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MEASUREMENT OF LIPID HYDROPEROXIDES IN NORMAL HUMAN BLOOD PLASMA USING HPLC-CHEMILUMINESCENCE LINKED TO A DIODE ARRAY DETECTOR FOR MEASURING CONJUGATED DIENES

ANNE E. HOLLEY† and TREVOR F. SLATER

Brunel University, Department of Biology and Biochemistry, Uxbridge, Middlesex, UB8 3PH, U.K.

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A modification of a method using high-performance liquid chromatography (HPLC) with chemiluminescence (CL) detection for the measurement of lipid hydroperoxides (LOOH) in human blood plasma has been developed. The system involves separation of different classes of LOOH using reverse-phase HPLC, and post-column detection of CL produced by isoluminol oxidation during the reaction of LOOH with microperoxidase. Complete ultra-violet absorption spectra are collected with an in-line diode-array detector and used to confirm a positive CL response due to LOOH, or other compounds, by the presence or absence, respectively, of the LOOH conjugated diene chromophore.

We have used the method to investigate the stability of exogenous 15(S)-HPETE (a hydroperoxide of eicosatetraenoic acid) and conjugated dienes (of both 15(S)-HPETE and its reduced metabolite, 15(S)-HETE) in human plasma stored at various temperatures. A large and rapid loss of the hydroperoxide occurred in plasma incubated at 0°C or 27°C, whereas only a small reduction in the level of conjugated dienes was found. 15(S)-HPETE in PBS was stable under the same conditions, and zero time recovery of the hydroperoxide from denatured plasma and from buffer containing albumin was identical to that of fresh plasma. Our data suggest that the observed temperature-dependent loss of exogenous hydroperoxide from fresh plasma results from a combination of enzymatic degradation to the hydroxy derivative and binding to plasma albumin.

15(S)-HPETE was found to be stable in plasma stored at −70°C for up to 2 weeks and in liquid nitrogen for 3 months in the presence of the antioxidants butylated hydroxytoluene (BHT) and desferal, with no significant loss of conjugated dienes.

KEY WORDS: lipid hydroperoxides, plasma, measurement, HPLC-chemiluminescence, stability.

INTRODUCTION

Lipid peroxidation has long been recognised as an important process in the oxidative degradation of foods and oils. In recent years it has been implicated as a major contributor to several types of toxic cell injury and more recently, suggestions have been made that products of lipid peroxidation have a significant role in the aetiology of a number of human diseases such as atherosclerosis and cancer.[1] Lipid peroxidation not only produces cell membrane disturbances and change in function but also gives rise to many different products that have powerful biological activity. Amongst these products are LOOH, known to affect the prostaglandin cascade and endothelium,

†To whom correspondence should be addressed.

lipid hydroxy acids, with a number of well characterised effects, and 4-hydroxy-alkenals which can inhibit DNA, RNA and protein synthesis and in relatively high concentration are cytotoxic.[2]

Various methods have been developed to measure LOOH in biological samples where the concentration can be expected to very low (less than 1 μM). These techniques include iodine liberation,[3] CL,[4,5] peroxidase[6] and cyclooxygenase activity.[7,8] Of these, methods combining HPLC and CL are the most advantageous with respect to pmol sensitivity and specificity for LOOH, and additionally allow determination of different classes of hydroperoxides. However, some complications have been reported[9,10] that make it important to identify CL with conjugated diene structure. This paper describes an HPLC-CL method to quantitate plasma LOOH based on that originally reported by Yamamoto *et al.*[4] in which CL is generated by isoluminol oxidation during reaction of hydroperoxides with microperoxidase – a haem peptide obtained by proteolysis of cytochrome C. The present method includes a different procedure to extract the hydroperoxides from plasma using antioxidants to prevent artifactual peroxidation. In addition, a diode-array detector has been incorporated to collect complete ultra-violet absorption spectra, rather than using single wavelength measurements. This enables us to obtain more precise information on conjugated diene absorption of the lipid hydroperoxides and hydroxy derivatives, which is found around 233–240 nm, and to confirm that a positive CL response is due to LOOH and not from other compounds which have been reported to react in this system.[9]

Plasma has very effective defence mechanisms against the accumulation of LOOH, and the recovery of exogenous free fatty acid hydroperoxides (FFAOOH) from plasma is reported to be low.[10] We have thus investigated the effect of temperature and storage conditions on 15(S)-HPETE added to fresh plasma, since this may have important implications in studies on plasma LOOH in both normal and diseased states.

