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Quantitative determination of cyclic phosphatidic acid in human serum by LC/ESI/MS/MS

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Abstract

An LC/ESI/MS/MS method for cyclic phosphatidic acid (cPA) quantification in serum is established in the present report. The limit of quantitation of the assay reaches low nanomolar level in human serum and the CV% are within 10%. Using this method, we successfully quantify the levels of two cPA species, 16:0 and 18:1, in human serum. We find that the concentrations of 16:0 cPA in the serum of normal subjects and post-surgery ovarian cancer patients are significantly higher than its corresponding concentration in pre-surgery ovarian cancer patients, supporting the observation that cPA has anti-cancer activity. Another discovery is that the addition of strong acids (such as hydrochloric acid) in human serum may lead to the production of artificial cPA. Therefore, strong acids should be avoided in the extraction of cPA present in a complex matrix. Based on this observation, a new lipid extraction method was developed and used to extract cPA. The extraction recovery is close to 80%, guaranteeing an accurate quantification of cPA by LC/ESI/MS/MS can be performed.

Keywords: Cyclic phosphatidic acid (cPA); cPA quantification; LC/ESI/MS/MS

1. Introduction

Cyclic phosphatidic acid (cPA) is a class of phospholipids with a unique structure in which a fatty acid moiety is located at the $sn-1$ position and a cyclic phosphate ring is connected to the $sn-2$ and $sn-3$ positions of the glycerol backbone (see Fig. 1A for the structure). Although its structure is close to the structure of lysophosphatidic acid (LPA, see Fig. 1C for the structure), its biological functions are quite different from those of LPA. LPA shows many of the functions of the hallmarkers of cancer [1], such as stimulating cell proliferation [2,3], cell migration [4], tumor cell invasion [5,6], and cell survival [7,8]. In contrast, cPA has been found to show some anti-cancer activities, such as, antimitogenic regulation of the cell cycle [9], inhibition of cancer cell invasion and metastasis [10]. In addition, it also plays some roles in regulation of actin stress fiber formation and rearrangement [11], regulation of differentiation and viability of neuronal cells [12], eliciting neurotrophin-like actions in embryonic hippocampal neurons [18], stimulating respiration without producing vasopressor or tachycardiac in rats [19], and mobilization of intracellular calcium [9].

cPA was first isolated from *Physarum polycephalum* by Murakami-Murofushi et al. [20]. Later it was found that this kind of lipids is present in human brain and serum [14]. Its biological origin and metabolic pathways remain to be elucidated. However, Friedman et al. [21] discovered that lysophospholipids can be converted to cPA by phospholipase D. Recently, Tsuda et al. [22] reported that cPA can also be produced by autotoxin in blood.

Brain is the richest source of cPA. However, cPA has also been found in mammalian serum with a concentration of 10^{-7} M. Due to its high anti-invasive activity, it may serve as an anti-cancer drug [9]. Moreover, it could also serve as a potential biomarker for diagnosing cancer. Therefore, it is significant to develop an accurate analytical method to quantify its levels in biological samples. In this report, an LC/ESI/MS/MS method is developed and used to quantify the levels of two cPA species (18:1 cPA and 16:0 cPA) in human serum. The limit of quantitation in human serum reaches low nanomolar level and the CV% are within 10%. Using this method, we successfully quantified

