60 TLC plates. A standard LPA mixture was also loaded on the plate, serving as a marker for scraping. The TLC plate was put into a solvent tank containing 65 ml chloroform, 35 ml methanol, and 5.5 ml ammonium hydroxide. The lipids were separated in this solvent tank for 90 min at room temperature. After the TLC separation, the TLC plate was removed from the tank and dried at room temperature for 3 min. The standard LPA marker lane of the TLC plate was sprayed with 0.1% 8-anilino-1-naphthalene-sulfonic acid to trace the movement of LPA. Then LPA species of each plasma sample on the plate was scraped into its own extraction tube according to the standard LPA marker lane. Two milliliters of 2:1 (v/v) methanol–chloroform was added into the recovered TLC powder containing LPA, which was vortexed and kept in ice for 1 h. The mixtures were vortexed and centrifuged at 3000 × g at 10 °C for 10 min. The top organic phase was transferred into another tube and dried under nitrogen. Then 1.5 ml 2:1 (v/v) methanol–chloroform was added into the TLC powder again for second extraction. The mixture was vortexed and centrifuged at 3000 × g at 10 °C for 10 min. The top organic layer was transferred into the same tube containing the organic phase and dried under nitrogen. The calibrations curves (see Table 3) of this TLC extraction method were established using varying amount of LPA and a fixed amount of [$^{13}C_{16}$] 16:0 LPA dissolved in 4% BSA in DPBS buffer, which were extracted following the procedure described above. The intra-day CV% of this extraction method were obtained using eight quality control samples in the same day while inter-day CV% of this extraction method

Table 1
Normalized peak areas of LPA species obtained using different mobile phase A

Mobile phase A	Normalized peak area[a]					
	22:6 LPA	20:4 LPA	18:0 LPA	18:1 LPA	18:2 LPA	16:0 LPA
Water	1.00 (9603)[b]	1.00 (11,430)	1.00 (12,232)	1.00 (17,754)	1.00 (6664)	1.00 (16,496)
50 μM ammonium acetate	1.29	1.20	1.00	1.15	1.19	1.18
100 μM ammonium acetate	1.28	1.22	1.01	1.16	1.21	1.19
200 μM ammonium acetate	1.11	1.16	0.80	0.98	1.06	1.02
400 μM ammonium acetate	0.93	1.00	0.69	0.83	0.92	0.82
1000 μM ammonium acetate	0.67	0.68	0.53	0.56	0.62	0.56
50 μM ammonium formate	1.14	1.06	0.65	0.93	1.06	1.05
100 μM ammonium formate	1.17	1.15	0.71	0.99	1.15	1.10
200 μM ammonium formate	1.05	1.04	0.64	0.85	1.03	0.96
400 μM ammonium formate	0.82	0.83	0.49	0.66	0.80	0.74
1000 μM ammonium formate	0.51	0.49	0.25	0.39	0.48	0.44
300 μM ammonium phosphate, pH 5.4	1.86	1.74	2.21	1.67	1.75	1.73

[a] Nomalized peak areas are calculated via dividing the peak area of one analyte obtained in one mobile phase A by the peak area of that analyte obtained in water. The data showed in this table are calculated by averaging the numbers obtained from three LC/ESI/MS/MS runs.
[b] The data in the parentheses are the peak areas obtained using water as the mobile phase A.

were obtained using 24 quality control samples in 3 days (eight quality control samples for each day).