MATERIALS AND METHODS

Materials

BHT, desferal, heparin, isoluminol, microperoxidase (MP-II) and all other chemicals of the highest grade available were purchased from Sigma (Poole, Dorset). HPLC grade methanol (MeOH) and hexane, and LiChrosorb RP18 HPLC column (4.6 × 250 mm) were obtained from BDH (Poole, Dorset). 2,2′-azobis (2,4-dimethyl-valeronitrile) (AMVN) and 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) were from Polysciences.

LOOH Standards

5(S)-, 12(S)- and 15(S)-HPETES were purchased from Cascade Biochem Ltd. (Reading, Berks) Cholesterol 5α and 7α-hydroperoxides were a kind gift from Professor L. L. Smith (Texas) and other LOOH standards were prepared by autooxidation with the azo-initiators AMVN and AAPH as described by Yamamoto *et al.*[11] except that liposomes were prepared by ultrasonication. Reduction of hydroperoxides to the hydroxy derivative with sodium borohydride ($NaBH_4$) was carried out as described by Thomas and Pryor.[12]

Preparation of CL Reagent

Isoluminol (1 mM) in MeOH/0.1 M sodium borate buffer, pH 10 (30 : 70, v/v) was prepared at least 2 days before use, to allow reduction of intrinsic CL. The solution was then added to microperoxidase freshly dissolved in 10 mM TMS, pH 7.4, to a final concentration of 1 μg MPx/ml, and filtered through a millipore 0.22 μm membrane prior to use.

HPLC Equipment

A schematic diagram of the HPLC-CL system for the assay of LOOH is shown in Figure 1. LOOH were separated by reverse phase HPLC using an eluting solvent of MeOH containing 0.02% triethylamine. UV absorption of the column effluent was monitored with a Philips 4021 Diode Array Detector set to scan between 190 and 390 nm (1.5 nm resolution). Data was analysed by an IBM computer system equipped with Philips PU 6003 analytical software. Standard curves were prepared by chromatographing known amounts of 15(S)-HPETE and measuring peak height at the absorption maximum.

After passing through the UV detector, the column eluate was mixed with the CL reagent via a T-joint and incubated in a 15 sec delay coil. The CL generated by

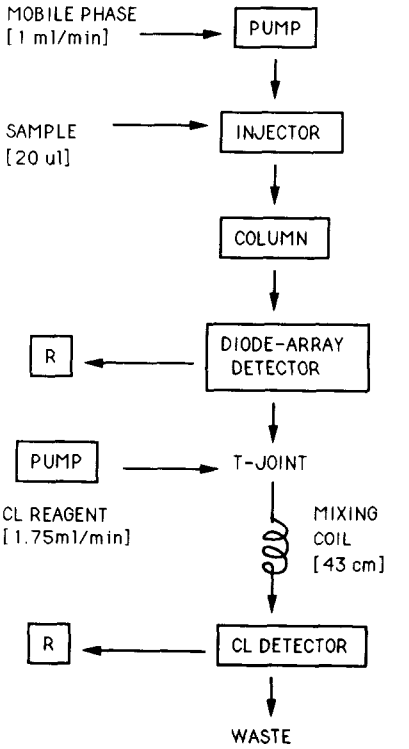

FIGURE 1 Schematic diagram of the HPLC-CL system for the assay of lipid hydroperoxides. [a]R = recorder and data handling system.

reaction with hydroperoxides was monitored with a B-RAM radio-HPLC monitor (Lablogic, Cambridge), equipped with a 200 μl spiral liquid flow cell inserted between 2 photomultiplier (PM) tubes. Since background CL (due to trace amounts of hydroperoxides in HPLC eluate and reaction solution) is high, photon emission was measured with both PM tubes in coincidence and followed with the lower energy counting channel. As peak areas are related to background CL, the counting windows were altered to adjust background counts to 1000 cpm. CL emission was analysed by a Walters computer system with B-RAM analytical software.

Standard curves were prepared by chromatographing known amounts of 15(S)-HPETE, and cholesterol 5α- and 7α-hydroperoxides and integrating CL counts of the peak area after subtraction of background counts.

