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High-performance liquid chromatography with mercury cathode electrochemical detection: application to lipid hydroperoxide analysis

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Abstract

Lipid hydroperoxide species can be analyzed with high sensitivity and specificity, using reversed-phase high-performance liquid chromatography with reductive mode electrochemical detection on a mercury drop cathode [HPLC–ED(Hg)]. The purpose of this study was to examine different variables in the operation of HPLC–ED(Hg) and to select optimal conditions for the analysis of several biologically relevant peroxides, including species derived from cholesterol, cholesteryl linoleate, oleate, linoleate, and two synthetic phosphatidylcholines. Parameters such as operating potential and mobile-phase solvent proportions, electrolyte composition, and ionic strength were evaluated for each peroxide class. Under optimal conditions, we have achieved baseline separation of four cholesterol hydroperoxide species, not only from one another, but also from phospholipid hydroperoxides; detection limits were < 0.3 pmol and < 30 pmol for the cholesterol and phospholipid hydroperoxides, respectively.

1. Introduction

Peroxidative modification of unsaturated lipids in cell membranes and lipoproteins is attracting considerable biomedical interest because of its possible role in pathological conditions such as inflammatory disease, ischemia-reperfusion injury, atherogenesis, and carcinogenesis [1,2]. This interest has prompted the development of highly sensitive techniques for assessing lipid peroxidation, most of which involve measurement of reactive intermediates and products such as hydroperoxides, aldehydes, and alkanes [2,3].

Though popular because of their relative simplicity, assays based on detection of thiobarbituric acid reactive compounds [4], conjugated dienes [5] or iodide-reducible hydroperoxides [6,7] have several limitations; e.g., they measure “bulk” lipid peroxidation only, lack absolute specificity, and may (depending on the assay) detect only part of the peroxide population or peroxidation process. These limitations have served as an impetus for the recent introduction of new approaches for the specific determination of intermediates/products such as lipid hydroperoxides (LOOHs). Included among these are several high-performance liquid chromatography (HPLC) approaches that incorporate highly specific peroxide assays, e.g. Fe²⁺/thiocyanate treatment with spectrophotometric detection [8],

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diphenyl-1-pyrenylphosphine treatment with fluorometric detection [9], cytochrome c/luminol [10] or microperoxidase/isoluminol [11,12] treatment with chemiluminescence detection, and reductive-mode [13–15] or oxidative-mode [16] electrochemical detection (ED). We have recently developed a new ED-based approach for peroxides, reversed-phase HPLC with reductive-mode mercury cathode detection [HPLC–ED(Hg)], which has several advantages over all other existing high-performance techniques [17,18]. These advantages include lower equipment cost, relative simplicity of operation, and fewer potential artifacts. The purpose of the present study was to examine several operating parameters in the HPLC–ED(Hg) technique (electrode potential, mobile-phase solvent composition, electrolyte concentration) and to optimize these parameters for the analysis of various biologically relevant fatty acid and lipid hydroperoxides.

2. Experimental

2.1. General chemicals

HPLC grade acetonitrile, methanol, and isopropanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Cholesterol (Ch), cholesteryl linoleate (CL), dimyristoyl phosphatidylcholine (DMPC), dicetylphosphate (DCP), linoleic acid, and oleic acid were from Sigma (St. Louis, MO, USA). 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) were supplied by Avanti Polar Lipids (Birmingham, AL, USA). Chloroaluminum phthalocyanine tetrasulfonate (AlPcS₄) was from Porphyrin Products (Logan, UT, USA) and merocyanine 540 (MC540) from Eastman Kodak (Rochester, NY, USA).

