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Technical note

Thin-layer chromatography blotting for the fluorescence detection of phospholipid hydroperoxides and cholesteryl ester hydroperoxides

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Abstract

A blotting technique was developed to specifically detect lipid hydroperoxides in thin-layer chromatography. Phosphatidylcholine hydroperoxides and cholesteryl linoleate hydroperoxides ranging from 0.1 to 0.5 nmol, which were prepared by reaction with soybean lipoxygenase, were visualized as fluorescent spots on the blotted membrane by immersing the plate into a blotting solvent containing 0.01% (w/v) diphenyl-1-pyrenylphosphine. This technique was applied successfully to monitor lipid peroxidation in human low-density lipoprotein in vitro.

Keywords: Phospholipid hydroperoxides; Cholesteryl ester hydroperoxides

1. Introduction

Lipid peroxidation attracts much attention because of its possible role in a variety of pathophysiological events. Lipid hydroperoxides are the primary products of lipid peroxidation and their detection has been frequently used as an effective tool for monitoring lipid peroxidation in biological systems in vivo and in vitro. Several high-performance liquid chromatography (HPLC) methods for the separation and quantification of lipid hydroperoxides have been reported in the literature [1–5]. Akasaka et al. [6,7] determined lipid hydroperoxides using HPLC with fluorescence detection. However, the HPLC technique is time-consuming and requires special instrumentation, although fluorescence detection is sensitive and reproducible. On the other hand, Taki et al. [8,9] have developed thin-layer chromatography (TLC) blotting as a simple isolation method for glycolipids from biological tissues. We therefore developed this TLC blotting technique for detecting lipid hydroperoxides and succeeded in the specific detection of lipid hydroperoxides on the blotting membranes.

2. Experimental

2.1. Materials

Egg yolk phosphatidylcholine (PC) and cholesteryl linoleate were obtained from Sigma (St. Louis, MO, USA). Phosphatidylcholine hydroperoxides (PC-OOH) were prepared from egg yolk PC by reaction with soybean lipoxygenase and were isolated using reversed-phase column chromatography

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according to the method described previously [10]. Cholesteryl ester hydroperoxides (CE-OOH) were also prepared with soybean lipoxygenase and isolated by silica gel column chromatography [11]. Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Dojindo Laboratories (Kumamoto, Japan). Polyvinylidenedifluoride (PVDF) membranes, polytrifluoroethylene (PTFE) membranes and glass fiber filters were obtained from ATTO (Tokyo, Japan). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako (Tokyo, Japan).

2.2. Analysis of lipid hydroperoxides by TLC blotting

An aliquot of lipid solution was charged onto a TLC plate (Silica gel 60 F254, 0.25 mm thickness, 5×5 cm) and developed with the solvent chloroform–methanol–0.2% aqueous $CaCl_2$ (65:35:5, v/v) for PC-OOH and with hexane–diethyl ether–acetic acid (70:30:1, v/v) for CE-OOH. PC-OOH or CE-OOH was blotted onto the PVDF membrane as follows. The TLC plate was dried thoroughly with a hair dryer and then dipped in 0.01% (w/v) DPPP-containing blotting solvent (isopropanol–0.2% aqueous $CaCl_2$–methanol, 40:20:7, v/v) for 30 s, after which it was placed on a glass fiber filter. Then the plate was covered with a PVDF membrane, a PTFE membrane and finally a glass fiber filter in layers. This TLC-blotting sandwich was pressed evenly for 60 s at 180°C using a TLC thermal blotter AC-5970 (ATTO) at a press level of 8. After blotting, the PVDF membrane was illuminated with a 150 W projection lamp for 20 min at room temperature. Then the PC-OOH or CE-OOH spot was visualized by fluorescence under UV irradiation using a fluorescence reader, Epi-light UV FA 500 (AISIN-COSMOS Institute,Tokyo, Japan).

2.3. Application of TLC blotting for monitoring lipid peroxidation in human LDL

Human LDL was prepared from healthy volunteers and an LDL suspension in PBS buffer (pH 7.4; 0.6 mg protein/2.7 ml) was added to 0.3 ml AAPH solution (final concentration 10 m*M*) and incubated at 37°C for 4 h. At given time intervals, LDL solution (600 μl) was taken out and extracted with chloroform–methanol (2:1, v/v). The resultant lipid extract was charged onto a TLC plate after concentration and then subjected to TLC blotting after development with a solution of hexane–diethyl ether–acetic acid (70:30:1, v/v).

