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High-performance liquid chromatographic determination of linoleic acid peroxide-derived radicals using electrochemical detection ☆

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Abstract

High-performance liquid chromatography–electrochemical detection (HPLC–ED) was applied to detect 13-hydroperoxide octadecadienoic acid (13-HPODE)-derived radicals such as the pentyl radical and octanoic acid radical. The 13-HPODE-derived radicals were successfully detected using HPLC–ED by the combined use of the spin-trapping technique with α-(4-pyridyl-1-oxide)-*N*-*tert*.-butylnitrone (4-POBN). The 4-POBN-pentyl radical adduct was detected at the retention time of 18.2±0.3 min on the elution profile of HPLC–ED with an ODS column (15 cm×4.6 mm I.D.) using a flow-rate of 1.0 ml/min with 50 m*M* ammonium acetate in 29% (v/v) aqueous acetonitrile. The 4-POBN–octanoic acid radical adduct was also detected at the retention time of 13.7±0.7 min using a flow-rate of 1.0 ml/min with 50 m*M* ammonium acetate in 14% (v/v) aqueous acetonitrile. The concentrations of the 4-POBN radical adducts were determined using HPLC–ED without an internal standard. HPLC–ED is 100 times as sensitive as HPLC–electron spin resonance (ESR) under the ESR and ED conditions employed here. Even 1.8 pmol of the 4-POBN–pentyl (or octanoic acid) radical adduct was detectable using HPLC–ED.

Keywords: Electrochemical detection; Electron spin resonance detection; Detection, LC; Spin trapping; Lindeic acid peroxide; Fatty acids

1. Introduction

Recently, high-performance liquid chromatography (HPLC) has been employed to detect, isolate, and identify radical adducts. HPLC–electron spin resonance (ESR) spectrometric detection has been utilized to detect, isolate, and identify radical adducts [1–6]. HPLC–ESR–MS have also been successfully used to detect and identify the pentyl radical, pentenyl radical, octanoic acid radical, 12,13-epoxylinoleic acid radical, 14,15-epoxyarachidonic acid radical, and dihydroxylinoleic acid radical in the reaction mixture of linoleic acid (or linolenic acid or arachidonic acid) with soybean lipoxygenase [4,7,8]. HPLC–ESR spectrometry has been shown to be a powerful method to identify radical species for complicated systems such as biological systems because retention behavior is characterized in addition to ESR hyperfine structures in HPLC–ESR. The ESR detector is, however, limited by its low sensitivity (>0.1 μ*M*).

To overcome this weak point of the HPLC–ESR,

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HPLC with electrochemical detection (ED) technique has been employed to detect the radical adducts. HPLC–ED has been applied to the detection of radical adducts of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) [9–11], a nitrosobenzene radical adduct [12], and α-(4-pyridyl-1-oxide)-*N*-*tert*.-butylnitrone (4-POBN)–phenyl radical adduct [13]. Floyd detected the DMPO–OH radical adduct by the HPLC–ED technique. Addition of ascorbic acid resulted in increase of the intensity of a peak which was assigned to the reduced form of the DMPO–OH radical adduct [9]. On the other hand, Towell and Kalyanaraman used an internal standard (2,2,5,5-tetramethylpyrrolidin-1-yloxy free radical) to determine the concentration of the DMPO radical adduct [11]. They also successfully employed isotope labeled compounds to identify the radical adduct [11]. However, no HPLC–ED analyses have been performed for the respective lipid-derived free radicals. In this study, two lipid-derived free radicals, i.e., the pentyl radical and octanoic acid radical, which form in the reaction of 13-hydroperoxide octadecadienoic acid (13-HPODE) with ferrous ions (Fig. 1), are detected using HPLC–ED. Furthermore, sensitivity of HPLC–ED is compared with that of HPLC–ESR.

