Chemiluminescent Determination of Cholesterol Hydroperoxides in Human Erythrocyte Membrane

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ABSTRACT: A method for separating, detecting, and quantifying cholesterol hydroperoxide (Ch-OOH) based on extraction, purification by solid-phase extraction cartridge, high-performance liquid chromatography with chemiluminescent detection (HPLC-CL), and liquid chromatography–mass spectrometry has been developed for human erythrocyte membrane. We prepared standard compounds of the cholesterol 5α-, 7α-, and 7β-hydroperoxides (Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH). An octyl silica column with methanol/water/acetonitrile 89:9:2 (by vol) as eluent was used to determine Ch-OOH. HPLC-CL that incorporated cytochrome c and luminol as the post-column luminescent reagent was used. We also investigated the optimal assay conditions and how to prevent formation of artifact Ch-OOH. Analysis of erythrocyte membranes from seven healthy volunteers identified Ch 7α-OOH and Ch 7β-OOH, but not Ch 5α-OOH, as commonly occurring components. The respective mean concentrations of Ch 7α-OOH and Ch 7β-OOH were 2.5 ± 1.6 and 5.4 ± 3.5 pmol/mL blood.
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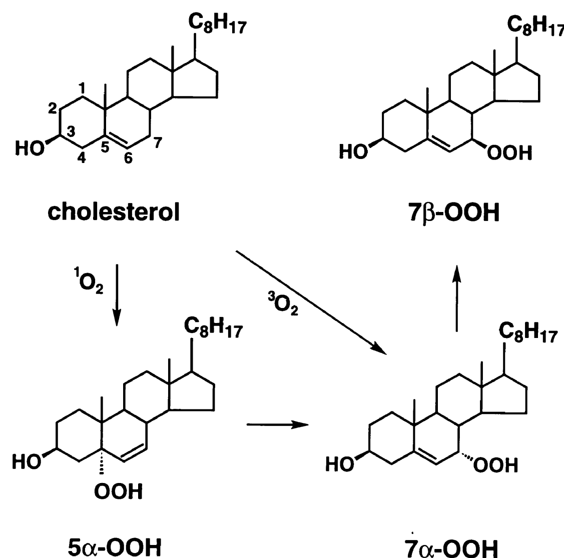
Lipid peroxidation has been linked to a number of pathological conditions and diseases, including ischemia–reperfusion injury, inflammation, and atherosclerosis. It has been monitored on the basis of the formation of thiobarbituric acid-reacting substances (1) and conjugated diene determination (2), but these lack specificity.

Highly sensitive, specific methods for direct measurement of lipid peroxides have now been developed. Phospholipid hydroperoxide levels have been determined in human blood plasma (3) and human red blood cells (4) by high-performance liquid chromatography with chemiluminescent detection (HPLC-CL). Cholesterol ester hydroperoxide in human blood plasma has been analyzed by HPLC-CL (5) and by HPLC with coulometric detection (6).

The possible production of cholesterol hydroperoxide from cholesterol is outlined in Scheme 1. Peroxidation of cholesterol can be induced by such active oxygen species as singlet oxygen, producing cholesterol 5α-hydroperoxide (Ch 5α-OOH) as the first step, then rearrangement of the hydroperoxide giving cholesterol 7α-hydroperoxide (Ch 7α-OOH), and finally epimerization of Ch 7α-OOH giving cholesterol 7β-hydroperoxide (Ch 7β-OOH) (7). In contrast, cholesterol may be autoxidized, producing cholesterol 7-hydroperoxide Ch 7-OOH (8). Ch 7α-OOH and Ch 7β-OOH were detected in the rat by HPLC-CL (9, 10), and in humans were found by HPLC combined with electrochemical detection from photooxidized erythrocyte ghosts (11), but not from the human erythrocyte ghost itself.