Extraction of Plasma LOOH

Blood (10 ml) from 2 healthy 29 year old volunteers was taken between 10 and 11.00 am from the ante cubital vein. The blood was added to heparin (1000 IU) and 10 μl each of aqueous BHT and desferal to give final concentrations of 20 μM, and immediately centrifuged (3000 rpm, 15 min, 4°C). All procedures were carried out on ice, and samples protected from light. Plasma (0.5 ml) was acidified to pH 3.3–3.4 with 0.25 ml 0.2 M citric acid and LOOH extracted with 6 ml *n*-hexane. After centrifugation (3000 rpm, 5 min) the upper hexane phase was removed and the residual aqueous phase was washed with 3 ml hexane. The combined hexane phases were evaporated under nitrogen and stored at −70°C until analysis. Dried extracts were dissolved in 100 μl MeOH and 20 μl was subjected to HPLC.

Stability of LOOH in Plasma

20 μl of 15(S)-HPETE in EtOH was added to 0.5 ml aliquots of plasma to give a concentration of 11.76 μM, and samples were incubated at 0°C or 27°C. At the time points indicated, the plasma was extracted with acidified hexane, as above. The zero-time value was obtained by adding 20 μl of the hydroperoxide solution to a mixture consisting of 0.5 ml plasma, 0.25 ml citric acid and 6 ml hexane.

For investigation of stability of 15(S)-HPETE during storage, plasma aliquots were frozen at −70°C or −196°C immediately after addition of 15(S)-HPETE (final concentration 5.88 μM).

RESULTS

Response of LOOH and Antioxidants

Figure 2 shows the calibration curve of CL counts integrated for peak area of 15(S)-HPETE obtained by the HPLC-CL system. Despite reports of a linear relationship between amount of hydroperoxide and CL response for linoleic acid hydroperoxide and phosphatidylcholine hydroperoxide[4,5] we obtained non-linear curves (Figure 2) for 15(S)-, 5(S)- and 12(S)-HPETE, cholesterol 5α- and 7α-hydroperoxide. The relative sensitivity for each hydroperoxide (defined as peak area/pmol hydroperoxide) differed (see legend to Figure 2) and the detection limit was 2 pmol for 15(S)-HPETE, which corresponds to a plasma concentration of 0.02 μM.

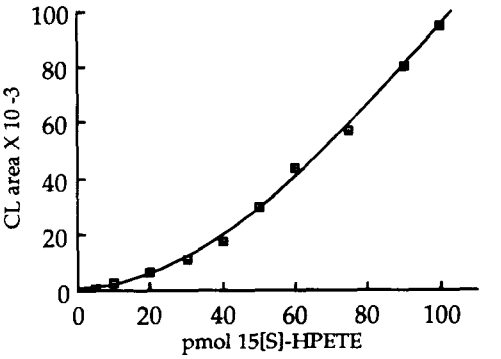

FIGURE 2 Calibration curve of 15(S)-HPETE in the CL assay. Similar curves were obtained for the different hydroperoxides examined. Relative sensitivity of hydroperoxides based on that of 15[S]-HPETE were: 15[S]HPETE (1.00); 12[S]-HPETE (0.65); 5[S]-HPETE (1.12); cholesterol 5α-hydroperoxide (0.93); cholesterol 7α-hydroperoxide (1.12).

The HPLC-CL system separated different classes of LOOH (Table I) and examination of UV spectra for the presence of conjugated dienes was used to confirm their identity. Although the system did not separate the various FFAOOH from each other, examination of UV absorption spectra can differentiate between arachidonate and linoleate hydroperoxides. Additional verification of LOOH was obtained by reduction to the hydroxy derivatives with $NaBH_4$, which resulted in loss of CL response, but not of conjugated diene absorption (not shown).

Table I also shows that compounds other than LOOH can produce a positive CL response. However, examination of UV spectra can verify their identity.

Antioxidants produce a negative peak in the HPLC-CL system by quenching background CL. Plasma antioxidants such as ascorbate and urate do not interfere at physiological concentrations since they are not extracted into the hexane phase, and α-tocopherol is separated from hydroperoxides (Table I). The antioxidant BHT and iron-chelator desferal were chosen for addition to plasma to prevent the formation and degradation respectively, of LOOH since they do not interfere with the assay.

