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Short communication

Determination of serum lysophosphatidic acid as a potential biomarker for ovarian cancer

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Abstract

A fast and selective analytical method, used to determine the different lysophosphatidic acid (LPA) species in serum, has been developed and validated. LPA species were quantitatively extracted from serum using methanol–chloroform (2:1, v/v). The proteins were precipitated by this solvent mixture and separated by centrifugation in one step. LPA levels were determined in clear extracts using the HPLC-MS/MS method. The linearity of this method was established in the concentration range between 0.1 and 16 μM for all LPA species with a correlation coefficient greater than 0.99. Recovery of all LPA species determined by the serum, fortified at approximately 1 μM and 2–3 μM, was between 93% and 111% with an average R.S.D. of less than 8%. This method was used to determine LPA in numerous sera of healthy controls, patients with benign ovarian tumours and ovarian cancer at different stages. Significantly higher total LPA levels were determined in the sera of patients with different types of tumours (benign and malignant).

Keywords: Lysophosphatidic acid; Ovarian cancer; Biomarker; Serum; High performance liquid chromatography; Tandem mass spectrometry

1. Introduction

Ovarian cancer leads to more mortalities than any other gynaecological cancer in the developed world. Its high mortality rate results from an inability to detect the cancer in its early curable stages. Most ovarian cancers are detected in the advanced stages, when metastases have already spread into the peritoneal cavity. It is therefore essential to develop a specific and sensitive method for early detection of ovarian cancer.

Lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate) is the simplest form of glycerophospholipid consisting of various species with both saturated (16:0, 18:0) and unsaturated (18:1, 18:2, 20:4) fatty acid tails. It is a normal constituent of the serum that is released by activated platelets during platelet aggregation [1,2].

LPA, originally identified as an intermediate in intracellular lipid metabolism, was later recognised as an important extracellular lipid mediator that signals through specific G-protein-coupled receptors [3–10]. It mediates a wide range of biological actions including stimulation of cell proliferation, survival, differentiation and motility [6–10]. Recently, it has been shown that ovarian cancer cells produce LPA and that LPA itself also acts as an ovarian cancer activating factor [7,11–12]. Increased levels of LPA were found not only in the ascites of ovarian cancer patients but also in the corresponding plasma samples [13–16].

Thus, many studies have attempted to develop a sensitive and specific method for the detection and quantification of LPA in different biological samples (ascites, plasma and

Abbreviations: ESI, electrospray ionisation; ESI-MS/MS, electrospray ionisation tandem mass spectrometry; HPLC, high performance liquid chromatography; GC, gas chromatography; LPA, lysophosphatidic acid; MRM, multiple reaction monitoring; R.S.D., relative standard deviation

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serum) [13–21]. The general approach for LPA determination involves using the modified Bligh and Dyer [22] method. Samples were acidified prior to LPA extraction. LPA was further separated from other interfering materials on a two-dimensional silica thin layer plate. Finally, individual LPA species were identified and quantified using negative electrospray ionisation tandem mass spectrometry (ESI-MS/MS) in different modes [14,15,17], capillary electrophoresis (CE) method using indirect ultraviolet (UV) detection [20] or methylated and determined using gas chromatography (GC) [11,13]. The sum of the LPA species represents the total LPA level. The major drawback of this method is the sample preparation time, since it takes several hours to prepare a sample. Consequently, several additional studies examining sample preparation used solid phase extraction [18] or multi-step solvent extraction [16,18,19,21]. However, most of these studies were not supported with the data of patients with ovarian cancer [17–21].

Especially the method by Yoon et al. is less time-consuming [16]. A two-step extraction was used in this method to separate LPA from the plasma and selective detection was by ESI-MS/MS in the multiple reaction monitoring (MRM) mode. This method was successfully performed on seven samples (four healthy controls and three patients with ovarian cancer). However, the problem for applying this method for routine analyses is connected with the two-step extraction, manipulation and separation of the lower chloroform extract.

Using increased levels of LPA in biological samples of patients with ovarian cancer compared to healthy individuals as an indicator/a biomarker of ovarian cancer is still disputable and raises doubts as to the utility of LPA as a potential biomarker for detection of ovarian cancer [13–16,23]. For this reason additional studies in this area are required.

The objective of the present research was to develop an analytical method using simple sample preparation and high performance liquid chromatography (HPLC) with ESI-MS/MS detection for determination of the LPA species. The method was used to determine and compare the LPA species in sera taken from healthy controls and patients with different types of ovarian tumours (benign and malignant).