We report a sensitive, simple method for determining cholesterol hydroperoxides (Ch-OOH) in erythrocyte membranes



SCHEME 1

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Abbreviations: 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; BHT, 3, 5-di-*tert*-butyl-4-hydroxytoluene; Ch-OOH, cholesterol hydroperoxides; Ch 5α-OOH, cholesterol 5α-hydroperoxide; Ch 7α-OOH, cholesterol 7α-hydroperoxide; Ch 7β-OOH, cholesterol 7β-hydroperoxide; CL, chemiluminescent, chemiluminescence; DMF, dimethylfuran; HPLC-CL, high-performance liquid chromatography with chemiluminescent detection; IS, internal standard; LC–MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; PCOOH, phosphatidylcholine hydroperoxide; UV, ultraviolet.

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of healthy volunteers using HPLC-CL and liquid chromatography–mass spectrometry (LC–MS) with an atmospheric pressure chemical ionization interface to identify Ch-OOH. Furthermore, to prevent formation of artifact Ch-OOH during extraction, we examined purification using a solid-phase cartridge, and the influences of light and antioxidants.

MATERIALS AND METHODS

Materials. Cholesterol was obtained from Sigma (St. Louis, MO). 3,5-Di-*tert*-butyl-4-hydroxytoluene (BHT), luminol (3-aminophthaloylhydrazine), and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH were synthesized as follows: A solution of cholesterol (960 mg) and rose bengal (10 mg) in pyridine (200 mL) was irradiated with a halogen lamp at 10°C with oxygen bubbling for 8 h. Subsequent evaporation of pyridine gave a crystalline residue, of which half was purified by medium-pressure column chromatography (silica gel) and recrystallized from benzene to give Ch 5α-OOH, m.p. 145–148°C. The product had an ^{1}H nuclear magnetic resonance (NMR) spectrum identical to that of an authentic sample (7). The remaining half of the crystals was dissolved in chloroform, and the resultant solution was stirred at room temperature for 3 d. Evaporation of the solvent gave a residue which was purified by medium-pressure column chromatography (silica gel), then recrystallized from ethyl acetate, affording Ch 7α-OOH, m.p. 144–147°C, and Ch 7β-OOH. Although Ch 7β-OOH was obtained as a 6:1 mixture with the 7α-isomer, both compounds had ^{1}H NMR spectra identical to those of authentic samples (12).

β-Sitosterol 5α-hydroperoxide was prepared by irradiating a solution of β-sitosterol (400 mg) and hematoporphyrin (7 mg) in pyridine (208 mL) with a high-pressure mercury lamp through a Pyrex filter at 10°C with oxygen bubbling for 9 h. The crude products were purified and recrystallized from ether to give β-sitosterol 5α-hydroperoxide. The product had a characteristic ^{1}H NMR spectrum as in the case of Ch 5α-OOH. β-Sitosterol 5α-hydroperoxide was the internal standard (IS).

HPLC-CL analysis. Ch-OOH were determined by reverse-phase HPLC with post-column chemiluminescent (CL) detection: HPLC was done in a column of TSK gel Octyl-80Ts (150 × 4.6 mm, i.d.) using an LC-10AD vp pump (Shimadzu, Kyoto, Japan). The column was kept at 40°C and flushed with methanol/water/acetonitrile (89:9:2), the mobile phase, at the flow rate of 0.7 mL/min. The premixed mobile phase was degassed by 5 min of sonication before use. A Rheodyne 7125 injector (100 μL, Cotati, CA) was used to inject the sample solution into the column. After passage through a SPD-6A spectrophotometric detector (Shimadzu) set at 210 nm, the eluate was mixed with a luminescent reagent in the post-column mixing joint, at the controlled temperature of 37°C, of a CLD-10A CL detector (Shimadzu). The luminescent reagent, prepared by dissolving cytochrome c and luminol in alkaline borate buffer (pH 10), was loaded with an LC-10AD vp pump at the flow rate of 0.5 mL/min. The CL generated by the reaction of the hydroperoxide with the luminescent reagent was measured with a CL detector.

