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Methyl- β -cyclodextrin modified micellar electrokinetic capillary chromatography with laser-induced fluorescence for separation and detection of phospholipids

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Abstract

Micellar electrokinetic capillary chromatography (MECC) with laser-induced fluorescence detection was applied to the separation of amino group-containing phospholipids including phosphatidylethanolamine (PE), phosphatidylserine (PS), lysophosphatidylethanolamine (LysoPE), and lysophosphatidylserine (LysoPS). A fluorogenic dye, 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), was successfully used to fluorescently label these phospholipids. 4-Fluoro-7-nitrobenzofurazan only produced fluorescent product from LysoPE and PE; signals were not observed from LysoPS and PS. A borax buffer containing sodium deoxycholate modified with methyl- β -cyclodextrin (methyl- β -CD) was an excellent MECC system for these phospholipids. Under the optimum conditions, four FQ-labeled phospholipid classes were separated within 8 min. Moreover, each of the PE, PS, LysoPE and LysoPS peaks split into two components corresponding to subclasses with different lengths of the fatty acid chains, but these subclasses were completely resolved only for LysoPE. Detection limits ranged from 0.18 to 1.1 fg (10^{-9} – 10^{-10} M), which was four- to five-orders of magnitude superior to previously reported CE methods. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, electrophoresis; Phospholipids; Lipids; Cyclodextrins; Furoylquinolinecarboxaldehyde

1. Introduction

The chemical and physical properties of cell membranes are largely dependent on their phospholipid composition [1]. Two common membrane phospholipids contain amino-groups, phosphatidylethanolamine (PE) and phosphatidylserine (PS). These phospholipids are involved in many important membrane-linked events such as fusion, phase separation and flip-flop movements [2].

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are the

most frequently used methods for analysis of phospholipids. However, TLC suffers from poor reproducibility and is not easily automated. HPLC is superior to TLC since it provides better resolution and reproducibility. In addition, its sample preparation and clean-up procedures are also simpler and less time-consuming [3]. For HPLC analysis of phospholipids, UV absorbance detection is often employed at 190–220 nm. However, this absorption is non-specific, weak, and obscured by solvents and additives. In order to improve the sensitivity and selectivity, fluorescence has been developed for HPLC detection of phospholipids, with detection limits of 2 to 20 pmol [4,5].

Capillary electrophoresis (CE) is a powerful tool for analysis of biomolecules. To date, most applica-

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tions are focused on the determination of amino acids, peptides, proteins and DNA. There are few reports dealing with phospholipid analysis [6–9]. Micellar electrokinetic capillary chromatography (MECC) with UV detection has been used to analyze phospholipids in a lecithin sample at a concentration of 200 mg/l, but the detection limit was not reported [6]. CE with indirect photometric detection (IPD) has also been applied to the determination of phospholipids [7].

In this work, a MECC–laser-induced fluorescence (LIF) detection method was developed for the analysis of phospholipids that contain a primary amine; the amine was labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). Our method provides fast separation, good resolution, and high sensitivity. The detection limit was four- to five-orders of magnitude superior to CE–IPD or MECC–UV methods.

2. Experimental

2.1. Materials and reagents

Fused-silica capillaries of 40 cm×20 μm I.D.×136 μm O.D. (Polymicro, Phoenix, AZ, USA) were used for all experiments. Sample injection was performed at 100 V/cm for 5 s. All separations were carried out by applying a voltage of 400 V/cm across the capillary.

PE, lysophosphatidylethanolamine (LysoPE), PS, 4-fluoro-7-nitrobenzofurazan (NBD-F), sodium deoxycholate (SDC), Brij 35, β-cyclodextrin (β-CD) and methyl-β-CD were purchased from Sigma (St. Louis, MO, USA). Lysophosphatidylserine (LysoPS) was purchased from Fluka (Oakville, Canada). FQ was obtained from Molecular Probes (Eugene, OR, USA). Sodium dodecyl sulfate (SDS) was purchased from BDH (Toronto, Canada). Lipid stock solutions (~0.6 mg/ml) were prepared with methanol and chloroform in a ratio of 5:1 and diluted with methanol to the desired concentration. For storage, FQ was dissolved in methanol, aliquots were dispensed, and the solvent was then removed under vacuum. NBD-F solution was prepared with methanol at a concentration of 150 mM.

