Determination of Lysophosphatidic Acids by Capillary Electrophoresis with Indirect Ultraviolet Detection

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Determination of lysophosphatidic acids by capillary electrophoresis with indirect ultraviolet detection

Yi-Lung Chen, Yan Xu

**Introduction**

Lysophosphatidic acid (LPA) is the simplest form of lysophospholipid (Lyso-PL) (Fig. 1). LPA belongs to the phospholipid (PL) family, and bears only one fatty acyl group. LPA is not only a membrane component and metabolic intermediate but also an intercellular messenger with a role in cell growth and motility [1]. LPAs have also been identified as the potent components in an ovarian cancer activation factor (OCAF) from ascitic fluid of ovarian cancer patients [2]. The OCAF that comprises various molecular species of LPA with different fatty acyl chains (i.e., palmitoyl, stearoyl, oleoyl, linoleoyl, arachidonoyl and docosahexarnoyl) stimulates calcium release, tyrosine phosphorylation and the proliferation of ovarian cancer cells. Recent studies [3,4] show that the elevated plasma LPAs may be used as potential biomarkers for the early detection of ovarian cancer.

Traditionally, gas chromatography (GC) and liquid chromatography (LC) have been used to measure PLs [5,6]. Despite their high analytical sensitivity, these methods often require laborious sample pretreatment, derivatization, or lengthy separation. Therefore, a simple and efficient analytical method is needed for PLs analysis.

Capillary electrophoresis (CE) is well known for its high separation efficiency, application versatility, and instrument simplicity. While the methodologies
sodium salt, LP A (S); 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate monosodium salt, LP A (O); and 1-decanoyl-2-hydroxy-sn-glycero-3-phosphate monosodium salt, LP A (D)); 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (synthetic, purity >99%), LPC (P); 1-palmitoyl-2-hydroxy-sn-glycero-phosphoethanolamine (synthetic, purity >99%), LPE (P); 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-serine] monosodium salt (synthetic, purity >99%), LPS (O) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). ADP (99%), and ATP (99%) were from Aldrich (Milwaukee, WI, USA). Human serum, HPLC grade methanol (>99.9%), acetonitrile (>99.9%) and 8-anilino-1-naphthalenesulfonic acid were obtained from Sigma (St. Louis, MO, USA). Boric acid (electrophoresis purity reagent) was from Bio-Rad Laboratories (Richmond, CA, USA). Chloroform, ammonium hydroxide, hydrochloric acid, and Whatman flexible-backed TLC plates (60-A silica gel medium without fluorescent indicator, Whatman no. 4410221) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

In this work, a CE method using indirect UV detection for the determination of molecular species of LP A has been developed. The optimum CE conditions were investigated and the internal calibration plots for LPAs were established. The method has been applied to the analysis of LPAs in serum. This paper is the first report on the quantitative analysis of LPAs by CE.

**Experiment**

**Chemicals and solutions**

Lysophosphatidic acids (synthetic, purity>99%) [1-myristoyl-2-hydroxy-sn-glycero-3-phosphate monosodium salt, LPA (M); 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate monosodium salt, LPA (P); 1-stearoyl-2-hydroxy-sn-glycero-3-phosphate mono-

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Fig. 1. The molecular structures of lysophospholipids.
solvent of 5% separation buffer in methanol–water (9:1 v/v).

**Instrumentation**

Open tubular fused-silica capillary (75-μm I.D., 354-μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ). The uncoated capillary was 47 cm in length (40 cm to the detection window). This capillary was mounted in a P/ACE cartridge that was connected to a temperature control system. A new capillary was conditioned sequentially with 0.1 M NaOH for 1 h, deionized water for 20 min, methanol for 20 min, and separation buffer for 30 min before use. As a daily routine procedure, the capillary was rinsed with 0.1 M NaOH for 5 min, deionized water for 2 min, and separation buffer for 15 min.

A Beckman (Fullerton, CA) P/ACE 2200 system and an IBM PC with System Gold software (Version 8.1) were used for CE separation. On-line indirect UV detection was performed at 254 nm, and the separation temperature was maintained at 25°C unless otherwise specified. Samples were introduced into the capillary by pressure injection at 3.45 kPa for 5 s. Separations were carried out under applied potential of 25 kV at the normal polarity (the cathode was placed at the outlet of the capillary) unless otherwise specified. Between runs, the capillary was rinsed with the separation buffer for 2 min.

A Hewlett-Packard (Wilmington, DE) HP 8453 UV–Visible spectrophotometer and a HP PC with ChemStation software were used for taking the UV spectra of the background electrolyte and LPA.