2. Experimental

2.1. Materials and reagents

Acyl-lysophosphatidic acids (LPA $C_{16:0}$, LPA $C_{17:0}$, LPA $C_{18:0}$, LPA $C_{18:1}$, LPA $C_{20:4}$) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Analytical grade ammonium acetate and GC grade 1-butanol were purchased from Fluka (Buchs, Switzerland). Methanol and acetonitrile were HPLC grade and purchased from J.T. Baker (Deventer, Holland). Analytical grade chloroform and formic acid were purchased from Merck (Darmstadt, Germany). Water purified with the Milli-Q gradient system (Millipore, Bedford, MA, USA) was used in all procedures.

2.2. Serum samples

Blood samples from the patients and healthy controls were collected from volunteers (Department of Gynaecology and Obstetrics, Medical Centre of Ljubljana, Slovenia) by venipuncture into standard red-top tubes with no additions and left at room temperature for 30 min. Serum was separated from the blood samples by centrifugation at $1500 \times g$ for 15 min, then stored in siliconised microcentrifuge tubes (Sigma–Aldrich, St. Louis, MO, USA) at −27 °C until analysis.

2.3. Calibration solutions

Stock solutions of LPA $C_{16:0}$, LPA $C_{18:0}$, LPA $C_{18:1}$ and LPA $C_{20:4}$ were prepared in methanol at concentrations of 20 and 100 μM and stored in glass flasks at −20 °C. LPA $C_{17:0}$ was used as an internal standard. The internal standard stock solution was prepared at a concentration of 70 μM and stored in a glass flask at −20 °C. Calibration set sera fortified with LPA species was prepared in the concentration range between 0.1 and 16 μM on each day of the analysis. The fortified sera were analysed according to the procedure described in Section 2.4.

2.4. LPA extraction procedure

The frozen sera were thawed and 300 μL of each sample was transferred into a glass centrifuge tube. Fifty microliters of internal standard stock solution and 2.0 mL of methanol–chloroform (2:1, v/v) were added to the sample. The tube was mixed vigorously for 15 s on a vortex-mixer and incubated at 4 °C for 20 min. After incubation the samples were warmed to room temperature and centrifuged at $2600 \times g$ for 10 min. The clear supernatant was transferred into a new glass tube, evaporated to dryness under a stream of nitrogen and redissolved in 200 μL of methanol for further analysis.

2.5. Chromatographic and mass spectrometric conditions

The chromatographic analysis was performed on the Waters Model 2790 separation system (Milford, MA, USA) using Phenomenex (Torrance, CA, USA) reversed phase Synergi MAX-RP column C12 (30 mm × 2.0 mm i.d., 4 μm particles) equipped with a guard column (Phenomenex C_8; 4 mm × 3.0 mm i.d.). The mobile phase was composed of acetonitrile–50 mM ammonium acetate (adjusted to pH 2.5 with formic acid) (80:20, v/v) and delivered isocratically at a flow rate of 0.4 mL min^{-1}. Twenty microliters of serum extract at a temperature of 4 °C was injected into HPLC by an autosampler.

The detection was performed on the Micromass Quattro II triple quadrupole mass spectrometer (Manchester, UK), using electrospray ionisation (ESI) and controlled by the Masslynx 4.0 software. ESI was performed in the negative ionisation mode with nitrogen as a nebulising gas at 360 °C. The temperature of the ion source was kept at 120 °C. The mass spectrometer was operated at a cone voltage of 40 V and a capillary voltage of 4 kV in the MRM mode using two product ions for each of the LPA species. The spectrometer was programmed to allow the [M-

H]$^-$ ions of the LPA species (*m/z* 409 for LPA $C_{16:0}$, *m/z* 423 for LPA $C_{17:0}$, *m/z* 433 for LPA $C_{18:2}$, *m/z* 435 for LPA $C_{18:1}$, *m/z* 437 for LPA $C_{18:0}$ and *m/z* 457 for LPA $C_{20:4}$) to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 22 eV using argon as a collision gas at a pressure of 0.4 Pa. The product ions for all LPA species were at *m/z* 79 (phosphoryl, PO_3^-) and at *m/z* 153 (glycerol phosphoryl, $C_3H_6O_2PO_3^-$). Product ions were monitored through the third quadrupole (Q3). The dwell time and scan delay were 0.2 s.

3. Results and discussion

3.1. Optimisation of HPLC-ESI-MRM

Since the method developed here used selective MRM detection, HPLC separation of the individual LPA species was not necessary. Nevertheless, different reversed stationary phases (amino, ciano, C_{18}, C_{12} and C_8) were tested. The optimal chromatographic conditions concerning peak shape of all investigated LPA species were obtained on a Synergy MAX-RP column with the C_{12} stationary phase. Different mobile phases were also tested. A mobile phase at pH 2.5 was used to prevent the dissociation of LPA. Using isocratic elution by the selected mobile phase and the selected detection resulted in sharp chromatographic peaks for each of LPA species, that were easy to quantitate.