3. Results and discussion

We selected the fluorescent derivative DPPP for visualizing lipid hydroperoxides on PVDF membranes. Akasaka et al. [6] originally developed this compound for the specific detection of lipid hydroperoxides in HPLC post-column assays. Its principle is the production of a fluorescent compound, diphenyl-1-pyrenylphoshine oxide, from DPPP by the reduction of lipid hydroperoxides [6,7]. This fluorescent reaction is simple and sensitive for the detection of lipid hydroperoxides [12]. Recently, DPPP was successfully applied as a fluorescent probe for lipid peroxidation of cultured cells [13]. Fig. 1A shows a typical result for the fluorescence detection of PC-OOH on the blotting membrane in the range 0.1 to 0.5 nmol. The PC-OOH spot in this range was clearly visualized on the membranes. Fluorescence detection of CE-OOH on TLC blotting shows that CE-OOH is visualized in the range 0.1 to 0.5 nmol, similar to PC-OOH (Fig. 1B). An advantage of the combination of DPPP and TLC blotting is that the fluorescent compound is formed during the thermal blotting by using a DPPP-containing blotting solvent. This indicates that an additional visualizing reaction on the membrane is not required. In TLC analysis, lipid hydroperoxides can be detected by spraying the reagents potassium iodide–starch, ammonium thiocyanate–ferrous sulfate, dimethylaminoaniline or dimethyl-*p*-phenylenediamine dihydrochloride [14,15]. However, the spraying procedure seems to reduce the sensitivity and reproducibility. For either PC-OOH or CE-OOH, the detection limit on the membrane is nearly 0.1 nmol. Although we tried colorimetric detection on TLC blotting, which was based on iodometry using potassium iodide and methylene blue [16], the detection limits for PC-OOH and CE-OOH by this colorimetric reaction were larger than 1.0 nmol.