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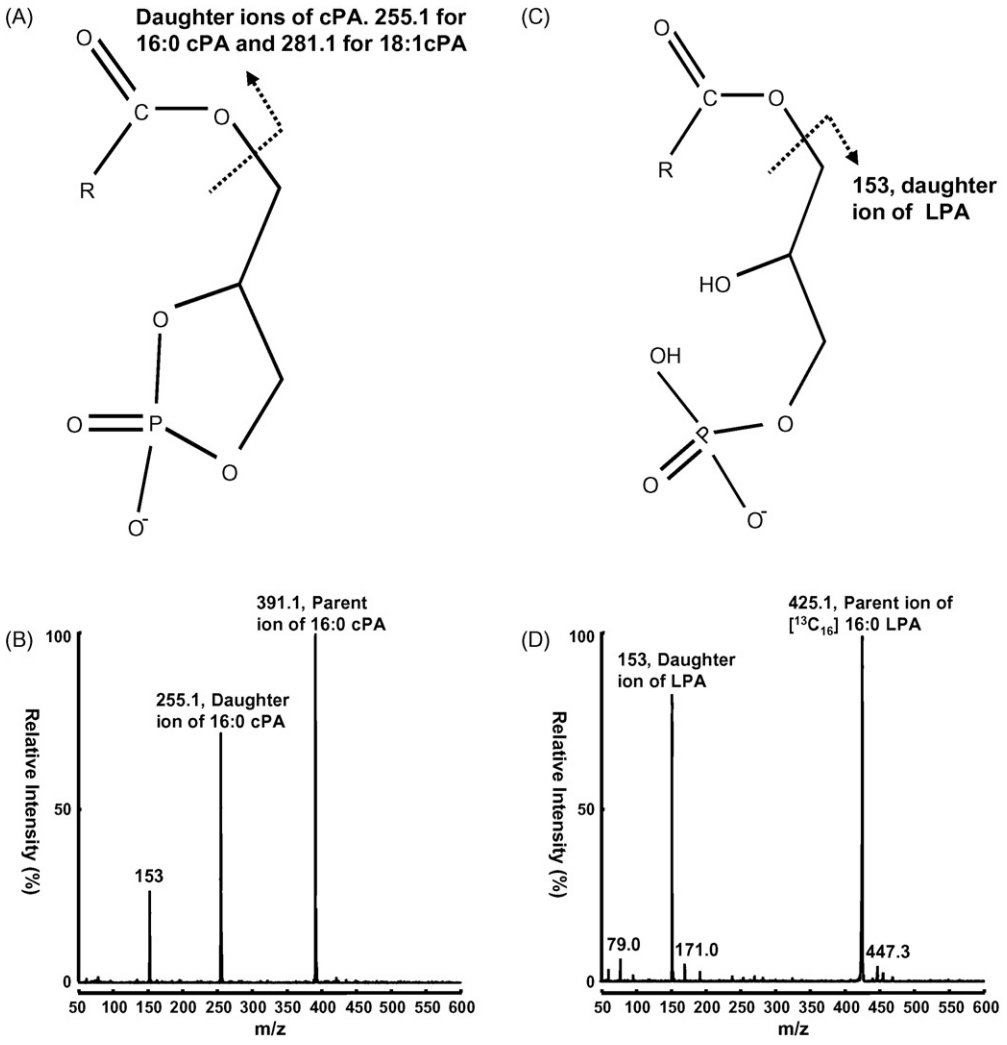

Fig. 1. Structures and fragmentation patterns of cPA (A) and LPA (C) as well as the full scan spectra of 16:0 cPA (B) and heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA (D). R is an alkyl group.

the levels of cPA in the serum of pre-surgery ovarian cancer patients, post-surgery ovarian cancer patients, as well as normal subjects and find that 16:0 cPA level in pre-surgery ovarian cancer serum is significantly lower than its level in either post-surgery ovarian cancer serum (p-value < 0.05) or normal subject serum (p-value < 0.003), supporting the observation that cPA has the anti-cancer activity in human body.

2. Materials and methods

2.1. Materials

16:0 cPA (cat. no.: 857323), 18:1 cPA (cat. no.: 857328), and heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA (cat. no.: 790661) were from Avanti Polar Lipids (Alabaster, AL, USA). 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), PBS buffer (phosphate-buffered saline, pH 7.4, cat. no.: P-3813), and BSA (bovine serum albumin, essentially fatty acid free, cat. no.: A7511) were from Sigma (St. Louis, MO, USA). Chloroform (cat. no.: C607-4, 99.9%) was from Fisher (Pittsburgh, PA, USA). DPBS buffer (Dulbecco's phosphate-buffered saline, w/o calcium & magnesium) was from Mediatech (Herndon, VA, USA). Methanol (cat. no.: HP702, HPLC grade) and acetonitrile (cat. no.: HP412, HPLC grade) were from Spectrum Chemical Mfg. (Gardena, CA, USA).