2.2. Hydroperoxide standards

Published procedures [15,19] were used for preparing and characterizing the following cholesterol B-ring hydroperoxides: 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide (5 α -OOH)

and 3 β -hydroxycholest-4-ene-6 β -hydroperoxide (6 β -OOH), which are singlet oxygen adducts [19]; and the epimeric pair, 3 β -hydroxycholest-5-ene-7 α -hydroperoxide and 3 β -hydroxycholest-5-ene-7 β -hydroperoxide (7 α /7 β -OOH), which are typically produced by free radical reactions [20]. Authentic 3 β -hydroxycholest-5-ene-25-hydroperoxide (25-OOH) was provided by Dr. J. van Lier as a gift. Hydroperoxides of POPC (POPC-OOH) and PLPC (PLPC-OOH) were prepared by AlPcS₄-sensitized photooxidation of unilamellar POPC–DMPC–DCP (1:1:0.25, mol/mol) and PLPC–DMPC–DCP (1:1:0.25, mol/mol) liposomes, respectively, as described previously [15,17]. Hydroperoxides of oleic acid, linoleic acid, and cholesteryl linoleate (Ole-OOH, Lin-OOH, and CL-OOH, respectively) were prepared by AlPcS₄-sensitized photooxidation of the respective parent compounds in chloroform–methanol (1:1, v/v). The peroxide products in each case were separated by means of semi-preparative HPLC–ED(Hg) [18]. The major phosphatidylcholine hydroperoxide fraction found in photooxidized L1210 leukemia cells was isolated as described [18]. Total hydroperoxide content of standards was determined by iodometric assay, using published specifications [15].

2.3. Instrumentation

The chromatographic equipment consisted of an Isco integrated HPLC system (Isco, Lincoln, NE, USA) interfaced with an EG&G-Princeton Model 420 electrochemical detector (Princeton, NJ, USA). The detector was equipped with a renewable mercury drop electrode that was operated in the cathodic (reductive) mode. Chromatography was carried out at 25–27°C, using a C₁₈ Ultrasphere column (4.6 × 150 mm; 5 μ m particles) from Beckman Instruments (San Ramon, CA, USA) and a C₁₈ guard cartridge (4.6 × 15 mm; 5 μ m particles) from Alltech Associates (Deerfield, IL, USA). For optimal separation of various peroxide analytes in various mixtures, three different mobile phases were used: 81% methanol, 10.5% acetonitrile, and 8.5% aqueous solution containing 10 mM ammonium acetate and 1.0 mM sodium perchlorate (Mobile Phase

I); 62% 2-propanol, 24% acetonitrile, 9.2% methanol, and 4.8% aqueous solution containing 10 mM sodium acetate and 1.0 mM sodium perchlorate (Mobile Phase II); 50% methanol, 22.5% acetonitrile, and 27.5% aqueous solution containing 10 mM sodium acetate and 1.0 mM sodium perchlorate (Mobile Phase III). (The designated proportions are by volume.) Each premixed mobile phase was sparged continuously with high-purity argon (>99.998%; BOC Gases, Chicago, IL, USA) that had been passed first through an OMI-1 oxygen scrubber (Supelco, Bellefonte, PA, USA) to reduce O₂ concentration to <10 ppb, and then through a presaturating mobile-phase scrubber. Typically, overnight purging, followed by column washing for 0.5 h was sufficient to achieve a background current in the range 0.4–1.0 nA (depending on the applied potential), which was satisfactory for most analyses. The mobile phase was delivered isocratically at a flow-rate of 1.0–2.0 ml/min against a back-pressure of 7.5–10.0 MPa, depending on the solvents used. Samples were dissolved in 2-propanol and injected through a 20- μ l loop connected to a Rheodyne valve. For high-sensitivity settings (0.1–0.2 nA full scale), samples were placed on ice and sparged with high purity helium for 30 s prior to injection. This minimized interference from dissolved O₂, which appeared as a peak near the solvent front. A mercury drop was dispensed and equilibrated for 3–4 min before each sample injection; this was typically sufficient to reduce background noise to a satisfactory level. The operating potential of the mercury cathode was measured against a Ag/AgCl reference. Data collection, manipulation and storage was accomplished with an IBM 486 clone and Isco ChemResearch software.