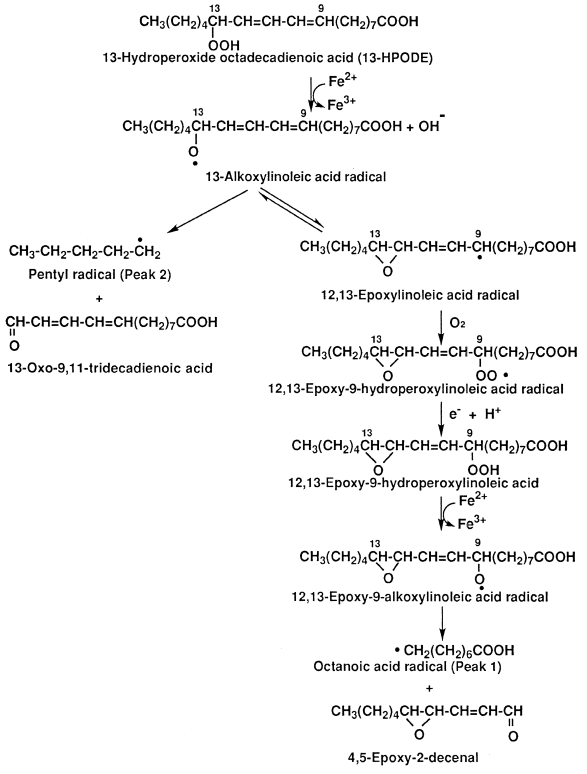

Fig. 1. Formation of pentyl radical and octanoic acid radical in the reaction of 13-HPODE with ferrous ions.

2. Experimental

2.1. Materials

Linoleic acid (9,12-octadecadienoic acid), 4-POBN, and soybean lipoxygenase (EC 1.13.11.12) Type V were obtained from Sigma (St. Louis, MO, USA). Ferrous ammonium sulfate was obtained from Kishida (Osaka, Japan). Pentylhydrazine oxalate was synthesized according to the method of Gever and Hayes [14]. All other chemicals used were of analytical-grade.

2.2. HPLC–ESR for purification of 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct

The HPLC system used in HPLC–ESR consisted of a model 7125 injector (Rheodyne, Cotati, CA, USA) with a 5-ml sample loop, a model 655A-11 pump with a model L-5000 LC controller (Hitachi, Ibaraki, Japan). A Waters μBondaPak C_{18} semi-preparative column (300 mm×10 mm I.D.) (Millipore, Milford, MA, USA) was used. The column was kept at 25°C. For HPLC–ESR, two solvents were used: solvent A, 50 m*M* ammonium acetate; solvent B, 50 m*M* ammonium acetate in 64% (v/v) aqueous acetonitrile. A combination of isocratic and linear gradient was used: 0–25 min, 100% A to 0% A (linear gradient) at a flow-rate of 2.0 ml/min; 25–35 min, 100% B (isocratic) at a flow-rate of 2.0 ml/min. The eluent was introduced into a model JES-FR30 free radical monitor (JEOL, Tokyo, Japan). The ESR spectrometer was connected to the HPLC with a PTFE tube, which passed through the center of the ESR cavity. The operating conditions of the ESR spectrometer were: power, 4 mW; modulation width, 0.2 mT; time constant, 1 s. The magnetic field was fixed at the third peak in the doublet-triplet ESR

spectrum (a^N=1.58 mT and $a^H\beta$=0.26 mT) of the 4-POBN radical adduct.

2.3. Preparation of 4-POBN–pentyl radical adduct

Pentyl radical was synthesized through the decomposition of pentylhydrazine. The reaction mixture contained, in a total volume of 1 ml, 0.1 *M* 4-POBN, 2.5 mg/ml pentylhydrazine oxalate, 0.2 m*M* $CuCl_2$, and 45 m*M* carbonate buffer (pH 10.0). After nitrogen gas was bubbled through the reaction mixture without $CuCl_2$ for 5 min, the reaction was started by adding $CuCl_2$. Reaction was performed for 2 h at 25°C. In order to purify the 4-POBN–pentyl radical adduct, 0.5 ml of the pentyl radical reaction mixture was mixed with 4.5 ml of 50 m*M* ammonium acetate and then applied to HPLC–ESR described in the Section 2.2. The peak fraction that appeared at 24 min on the HPLC–ESR elution profile was collected. Identification of the 4-POBN–pentyl radical adduct has described in a previous paper [15].