The concentrations of cytochrome c and the luminol prepared, respectively, 10 and 2 μg/mL, were the same as those employed by Miyazawa *et al.* (13,14). The luminescent solution was 20 mM $H_3BO_3 \cdot Na_2CO_3$ buffer at pH 10. The effect of the flow rate of this reagent was examined between 0.2 and 0.8 mL/min. The injection volume and supply voltage also were examined in terms of the hydroperoxide-dependent CL intensities.

Standard curves were prepared by the analyses of 1, 2, 4, and 10 ng (2.39, 4.78, 9.58, and 23.9 pmol) of Ch 5α-OOH and of Ch 7β-OOH, and 0.5, 1, 2, and 5 ng (1.20, 2.39, 4.78, and 12 pmol) of Ch 7α-OOH with 2.5 ng of the IS. Individual peak areas were calculated with an integrator (Chromatopac C-R4A; Shimadzu). The ratios of the hydroperoxides to the IS also were calculated for the standard compounds and lipid extracts of the specimens. The recoveries from the sample extracts were determined by comparison of the peak areas obtained after injection of a sample extract spiked with a known concentration. The recoveries of Ch 7α-OOH and IS varied from 55 to 65%.

HPLC–MS analysis. An L-7000 series (Hitachi, Tokyo, Japan) liquid chromatography system fitted with spherisorb ODS-2-5 (250 × 4.6 mm, i.d.) and a model M-1200AP LC–MS system that incorporated an atmospheric chemical ionization system (Hitachi) were used. The mobile phase, methanol containing 0.1 M ammonium acetate or methanol alone, was delivered at the flow rate of 0.7 mL/min. By adding ammonium acetate to the methanol as the mobile phase, an ion appeared at *m/z* 401 so that Ch-OOH were distinguishable from the 7-hydroxycholesterols as shown in Figure 1, although most had similar retention times. Application parameters for the mass spectrometer were positive-ion measurement mode, a nebulizer temperature of 170°C, a desolvator temperature of 400°C, and a needle-electrode voltage of 3000 V.

Artifact formation. Two milligrams of cholesterol was extracted twice with 9 mL chloroform/methanol (2:1) containing 0.005% (50 ppm) BHT by the modified method of Folch *et al.* (15). The combined chloroform layer was concentrated in a rotary evaporator, then dried under a nitrogen stream. The residue was dissolved in 1 mL of methanol and a 10 μL portion injected into the HPLC column (Method A). A silica column (Bond Elut® Varian, Harbor City, CA) of 3-mL capacity, containing aminopropyl- derivatized silica ($-NH_2$) as packing material, was conditioned by washing it with 5 mL of acetone and 10 mL of *n*-hexane, after which 2 mg of cholesterol in a small amount of chloroform was passed through, followed by elution with a mixture of 2 mL chloroform and 1 mL isopropanol. The eluate was concentrated, and the residue subjected to HPLC (Method B), or after concentration it was passed through another Bond Elut® column with elution using 3 mL of 10% of ethyl acetate/*n*-hexane. The eluate was concentrated and the residue subjected to HPLC (Method C). When two cartridges of Bond Elut® were conditioned, 0.1%