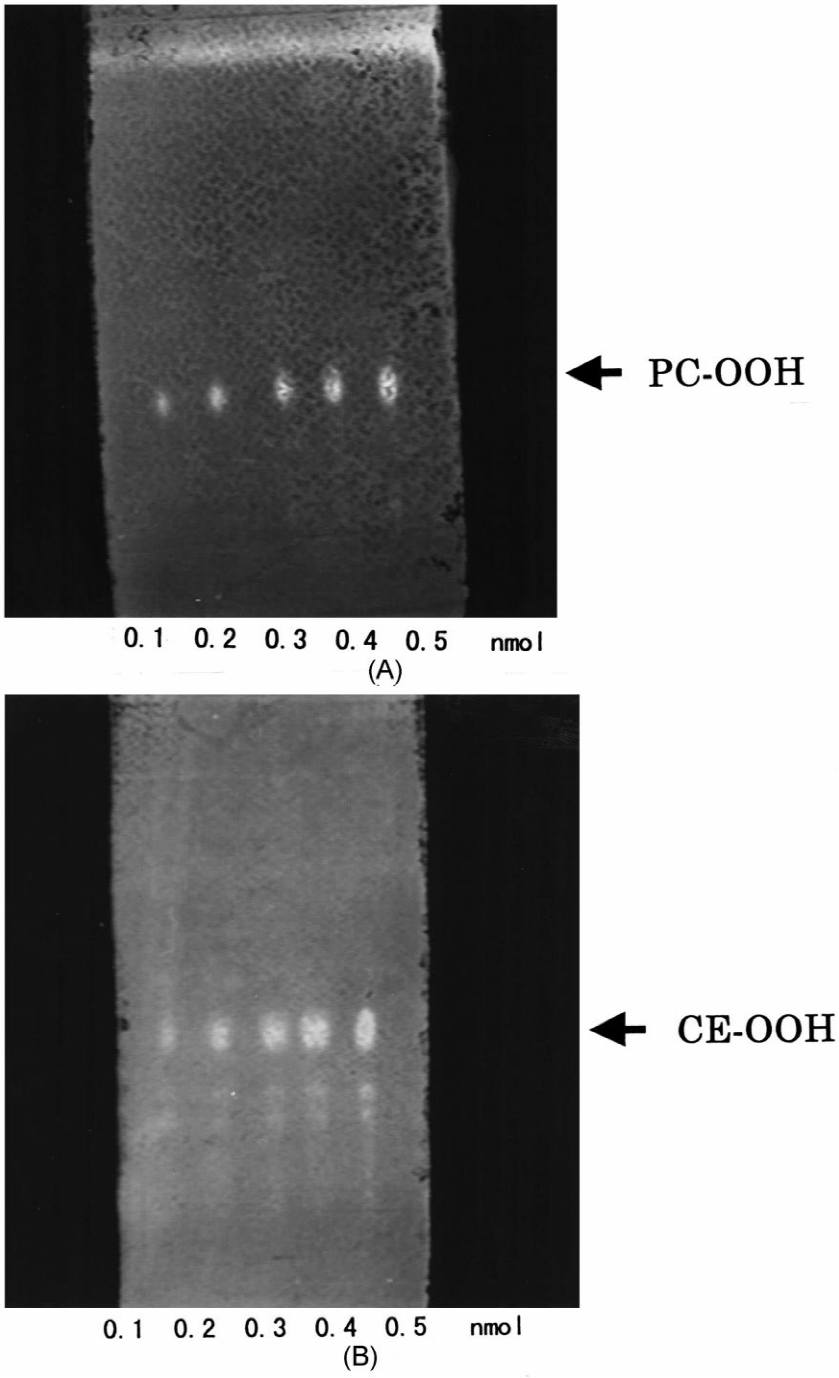

Fig. 1. TLC blotting of standard PC-OOH (A) and CE-OOH (B) at different levels. The arrow shows the PC-OOH (A) and CE-OOH (B) spots.

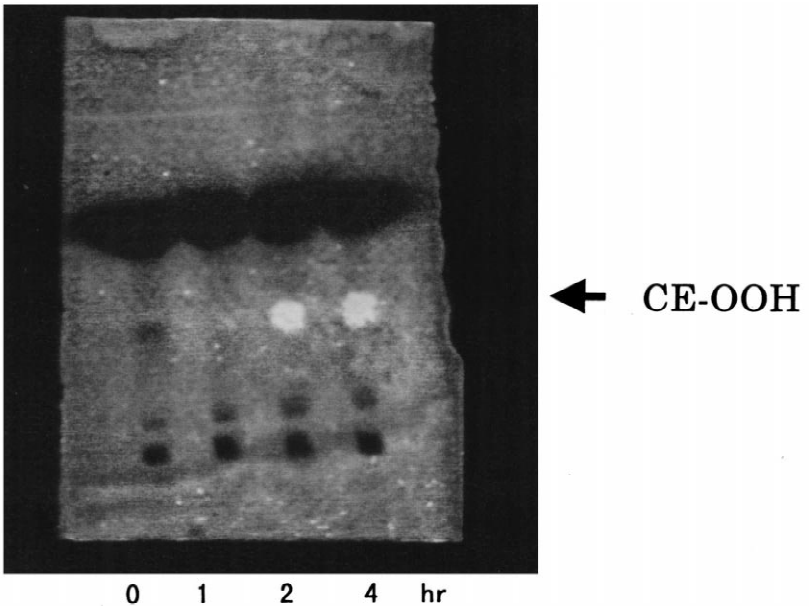

Fig. 2. TLC blotting of the lipid extracts from oxidized human LDL. The arrow shows the CE-OOH spots.

Next, we applied DPPP-TLC blotting to the detection of lipid hydroperoxides from oxidized human LDL to assess whether this technique is useful for monitoring lipid peroxidation in biological samples. Human LDL was prepared from healthy volunteers and oxidized with a water-soluble azo compound, AAPH, by the method described previously [17]. Fig. 2 shows the results for lipid extracts from LDL before and after 1, 2 and 4 h oxidation. The CE-OOH spot appeared in the lanes after 2 and 4 h oxidation. Among the lipid hydroperoxides, CE-OOH has frequently been used in HPLC analyses for monitoring the degree of lipid peroxidation of plasma lipoproteins [18]. It is therefore likely that this technique is an efficient tool for monitoring lipid peroxidation of LDL in vitro.

In conclusion, the combination of fluorescence detection using DPPP and TLC blotting serves as a simple and sensitive technique for the detection of lipid hydroperoxides from biological samples. In addition, this technique may be useful for isolating lipid hydroperoxides from biological samples after TLC separation.

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