2.2. Serum sample

Human serum samples were obtained from 51 pre-surgery ovarian cancer patients, 52 post-surgery ovarian cancer patients, and 40 normal subjects in 2001. During sample collection, 100 μM (after mixing with serum samples) BHT (2,6-di-*tert*-butyl-4-methylphenol) 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 recovery study for Bligh–Dyer method

To get the extraction recovery of cPA at different pH conditions using Bligh–Dyer [13] method, 10 pmol 16:0 cPA and 18:1

cPA were added into a 4% 500 μl BSA aqueous solution, followed by the addition of 2 ml of 2:1 (v/v) methanol–chloroform. The pH of the mixture were adjusted using 100 μl PBS buffer (pH 7.4, containing 100 mM phosphate, 138 mM NaCl, and 2.7 mM KCl), 100 μl 1 M HCl, 100 μl 3 M HCl, 100 μl 6 M HCl, 100 μl 9 M HCl, and 100 μl 12 M HCl, respectively. The mixture was vortexed and kept at room temperature for 10 min. One millilitre of chloroform and 1 ml water were then added. After vortexed, the mixture was centrifuged at $3000 \times g$ at 10 °C for 10 min. Three layers could be seen in this mixture. The top layer was a mixture of water and methanol, the middle layer was a protein pellet, and the bottom layer was chloroform. 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. Another 1 ml chloroform was added into 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 0.1 M ammonium acetate in methanol containing 200 pmol heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA, transferred into a microcentrifuge tube, and centrifuged at 9000 rpm for 5 min. The supernatant was injected and analyzed by LC/ESI/MS/MS. The amount of recovered cPA was calculated using a calibration curve (this calibration curve is different from the calibration curve for serum samples) which is obtained by directly injecting a series of standard solution (dissolved in 200 μl 0.1 M ammonium acetate in methanol) with varying amount cPA and a fixed amount (200 pmol) of heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA into the LC/ESI/MS/MS system. The recovery was calculated by dividing the amount of recovered cPA by the original amount of cPA (10 pmol).

2.4. Extraction of cPA from human serum

cPA in serum was extracted using a modified Bligh–Dyer method, which follows the procedure described below: First mix 200 pmol heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA with 500 μl serum. The mixture was vortexed and 2 ml 2:1 (v/v) methanol–chloroform was added. The mixture was vortexed again and kept at room temperature for 10 min. Then it was centrifuged at $3000 \times g$ at 10 °C for 10 min. Two layers could be seen in this mixture. The top layer is a mixture of water, methanol, and chloroform while the bottom layer is the protein pellet. The top liquid layer was transferred into another tube and dried under nitrogen. The dried pellet was dissolved in 200 μl 0.1 M ammonium acetate in methanol, transferred into a microcentrifuge tube, and centrifuged at 9000 rpm for 5 min. The supernatant was injected and analyzed by LC/ESI/MS/MS.

The recovery of cPA extraction using this method was obtained following the procedure described below: First, 10 pmol 16:0 cPA and 18:1 cPA were added into a 4% 500 μl BSA in DPBS buffer and extracted using the method described above. The dried pellet was then dissolved in 200 μl 0.1 M ammonium acetate in methanol containing 200 pmol heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA, transferred into a microcentrifuge tube, and centrifuged at 9000 rpm for 5 min. The supernatant was injected and analyzed by LC/ESI/MS/MS. The amount of recovered cPA was calculated using a calibration curve (this calibration curve is different from the calibration curve for serum samples) which is obtained by directly injecting a series of standard solution (dissolved in 200 μl 0.1 M ammonium acetate in methanol) with varying amount cPA and a fixed amount (200 pmol) of heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA into the LC/ESI/MS/MS system. The recovery was calculated by dividing the amount of recovered cPA by the original amount of cPA (10 pmol).