2.5. LC/ESI/MS/MS analysis of LPA

LC/ESI/MS/MS analysis of LPA species was performed using a Quattro Micro mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) probe and interfaced with a Shimadzu SCL-10Avp HPLC system (Shimadzu, Tokyo, Japan). Lipids were separated with a Betabasic-18 column (20 mm × 2.1 mm, 5 μm, Thermo Electron, Waltham, MA), protected by a Betabasic 18 pre-column (10 mm × 2.1 mm, 5 μm, Thermo Electron, Waltham, MA). Several different solvents were used for mobile phase A, depending on the experiment (see Table 1 for details), while 9:1 (v/v) methanol–acetonitrile was used as mobile phase B. The gradient used was as follows: the column was first equilibrated with 70% B (30% A) at 200 μl/min, followed by a linear change from 70% B (30% A) to 100% B (0% A) at 200 μl/min in the first 2 min. The gradient was kept at 100% B (0% A) at 200 μl/min in the following 1.4 min. The flow rate was then increased to 500 μl/min in the subsequent 1.1 min. In the following 2.5 min, the gradient was changed back to 70% B (30% A) at 200 μl/min to re-equilibrate the column. Mass spectrometric analyses were performed online using electrospray ionization tandem mass spectrometry in the negative multiple reaction monitoring (MRM) mode. The MS parameters are: capillary voltage, 3.0 kV; cone voltage, 35 V; source temperature, 100 °C; desolvation temperature, 350 °C; flow rate of desolvation gas, 500 l/h; flow rate of cone gas, 50 l/h; mass resolution of both parent and daughter ions, 15.0; multiplier, 650. The MRM transitions used to detect LPA were the mass to charge ratio (*m/z*) for the molecular anion M^- and their daughter ion *m/z* 153 (collision energy 22 eV, see Fig. 1 for the details) or 79 (collision energy 65 eV, see Fig. 2 for the details).

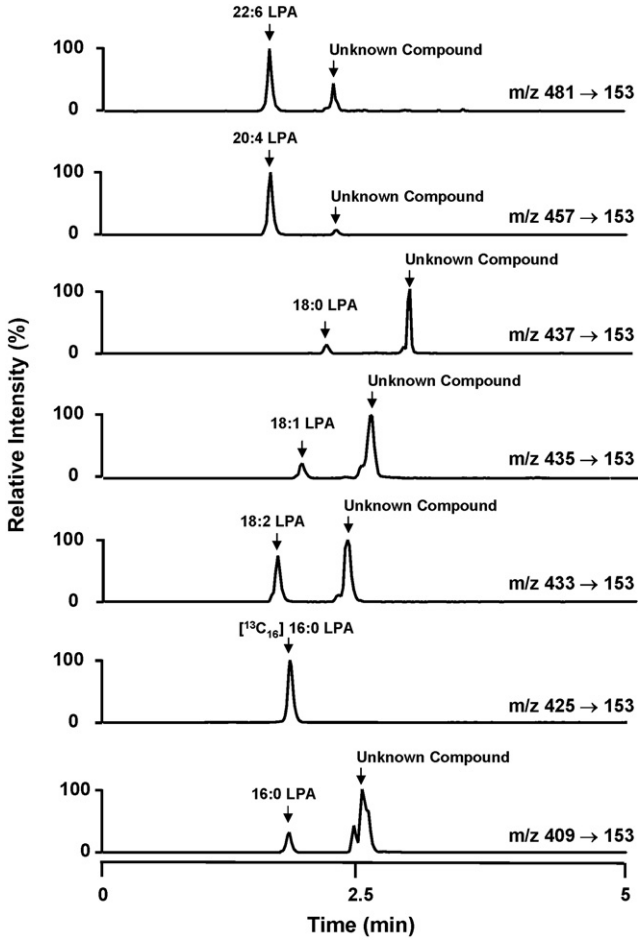

Fig. 1. MRM chromatograms of LPA extracted from human plasma samples. The MRM transitions used to detect LPA were the mass to charge ratio (*m/z*) for the molecular anion M^- and their daughter ion *m/z* 153. The experimental condition has been described in Section 2.5.