3.2. Optimization of LPA extraction

Solvents previously used for LPA extraction were examined including 1-butanol [21], chloroform/methanol/water [22] and chloroform/methanol/water using acidified samples [13–16]. Extraction with 1-butanol and chloroform/methanol/water resulted in low recovery of LPA. Two-step extraction of LPA from acidified samples resulted in a better recovery. However, manipulation of the lower phase of the chloroform extract was difficult to perform. Therefore, this procedure is less appropriate for routine analyses. The presented extraction was shortened in comparison with previously described procedures [13–16]. Using a methanol–chloroform mixture (2:1, v/v) without acidification, the proteins were precipitated and separated from solvents by centrifugation in one step. LPA species were quantitatively extracted from the sera with good recoveries (Table 1). Clear extracts were obtained that did not require further cleaning.

3.3. Quantitative analysis of LPA

3.3.1. Linearity of the method

The linearity of this method was tested by analysis of fortified sera at eight levels. A relative peak area of each LPA to that of the internal standard was presented according to each LPA concentration. Linear relationships of the relative peak area for all of the LPA species examined here were within the concentration range from 0.1 to 16 μM. The correlation coefficients (r^2) for all LPA were more than 0.99. The characteristics of the calibration curves and detection limits (LODs), established by analysis of the standard solutions of LPA species in methanol, are presented in Table 1. This method showed approximately five-fold lower sensitivity, but better specificity in comparison with the method of Yoon et al. [16] due to use of the LPA $C_{17:0}$ as an internal standard and two MRM transitions monitoring for each of LPA species. The calibration curves were prepared daily. The stability of the extraction solutions was also tested. We showed that extracts were stable for at least 24 h at 4 °C.

3.3.2. Precision and recovery of the method

A serum with a known initial concentration of LPA species was used to estimate the level of recovery. The initial concentration of LPA was determined by six repetitive analyses. A R.S.D. of less than 7% confirms that the method is precise (data not shown). This serum was fortified with the LPA species at two concentration levels (approximately 1 μM and 2–3 μM). The added concentrations of LPA species in the serum as well as the recoveries with corresponding R.S.D.s are shown in Table 1. The recoveries were between 93% and 111% with an average R.S.D. of less than 7% confirming that this method is accurate as well as precise.

Table 1
Recovery, precision, linearity and LOD data for different LPA species

LPA	Recovery and precision[a]				Calibration curves[b]			Limit of detection, LOD (μM)
	Concentration added (μM)	Concentration found (μM)	Recovery (%)	%R.S.D.	Slope *k*	Intercept *n*	Correlation coefficient r^2	
LPA $C_{16:0}$	1.38 (*n* = 3)	1.53	111	3.4				
	2.75 (*n* = 6)	2.77	101	4.7	0.171	0.016	0.9972	0.02
LPA $C_{18:1}$	1.29 (*n* = 3)	1.37	106	4.6				
	2.58 (*n* = 6)	2.57	100	3.1	0.259	−0.015	0.9901	0.01
LPA $C_{18:0}$	1.18 (*n* = 3)	1.30	110	1.3				
	2.37 (*n* = 6)	2.48	105	2.0	0.151	0.037	0.9958	0.02
LPA $C_{20:4}$	1.16 (*n* = 3)	1.27	109	7.3				
	2.33 (*n* = 6)	2.17	93	3.6	0.126	−0.025	0.9929	0.03

R.S.D.: Relative standard deviation.

[a] Calculated as the average concentration of the fortified serum minus the average concentration of the unfortified serum (initial concentration).

[b] $y = kC + n$: the average relative LPA peak area of the unfortified serum was subtracted from the average relative LPA peak area of the fortified serum.

3.3.3. Application of the method

The method described above was used to determine and compare the level of LPA in sera from two groups of women. The healthy control group consisted of 55 women of different ages in a reproductive and postmenopausal state (20–65 years old). The patient group consisted of 50 patients with different stages of ovarian malignant cancer (including early curable stages I and II, when metastases had not spread into the peritoneal cavity). The benign patient group consisted of 65 women. Fig. 1 shows the representative MRM chromatogram of LPA $C_{16:0}$ species in the serum of the control group compared to the serum of a patient with ovarian cancer. Chromatograms for the other LPA species are similar. Thus, only the data for LPA $C_{16:0}$ is presented.