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

LC/ESI/MS/MS analysis of cPA 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). Three hundred micromolars of ammonium phosphate, pH 5.4 aqueous solution was used as mobile phase A and 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 4 min. The gradient was kept at 100% B (0% A) at 200 μl/min in the following 5 min. In the following 3 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 as follows: 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 16:0 cPA and 18:1 cPA were the mass to charge ratio (m/z) for their molecular anion M^- (391 and 417, respectively) and their daughter ion (255 and 281, respectively, collision energy 22 eV, see Figs. 3 and 4 for the details). The fragmentation patterns of cPA and LPA are shown in Fig. 1A and 1C, respectively. The full scan spectra of 16:0 cPA and heavy isotope-labeled [$^{13}C_{16}$] 16:0 LPA are shown in Fig. 1B and 1D, respectively.

2.6. Data analysis

To identify group differences, data analysis were performed using Student's t-test. All data were considered statistically significant at the 95% confidence level ($p < 0.05$).

3. Results and discussion

3.1. Hydrochloric acid converts some unknown compounds present in serum to form cPA

Due to its simplicity in use and high extraction recovery for lipids, Bligh–Dyer method is widely utilized in lipid extraction [13]. However, since a cyclic phosphate group is present in cPA, the pH of the serum has to be adjusted to acidic in order to get a satisfactory extraction recovery for cPA if Bligh–Dyer extraction method is used. Thus, an experiment was done to test how much hydrochloric acid should be added in order to get the highest extraction recovery.

First, 10 pmol of 16:0 cPA and 18:1 cPA were dissolved in 4% BSA aqueous solution. After the addition of 2 ml mixture of methanol and chloroform (2:1, v/v), the pH of this cPA solution was adjusted by using 100 μl PBS buffer (pH 7.4), 100 μl 1 M HCl, 100 μl 3 M HCl, 100 μl 6 M HCl, 100 μl 9 M HCl, and 100 μl 12 M HCl, respectively (see Section 2.3 and Table 1). The cPA species were extracted using the Bligh–Dyer method described in Section 2.3 and quantified using the LC/ESI/MS/MS method described in Section 2.5. The data for each pH condition were obtained by three sets of experiments and expressed as extraction recovery ± standard deviation. Listed in Table 1 is the extraction recovery of the two cPA species obtained at the six different pH conditions described above. At neutral pH (PBS buffer is used to adjust the pH), the extraction recovery for cPA is below 50%. However, the addition of HCl greatly increases the extraction recovery, demonstrating that the adjustment of pH is necessary for cPA extraction. Generally, after the pH reaches highly acidic, the extraction recovery cannot be greatly improved with the addition of more HCl. In our experiment, use of 100 μl 6 M HCl hydrochloric acid seems to give the highest extraction recovery. After this, too much hydrochloric acid cannot improve the extraction recovery. Actually the extraction recovery decreases if 100 μl 9 M or 12 M HCl was used.