3. Results and discussion

3.1. Studies of the effect of mobile phases on LPA detection

In order to get the best peak shape and highest sensitivity, several different solvents were used for mobile phase A (see Section 2.5 and Table 1 for detailed information) to optimize the HPLC conditions for quantitative determination of LPA, while 9:1 (v/v) methanol–acetonitrile was used as mobile phase B. Shown in Table 1 are the normalized peak areas obtained by injecting 1.5 pmol of each LPA species (including 22:6, 20:4, 18:0, 18:1, 18:2, and 16:0 LPA). The HPLC conditions have been described in Section 2.5. If water is used as mobile phase A, the chromatograms of LPA (chromatograms not shown) are acceptable, with the peak areas reaching the range of 6600–18,000, depending on the LPA species. Use of low concentration ammonium acetate or ammonium formate solutions increases the sensitivity of the assay. For example, using 100 μM ammonium acetate can increase the peak areas of most of the LPA species by about 20% over water. The highest sensitivity is obtained by using 300 μM ammonium phosphate buffer (pH 5.4). It has been suggested that ammonium phosphate is not a suitable buffer species for LC/ESI/MS/MS because it is not quite volatile and will contaminate the MS source. MS source contamination does happen in this assay, however, because 300 μM is not a very high concentration and Z-spray is employed in our assay, the MS source does not require to be cleaned frequently. In our observation, if the instrument is used 24 h a day, the source only requires cleaning once after 2 day's use. The utilization of ammonium phosphate greatly increases the sensitivity and improves the peak shape of LPA. Compared to water, use of 300 μM ammonium phosphate increases the peak areas of LPA 67–121%, depending on the LPA species. Also, use of ammonium phosphate produces better peak shape for LPA. Thus, in the following studies, we use 300 μM ammonium phosphate buffer, pH 5.4 solution as the mobile phase A.

3.2. Extraction recovery, detection limits, calibration curves and CV%

Listed in Table 2 is the extraction recovery obtained using Bligh–Dyer extraction method mentioned in Section 2.3 at neutral and acidic pH conditions. The extraction recovery was obtained by averaging the results from three extractions for both

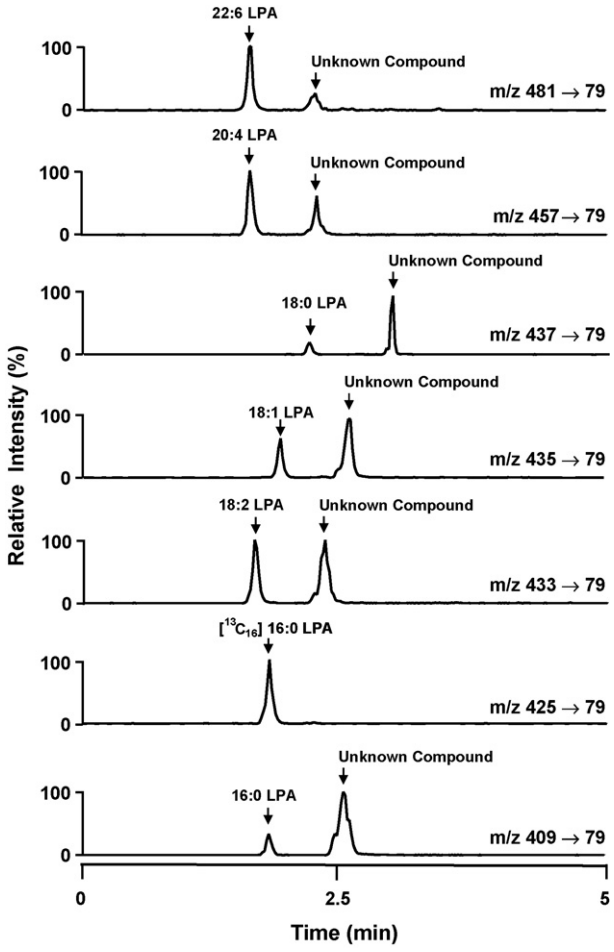

Fig. 2. MRM chromatograms of LPA extracted from human plasma samples. The MRM transitions used to detect LPA were the mass to charge ratio (*m/z*) for the molecular anion M^- and their daughter ion *m/z* 79. The experimental condition has been described in Section 2.5.

the neutral and acidic pH conditions. At the physiological pH, 7.4, the extraction recovery for LPA is very low, below 5% for each LPA species. However, if 6 M HCl is used in the extraction, the recovery is greatly increased, reaching 88–93%. Therefore, in order to get a high extraction recovery using Bligh–Dyer method, strong acid is used to adjust the pH of the sample to highly acidic.