2.2. Apparatus

Experiments were performed using a laboratory-built CE instrument, equipped with a post-column sheath-flow cuvette LIF detector as described elsewhere [10–12]. High voltage (0–30 kV) was provided by a Spellman CZE 1000 power supply (Plainview, NY, USA). Fluorescence excitation was provided by an argon ion laser (Uniphase, San Jose, CA, USA) operating at 488 nm with a power of 12 mW. Fluorescence collected passed through a 630DF30 (for FQ derivatives) or a 535DF30 (for NBD-F derivatives) bandpass filter (Omega Optical, Brattleboro, VT, USA) to a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ, USA), which was biased at 900 V.

2.3. Derivatization of phospholipids

2.3.1. FQ labeling

Typically, a 2-μl aliquot for each lipid (60 μg/ml each) was added to a 500-μl vial containing 100 nmol dried FQ. Then 2 μl of 25 mM KCN was added. The reaction temperature varied from 25 to 65°C, and the reaction time varied from 2 to 55 min. Finally, 90 μl of the running buffer was added to stop the labeling reaction. The product was stable over at least 1 month if stored at 4°C.

2.3.2. NBD-F labeling

The protocol was similar to that for FQ labeling except that KCN was not used for NBD-F labeling. After 2-μl aliquots (both 6 μg/ml) for LysoPE and PE were mixed, 2 μl NBD-F solution was added. The final concentration of NBD-F was 30 mM. The mixture was then incubated at 55°C for 5 min. Finally, 94 μl of the running buffer was added to stop the reaction.

3. Results and discussion

As far as we know, there has been no report on the use of CE–LIF to analyze phospholipid classes. To perform LIF detection, the phospholipid must be labeled with a fluorescent dye. So the feasibility of labeling phospholipids by several fluorophores was first explored. Considering the compatibility with the

laser excitation line (488 nm) employed in this work, two fluorophores, FQ and NBD-F, were tested.

3.1. Labeling with FQ

FQ is a fluorogenic reagent. It should produce a much lower background signal compared to a fluorescent reagent such as FITC. FQ has been used for highly sensitive detection of proteins [12]. However, its use has not been reported for the labeling of phospholipids. Here we employed FQ as the pre-column labeling reagent for highly sensitive LIF detection of four phospholipid classes.

3.1.1. Optimization of reaction conditions

3.1.1.1. Reaction temperature

Generally, reaction temperature influences the derivatization efficiency [13]. In our experiments, reactions were conducted at 25, 35, 45, 55 and 65°C. As shown in Fig. 1, phospholipids reacted with FQ even at room temperature (25°C) although with low efficiency. The signal-to-noise ratio (S/N) maximizes near 55°C. A reaction temperature of 55°C was used for the following experiments.

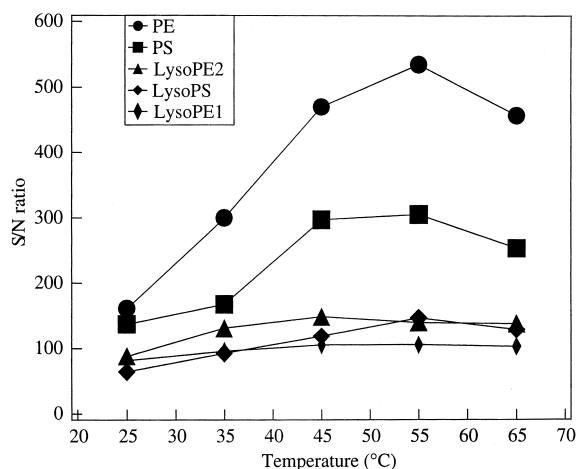


Fig. 1. S/N ratio of the phospholipids as a function of reaction temperature. Derivatization time: 15 min. CE conditions: background electrolyte, 10 mM borax with 35 mM SDC and 0.3% Brij 35, pH 9; injection, 100 V/cm for 5 s; separation voltage, 400 V/cm; lipid concentrations, 1.2 $\mu\text{g/ml}$ each.

3.1.1.2. Reaction time

Reaction time also affects the derivatization efficiency. Reactions were carried out for 2 to 55 min. Fig. 2 illustrates the S/N ratio for the phospholipids as a function of reaction time. For most of the phospholipids, derivatization efficiency was maximized by 15 min. A reaction time of 15 min was used for further experiments.