To test if the addition of 100 μl 6 M HCl could get the highest extraction recovery for cPA species in serum samples, the following experiment was then preformed: First, the pH of 500 μl serum was adjusted using 100 μl PBS buffer, pH 7.4, 100 μl 1 M HCl, 100 μl 3 M HCl, 100 μl 6 M HCl, 100 μl 9 M HCl, and 100 μl 12 M HCl, respectively. Then 2 ml mixture of methanol and chloroform (2:1, v/v) was added into the mixture. The cPA species were extracted using the Bligh–Dyer method described in Section 2.3 and quantified using the LC/ESI/MS/MS method described in Section 2.5, with the result shown in Fig. 2. Obviously, with the increased amount of HCl added, cPA levels greatly increase. For example, cPA levels extracted using 12 M HCl is about 2.6-fold (for 18:1 cPA) to 3-fold (for 18:0 cPA) of cPA levels extracted using 6 M HCl. However, in Table 1, it shows that the extraction recovery using 6 M HCl is higher than 12 M HCl. Such a huge increase of cPA cannot be explained just by the improvement of extraction recovery by adding more HCl. (A pH meter is used to measure the pH after 100 μl 6 M HCl or 100 μl 12 M HCl is added into 500 μl serum and the mixtures are vortexed. Both of their pHs are below 0.5.) We postulate that the addition of HCl possibly leads to the production of artificial cPA in human serum. Therefore, in the following experiments the extraction of cPA was performed using a modified Bligh–Dyer method which was described in Section 2.4.

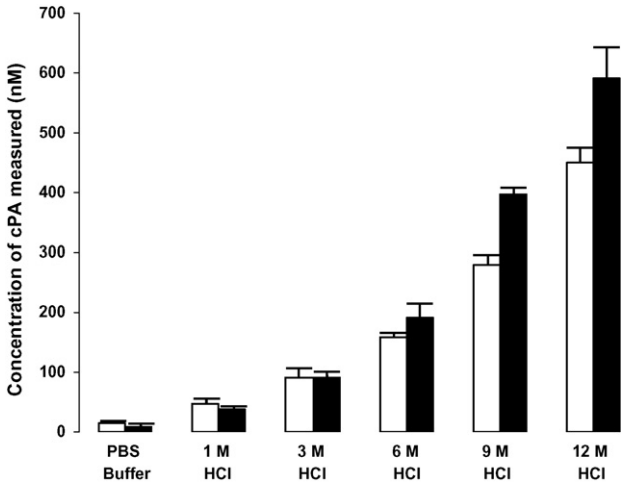

Fig. 2. Concentrations of two cPA species, 18:1 (represented by white bar) and 16:0 (represented by black bar), in human serum extracted in six different pH conditions and measured by the LC/ESI/MS/MS method described in Section 2.5. cPA were extracted using the Bligh–Dyer method described in Section 2.3 under six different pH conditions (see Sections 2.3 and 3.1 and Table 1 for experimental details). The data for each pH condition were obtained by three sets of experiments. The concentrations of cPA were expressed as average (bars) ± standard deviation (the vertical lines at the top of the bars).

Table 1
Extraction recovery[a] of 16:0 cPA and 18:1 cPA obtained using Bligh–Dyer method at six different pH conditions

Reagent used to adjust pH	Extraction recovery (%)	
	16:0 cPA	18:1 cPA
PBS buffer, pH 7.4	40.3 ± 3.1	34.5 ± 3.3
1 M HCl	83.3 ± 4.7	77.3 ± 2.9
3 M HCl	87.6 ± 2.1	82.3 ± 3.2
6 M HCl	93.3 ± 1.2	87.4 ± 2.7
9 M HCl	87.5 ± 2.8	86.2 ± 1.7
12 M HCl	82.6 ± 1.6	80.1 ± 4.3

[a] See Section 2.3 for experimental detail.

3.2. Extraction recovery, limit of detection, limit of quantitation, calibration curves, and CV% of the LC/ESI/MS/MS method for cPA quantification

Due to the possibility that artificial cPA can be produced in human serum by the addition of HCl to interfere cPA quantification, a modified Bligh–Dyer method is used to extract cPA from serum (see Section 2.4) and the levels of cPA are quantified by the LC/ESI/MS/MS described in Section 2.5. Listed in Table 2 are the extraction recovery, limit of detection, limit of quantitation, calibration equation, as well as intra-day and inter-day CV% of cPA. Although the pH is not adjusted to acidic, the extraction recovery of cPA using this modified Bligh–Dyer method are still very high, reaching 77% for 16:0 cPA and 84% for 18:1 cPA. Compared to conventional Bligh–Dyer method using strong