Table 2
Extraction recovery of LPA using Bligh–Dyer method at neutral and acidic pH conditions

LPA species	Extraction recovery* ± S.D. (%)	
	PBS buffer, pH 7.4	6 M HCl
22:6 LPA	3.1 ± 1.7	92.1 ± 2.7
20:4 LPA	2.6 ± 2.2	91.9 ± 3.5
18:0 LPA	3.6 ± 1.2	88.1 ± 4.1
18:1 LPA	3.3 ± 2.1	90.3 ± 2.8
18:2 LPA	4.6 ± 2.2	92.5 ± 3.3
16:0 LPA	3.8 ± 1.7	88.4 ± 2.5

* Extraction recovery was calculated by averaging the numbers of three extractions. See Section 2.3 for experimental detail.

The matrix effect was also evaluated by comparing the signal intensity produced by the same amount of LPA spiked into an extracted plasma sample and spiked into methanol. Due to the presence of the matrix effect, the signal intensity of LPA was greatly depressed. The signal intensity of 22:6, 20:4, 18:0, 18:1, 18:2, and 16:0 LPA in the presence of the plasma matrix only reaches 38%, 40%, 17%, 12%, 38%, and 17% of the signal intensity obtained using the neat sample. However, because a heavy isotope labeled LPA internal standard is used in the present application, the precision and accuracy of the assay will not be affected significantly.

Listed in Table 3 are limit of detection, limit of quantitation of the LC/ESI/MS/MS method, calibrations curves of the assays using or without using TLC, as well as their corresponding intra-day and inter-day CV%. This LC/ESI/MS/MS method is very sensitive and can detect LPA species at fmol level. The calibration curves for the assays using TLC and without using TLC were established using varying amount of LPA and a fixed amount of [$^{13}C_{16}$] 16:0 LPA dissolved in 4% BSA in DPBS buffer, which were extracted following the procedures described in Sections 2.3 and 2.4. Theoretically, the calibration curves of the assays using and without using TLC should be close. This equality is only true for 16:0 LPA and 18:0 LPA, not for the other four LPA species (18:1, 18:2, 20:4, and 22:6 LPA). The inequity may be due to the structure of ^{13}C 16:0 LPA is closer to the structures of 16:0 LPA and 18:0 LPA than the structures of the other four LPA species and it imitates the behavior of 16:0 and 18:0 LPA better than the behavior of the other four LPA species, making it a better internal standard for 16:0 LPA and 18:0 LPA. It is supposed that CV% of the assay without using TLC should be better than CV% of the assay using TLC because more processing steps are used in the assay using TLC, which introduces more error. This is proven by the data listed in Table 3. Even though TLC is used, the CV% of most of the LPA species is still acceptable, within or close to 10%, except for 22:6 LPA, whose intra-day and inter-day CV% reaches 14.5 % and 17.9% respectively. This can be explained by the fact that the structure of 22:6 LPA is not as close to the structure of the internal standard as the other LPA species; additionally, it is a minor component in plasma, making the relative quantification error larger.

3.3. Quantification of LPA in plasma without using TLC

Shown in Fig. 1 are the chromatograms of six LPA species extracted from plasma using Bligh–Dyer method detailed in Section 2.3. The LPA species were detected using the MRM transition of the mass to charge ratio (*m/z*) for the molecular anion M^- and their daughter ion *m/z* 153. The calibration curves and CV% of the assays have been described in the last section (see Table 3). Because the extracted lipids were not purified with TLC, second peaks with the same parent-to-daughter ion transitions as LPA species produced by unknown compounds also appeared in the chromatograms. In flow injection LC/ESI/MS/MS, these unknown compounds will reach the mass spectrometer at the same time as LPA and will interfere the quantification of LPA if the extracted lipids containing LPA from plasma are not purified by TLC. Therefore, if flow injection

Table 3
Limit of detection, limit of quantification, calibration equations, and CV% of LPA obtained by the LC/ESI/MS/MS method