3. Results

3.1. Optimization of the chromatographic conditions

In order to establish optimal HPLC conditions for the separation and detection of various lipid and fatty acid hydroperoxides, we examined

variables such as solvent composition and electrolyte (sodium perchlorate, ammonium acetate) concentration of the mobile phase. Various ternary and quaternary phases consisting of methanol, acetonitrile, 2-isopropanol, and water in different proportions were tested. We found that cholesterol B-ring hydroperoxides (5 α -OOH, 6 β -OOH, 7 α /7 β -OOH) and a side-chain hydroperoxide (25-OOH) could be separated from one another with baseline resolution using Mobile Phase I (Fig. 1A). This system also permitted certain groups of phospholipid hydroperoxides, e.g. those derived from the synthetic phosphatidylcholines POPC and PLPC, to be separated from each other (Fig. 1B) and also from most of the cholesterol hydroperoxide species. The effect of varying the ammonium acetate concentration in Phase I on POPC-OOH separation was tested. Over the concentration range examined (0–2 mM in bulk phase), 0.8–1.0 mM ammonium acetate was found to be sufficient for maximal (albeit still partial) resolution of the two POPC-OOH peaks. The retention time of CL-OOH was found to be >60 min with Mobile Phase I. Switching to a lower polarity system, Mobile Phase II, shortened this retention time considerably (Fig. 1C). Optimal analysis of peroxidized fatty acids was accomplished with a relatively polar solvent system, Mobile Phase III. This system increased the retention time of fatty acid hydroperoxides, thereby preventing them from being obscured by the trace O₂ peak that inevitably appeared near the solvent front. An excellent separation of two fatty acid hydroperoxide families, Ole-OOH and Lin-OOH, was achieved with Mobile Phase III (Fig. 1D). The optimal solvent compositions for resolving various hydroperoxide species along with corresponding capacity factors are summarized in Table 1.

3.2. Optimization of the detector response

The optimal working potential of the mercury cathode for any given hydroperoxide or hydroperoxide family was determined by examining its hydrodynamic voltammogram under the chromatographic conditions described in Section 3.1.