Plasma Extracts

Studies on the effect of the antioxidants BHT and desferal on the measurement of both endogenous and exogenous LOOH showed that the optimal concentration of these antioxidants was 20 μM. Higher concentrations of BHT interfered with the CL assay by producing a very large negative peak. In the absence of the antioxidants, small amounts of an unidentified component was sometimes found in plasma extracts. This component eluted at about 4 min in the CL assay but no corresponding UV absorption could be detected (not shown). The CL peak was also found when plasma extracts supplemented with 15(S)-HPETE were dried under vacuum on a rotary evaporator but not when the extracts were N_2 dried (both in the presence of BHT and desferal). In addition, rotary evaporation resulted in a lower recovery of 15(S)-HPETE compared to N_2 drying (not shown). These observations suggest artifactual production of some component, possibly from 15(S)-HPETE, which can be prevented by the addition of antioxidants to plasma and drying the extracts under N_2.

TABLE I
Retention Times (T_R) of hydroperoxides, antioxidants and other compounds as monitored by CL and corresponding UV maxima

Compound	T_R (min)	λ max (nm)
LOOH		
5(S)-HPETE	1.54	239
12(S)-HPETE	1.52	239
15(S)-HPETE	1.50	239
18:2 OOH	1.57	234
H_2O_2	NE[b]	–
Chol 5α-OOH	5.28	–
Chol-7α-OOH	5.07	–
PCOOH[a]	3.10; 4.30	233; 235
PEOOH[a]	2.44; 2.14	235
PSOOH	1.88	
Ch18:4	32.23	237
Ch 18:2	37.04	233
TG 18:2	21.10; 8.56	234; 235
Antioxidants		
Ascorbate	2.04	272
GSH	NE	–
Urate	2.01	241, 296
α-Tocopherol	8.40	228, 290
BHA	3.04	231, 294
BHT	3.37	224, 282
Desferal	NE	–
EDTA	NE	–
Trolax	2.03	222, 295
Other Compounds		
B carotene	1.58; 2.42	–; 224
Omnipaque[c]	2.45	246
Naproxen	–	235
$NaBH_4$	4.43	No UV absorption
Ubiquinone 50	2.43; 7.09	No UV absorption

18:2 OOH = Linoleic acid OOH, PC-, PE-, and PS-OOH = phosphatidyl-choline, -ethanolamine and -serine hydroperoxide, respectively, Ch 18:4 = cholesterylarachidonate OOH, Ch 18:2 = cholesterylinoleate OOH and TG 18:2 = trilinolein OOH.

[a]The oxidation of PC, PE and TG 18:2 gave 2 hydroperoxide fractions. The T_R of the major fraction is given first.

[b]Not eluted.

[c]Dye used for X-ray contrast in angioplasty operations.

We used the method to analyse the LOOH profile of plasma from 16 normal healthy subjects, and of some hospitalized patients. LOOH were undetectable in control plasma i.e. if present, the concentration was less than 0.02 μM, but were found at levels between 0.02 and 3.30 μM in patients with adult respiratory distress syndrome[13] and up to 0.92 μM in angioplasty patients.[14] In each of these groups, both FFA- and cholesterol-hydroperoxides were detected.

Figure 3 shows a representative recording of the CL of an extract of normal plasma supplemented with 15(S)-HPETE as an internal standard. The trace shows that the method separates 15(S)-HPETE (which appears as a positive peak) from the

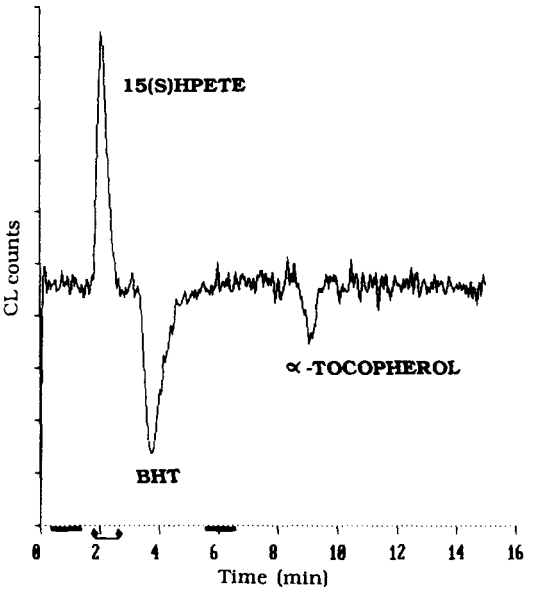

FIGURE 3 HPLC-CL trace of an extract of normal plasma supplemented with 15[S]HPETE.