2.4. Preparation of 13-hydroperoxide octadecadienoic acid (13-HPODE)

The reaction mixture contained, in a total volume of 25 ml, 1.5 mg/ml linoleic acid, 440 units/ml soybean lipoxygenase, and 0.2 *M* boric acid (pH 9.0). The reaction was performed at 25°C under air for 1 h. After 1 h, 0.4 ml of the reaction mixture was mixed with 3.6 ml of 0.2 *M* borate buffer (pH 9.0), and then injected onto an HPLC–UV system. The HPLC–UV system used consisted of a model 7125 Rheodyne injector with a 5-ml sample loop (Rheodyne), a model Hitachi 655A-11 pump with a model L-5000 LC controller (Hitachi), a Waters μBondaPak C_{18} semipreparative column (Millipore) (300 mm×10 mm I.D.), and a model SPD-M10AVP diode array detector (Shimadzu, Kyoto, Japan) with a model CLASS-LC10 LC workstation (Shimadzu). The SPD-M10AVP diode array detector was operated from 200 to 350 nm in the HPLC–UV system. Two solvents were used in the HPLC–UV: A, water; B, methanol. A combination of isocratic and linear gradient was used for the HPLC–UV: 0–20 min, 0% B to 90% B (linear gradient) at a flow-rate of 2.0 ml; 20–30 min, 90% B (isocratic) at a flow-rate of 2.0 ml/min. The column was kept at 25°C. A prominent peak was observed at a retention time of 20.3 min when the HPLC profile was monitored at 235 nm. The peak fraction was collected. The methanol contained in the fraction was removed using a model CC-105 centrifugal concentrator with a model TU-055 low temperature trap (TOMY, Yokohama, Japan). The water solution of the fraction was used as a stock solution of 13-HPODE. The concentration of 13-HPODE was determined from its absorbance at 234 nm (ϵ=25 600 cm^{-1} M^{-1}) [16].

2.5. Reaction mixture for the preparation of 4-POBN–octanoic acid radical adduct

The reaction mixture for the preparation of 4-POBN–octanoic acid radical adduct contained, in a total volume of 5 ml, 140 μ*M* 13-HPODE, 0.33 m*M* $FeSO_4(NH_4)_2SO_4$, 0.1 *M* 4-POBN, 1 m*M* EDTA, and 38 m*M* phosphate buffer (pH 7.4). The reaction was started by adding $FeSO_4(NH_4)_2SO_4$. The reaction was performed for 2 min at 25°C. In order to purify the 4-POBN–octanoic acid radical adduct, the reaction mixture was applied to the HPLC–ESR described in the Section 2.2. A prominent peak appeared at the retention time of 16 min on the HPLC–ESR elution profile. The peaks from four HPLC–ESR chromatographs were collected and combined. Chromatography was performed again using the same HPLC–ESR conditions after the volume of the combined sample had been reduced to about 2 ml. Peak fraction on the second chromatogram showed the same retention time as those on the initial HPLC–ESR chromatogram indicating that the radical adducts were stable during the isolation procedures. Identification of the 4-POBN–octanoic acid radical adduct has described in a previous paper [15].

2.6. HPLC-ESR analyses for 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct

The HPLC used in the HPLC–ESR consisted of a model 7125 injector (Rheodyne) with a 0.2-ml sample loop, a model LC-10ATVP pump with a model DGU-3A degasser (Shimadzu). The column (150 mm×4.6 mm I.D.) packed with TSKgel ODS-

120T (5 μm particle size) (Tosoh, Tokyo, Japan) was used at a flow-rate of 1.0 ml/min with 50 m*M* ammonium acetate in 29% (v/v) aqueous acetonitrile for 4-POBN–pentyl radical adduct or with 50 m*M* ammonium acetate in 14% (v/v) aqueous acetonitrile for 4-POBN–octanoic acid radical adduct. The TSKgel ODS-120T is a non-silanol type disposed with an end cap to a remaining silanol. The pore size of the TSKgel ODS-120T is 120 Å. The column was kept at 35°C throughout the analyses. ESR conditions were as described in Section 2.2.

2.7. HPLC–ED analyses for 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct

The HPLC used for the HPLC–ED was as described in Section 2.6. The electrochemical detector employed here was a model 5100 A Coulochem electrochemical detector with a model 5010 analytical cell (ESA, Bedford, MA, USA). The analytical cell is a low volume flow-through cell containing a reference electrode (Pd), a counter, and two porous graphite electrodes.

2.8. Determination of the concentration of 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct using HPLC–ED

The concentration of the 4-POBN–pentyl (or octanoic acid) radical adduct was estimated using the HPLC–ED. Because 100% of the radical form of the 4-POBN–pentyl (or octanoic acid) radical adduct, which flow through the analytical cell, is oxidized on the model 5010 analytical cell of Coulochem electrochemical detector (Fig. 2), the concentrations of the purified 4-POBN–pentyl and 4-POBN–octanoic acid radical adducts were estimated to be 9.1 and 3.6 μmol/l, respectively, under the assumption of quantitative electrolysis.

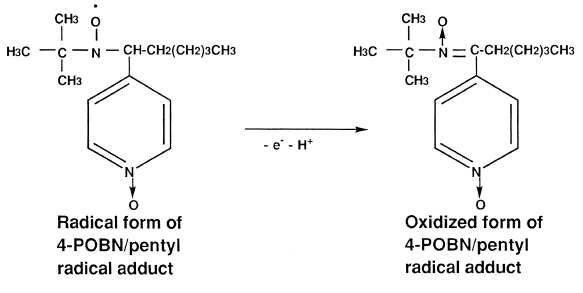

Fig. 2. Oxidation of radical form of 4-POBN–pentyl radical adduct.