3.1.2. Separation conditions

Most phospholipids are sparingly soluble in water. It is difficult to separate these lipids in aqueous CE systems because they tend both to co-elute and to adsorb on the capillary wall. Hence, choosing a suitable buffer system is critical for separation of phospholipids. Two approaches are useful to solve this problem. One is to use a nonaqueous medium for lipid separation [9]. We demonstrate another approach that uses detergents to enhance the solubility of phospholipids. In our experiments, 10 mM borax containing 10 mM SDS buffer was first tried as the separation buffer. However, as shown in Fig. 3, the four FQ-labeled phospholipids co-eluted in the borax–SDS buffer. Further experiments also showed that SDS did not influence the resolution significantly for concentrations up to 50 mM. A nonionic surfactant, Brij 35, was also tried as a buffer additive, but the resolution was not improved.

The resolution was greatly improved when SDC was used as the additive to borate buffer instead of

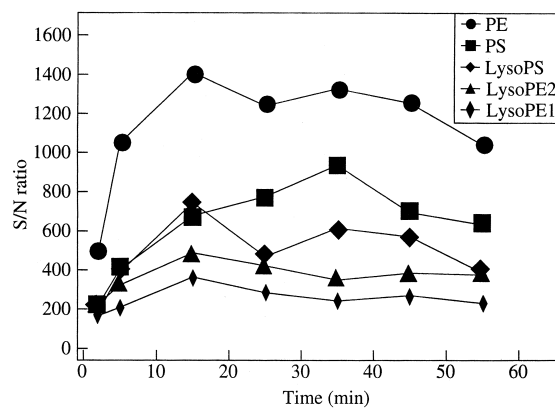


Fig. 2. S/N ratio of the phospholipids as a function of reaction time. Derivatization temperature: 55°C. CE conditions as in Fig. 1, lipid concentrations, 3 $\mu\text{g/ml}$ each.

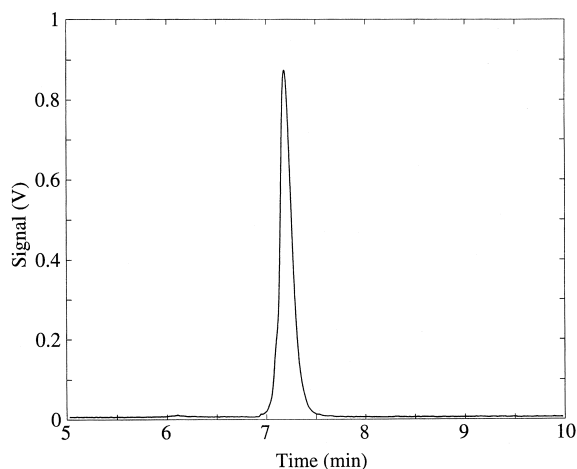


Fig. 3. Electropherogram of FQ labeled phospholipids obtained in 10 mM borax with 10 mM SDS. Injection, 100 V/cm for 5 s; separation voltage, 400 V/cm; lipid concentrations, 1.2 $\mu\text{g}/\text{ml}$ each.

SDS (Fig. 4). SDC is a bile salt surfactant with a critical micellar concentration of 6 mM [14]. SDC, sodium cholate, and sodium taurocholate are important surfactants for separation of highly hydrophobic analytes in MECC. As shown in Fig. 4, peaks of LysoPE split into two for 35 mM SDC concentration. This peak splitting could be due to the presence of heterogeneous components with different fatty acid chains that make up LysoPE; the manufacturer specifies that this lipid consists of two subclass-

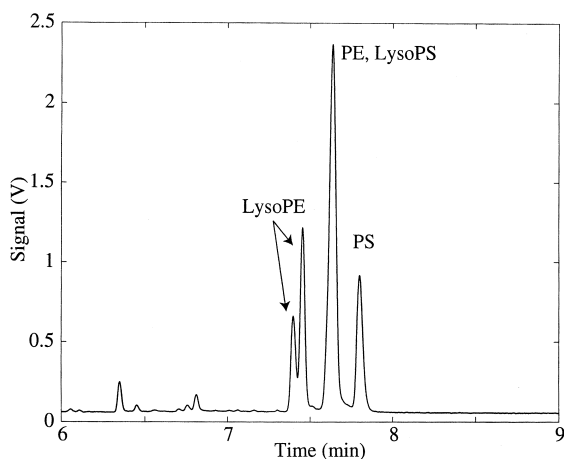


Fig. 4. Electropherogram of FQ labeled lipids obtained in 10 mM borax with 35 mM SDC. Other conditions as in Fig. 3.

ses with C_{16} and C_{18} fatty acid tails. On the other hand, a single peak was observed for LysoPS and PE when analyzed with this buffer. Further increasing the concentration of SDC did not improve the resolution.