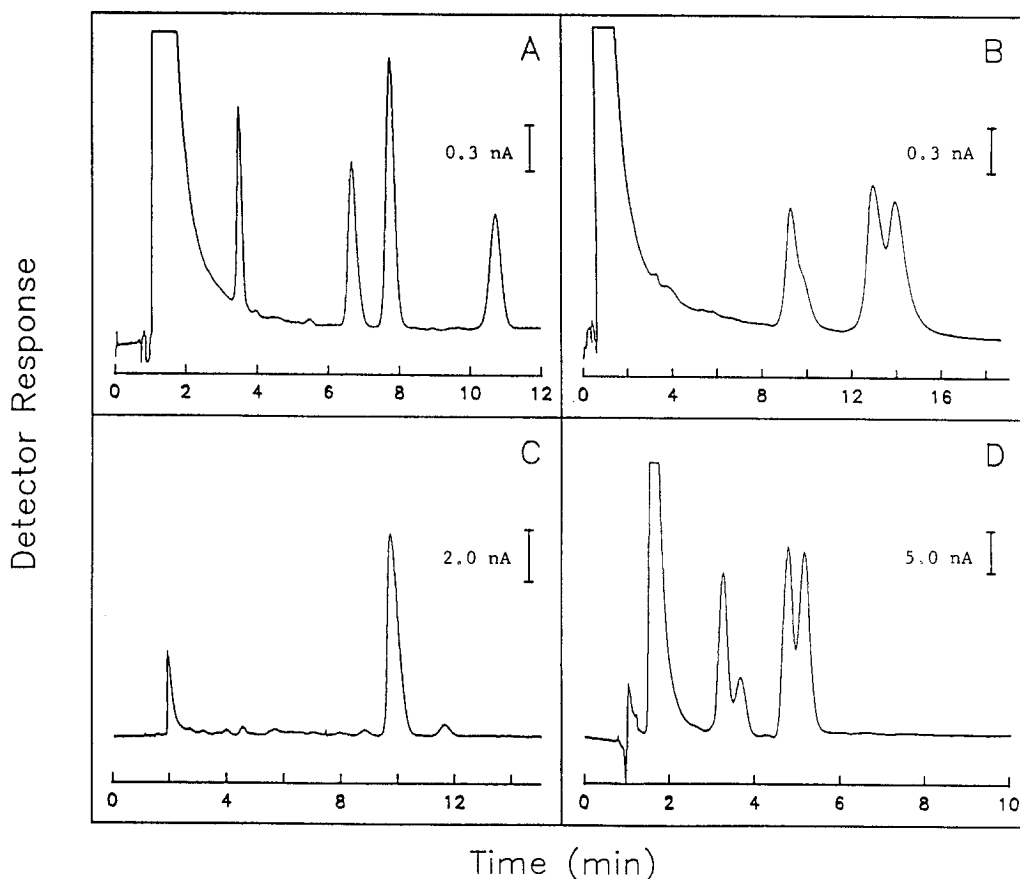


Fig. 1. HPLC-ED(Hg) profiles of photochemically generated lipid hydroperoxide standards. (A) 25-OOH, 50 pmol (3.6 min); 7 α /7 β -OOH, 35 pmol (6.7 min); 5 α -OOH, 65 pmol (7.8 min); 6 β -OOH, 45 pmol (10.7 min). (B) PLPC-OOH, 0.5 nmol (9.8 min); POPC-OOH, 1.0 nmol (13.8 min, 14.2 min). (C) CL-OOH, 0.3 nmol (9.7 min). (D) Lin-OOH, 0.5 nmol (3.3 min, 3.7 min); Ole-OOH, 1.0 nmol (4.8 min, 5.2 min). The following solvent systems were used: (A, B) Mobile Phase I, flow-rate 1.7 ml/min; (C) Mobile Phase II, flow-rate 1.0 ml/min; (D) Mobile Phase III, flow-rate 1.5 ml/min. Full-scale sensitivities were as follows: (A, B) 2 nA; (C) 20 nA; (D) 50 nA. Operating potential was -300 mV (A, B) and -150 mV (C, D).

Current-potential profiles for various cholesterol hydroperoxides are shown in Fig. 2. The peak-height plateau for 5 α -OOH, 6 β -OOH, and 7 α /7 β -OOH was reached at approximately -250 mV (Fig. 2A) and for 25-OOH at approximately -300 mV (Fig. 2B). In striking contrast, the maximal response for CL-OOH was obtained at the lowest applied potential (0 mV) and remained constant down to at least -400 mV (Fig. 2C). Similar behavior was observed for Ole-OOH and Lin-OOH (data not shown). For CL-

OOH and the fatty acid hydroperoxides, the constant signal with applied potential translates into an analytical advantage, since the less negative the working potential, the less interference from background noise or O₂. Similar reasoning applies to the cholesterol hydroperoxides (Figs. 2A, 2B), since their responses fell off rather gradually with potential below the -250 to -300 mV range. The POPC-OOH signal, like that of 25-OOH, maximized at about -300 mV (Fig. 3). However, POPC-OOH was much more sensitive

Table 1
Mobile phase compositions and capacity factors for HPLC–ED(Hg) analysis of various lipid and fatty acid hydroperoxides

Mobile phase ^a	Analyte ^b	Capacity factor (k') ^c
I	5 α -OOH	8.80
	6 β -OOH	12.48
	7 $\alpha/7\beta$ -OOH	7.42
	25-OOH	3.89
	POPC-OOH	14.18 (Peak I) 15.29 (Peak II)
II	PLPC-OOH	10.01
	CL-OOH	4.08
III	Ole-OOH	5.06 (Peak I) 5.52 (Peak II)
	Lin-OOH	3.16 (Peak I) 3.69 (Peak II)

^a Mobile phase compositions (% by volume): (I) 81% methanol, 10.5% acetonitrile, and 8.5% aqueous solution containing 10 mM ammonium acetate and 1.0 mM sodium perchlorate; (II) 62% isopropanol, 24% acetonitrile, 9.2% methanol, and 4.8% aqueous solution containing 10 mM ammonium acetate and 1.0 mM sodium perchlorate; (III) 50% methanol, 22.5% acetonitrile, and 27.5% aqueous solution containing 10 mM ammonium acetate and 1.0 mM sodium perchlorate.