**Serum samples preparation**

Extraction of serum lipids was performed at 0–4°C to minimize damage to ester bonds. The procedure developed by Bligh and Dyer [12] was used with modifications: 1 ml serum spiked with 100 μM LPA (S), LPA (O), LPA (P), LPA (M) and LPA (D) was acidified by 0.2 ml of 12 M HCl, and mixed with 4 ml of methanol–chloroform (2:1 v/v) for 10 min. After adding 1 ml of chloroform and 1.25 ml of water, the mixture was centrifuged at 1000 g for 10 min. The methanol–chloroform phase (lower phase) containing lipids was pipetted, evaporated under N₂ until remaining about 50 μl, and loaded on a TLC plate. The spot containing LPAs was separated from other lipids by TLC using the solvent system of chloroform–methanol–ammonium hydroxide (65:35:5.5 v/v/v) [3]. To conform the position of LPA spot on the TLC plate for the first time, 0.1% of 8-anilino-1-naphthalenesulfonic acid in water was used as spray on the standard LPA and the lipid extraction after TLC separation. The LPA spot was visualized in a dark room under UV light. For subsequent separations, LPA spot was detected by water spray to prevent the contamination, scraped and extracted [12] from plate. The extract was evaporated under N₂ to dryness. The dry powder was reconstituted in 1 ml of 5% separation buffer in methanol–water (9:1 v/v), and the solution was subjected to CE analysis.

**Results and discussion**

**Indirect UV detection and UV-absorbing background electrolyte**

Direct UV spectrophotometry has been used for detection of PLs at the absorptive range of 200 to 210 nm [6]. However, due to the low UV absorptivity of carbonyl group of Lyso-PLs, direct UV detection of Lyso-PLs is not more sensitive than indirect UV detection. Particularly, when methanol and chloroform were used as solvents in this study, which exhibit absorptivities at around 200 nm. Hence, indirect UV detection was used for this study.

In indirect UV detection, a chromophore is necessarily included in background electrolyte (BGE) to generate high background absorption at the UV detector. The displacement of UV-absorbing additive by the non-absorbing analytes results in negative analytical peaks [13].

For this work, 5 mM AMP was chosen as the UV-absorbing additive for detection because of its high molar absorptivity, large ratio of background absorbance to background noise (dynamic reserve), and favorable transfer ratio (the ratio of the solute charge to the charge of the UV-absorbing additive) [14], as well as closely matched mobility with Lyso-PLs. As shown in Fig. 2, the BGE (a 250-fold dilution of separation buffer) gave two maximum absorption peaks at 206 and 259 nm, and the
Separation of lysophosphatidic acids

Effect of boric acid

Boric acid was used as the BGE in this work, which affects migration and detection of the LPAs. The effects of boric acid concentration on migration, resolution, and indirect signal response were shown in Fig. 3. Theoretically, an increase in BGE concentration lowers the $\zeta$ potential on fused-silica capillary wall. Therefore, it decreases the electro-osmotic flow (EOF) and lengthens the migration time of analytes, which may improve or worsen the separation resolution. In addition, the signal response in indirect detection is directly related to the concentration ratio of UV additive and BGE. In this study, at the fixed concentration of UV additive (5 mM AMP), as the concentration of boric acid increased from 10 to 50 mM, the migration time of LPA (M) increased ca. 1.3 min, and the resolution of LPAs worsened due to the Joule heating at higher currents. Further, the indirect signal responses for LPAs were slightly lower in 50 than in 10 mM boric acid.

Organic solvent

It is worth noticing that the use of 90% organic solvent in separation buffer is essential for the solubility and selectivity of PLs [11]. Methanol was used as the organic solvent in our experiments, because its solvation properties can reduce the hydrophobic interaction among the Lyso-PLs and its dielectric constant and viscosity can help to achieve desirable resolution [15]. The use of 10% acetonitrile and 80% methanol in the separation buffer was also tested for the separation of LPAs and the results were compared with those obtained from the buffer containing 90% methanol alone. Although the migration times for all LPAs were shorter (within 9 min) and the peaks were sharper than the separation performed in the buffer containing 90% methanol due to the lower viscosity of acetonitrile, the use of acetonitrile in the separation buffer resulted in poor resolutions (data not shown). Therefore, methanol–water (9:1 v/v) was used as the solvent for all subsequent separations.