Table 2
cPA extraction recovery of the modified Bligh–Dyer method and the limit of detection, limit of quantitation, calibration equations, and CV% obtained using the LC/ESI/MS/MS method

	16:0 cPA	18:1 cPA
Extraction recovery[a] ± standard deviation	77.1 ± 1.7%	84.2 ± 3.6%
Limit of detection[b] (nM)	0.7	1.8
Limit of quantitation[c] (nM)	2.5	6.0
Calibration equation	y[d] = 1.612x[e] + 0.0092 (R^2 = 0.9972)	y = 0.586x + 0.0138 (R^2 = 0.9965)
Relative standard errors in slope	11.3%	14.1%
CV%	Intra-day: 7.3%; inter-day: 9.3%	Intra-day: 7.7%; inter-day: 9.8%

[a] Extraction recovery was obtained using the data obtained with three sets of experiments and expressed as average ± standard deviation.
[b] Limit of detection is set at the signal to noise ratio equaling to 3.
[c] Limit of quantitation is set at the signal to noise ratio equaling to 10.
[d] y is peak area ratio of analytes and ^{13}C 16:0 LPA.
[e] x is mole ratio of analytes and ^{13}C 16:0 LPA.

acid, this extraction method is much simpler and less labor-intensive. However, the extraction recovery is comparable to the extraction recovery obtained with conventional Bligh–Dyer method using strong acid. The most important aspect is it avoids using strong acid in the extraction, which possibly leads to form artificial cPA, making the accurate quantification of cPA in serum using LC/ESI/MS/MS method possible. One disadvantage of this extraction method is that a lot salts are present in the extracted lipids and may contaminate the source of the electrospray mass spectrometer. However, this problem can be resolved by directing the first 2 min eluent to the waste using a switching valve. After the salts are eluted out, the eluent is then directed to the mass spectrometer for cPA quantification.

Shown in Fig. 3 are the chromatograms of blank and the chromatograms with spikes of cPA at limit of quantitation (Fig. 3a and 3b are the chromatograms of blank run for 18:1 cPA and 16:0 cPA, respectively, while Fig. 3c and 3d are the chromatograms with spikes at limit of quantitation for 18:1 cPA and 16:0 cPA, respectively). The limit of quantitation of this LC/ESI/MS/MS method reaches low nanomolar level (2.5 nM for 16:0 cPA and 6.0 nM for 18:1 cPA, see Table 2), ensuring that this method is sensitive enough to quantify cPA in serum. The calibration curves for the assays were established using varying amount of cPA and a fixed amount of [$^{13}C_{16}$] 16:0 LPA dissolved in 4% BSA in DPBS buffer, which were extracted out following the procedure described in Section 2.4. The calibration equations and relative standard errors in slopes are listed in Table 2. The intra-day CV% was obtained by running eight same serum samples in the same day, while the inter-day CV% was obtained by running 24 same serum samples in 3 days. Although cPA levels in serum is very low, the CV% obtained by this LC/ESI/MS/MS method is still within 10% (see Table 2), guaranteeing the quantification of cPA is precise enough.