antioxidants BHT and α-tocopherol (which produce negative peaks). Figure 4 shows the UV absorption data for the same sample. The conventional chromatogram recorded at 236 nm (Figure 4a) shows that 4 components appear to have conjugated diene absorption (at retention times of 1.30, 2.40, 2.58 and 3.57 min). The corresponding contour plot recorded between 200 and 300 nm (Figure 4b), however, shows that the absorption maxima of these components are in fact 236, 231, 222 and 230 nm respectively, and that the chromatogram at 236 nm (Figure 4a) is detecting tailing absorption of these peaks. The data which can be obtained from a contour plot demonstrate the advantage of the diode-array detector over single wavelength monitoring for conjugated diene absorption to verify that a CL peak is actually due to a hydroperoxide.

Stability of LOOH with Temperature

Data in all experiments have been expressed as a % of zero-time recovery of 15(S)-HPETE or conjugated dienes added to plasma. Zero time recovery of 15(S)-HPETE was 36.9 ± 1.1% (n = 7) for subject 1 and 39.4 ± 3.8 (n = 6) for subject 2. Zero time recovery from biffer was 80% (see below).

The effect of temperature on 15(S)-HPETE added to fresh plasma containing BHT and desferal is shown in Figure 5. The hydroperoxide was very rapidly lost at 0°C, with a 50% reduction in the amount recovered after 10 s. Further incubation resulted in a more gradual loss of 15(s)-HPETE, with 72% degraded by 60 min. Degradation was greater at 27°C with 93% loss of the added 15(S)-HPETE by 60 min. The hydroperoxide was very stable in PBS at 0°C for up to 60 min although some loss occured at 27°C (14.8 ± 2.2% at 60 min) indicating that its loss in plasma was not due to chemical instability.

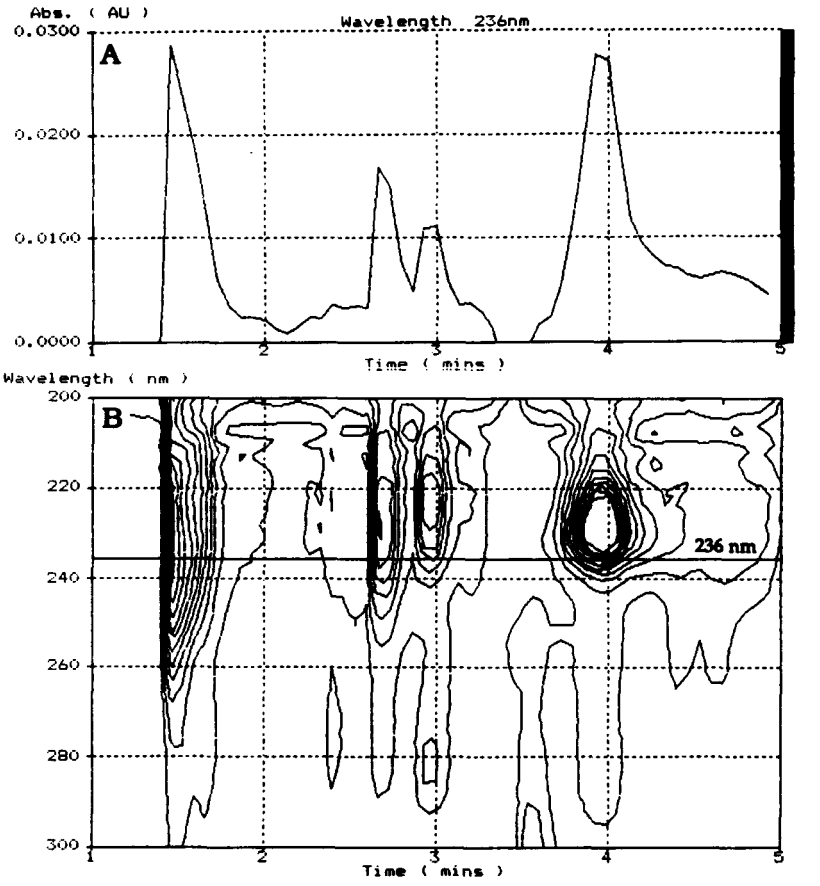

FIGURE 4 HPLC of an extract of normal plasma supplemented with 15[S]-HPETE. Figure 4a is a chromatogram of the sample recorded at 236 nm, while figure 4b is a counter plot of the same sample scanned and recorded between 200 and 300 nm, showing absorption maxima of the 4 components at 236, 231, 222 and 230 nm.