Name	Limit of detection[a] (fmol)	Limit of quantitation[b] (fmol)	Assay without using TLC			Assay using TLC		
			Calibration equation	CV%[c]		Calibration equation	CV%	
				Intra-day	Inter-day		Intra-day	Inter-day
16:0 LPA	50	160	$y^{d} = 1.010x^{e}$ ($R^2 = 0.9991$)	5.7	6.9	$y = 0.952x$ ($R^2 = 0.9986$)	7.9	8.4
18:2 LPA	200	650	$y = 0.379x$ ($R^2 = 0.9979$)	5.1	5.2	$y = 0.257x$ ($R^2 = 0.9994$)	6.9	10.9
18:1 LPA	80	250	$y = 0.999x$ ($R^2 = 0.9980$)	5.1	5.5	$y = 0.772x$ ($R^2 = 0.9976$)	7.4	7.9
18:0 LPA	100	350	$y = 0.543x$ ($R^2 = 0.9910$)	7.1	7.4	$y = 0.582x$ ($R^2 = 0.9982$)	8.9	12.4
20:4 LPA	150	500	$y = 0.546x$ ($R^2 = 0.9980$)	4.8	5.2	$y = 0.350x$ ($R^2 = 0.9978$)	6.7	9.3
22:6 LPA	200	650	$y = 0.455x$ ($R^2 = 0.9974$)	9.0	9.3	$y = 0.263x$ ($R^2 = 0.9912$)	14.5	17.9

[a] Limit of detection is set at the signal to noise ratio equaling to 3.
[b] Limit of quantitation is set at the signal to noise ratio equaling to 10.
[c] The precision was assessed using a pooled plasma sample with the concentrations of LPA listed in the following: 22:6 LPA, 0.049 μM; 20:4 LPA, 0.179 μM; 18:0 LPA, 0.059 μM; 18:1 LPA, 0.078 μM; 18:2 LPA, 0.649 μM; and 16:0 LPA, 0.142 μM.
[d] y is the peak area ratio of analytes and [$^{13}C_{16}$] 16:0 LPA.
[e] x is the mole ratio of analytes and [$^{13}C_{16}$] 16:0 LPA.

LC/ESI/MS/MS is employed, TLC must be used to remove these unknown compounds in order to get an accurate quantification of LPA. In our LC/ESI/MS/MS assay, these unknown compounds are well separated with LPA by the C18 column, ensuring that an accurate quantification can be performed without using TLC.

Another experiment using the MRM transition of the mass to charge ratio (m/z) for the molecular anion M^- and their daughter ion m/z 79 was also done, with the chromatograms shown in Fig. 2. The extraction procedure and LC/ESI/MS/MS method have been described in Section 2. Compared to the previous MRM method, the sensitivity of this MRM method is much lower and the limit of detection is fourfold higher. However, even though the MRM transitions are changed, the peaks produced from the unknown compounds are still present. Therefore, if a flow injection LC/ESI/MS/MS method is used, these unknown compounds can interfere with the quantification of LPA analyzed by this MRM method, meaning that the LPA levels measured by Yoon et al. [24] may not have been the true levels of LPA in plasma. Potentially, the LPA levels they measured were the levels of a combination of native LPA and unknown compounds. This can explain why the LPA levels they measured were much higher than Baker et al. [22] and Sutphen et al. [26]

Using this method, the LPA levels in plasma samples of 5 ovarian cancer patients and 5 normal women were quantified, with the result listed in Table 4. In our result, 18:2 LPA is the major LPA species present in human plasma, accounting for about 50% of the total LPA in plasma, consistent with the result obtained by Baker et al. [22]. Although the levels of each LPA species vary from sample to sample, in our result the concentrations of LPA in human plasma generally follow the order 18:2 > 16:0, 20:4 > 18:1, 22:6, and 18:0. The total levels of LPA determined here are higher than the result obtained by Baker et al. [22], but much lower than the result obtained by Yoon et al. [24].