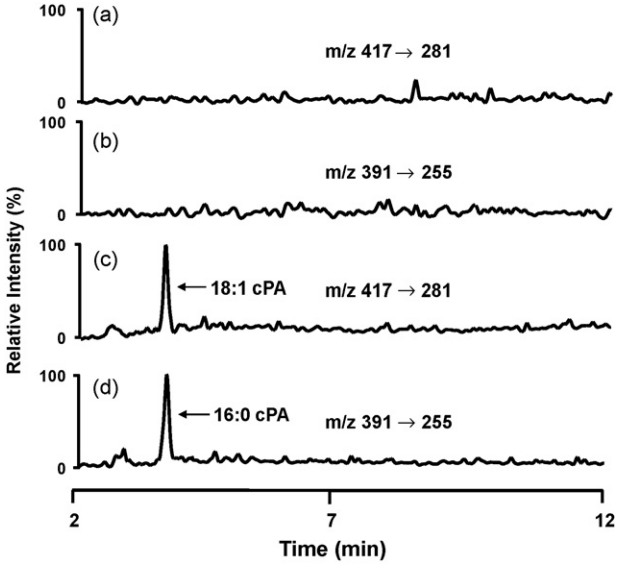

Fig. 3. The chromatograms of blank ((a) for 18:1 cPA and (b) for 16:0 cPA) and chromatograms with spikes of cPA close to the limit of quantitation ((c) for 18:1 cPA and (d) for 16:0 cPA). The MRM transitions and experimental conditions have been described in Section 2.5.

3.3. Quantitative determination of cPA in serum samples

Using the extraction method described in Section 2.4 and LC/ESI/MS/MS method described in Section 2.5, two cPA species, 16:0 and 18:1, were successfully detected in human serum. Shown in Fig. 4 are the chromatograms of 16:0 cPA, 18:1 cPA of a human serum sample and their internal standard, [$^{13}C_{16}$] 16:0 LPA. Under the HPLC condition used, their retention times are very close. However, due to its unique feature

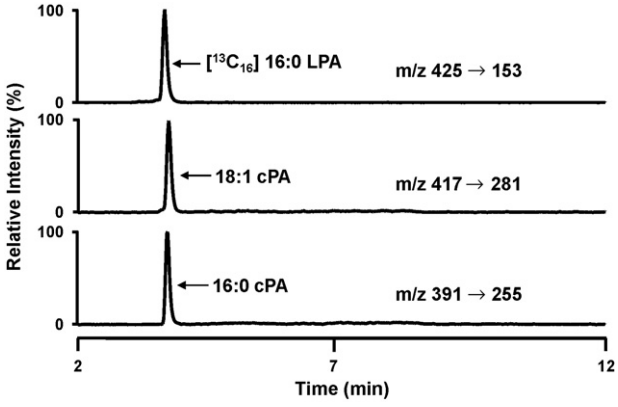

Fig. 4. MRM chromatograms of cPA and their internal standard [$^{13}C_{16}$] 16:0 LPA extracted from human serum sample. The MRM transitions and experimental conditions have been described in Section 2.5.

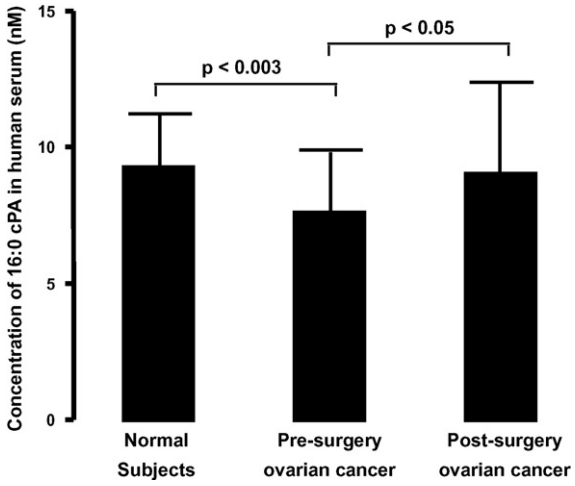

Fig. 5. Concentrations of 16:0 cPA in human serum measured by the LC/ESI/MS/MS method described in Section 2.5. Lipid was extracted using the modified Bligh–Dyer method described in Section 2.4. The concentrations of 16:0 cPA were expressed as average of each group (bars) ± their corresponding standard deviation (vertical lines at the top of the bars).

to use parent to daughter ion transitions to quantify analytes, LC/ESI/MS/MS quantification of cPA will not be interfered by this problem. This is one of the advantages of LC/ESI/MS/MS over HPLC. Although cPA concentration in human serum is at low nanomolar level, the chromatograms (Fig. 4) show that very symmetrical and clean sharp peaks could still be obtained, guaranteeing that the quantification would not be compromised by bad HPLC peaks or other unfavorable HPLC background.