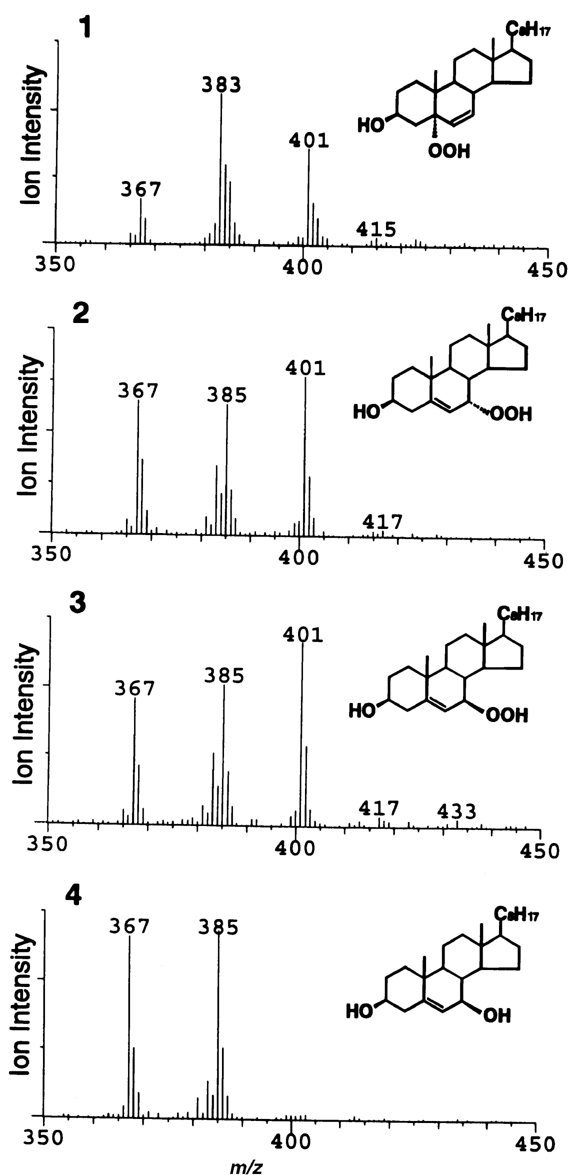

FIG. 1. Mass spectra of the cholesterol 5α-, 7α-, and 7β-hydroperoxides (M = 418) and 7β-hydroxycholesterol (M = 402) were detected by liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface. Methanol containing 0.1 M ammonium acetate was used as a mobile phase.

EDTA · 2Na was used before water and acetone (Method D). Dark test tubes were used during solid extraction with Bond Elut® (Method E). Two milligrams of cholesterol was extracted twice with 9 mL chloroform and methanol containing BHT and dimethylfuran (DMF) to give final concentrations of 10, 20, 50, 100, 400, and 1000 ppm BHT. The concentrations of DMF were 0, 15, 60, and 150 ppm. The chloroform layer was evaporated, and the residue was applied to a Sep-Pak® (Waters, Milford, MA) of 3-mL capacity containing aminopropyl-derivatized silica (-NH_2) packing material (Method F).

Extraction. Seven healthy male volunteers (39.3 ± 12.2 yr) participated in the study. After obtaining their informed consent, venous blood samples were drawn. Five milliliters of blood was collected in a glass test tube containing 0.5 mg EDTA · 2Na, then centrifuged at 4°C and 800 × *g* for 10 min; the erythrocytes were then fractionated. A method for erythrocyte ghost preparation was used (16). IS (200 pmol in 100 µL chloroform) was added to lipids from the white ghosts and extracted twice with 9 mL chloroform and methanol containing 0.005% BHT at room temperature, essentially by the method of Folch *et al.* (15). The combined chloroform layer was concentrated, and the residue applied to Sep-Pak®. Each column was conditioned by washing it with 5 mL of acetone and 10 mL of *n*-hexane. Before the column became completely desiccated, the crude membrane lipids dissolved in a small amount of chloroform were applied and drawn through, followed by elution with a mixture of 2 mL chloroform and 1 mL isopropanol. The eluate was concentrated. The residue was dissolved in 200 µL of methanol, and a 10-µL portion injected to an HPLC column.

Statistical analysis. Mann-Whitney's U-test was used to determine the statistical significance of the difference between group means. *P*-values of <.05 were considered statistically significant.

RESULTS

Figure 2 shows typical HPLC chromatograms obtained with a mixture of standard Ch-OOH and hydroxycholesterols. The 3 Ch-OOH (7β-, 7α, and 5α-OOH) separated when methanol/water/acetonitrile was the mobile phase, as shown by CL, and the two hydroxycholesterols also were separated as seen by ultraviolet (UV). The retention times were Ch 7β-OOH, 6.8 min; Ch 7α-OOH, 7.3 min; Ch 5α-OOH, 7.8 min; IS, 9.5 min; 7β-hydroxycholesterol (7β-OH), 6.6 min; and 7α-hydroxycholesterol (7α-OH), 7.1 min. Because CL detection is specific, the Ch-OOH were distinguishable from peaks 7α-OH and 7β-OH despite the similar retention times. Although Ch-OOH theoretically are detectable by CL and UV, no Ch-OOH peaks appeared on UV because of the small amount used. The CL detector response was about 300 times greater for Ch 7α-OOH than the UV detector response.