In contrast, conjugated dienes were found to be more stable in plasma. Levels decreased by approximately 15% after 10 sec at 0°C, but no further loss was observed with time. Interestingly, levels increased to zero time values after 60 min at 0°C, and even higher levels were found after 15–60 min incubation at 27°C.

The temperature-dependent loss of 15(S)-HPETE and also the low zero-time recovery values from fresh plasma compared to Tris buffer (approximately 80%) prompted us to investigate the recovery of hydroperoxides from denatured plasma. No significant difference was found between the recovery of either 15(S)-HPETE or cholesterol 7α-hydroperoxide added to fresh plasma compared to plasma heated for 30 min at 70°C (Table II). This suggested the low zero time plasma recovery was not due to enzymatic degradation. Indeed, we found reduced 10 sec recovery compared to zero time values with heated plasma which were of a similar magnitude to that observed with fresh plasma (not shown). We subsequently demonstrated that

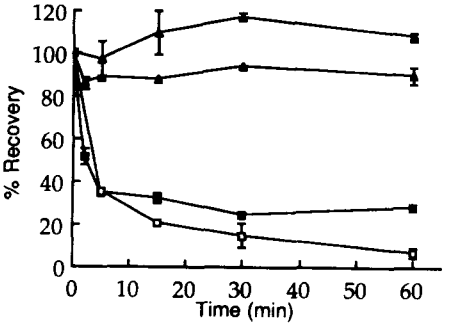

FIGURE 5 Stability of 15[S]-HPETE and conjugated dienes in human plasma. 15[S]-HPETE (11.76 μM) was incubated in fresh plasma containing 10 μM BHT and 20 μM desferal at 0°C or 27°C. Mean values ± SEM for 2 experiments are given as a percent of the initial concentration. ■, LOOH 0°C, □, LOOH 27°C, ▲, dienes 0°C, △, dienes 27°C)

TABLE II
Recovery of LOOH added to 0.5 ml of plasma or buffer containing albumin

Sample	Recovery (%)	
	15(S)-HPETE	Cholesterol 7α-Hydroperoxide
Fresh plasma	49.7 ± 1.5 (11)	17.6 ± 1.6 (10)
Heated plasma (30 min at 70°C)	54.5 ± 1.9 (10)	9.9 ± 0.9 (8)
0.1 M Tris containing albumin	48.7 ± 0.8 (3)	15.0 ± 1.2 (3)

15(S)-HPETE and cholesterol 7α-hydroperoxide were added at concentrations of 5.88 and 3.3 μM respectively and extracted as described in the methods sections. Results are expressed as mean ± SEM for the number of samples indicated in parenthesis.

recovery of 15(S)-HPETE and cholesterol 7α-hydroperoxide from Tris buffer containing human albumin (at the concentration found in plasma) was very similar to the amounts recovered from fresh plasma (Table II).

Stability of 15(S)-HPETE in stored plasma

We investigated the stability of 15(S)-HPETE added to plasma, with or without BHT and desferal, and then stored at either − 70°C or in liquid nitrogen. Table III shows that 15(S)-HPETE is stable for up to 2 weeks in plasma stored at − 70°C in both the presence or absence of BHT and desferal. However, with longer storage time there is a considerable decrease in the amount of 15(S)-HPETE recovered. Doubling the concentrations of BHT and desferal did not prevent the loss of 15(S)-HPETE observed at 1 months storage at − 70°C. In plasma stored for 1 month or longer in the absence of BHT and desferal, we found, in addition to 15(S)-HPETE, a second, unidentified CL peak when samples were analyzed using the HPLC-CL system. This peak was not present in samples containing BHT and desferal, indicating that it may have been produced by some kind of oxidant action on plasma components.