![Fig. 2. The UV spectrum of BGE containing 20 $\mu$M AMP and 40 $\mu$M $\text{H}_3\text{BO}_3$ at pH 5.4 in the solvent, methanol–water (9:1 v/v), compared with the spectrum of 20 $\mu$M LPA (M) in the solvent. In these spectra, the solvent was used as the blank.](image)

![Fig. 3. The effect of boric acid on the separation of LPAs. The separation buffer was 5 mM AMP and 10–50 mM boric acid at pH 5.4 in methanol–water (9:1 v/v).](image)
LP As decreased as applied voltage increased. The last LP A in the migration sequence was LP A (M). Its migration time decreased from 14.5 to 9.4 min as the applied voltage increased from 20 to 30 kV. However, the resolution of LP As varied according to the applied voltage. The effect of applied voltage on the resolution of the two closely related LPA peaks, LPA (O) and LPA (P), was summarised in Table 1. The optimum resolution was obtained at 25 kV. Therefore, an applied voltage of 25 kV was used for all subsequent separations.

**Effect of temperature**

Temperature affects many variables including pH, injection volume, viscosity, EOF, sensitivity and resolution, current, ionic strength, and electrophoretic mobility. The effect of temperature on the separation of LP As was shown in Fig. 5. The influence of temperature on resolution and migration time was summarized in Table 1. The resolution of the LP As decreased as temperature increased, which is probably attributed to the higher diffusion rates at higher temperatures. The migration time of the LP As also decreased as temperature increased, which might be caused by the higher EOF at higher temperatures. A temperature of 25°C was chosen as for all subsequent studies.

**Effect of buffer pH**

Buffer pH is one of the most important parameters for the separation of ionic species. In this work, the pH influences not only the degrees of dissociation of boric acid, AMP and LP As, but also the EOF of the capillary. Hence, it affects the apparent mobilities of LP As, as well as the indirect signal response.

The effect of buffer pH on the separation of LP As decreased as applied voltage increased. The last LPA in the migration sequence was LPA (M). Its migration time decreased from 14.5 to 9.4 min as the applied voltage increased from 20 to 30 kV. However, the resolution of LP As varied according to the applied voltage. The effect of applied voltage on the resolution of the two closely related LPA peaks, LPA (O) and LPA (P), was summarised in Table 1. The optimum resolution was obtained at 25 kV. Therefore, an applied voltage of 25 kV was used for all subsequent separations.

Table 1

<table>
<thead>
<tr>
<th>Effect</th>
<th>Applied Voltage (kV)</th>
<th>Temperature (°C)</th>
<th>Buffer pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution of LPA (O) and LPA (P)</td>
<td>1.18 1.31 1.24</td>
<td>1.32 1.31 1.31 1.31</td>
<td>1.31 1.31 1.52 2.08</td>
</tr>
<tr>
<td>Migration time of LPA (M) (min)</td>
<td>14.5 12.2 9.4</td>
<td>13.5 12.9 12.2 11.0</td>
<td>12.2 13.8 19.2</td>
</tr>
</tbody>
</table>
was shown in Fig. 6. It is remarkable that the higher the pH, the slower were the migrations of LPAs. According to the manufacturer, the values of $pK_{a,1}$ and $pK_{a,2}$ for LPAs are 3–4 and 8–9, respectively. As the pH value increased from 5.4 to 7.0, the molecular charges of LPAs increased from 1 negative to partial 2 negative. Moreover, the EOF also increased with increasing pH, which was in the opposite direction of the electrophoretic forces of LPAs. Compared to the increased electrophoretic forces, the increased EOF was smaller in magnitude. Therefore, the apparent mobilities of LPAs were actually decreased under the higher pH values. The lower pH resulted in shorter migration time for the LPAs. However, if pH is below 5.4, it could cause problems such as current error and reduced solubility of AMP.

Although at higher pH values the signal responses became broader and shorter due to the longer diffusion at slower migration and the charge displacement of UV-absorbing additive and BGE by analytes [13], the resolution of LPAs increased with increasing pH and migration time. As shown in Table 1, the resolution of LPA (O) and LPA (P) increased from 1.31 to 2.08 with increasing pH from 5.4 to 7.0 and migration time from 12.2 to 19.2 min. A pH of 5.4 was used for all subsequent studies.

Separation of lysophosphatidic acids in presence of other lysophospholipids

The optimum conditions were applied to the separation of LPAs in the presence of other anionic Lyso-PLs (i.e., LPI, LPS, LPG). As shown in Fig. 7a, the migration order was LPI > LPS > LPG > LPA. The species with the least negative charge-to-mass ratio eluted first and the species with the most negative charge-to-mass ratio eluted last at the outlet of the capillary where the cathode was placed. It is worth noted that there were considerable capacities among the LPI, LPS, LPG peaks for the further separation of these Lyso-PLs with various length of fatty acyl groups. Separation of neutral Lyso-PLs, LPC and LPE, were attempted under the same conditions, but there were no separation. These neutral Lyso-PLs eluted together with the system peak (ca. 5 min) which had the same mobility as the EOF.
Table 3
The performance of capillary column