Using the calibration curves shown in Table 2, the concentrations of 16:0 cPA and 18:1 cPA in the serum of 40 normal subjects, 51 pre-surgery ovarian cancer patients, and 52 post-surgery ovarian cancer patients were calculated, with the results shown in Figs. 5 and 6. The average concentration of 16:0 cPA in the serum of 40 normal subjects is 9.25 ± 1.92 nM, which is 22% higher than the average concentration of 16:0 cPA in the serum of 51 pre-surgery ovarian cancer, 7.58 ± 2.21 nM (see Fig. 5). The *p*-value of the two groups is less than 0.003, demonstrating that the difference of 16:0 cPA levels between these two groups reaches statistical significance. The average concentration of 16:0 cPA in the serum of 52 post-surgery ovarian cancer patients is 9.01 ± 3.17 nM, which is 19% higher than the average concentration of 16:0 cPA in the serum of pre-surgery ovarian cancer. The *p*-value between these two groups of patients is 0.04, still reaching statistical significance (see Fig. 5). To the contrary, there is no significant difference of 18:1 cPA levels in these three groups of people (see Fig. 6) and the average 18:1 cPA concentrations in the serum of these three groups are 16.1 nM (normal subjects), 17.2 nM (pre-surgery ovarian cancer patients), and 16.1 nM (post-surgery ovarian cancer patients), respectively.

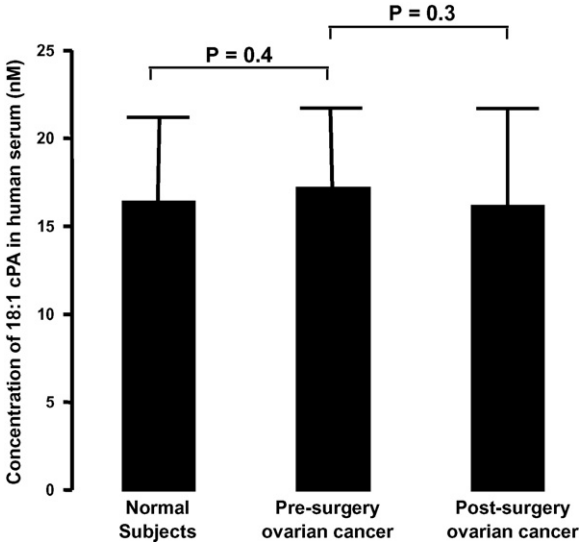

Fig. 6. Concentrations of 18:1 cPA in human serum measured by the LC/ESI/MS/MS method described in Section 2.5. Lipid was extracted using the modified Bligh–Dyer method described in Section 2.4. The concentrations of 18:1 cPA were expressed as average of each group (bars) ± their corresponding standard deviation (vertical lines at the top of the bars).

Although only two cPA species were detected and quantified in the present report, other cPA species such as 18:0, 18:2, 20:4, and 22:6 cPA species should also be present in human serum. However, we cannot quantify their levels because the pure standard of these compounds are not available.

cPA has been found to have anti-cancer activities, especially in inhibiting tumor invasion and metastasis [10,15,16]. It is hypothesized that its levels in cancer patients are lower than its levels in normal healthy people. This hypothesis is confirmed by the results obtained for 16:0 cPA species. However, there is no significant difference of 18:1 cPA concentrations in the three groups of people, which is possibly caused by the structural difference between 16:0 cPA and 18:1 cPA. This phenomenon has also been found in other lipid compounds, such as LPA, which has been reported that different species exhibits quite different biological activities [17]. Another interesting phenomenon is that the 16:0 cPA concentration in the serum of post-surgery patients is also significantly higher than its corresponding concentration in the serum of pre-surgery patients, showing that surgery can affect the serum cPA levels of ovarian cancer patients.

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