15(S)-HPETE added to plasma was more stable when stored in liquid nitrogen. Only 5% loss was observed in plasma from subject 1, and 11% in that from subject 2

TABLE III
Stability of 15(S)-HPETE in plasma stored at −70°C

Storage time	Recovery (%)	
	+ BHT/desferal	− BHT/desferal
none (fresh)	100	100
3 days	115.4 ± 2.5	115.9 ± 3.9
1 week	102.9 ± 10.5	104.3 ± 13.6
2 weeks	111.2 ± 5.7	118.1 ± 1.5
1 month	45.1 ± 6.4	47.2 ± 1.2
3 months	29.9 ± 9.1	15.9 ± 4.4

15(S)-HPETE (5.88 μM) was added to plasma aliquots from 2 healthy subjects, and stored at −70°C for the times indicated in the presence or absence of BHT and desferal. After thawing on ice, samples were extracted in the presence of BHT and desferal, as described in the methods section. Results represent the mean ± SEM of duplicate samples for each subject.

after 3 months, in the presence of antioxidants (not shown). Examination of conjugated diene levels showed that these too were stable in liquid nitrogen, with 99.1 ± 4.4 (n = 7) recovery after 3 months.

DISCUSSION

Many techniques are available to measure LOOH in biological samples such as plasma but often these are indirect and non-specific. For example, the commonly used TBA assay indirectly quantitates LOOH by measuring aldehyde breakdown products of LOOH. However, this method does not measure cholesterol hydroperoxide and additionally various compounds including endoperoxides, some sugars, aldehydes and sialic acid are reactive.[14] Methods which allow direct estimation of the hydroperoxides provide more useful information. HPLC-CL techniques using isoluminol or luminol and a haem catalyst are probably the most sensitive methods available. They separate LOOH from interfering antioxidants and furthermore, allow quantification of hydroperoxides in each lipid class − an important factor for assessing the contribution to disease of lipid peroxidation and oxidative stress. Nevertheless, HPLC-CL techniques are subject to false positives in the analysis of plasma LOOH: Yamamoto *et al.*[4] originally reported 4.83 μM hydrogen peroxide in human plasma and subsequently admitted this was an artefact.[9] Artifactual peroxidation during the extraction procedure is prevented in our modified method by the addition to plasma of the antioxidants BHT and desferal − we have detected an unidentified component which produces CL in the assay in plasma samples extracted in the absence of these antioxidants. Furthermore, we found that it is important that the plasma extracts are dried under nitrogen rather than under vacuum on a rotary evaporator, as reported by Yamamoto *et al.*[4] since the latter resulted in a lower recovery of exogenous 15(S)-HPETE and appearance of an additional, unidentified, CL peak.

A very important feature of our method is the incorporation of a diode-array detector into the HPLC system for the confirmation of LOOH detected by the CL reaction. Others have used single or dual wavelength monitoring[5,10] which has the disadvantage that in addition to the conjugated diene chromophore, tailing absorption of other compounds is detected (see Figure 4). Alternatively, the time-consuming

procedure of eliminating the CL response by treatment of plasma extracts with reducing agents has been used to identify hydroperoxides.[9,15] Identification of a LOOH conjugated diene chromophore or other compounds is simplified with the diode-array detector, as complete UV absorption data is collected between 190 and 390 nm and can be used to determine the exact absorbing wavelength of each component eluted from the column. By examination of the UV data (see Table I) and without the need for $NaBH_4$ reduction, our system could thus be easily used to identify the hydroquinones, ubiquinol-9 and ubiquinol-10, reported to be present in plasma and to produce a positive CL response.[9] We have found some drugs which produce a positive CL response in our system, for example Omnipaque which can be identified by its UV absorption spectrum (Table I). This finding highlights the importance of considering patient medication when analysing plasma LOOH.

Using our modified method we have shown, in agreement with Longhi *et al.*[10] that healthy human plasma does not contain LOOH in concentrations greater than 0.02 μM. This is in contrast to the findings of Miyazawa[5] and Yamamoto and Niki[15] who reported up to 0.5 μM phosphatidyl – choline hydroperoxide and about 3 nM cholesteryl ester hydroperoxide, respectively, in human plasma using HPLC-CL methods. We could not reproduce Miyazawa's results when we used his method on plasma from caucasian subjects. It is interesting to note that plasma hydroperoxides have only been reported by workers in Japan and that the method used by Yamamoto and Niki[15] is practically identical to that of Frei *et al.*,[9] who did not detect plasma hydroperoxide in American subjects. Our method is shown to be viable since we were able to detect high levels (up to 3.3 μM) of both FFA and cholesterol hydroperoxides in patients with ARDS[13] or undergoing angioplasty.[14]