^b Analytes were as follows: 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide (5 α -OOH); 3 β -hydroxycholest-4-ene-6 β -hydroperoxide (6 β -OOH); mixture of 3 β -hydroxycholest-5-ene-7 α -hydroperoxide and 3 β -hydroxycholest-5-ene-7 β -hydroperoxide (7 $\alpha/7\beta$ -OOH); 3 β -hydroxycholest-5-ene-25-hydroperoxide (25-OOH); 1-palmitoyl-2-oleoyl, phosphatidylcholine hydroperoxides (POPC-OOH); 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxides (PLPC-OOH); CL-OOH, cholesteryl linoleate hydroperoxides; Ole-OOH, oleic acid hydroperoxides; Lin-OOH, linoleic acid hydroperoxides.

^c The dead retention time used for determining k' was the interval between sample injection and appearance of solvent front.

to potential change over the 0 to –200 mV range than any of the cholesterol peroxides, including 25-OOH.

Based on earlier work [14,15,17], we included sodium perchlorate as a supporting electrolyte in our systems. The effect of NaClO₄ concentration on detector response is shown in Fig. 4. As

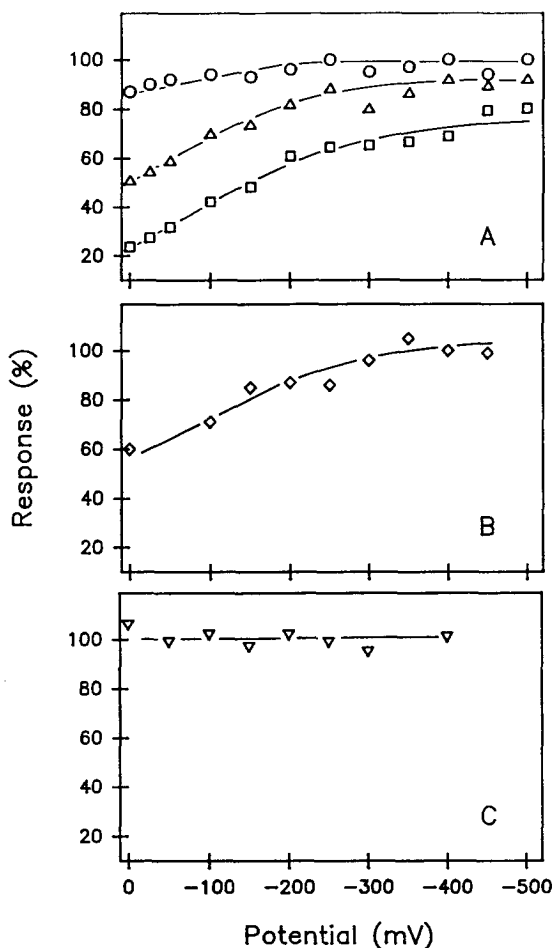


Fig. 2. Hydrodynamic voltammograms of cholesterol and cholesteryl ester hydroperoxides. (A) 5 α -OOH (Δ), 6 β -OOH (\square), 7 $\alpha/7\beta$ -OOH (\circ); (B) 25-OOH; (C) CL-OOH. Chromatographic conditions were as described in Fig. 1. Plotted values are means of triplicate injections; maximal deviations were <5%.

anticipated, sensitivity increased with electrolyte concentration for each of the peroxides tested. The responses appear to begin leveling off at ~100 μ M NaClO₄.

3.3. Stability of electrode response

A major disadvantage of an earlier HPLC–ED approach employing a glassy carbon or gold

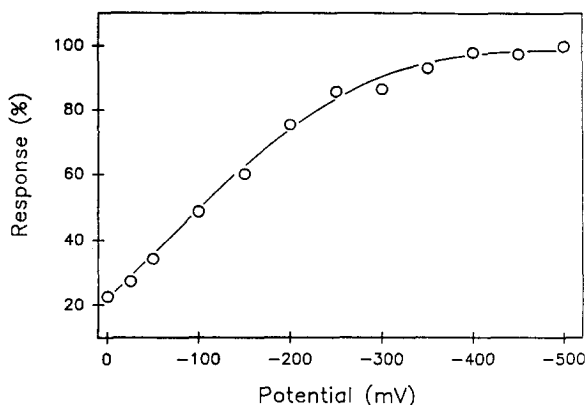


Fig. 3. Hydrodynamic voltammogram of peroxidized 1-palmitoyl-2-oleoyl phosphatidylcholine. Chromatographic conditions were as described in Fig. 1. Plotted values are means of triplicate injections; maximal deviations were $<5\%$.