LPA induces cell proliferation, migration and survival, so its actions are concordant with many of the hallmarks of cancer [25]. LPA was first reported to serve as a plasma biomarker for diagnosing ovarian cancer by Xu et al. [17] and later was confirmed by Yoon et al. [24] and Sutphen et al. [26]. However, negative result was also reported by Baker et al. [27]. Our results listed in Table 4 do not discriminate the LPA levels of ovarian cancer patients from healthy women. However, we cannot make a final conclusion that LPA is not a plasma biomarker for diagnosing ovarian cancer because our samples have been stored

Table 4
Concentrations of LPA measured in the plasma samples of 5 ovarian cancer patients and 5 healthy women by the LPA assay without using TLC

Sample	Concentration (μM)						
	22:6 LPA	20:4 LPA	18:0 LPA	18:1 LPA	18:2 LPA	16:0 LPA	Total LPA
Cancer 1	0.0620	0.140	0.0295	0.0603	0.490	0.218	1.000
Cancer 2	0.0663	0.132	0.0218	0.0755	0.646	0.220	1.161
Cancer 3	0.0769	0.166	0.0328	0.0672	0.585	0.178	1.106
Cancer 4	0.118	0.246	0.0469	0.0820	0.824	0.372	1.689
Cancer 5	0.140	0.196	0.0393	0.0518	0.358	0.245	1.030
Healthy 1	0.0661	0.178	0.0251	0.0770	0.553	0.203	1.102
Healthy 2	0.0663	0.152	0.0213	0.0707	0.441	0.199	0.951
Healthy 3	0.0839	0.217	0.0142	0.0524	0.456	0.208	1.031
Healthy 4	0.101	0.278	0.0284	0.129	1.163	0.327	2.026
Healthy 5	0.104	0.119	0.0342	0.0836	1.166	0.214	1.722

Table 5
Concentrations of LPA measured in the plasma samples of 5 ovarian cancer patients and 5 healthy women by the LPA assay using TLC

Sample	Concentration (μM)						
	22:6 LPA	20:4 LPA	18:0 LPA	18:1 LPA	18:2 LPA	16:0 LPA	Total LPA
Cancer 1	0.0449	0.160	0.0746	0.101	0.483	0.197	1.060
Cancer 2	0.0951	0.128	0.100	0.0755	0.490	0.202	1.090
Cancer 3	0.116	0.221	0.122	0.121	0.565	0.271	1.416
Cancer 4	0.0462	0.0788	0.0750	0.0601	0.427	0.160	0.847
Cancer 5	0.0291	0.0498	0.0463	0.0396	0.228	0.149	0.542
Healthy 1	0.124	0.166	0.0906	0.0736	0.531	0.192	1.177
Healthy 2	0.168	0.351	0.102	0.136	1.380	0.270	2.407
Healthy 3	0.0621	0.124	0.0443	0.0560	0.506	0.124	0.917
Healthy 4	0.0449	0.143	0.105	0.0791	0.534	0.270	1.175
Healthy 5	0.0515	0.120	0.138	0.0799	0.405	0.242	1.036

frozen for over 3 years. The samples may have already deteriorated and the levels of LPA measured may not reflect the original levels of LPA in these samples. More studies are required using fresh human plasma samples.

3.4. Quantification of LPA in plasma using TLC

Due to the fact that these results obtained above do not discriminate ovarian cancer patients and healthy women, we examined the extraction procedure reported by Xu et al. [23] to see if using TLC will improve our result. In this experiment, LPA was extracted from the plasma samples using Bligh–Dyer method in highly acidic condition and then purified by TLC (see Section 2.4 for experimental details). The levels of LPA in the plasma samples of 5 ovarian cancer patients and 5 healthy women were quantified by our LC/ESI/MS/MS method. We obtained very clean and symmetric peaks for each LPA species (chromatograms are not shown) because the unwanted compounds present in human plasma have been removed with TLC. The calibration curves of LPA for this assay are shown in Section 3.2 (see Table 3). The LPA levels of the 5 ovarian cancer patients and 5 healthy women are listed in Table 5. Again, 18:2 LPA is the major LPA species measured by this method. The order of the concentrations of LPA species is consistent with that obtained without using TLC. However, our result still does not discriminate ovarian cancer patients and healthy women probably due to the same reason discussed in Section 3.3.

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