The injection volume of Ch 7α-OOH (20 pmol) vs. peak areas (CL intensity) was linear. We usually injected 10 µL of the sample to the HPLC. Standard curves (ratio of the IS vs. the amount of Ch-OOH injected) for Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH were linear. The detection limit for Ch 5α-OOH was 0.7 pmol, that for Ch 7α-OOH 0.3 pmol, and that for Ch 7β-OOH 0.3 pmol at the signal-to-noise ratio of six.

We investigated the effects of the supply of the chemiluminescent detection voltage (–0.7 to –0.9 kV) and the flow rate of the chemiluminescent reagent (0.2 to 0.8 mL/min) on the chemiluminescence response. At the supply voltage of –0.9 kV, Ch 7α-OOH (20 pmol) gave its maximum response. We used –0.7 kV as the supply voltage. At the flow rate of 0.5 mL/min, Ch 7α-OOH (20 pmol) gave its maximum CL response.

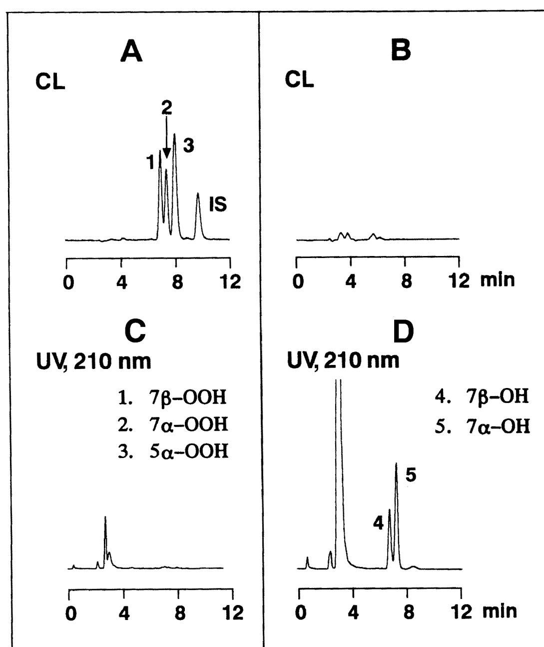

FIG. 2. Chromatographic separation of cholesterol hydroperoxides (Ch-OOH) and corresponding diols in standard mixtures. (A,C) A 10-μL sample containing known Ch-OOH in methanol [2 pmol cholesterol 7α-hydroperoxide (Ch 7α-OOH), 4 pmol cholesterol 5α-hydroperoxide (Ch 5α-OOH), and cholesterol 7β-hydroperoxide (Ch 7β-OOH)]. (B,D) A 10-μL sample containing known hydroxycholesterol in methanol [50 nmol 7α-hydroxycholesterol (7α-OH) and 25 nmol 7β-hydroxycholesterol (7β-OH)]. CL, chemiluminescent detection; UV, ultraviolet detection.

Table 1 compares artifact formation by Methods A to F. Method A, extraction of cholesterol itself by the method of Folch *et al.* (15), did not cause artifact formation, nor did passing cholesterol through one cartridge of Bond Elut® result in artifact formation (Methods B). Passing cholesterol through two cartridges (Method C), however, sometimes caused artifact formation, and formation was prevented neither by washing the cartridge with EDTA · 2Na (Method D) nor by shading it with a dark test tube (Method E). When we used DMF as well as BHT as the antioxidants in the extraction solvent (Method F), to prevent artifact formation 0.1% (1000 ppm) BHT and 0.015% (150 ppm) DMF were necessary. A large negative peak appeared, which disturbed the Ch-OOH peak. When BHT alone was the antioxidant in the extraction solvent (Method F), artifact formation did not occur at any concentration (10 to 100 ppm). We therefore did the extraction with chloroform and methanol containing 0.005% (50 ppm) BHT as the antioxidant, and followed it by purification with Sep-Pak® (Method F).