amalgamate cathode [14,15] was fouling of the electrode surface due to progressive adsorption of reduced analytes. To examine the HPLC-ED(Hg) system in this regard, we checked the within-drop reproducibility of ED signals by repetitively injecting a constant amount of a hydroperoxide standard over a period of 1 h without a drop change. As shown in Fig. 5 for CL-OOH (which is representative of all peroxides examined), no significant reduction of signal strength occurred over this time frame. The standard deviation for the within-drop response shown was $<2.8\%$. The implication of these results is that electrode sensitivity for analytes in a complex peroxide mixture is maintained over at least a 1-h separation period.

3.4. Calibration, linearity, and limit of detection

For all hydroperoxide standards, a linear relationship between amount of analyte injected and detector response was observed over at least a 1000-fold concentration range. The analytical parameters are represented in Table 2. Our approach was validated by performing replicate analyses (0.1 nmol of any given standard per injection; $n = 5$). Reproducibility was reasonably good for all compounds, the standard deviations being $<6\%$.

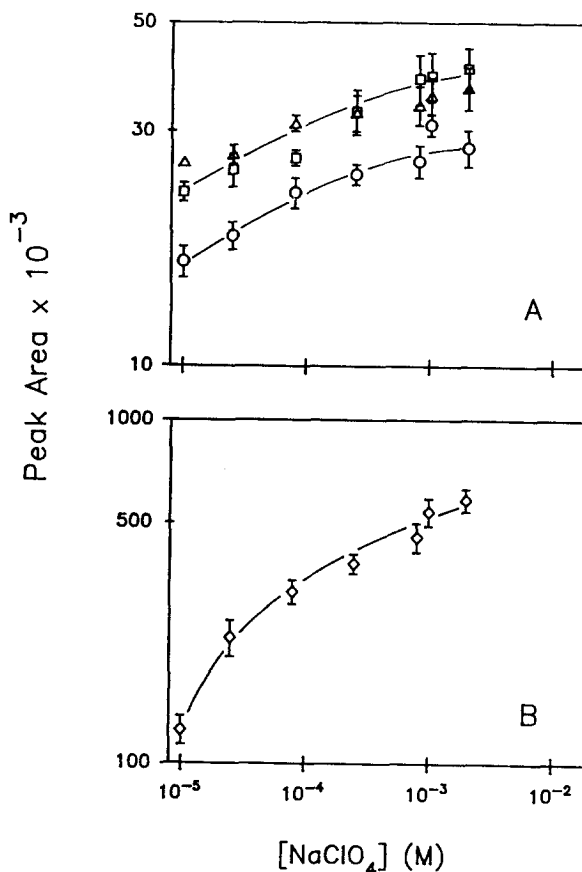


Fig. 4. Effect of mobile phase sodium perchlorate concentration on detector response of different lipid hydroperoxides. (A) Cholesterol hydroperoxides: 5α -OOH (Δ); 6β -OOH (\circ); $7\alpha/7\beta$ -OOH (\square). (B) Phospholipid hydroperoxides: POPC-OOH (\diamond). Mobile Phase I with varying NaClO_4 concentration was used for each separation; flow-rate was 1.7 ml/min. The operating potential was -300 mV.

3.5. Recovery and reproducibility

The recovery of added hydroperoxides was ascertained by adding known amounts of various standards to Ch-DMPC-DCP (1:1:0.5, mol/mol) liposomes or to a suspension of murine L1210 cells ($2 \cdot 10^6$ /ml). In both systems, the mean extraction and processing recoveries for CL-OOH, cholesterol hydroperoxides, and phospholipid hydroperoxides ranged from 94% to 103%. Slightly lower values were observed for fatty acid hydroperoxides. Results of the cell experiments are summarized in Table 3.