The concentration of LPA in different sera was determined using the calibration curves prepared by analysis of the fortified sera. Since the LPA $C_{18:2}$ standard was not commercially available, the calibration curve of the LPA $C_{18:1}$ was used to calculate the level of LPA $C_{18:2}$ in samples with the assumption that they have the same ionisation properties. The total level of LPA was calculated as the sum of the individual LPA species.

Significantly lower total LPA levels were found in the sera of the healthy controls in comparison with the sera of the ovarian cancer patients (Figs. 1 and 2). The mean level of LPA in the sera of healthy controls was 2.9 μM. The cut off value for presence of ovarian tumours was set to 3.9 μM. In 92% of patients with ovarian cancer and in 72% of patients with benign ovarian tumours, the LPA level was above 5 μM. The mean LPA level for the malignant group was 8.4 and 8.0 μM for the benign group. The stage of the disease did not correlate with the total LPA levels. The mean LPA level in the sera of the patients with benign tumours was not significantly different from the mean LPA level observed in patients with malignant tumours. On Fig. 2 there is an overlap of healthy controls with patients with benign ovarian tumours. Indeed, 12 women with benign ovarian tumours that needed no operation were misclassified as healthy. Such exceptions were observed in other comparable tests, too [24].

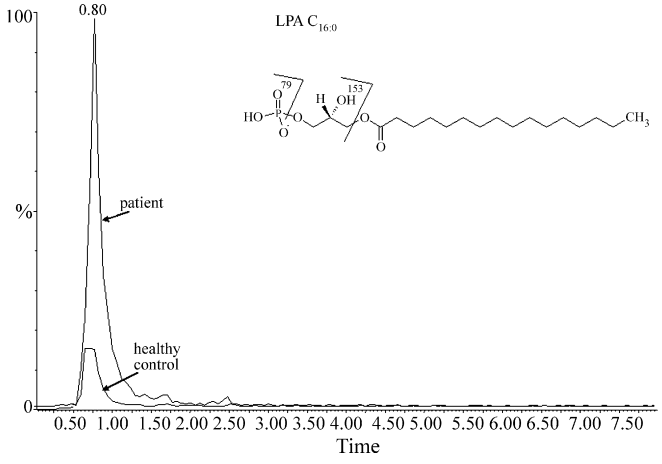

Fig. 1. Representative MRM chromatogram of LPA $C_{16:0}$ (MRM transitions: 409 > 153, 409 > 79) in the serum extract of a healthy control compared to a patient with ovarian cancer. The chemical structure and product ions of LPA $C_{16:0}$ are also presented.

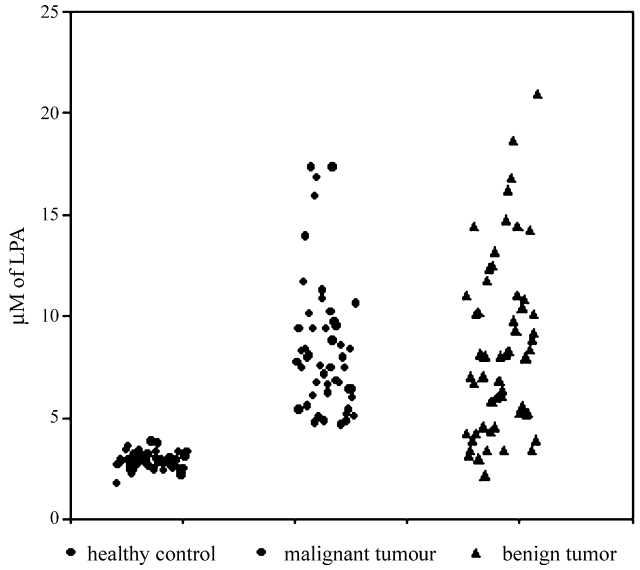

Fig. 2. Total LPA levels (in (M) in the sera of patients with ovarian tumours compared to the healthy controls.

4. Conclusion

A highly precise and accurate simple method has been developed to determine the levels of LPA in sera. Using a methanol–chloroform mixture, LPA species were extracted from the sera in one step. Furthermore, additional cleaning of the extract was not required. LPA was determined using ESI HPLC-MS/MS in the MRM mode. It enabled easier quantitation (considering peak area) in comparison with a direct injection of extracts and further MRM analysis. Due to the simplicity and shorter time of analysis, the method presented here could be applied in routine analyses.

This method was used to determine LPA levels in 170 different sera taken from healthy individuals and patients with ovarian tumours. We show that significantly lower total LPA levels were found in the sera of the healthy controls in comparison with the LPA levels in the sera of the ovarian cancer patients. No difference in LPA levels was observed between malignant and benign tumours. In conclusion, LPA levels in serum samples could be used as a potential biomarkers of ovarian cancer even in its very early stages, even though the marker cannot distinguish between malignant and benign tumours.

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