Figure 3 shows a typical HPLC chromatogram of artifact formation. Peak 1 seemed to be Ch 7β-OOH, peak 2 Ch 7α-OOH, and peak 3 Ch 5α-OOH because the retention times corresponded to these standard compounds. Peaks A, B, and C could not be determined.

Figure 4 shows chromatograms for the erythrocyte sample from a healthy volunteer, a mixture of standard Ch-OOH, and IS. Standard Ch 7β-OOH, Ch 7α-OOH, Ch 5α-OOH, and IS appeared, respectively, at 6.8, 7.3, 7.8, and 9.5 min. The extracts from human erythrocyte membranes contained Ch 7β-OOH and Ch 7α-OOH, but not Ch 5α-OOH. The addition of the standards Ch 7β-OOH and Ch 7α-OOH independently confirmed the peak identities.

The mean levels of Ch 7β-OOH and Ch 7α-OOH in erythrocyte membranes of seven healthy volunteers were 5.4 ± 3.5 (range 1.9–9.2) and 2.5 ± 1.6 (range 1.1–5.3) pmol/mL blood (means ± standard deviations). The concentration of Ch 7β-OOH was not significantly higher than that of Ch 7α-OOH.

DISCUSSION

There are many recent reports of analytical methods for determining phospholipid hydroperoxides and cholesteryl ester hydroperoxides in biological samples, but only a few researchers have given an analytical method for Ch-OOH and showed the presence of Ch-OOH. To develop an analytical

TABLE 1
Artifact formation by Methods A to F[a]

Method	BHT (ppm)	DMF (ppm)	Formation/sample
A: Extraction	50	0	0/2
B: Bond Elut®[b]	0	0	0/3
C: Bond Elut® + Bond Elut®	0	0	5/7
D: EDTA · 2Na + (Bond Elut® + Bond Elut®	0	0	2/4
E: Method D with dark test tube	0	0	2/3
F: Extraction + Sep-Pak®[c]	1000	150	0/6
Extraction + Sep-Pak®	400	60	2/3
Extraction + Sep-Pak®	100	150	2/2
Extraction + Sep-Pak®	100	15	2/3
Extraction + Sep-Pak®	100	0	0/3
Extraction + Sep-Pak®	50	0	0/3
Extraction + Sep-Pak®	20	0	0/2
Extraction + Sep-Pak®	10	0	0/2

[a]Abbreviations: BHT, 3,5-di-tert-butyl-4-hydroxytoluene; DMF, dimethylfuran.
[b]Varian Harbor City, CA.
[c]Waters, Milford, MA.

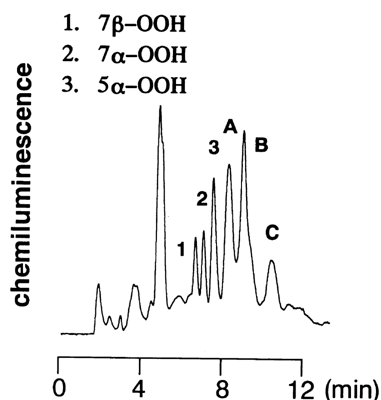

FIG. 3. Typical high-performance liquid chromatograms of artifact formation produced by passing cholesterol in chloroform through two cartridges of Bond Elut®-NH_2. A, B, C, unknown; for abbreviations see Figure 2.

method for Ch-OOH, we first prepared the standard compounds Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH. The standard Ch-OOH were subjected to LC–MS using an atmospheric pressure chemical ionization interface. It is important to confirm the chemical structure of the Ch-OOH, and this has not been previously achieved.