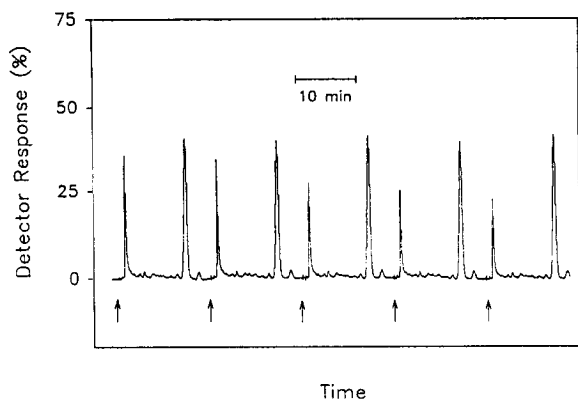


Fig. 5. Stability of detector response during repetitive injections of cholesteryl linoleate hydroperoxide. A single mercury drop set at -150 mV vs. Ag/AgCl was used. Time points for five injections are indicated with arrows. Full-scale sensitivity was 20 nA. Mobile Phase II was used at a flow-rate of 1.0 ml/min.

4. Discussion

Many different HPLC-based methods for determining LOOHs have been described in recent years [8–18]. These generally provide more specificity and sensitivity than methods in which total hydroperoxide content is measured. The peroxide detection limit for a “bulk” method such as the iodometric assay is ca. 1 nmol/ml [15]. Use of HPLC in conjunction with post-column reactions that generate chemiluminescence (CL) signals lowers the detection limit to

0.5–1.0 pmol for fatty acid- and lipid-derived hydroperoxides, but is technically quite demanding in requiring postcolumn manipulations that need to be carefully standardized [10–12]. Moreover, sample complexity and the presence of antioxidants that interfere with the CL reaction can pose quantitation problems, especially for peroxides with retention times similar to those of the interfering compounds. Antioxidants that give negative responses in the HPLC–CL approach (e.g. α -tocopherol, ascorbate) are ED-silent and, thus do not interfere with ED detection. An additional advantage of ED over CL detection is that it does not require postcolumn reactions for signal development; thus, potential problems associated with mobile-phase incompatibility, mixing inaccuracy and reagent instability are avoided, or at least minimized. Detection limits for both methods are exceedingly low, but the use of reversed-phase vs. normal-phase chromatography can make a difference. HPLC–CL is compatible with both normal- and reversed-phase solvent systems [10–12], whereas HPLC–ED(Hg) requires the high polarity of reversed-phase. In the case of phospholipid hydroperoxides, this puts HPLC–CL at an advantage over HPLC–ED(Hg), since these species elute relatively slowly by reversed-phase and appear as broad peaks. This makes quantitation more difficult and results in relatively high detection limits. On the other hand, cholesterol and

Table 2
Linear regression analysis and detection limits for various lipid and fatty acid hydroperoxides

Analyte	Range (pmol)	Slope ($\times 10^{-3}$) ^a	y-Intercept ($\times 10^{-3}$)	Detection limit (pmol) ^b
5 α -OOH	0.4–850	2.35 \pm 0.04	1.2	0.2
6 β -OOH	0.7–1200	1.90 \pm 0.05	6.6	0.3
7 α /7 β -OOH	0.3–650	2.55 \pm 0.04	–0.8	0.1
CL-OOH	0.1–10000	3.92 \pm 0.04	1.1	0.1
25-OOH	0.2–4000	1.22 \pm 0.04	–5.0	0.1
POPC-OOH	30–700	1.10 \pm 0.05	–16.7	30
PLPC-OOH	20–700	1.09 \pm 0.05	15.1	20
Ole-OOH	0.1–1000	1.80 \pm 0.09	–0.7	0.1
Lin-OOH	0.1–1000	2.62 \pm 0.10	–0.6	0.1

^a Values are means \pm S.E. ($n = 9$); correlation coefficient was > 0.999 for each slope.