In order to improve the resolution of LysoPS and PE, some other compounds were added to the running buffer. First, methanol was added to the 10 mM borax and 35 mM SDC buffer. The resolution for LysoPS and PE improved slightly as the concentration of methanol changed from 5% to 15% but at the expense of baseline instability and much longer separation times (data not shown). Cyclodextrin (CD) is an important additive in MECC system, which is often called CD-modified MECC [15,16]. Unfortunately, addition of 10 mM β -CD to the 10 mM borax and 35 mM SDC buffer did not improve the resolution. Further, as the concentration of β -CD was increased to 25 mM, peaks of all the lipids were distorted (data not shown). Brij 35 was also added to the above MECC system. Fig. 5 is the electropherogram obtained in borax buffer with SDC and 0.3% Brij 35 (g/ml). It can be seen that LysoPE and PS are partly separated without a loss in efficiency. However, the resolution did not improve as the concentration of Brij 35 was increased to 1% (data not shown).

As shown in Fig. 6, complete separation of

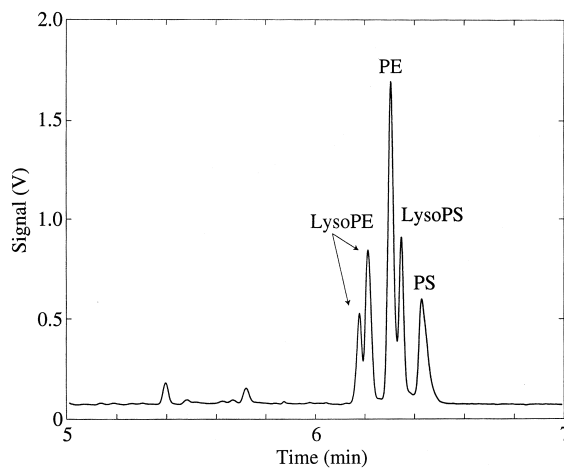


Fig. 5. Electropherogram of FQ labeled lipids obtained in 10 mM borax with 35 mM SDC and 0.3% Brij 35. Other conditions as in Fig. 3.

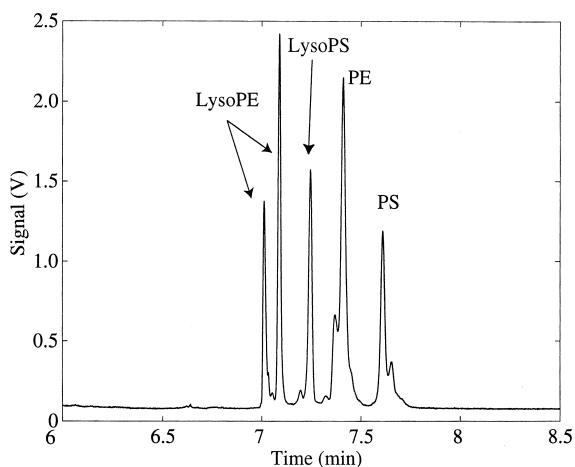


Fig. 6. Electrophoregram of FQ labeled lipids obtained in 10 mM borax with 35 mM SDC and 7.5 mM methyl- β -CD. Other conditions as in Fig. 3.

individual phospholipid classes was obtained as 7.5 mM methyl- β -CD was added to modify the MECC system containing borax and SDC. When we lowered the concentration of methyl- β -CD to 2.5 mM, the peaks of LysoPS and PE overlapped. If the concentration of methyl- β -CD was increased to 12.5 mM, the peak shape deteriorated. For intermediate methyl- β -CD concentration (Fig. 6), the peak of each phospholipid split into two, which was most probably due to two components with different fatty acid chains, although the minor peak before Lyso-PS may be due to an impurity. Although no detailed information on the fatty acid composition for PE, PS and LysoPS was available from the manufacturer, we would expect similar heterogeneity of these three lipids with that of LysoPE.