We then investigated the optimal assay conditions for HPLC-CL. We separated Ch 5α-OOH, Ch 7α-OOH, and Ch 7β-OOH from one another in a C8 column with methanol/water/acetonitrile, under similar conditions to those of Korytowski *et al.* (11). They, however, used HPLC combined with electrochemical detection, establishing the detection limit for Ch-OOH as ~25 pmol, so that they could analyze the Ch-OOH generated by photodynamic action, not from erythrocyte ghosts. Because of the report of Zhang *et al.* (17) that the HPLC-CL system employed by Miyazawa *et al.* (13,18,19) and them (20) combined with a cytochrome c–luminol cocktail was 16- to 100-fold more sensitive to phosphatidylcholine hydroperoxide (PCOOH) than the microperoxidase–isoluminol cocktail used by Yamamoto *et al.* (21), we used the cytochrome c–luminol cocktail. Brown *et al.* (22) reported a normal-phase HPLC method with UV detection that could resolve all the cholesterol products oxygenated at the 7-position: 7-ketocholesterol, Ch 7α-OOH, Ch 7β-OOH, 7α-OH, and 7β-OH, but they detected Ch-OOH from the lipid extract of atherosclerotic plaque only in trace amounts because of the low detection limit.

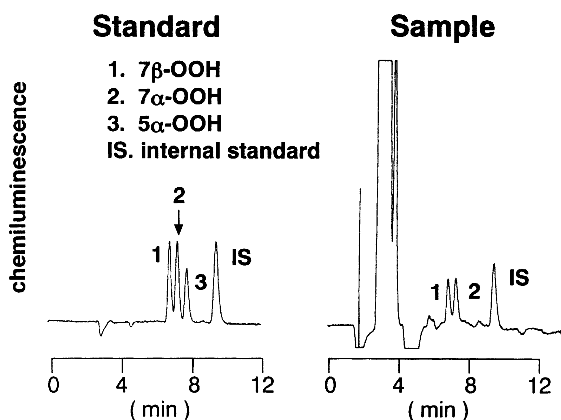

FIG. 4. Chromatograms from high-performance liquid chromatography with chemiluminesent detection for the erythrocyte sample from a healthy volunteer, a mixture of standard Ch-OOH, and the IS.

Lastly, we examined the extraction procedure and some antioxidants to avoid artifact formation. Cholesterol in low density lipoprotein was easily oxidized with the help of the metal ion Cu^{2+} (22,23) or a metal-independent peroxy-radical generated system (AAPH) (22) and yielded Ch 7α-OOH and Ch 7β-OOH. BHT (0.1%) and DMF (0.015%) are reported to be absolutely necessary as antioxidants to prevent artifact formation during the assay process (9,10), but both antioxidants at a high level produced a negative peak, thereby disturbing the Ch-OOH peak. In contrast, we found that the presence of BHT alone was sufficient to prevent artifact formation. Ozawa *et al.* (9,10) purified the lipid extract from rat skin using two Bond Elut® cartridges, but artifact formation might occur on passage through the two cartridges due to metal contaminants. Unknown peak A in the chromatograms of artifact formation (Fig. 3) was assumed to be cholesterol 6β-hydroperoxide based on a comparison with the chromatograms of Korytowski *et al.* (11).

In conclusion, for the first time we detected Ch 7α-OOH and Ch 7β-OOH in the erythrocyte membranes of healthy volunteers. The respective mean concentrations of Ch 7β-OOH and Ch 7α-OOH were 5.4 and 2.5 pmol/mL blood. Healthy human plasma is reported to contain 0.5 μM of PCOOH (18), 3 nM (5,24) and 4.2 nM (25) of cholesterol ester hydroperoxide, and less than 20 nM hydroperoxide of eicosatetraenoic acid (26). Plasma glutathione peroxidase was reactive with linoleic acid hydroperoxide but had a low reducing activity toward Ch 7α-OOH and no detectable activity with the Ch 5α-OOH (27); therefore, PCOOH might be absent but Ch-OOH might be present in human plasma. The concentration of total Ch 7-OOH in rat skin was 20–150 μmol/g skin (7), which was higher than the values we found, probably because lipid peroxides easily accumulate in the skin of the rat.

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