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative migration time</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± %C. V.</td>
<td></td>
</tr>
<tr>
<td>LPA (M)</td>
<td>0.892 ± 0.0049</td>
<td>0.55  115 000</td>
</tr>
<tr>
<td>LPA (P)</td>
<td>0.852 ± 0.0061</td>
<td>0.72  117 000</td>
</tr>
<tr>
<td>LPA (O)</td>
<td>0.837 ± 0.0065</td>
<td>0.78  116 000</td>
</tr>
<tr>
<td>LPA (S)</td>
<td>0.818 ± 0.0070</td>
<td>0.86  120 000</td>
</tr>
</tbody>
</table>

\(^a\) n = 18.
\(^b\) \(W_{1/2}\) was corrected by velocity of each species. \(W_{1/2} = \text{area} \times L_p/t/\text{peak height}\).

Quantitative analysis of lysophosphatidic acids

Quantitative analysis of LPAs was carried out with the method of internal calibration. Internal calibration usually results in better precision compared to direct calibration because neither the injection quantities nor the detector response is required to remain constant. Therefore, it can eliminate the errors caused by the variation of sample injection and UV lamp intensity, as well as high volatility of solvent (e.g., methanol and chloroform). In this work, LPA (D) was chosen as the internal standard (I.S.) because it is not an endogenous compound. Fig. 7b showed the separation of standard LPAs from I.S. The complete separation of LPA (S), LPA (O), LPA (P), LPA (M), and LPA (D) was accomplished in less than 14 min.

The merits of analytical performance of the method were summarized in Table 2. The calibration plot for each molecular species of LPA was linear from 2.8 to 75 \(\mu M\) determined by the mean of three measurements at each of the six concentrations (2.8, 4.5, 11, 29, 47 and 75 \(\mu M\)). The linear correlation coefficients were greater than 0.999. The detection limits of LPAs were ranged from 1.2 to 2.3 \(\mu M\). The repeatabilities of the area ratio (the peak area of LPA vs. the peak area of I.S.) calculated as %C.V. were less than 10%.

The performance of capillarity column was given in Table 3. The relative migration time is defined as the migration time of a LPA peak divided by the migration time of a reference peak. Using the I.S.
peak as a reference, the reproducibility of relative migration time for each LPA peak was very good (CV = 0.86). The separation efficiency calculated based on the equation, \( N = 5.54\left(\frac{L_d}{W_{1/2}}\right)^2 \) (where \( L_d = 40 \text{ cm} \), the length of the capillary from inlet to the detector, and \( W_{1/2} = \text{the peak width at half-height} \) was 115 000–120 000 at each LPA concentration of 11 \( \mu \text{M} \).

**Serum sample analysis**

Human serum fortified with LPA (S), LPA (O), LPA (P), LPA (M) and internal standard LPA (D) was prepared by liquid–liquid extraction and TLC according to the procedure described in the Section 2.3. The sample was then analyzed by the CE method. As shown in Fig. 8b, the separation of LPAs in serum was successfully achieved. Although the migration times of LPAs had shifted for ca. 1 min (due to the matrix effect) compared to those in Fig. 7b, the relative migration times remained the same. The relative recoveries [defined as the area ratio of the serum sample (the peak area of LPA vs. the peak area of I.S.) divided by that of the standard solution] for LPA (S), LPA (O), LPA (P), and LPA (M) were 83, 110, 112, and 111% respectively. As seen in Fig. 8b, there were trace amounts of other anionic Lyso-PLs present in the electropherogram because the TLC separation was not complete, and these anionic Lyso-PLs did not interfere with the LPA detection by the CE method. This method shows great potential for clinical application. The authors also believe that the procedure used for serum pretreatment was laborious and time-consuming. Therefore, effort is being taken for developing one-step solid-phase extraction procedure for blood samples.

**Conclusions**

This paper has reported a simple CE method for the quantitative analysis of molecular species of LPA. The separation of LPAs was achieved within 14 min in an adenosine monophosphate-borate–methanol–water solution, and the measurement was accomplished by indirect UV detection. The method was optimized in terms of UV-absorbing additive, organic modifier, background electrolyte, applied potential, temperature, and pH. With internal calibration, the method has linear calibration ranges for LPAs from 2.8 to 75 \( \mu \text{M} \) with correlation coefficients greater than 0.999. The detection limits for various molecular species of LPA were from 1.2 to 2.3 \( \mu \text{M} \) by the pressure injection at 3.45 kPa for 5 s. The method may be used in clinical diagnosis of ovarian cancer.

**Acknowledgements**

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**References**


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