3. Results

3.1. The HPLC–ESR and HPLC–ED analyses of 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct

The HPLC–ESR and HPLC–ED analyses of 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct were performed (Fig. 3). A 20-μl aliquot (182 pmol) of the purified 4-POBN–pentyl radical adduct (9.1 μmol/l) was applied to the HPLC–ESR and HPLC–ED with +0.5 V of applied potential. Compared with the HPLC–ESR (Fig. 3A), a very good *S*/*N* peak was observed on the elution

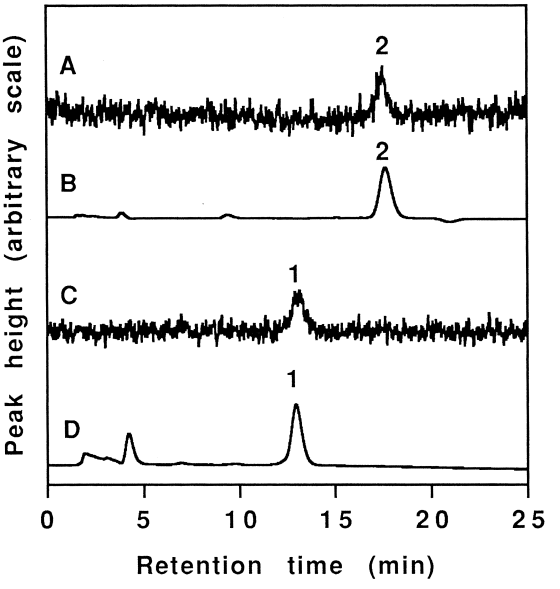

Fig. 3. HPLC–ESR and HPLC–ED analyses of 4-POBN–pentyl and 4-POBN–octanoic acid radical adducts. (A) HPLC–ESR analysis of 4-POBN–pentyl radical adduct (20 μl of 9.1 μmol/l). (B) HPLC–ED analysis of 4-POBN–pentyl radical adduct (20 μl of 9.1 μmol/l) at+0.5 V. (C) HPLC–ESR analysis of 4-POBN–octanoic acid radical adduct (50 μl of 3.6 μmol/l). (D) HPLC–ED analysis of 4-POBN–octanoic acid radical adduct (50 μl of 3.6 μmol/l) at +0.5 V.

profile of HPLC–ED chromatography at the retention time of 18.2±0.3 min (Fig. 3B). On the other hand, 50 μl (180 pmol) of the purified 4-POBN–octanoic acid radical adduct (3.6 μmol/l) was applied to the HPLC–ESR and HPLC–ED with +0.5 V of applied potential (Fig. 3C and D). A very good *S/N* peak was also observed on the elution profile of HPLC–ED chromatography at the retention time of 13.7±0.7 min (Fig. 3D). The solution of the 4-POBN–octanoic acid radical adduct was diluted by one hundredth and applied to the HPLC–ED with +0.5 V of applied potential. Even 1.8 pmol of 4-POBN–octanoic acid radical adduct was detectable on the elution profile of the HPLC–ED (Fig. 4A). The case for 4-POBN–pentyl radical adduct also gave similar results (data not shown).

3.2. HPLC–ED analyses of 4-POBN–pentyl radical adduct and 4-POBN–octanoic acid radical adduct with various applied potentials

The peak heights of the 4-POBN–pentyl radical adduct and the 4-POBN–octanoic acid radical adduct were monitored on the HPLC–ED with various applied potentials (+0.8 V to +0.2 V for the 4-POBN–pentyl radical radical adduct and +0.7 V to +0.2 V for the 4-POBN–octanoic acid radical adduct) (Fig. 5). The two radical adducts showed similar applied potential dependences of the peak heights. Half of the radical adducts were oxidized at +0.40 V of the applied potential for the 4-POBN–pentyl radical adduct and +0.42 V for the 4-POBN–octanoic acid radical adduct, respectively.