3.2. NBD-F derivatives

NBD-F has been used as a fluorescence reagent for the analysis of amino acids and peptides in CE [17,18]. To the best of our knowledge, there are no reports using NBD-F as the derivatization reagent for phospholipids in HPLC or CE. As shown in Fig. 7, fluorescent signals were obtained only for LysoPE and PE; the reason why no signals were observed for PS and LysoPS needs to be investigated. NBD-F labeling provided rapid and high-sensitivity analysis

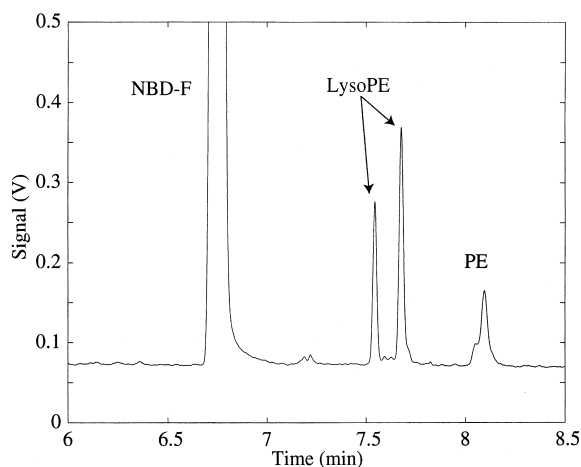


Fig. 7. Electrophoregram of NBD-F labeled lipids obtained in 10 mM borax with 35 mM SDC and 7.5 mM methyl- β -CD. Lipid concentrations: 0.12 μ g/ml each. Other conditions as in Fig. 3.

of LysoPE and PE. The derivatization was conducted for 5 min and the separation was finished within 9 min.

3.3. Comparison of phospholipid labeling reagents in CE-LIF and HPLC-LIF

In earlier reports, succinimidyl 2-nathoxyacetate [4] and 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl) were used as the pre-column derivatization reagents for phospholipid analysis in HPLC-LIF [5]. Table 1 compares several labeling reagents for phospholipids in HPLC and our CE work. FQ reacts rapidly with the four lipids and produces low background signal. FQ phospholipid derivatives are more stable than Dns-Cl and succinimidyl 2-nathoxyacetate phospholipid derivatives.

3.4. Detection limits

Detection limits ($\sigma=3$) were estimated by serial dilution of the fluorescently labeled phospholipid classes and are listed in Table 2. The mass detection limits are four- to five-orders of magnitude superior to those in CE-IPD [7], and six- to seven-orders of magnitude superior to those in HPLC [4].

Table 1
Comparison of labeling reagents for phospholipids in CE–LIF and HPLC–LIF

	Analytes			
	PE, PS, LysoPE, LysoPS	PE, PS, LysoPE, LysoPS	PE, PS, LysoPE, LysoPS	PE, LysoPE
Separation method	HPLC	HPLC	CE	CE
Labeling reagent	Dns-Cl	Succinimidyl 2-nathoxyacetate	FQ	NBD-F
Fluorescent or fluorogenic	Fluorescent	Fluorescent	Fluorogenic	Fluorescent
Reaction temperature	50°C	Room temperature	55°C	55°C
Reaction time	3 h	2 h	15 min	5 min
Stability of derivatives	At least 24 h at –20°C	Several days at –20°C	At least 1 month at 4°C	Several days at 4°C

Table 2
Detection limits (3σ) for phospholipids obtained with FQ labeling and NBD-F labeling

Lipid	FQ labeling		NBD-F labeling	
	CDL ^a (ng/ml)	MDL ^b (fg)	CDL ^a (ng/ml)	MDL ^b (fg)
LysoPE	2.5	0.89	0.54	0.18
LysoPS	2.1	0.73	–	–
PE	1.9	0.65	2.7	0.87
PS	3.4	1.1	–	–

^a Concentration detection limit (CDL).

^b Mass detection limit (MDL).

4. Conclusion

This is the first study of using MECC–LIF with FQ labeling for analysis of phospholipids. Different MECC systems were studied in terms of their ability to resolve FQ-labeled phospholipids. Fast separation and good resolution for the four phospholipids were obtained by using methyl- β -CD modified SDC–MECC system. Compared with HPLC–LIF analysis, the manipulation of experimental conditions in MECC is much easier since gradient elution is not required. Most importantly, the high sensitivity obtained with this method shows a great potential for the analysis of trace phospholipids in the biological microenvironment.

Acknowledgements

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