^b Measured at a signal-to-noise ratio of 3.

Table 3
Recovery of hydroperoxide standards after addition to a cell suspension

Analyte	Added amount ^a (nmol)	Observed amount ^b (nmol)	Recovery (%)
5 α -OOH	0.7	0.7 \pm 0.05	100
6 β -OOH	0.7	0.7 \pm 0.06	100
7 α /7 β -OOH	0.7	0.7 \pm 0.06	100
25-OOH	1.0	1.03 \pm 0.10	103
CL-OOH	0.7	0.68 \pm 0.05	97
POPC-OOH	2.0	1.88 \pm 0.10	94
Ole-OOH	1.0	0.89 \pm 0.10	89

^a Each lipid or fatty acid hydroperoxide (in 20 μ l of 2-propanol) was added to a 0.5-ml suspension of L1210 cells ($2 \cdot 10^6$ /ml) in 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.4) containing 50 μ M desferrioxamine, and extracted with 0.8 ml of chloroform-methanol (2:1, v/v). A 0.4-ml sample of the organic phase was recovered, dried under argon, resuspended in 50 μ l of 2-propanol and analyzed by HPLC-ED(Hg).

^b Mean \pm S.E. ($n = 5$).

cholesteryl ester hydroperoxides elute from ODS columns as sharp, symmetric peaks, and can be detected by ED(Hg) at levels considerably lower than those reported for the CL approach [11,12]. This might be of great advantage when trace amounts of cholesterol-derived hydroperoxides in lipoproteins or oxidatively stressed cells are to be analyzed. As an illustration, the HPLC-ED(Hg) detection limit for cholesteryl ester hydroperoxide, the most abundant LOOH class in low-density lipoprotein, is ca. 0.1 pmol [21]. This represents at least a 20-fold-greater sensitivity than that reported for HPLC-CL [22].

The detector response for the CL and ED(Hg) modalities is a function of hydroperoxide concentration and yield of the analytical reaction. This yield can be quite variable for different molecular species. Thus, for accurate quantitation, standardization is necessary for each molecular species analyzed. This would be difficult, if not impossible, for poorly resolved peroxides, which often elute as composites of several molecular species. We have recently shown that phosphatidylcholine hydroperoxides, including POPC-OOH, PLPC-OOH, and species derived from natural sources (egg yolk, leukemia cells) exhibit almost identical ED(Hg) responses [17]. This suggests that at least for phosphatidylcholine hydroperoxides in complex samples, quantitation

based on the amperometric behavior of simple standards is justified.

In earlier work involving reductive-mode HPLC-ED for LOOH analysis, we [15] and others [13,14] used a glassy carbon indicator electrode. A major disadvantage of the glassy carbon electrode is progressive loss of responsiveness due to analyte deposits and deterioration of the electrode surface. We have now determined that under normal ED(Hg) operating conditions, several samples can be injected consecutively on a single mercury drop with no measurable loss of detector sensitivity. Nevertheless, when analyzing complex samples [17,18], we customarily dispense a new drop before each injection. This avoids any possible loss of responsiveness, but does not prolong analysis unduly, since reequilibration time is reasonably short. With a scrupulously deoxygenated mobile phase, the mercury drop equilibrates within 2–3 min, i.e. background current drops to less than 1 nA within this period. A carefully deoxygenated system is absolutely necessary for high-sensitivity measurements at low operating potentials (< -200 mV). However, examination of current-potential profiles for several hydroperoxide families has revealed that fatty acid and cholesteryl ester hydroperoxides are unique in being relatively insensitive to applied potential up to approxi-

mately 0 mV vs. Ag/AgCl. This would constitute an advantage for routine analysis of fatty acid or cholesteryl ester hydroperoxides in plasma samples, since O₂ can be present when working at relatively high potentials.

We have demonstrated in this and preceding [17,18,21] work that HPLC–ED(Hg) is an extraordinarily sensitive and precise approach for analyzing a wide variety of LOOHs. As it continues to be developed and refined, this technique should prove to be extremely valuable for assessing LOOH status in cells, tissues and biological fluids.

Acknowledgements

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