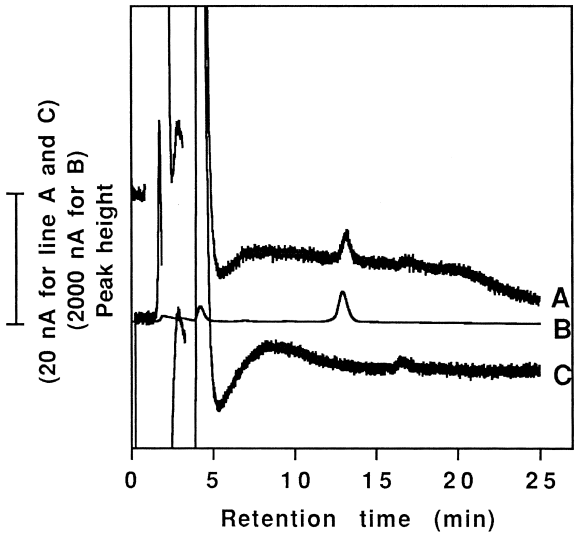

Fig. 4. HPLC–ED of 1.8 pmol of 4-POBN–octanoic acid radical adduct at +0.5 V. (A) purified 4-POBN–octanoic acid radical adduct (50 μl of 36 nmol/l). (B) purified 4-POBN–octanoic acid radical adduct (50 μl of 3.6 μmol/l). (C) water.

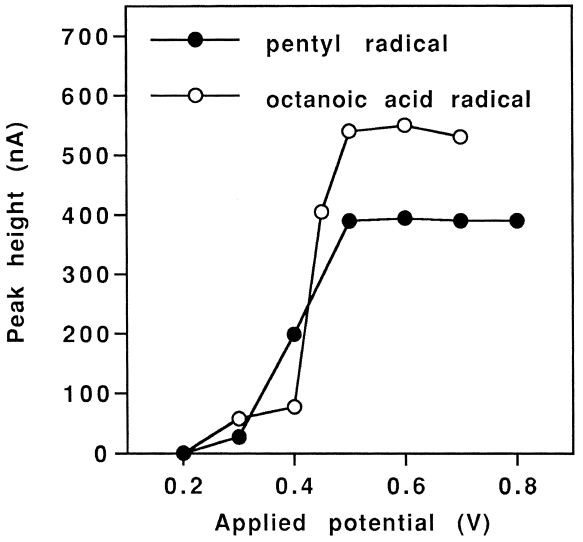

Fig. 5. Applied potential dependence of the HPLC–ED peak height. ○, 4-POBN–octanoic acid radical adduct (50 μl of 3.6 μmol/l). ●, 4-POBN–pentyl radical adduct (20 μl of 9.1 μmol/l).

3.3. Injection amount dependence of the HPLC–ED peak heights of the 4-POBN–pentyl radical adduct

Various amounts (18–91 pmol) of 4-POBN–pentyl radical adduct were applied to the HPLC–ED with +0.5 V of applied potential (data not shown). A linear relationship exists between the amounts of 4-POBN–pentyl radical adduct injected and peak area, which correspond to the total current during the oxidation process of the 4-POBN–pentyl radical adduct on the analytical cell. Furthermore, the linear line passes the original point of the graph, suggesting that all the 4-POBN–pentyl radical adduct injected was oxidized on the analytical cell. Indeed, no peak was detected by electrode 2 when +0.5 V of applied potential was applied on electrodes 1 and 2 in the analytical cell. Thus concentrations of the 4-POBN

radical adducts can be determined using procedures described in Section 2.

4. Discussion

The pentyl radical and octanoic acid radical have been detected in the reaction mixture of soybean lipoxygenase with linoleic acid (or arachidonic acid) and in the reaction mixture of 13-HPODE with ferrous ions using HPLC–ESR–MS spectrometry with the spin-trapping technique [7,8,15]. These radicals possibly form in vivo under several oxygen stresses. In order to detect the 4-POBN–pentyl radical adduct (or octanoic acid radical adduct), large amounts of reaction mixtures had to apply to the HPLC–ESR–(MS) because of low ESR sensitivity. In this study, however, even 1.8 pmol of the 4-POBN–pentyl radical adduct (or octanoic acid radical) was shown to be detectable using HPLC–ED. Thus HPLC–ED can be applicable for biological systems where there are very low concentration of radical forms. HPLC–ED is 100 times as sensitive as HPLC–ESR under the ESR and ED conditions employed here. The sensitivity afforded by electrochemical detection combined with the separation methods of HPLC seems ideal for the detection and quantification of free radical production in biological systems. Thus HPLC–ED and HPLC–ESR are shown to be complementary techniques to detect free radicals in biological systems. Furthermore, with the amount of 4-POBN–pentyl radical adduct (or 4-POBN–octanoic acid radical adduct) used, it is possible to determine using HPLC–ED without an internal standard.

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