Recovery of 15(S)-HPETE added to plasma is reportedly much lower than from normal saline or buffer. Terao *et al.*[16] and Longhi *et al.*[10] reported recoveries of about 20% and 30% respectively; we achieved approximately 50% recovery from our zero time samples (probably due to a different extraction procedure) but this is still considerably less than the amount which can be extracted from buffer (about 80%). It has been suggested that FFAOOH is rapidly degraded by plasmatic reducing systems such as glutathione peroxidase.[16] This, however, is not supported by our finding of identical recovery of 15(S)-HPTE from fresh and denatured plasma. Our results indicate that low plasma recovery of exogenous hydroperoxides may arise from their binding to plasma albumin. Albumin is a transport protein for FFA, so binding of FFAOOH to this protein is perhaps not surprising and could conceivably occur by a direct exchange mechanism. It is, however, more difficult to explain the binding of cholesterol hydroperoxide to plasma albumin. It is possible that there is some interaction between cholesterol hydroperoxide and the albumin-bound FFA since we detected very low levels of FFAOOH (about 0.025–0.15 μM) in plasma and in albumin solutions in TRIS buffer supplemented with 3.3 μM cholesterol hydroperoxide before extraction (not shown). However, the very low amount of FFAOOH measured indicates this is only a minor mechanism. A more likely explanation is that cholesterol hydroperoxide binds at another binding site on the albumin.

In addition to the low zero time recovery, we observed a very rapid reduction (about 50% in 10 s) in the amount of 15(S)-HPETE which could be recovered from plasma at 0°C, which was followed by a more gradual loss with incubation time. In contrast, there was only 13% reduction in the recovery of conjugated dienes (which is a measure of both 15(S)-HPETE and its reduced metabolite, 15(S)-HETE) after 10 s incubation, and levels subsequently remained relatively constant (Figure 5). Lower

recovery of FFAOOH compared to conjugated dienes has previously been found in plasma and rat liver homogenate[16,17] and additionally Frei *et al.*[18] reported linoleic acid hydroperoxide is rapidly degraded by plasma. In view of these reports and our finding of a greater reduction in hydroperoxide levels at 27°C compared to 0°C, our initial large loss of hydroperoxide probably reflects a combination of binding to plasma albumin and metabolism of 15(S)-HPETE for example by glutathione peroxidase[16] or some other peroxidase activity.[9] The later small reduction in hydroperoxide levels is likely to result from metabolism alone.

In vivo, circulating LOOH, if present, are at a steady-state level. In view of their rapid degradation *in vitro*, even in the presence of antioxidants, (Figure 5) it is obvious that plasma samples must be extracted as quickly as possible to obtain realistic data. In the study of LOOH in disease, it is often impractical to extract samples immediately. They should thus be treated identically; the blood should be added to BHT and desferal (20 μM final concentration) and the plasma frozen immediately after preparation. The samples can then be stored for up to 2 weeks at -70°C or 3 months in liquid nitrogen, and extracted immediately after thawing on ice.

In conclusion, we have reported a very sensitive, specific method for the quantitative and qualitative measurement of LOOH in plasma, which we are currently using to clarify the physiological and pathological effects of hydroperoxides in diseases associated with oxidative stress.

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References

1. H. Esterbauer and K.H. Cheeseman (1987) *Chemistry and Physics of Lipids*, **45,** 103–371.
2. H. Esterbauer, H. Zoliner, R.J. Shaur (1988) Hydroxy-alkenals: cytotoxic products of lipid peroxidation. *ISI Atlas of Science: Biochemistry*.
3. S.M. Thomas, W. Jessop, J.M. Gebicki and R.T. Dean (1989) A continuous flow automated assay for the iodometric estimation of hydroperoxides. *Analytical Biochemistry*, **176,** 353–359.
4. Y. Yamamoto, M.H. Brodsky, J.C. Baker and B.N. Ames (1987) Detection and characterization of lipid hydroperoxides at picomole levels by high performance liquid chromatography. *Analytical Biochemistry*, **160,** 7–13.
5. T. Miyazawa (1989) Determination of phospholipid hydroperoxide in human blood plasma by a chemiluminescence-HPLC assay. *Free Radicals in Biology and Medicine*, **7,** 209–217.
6. W.A. Pryor and L. Castle (1984) Chemical methods for the detection of lipid hydroperoxides. *Methods in Enzymology* **105,** 293–299.
7. P.J. Marshall, M.A. Warso and W.E.M. Lands (1985) Selective microdetermination of lipid hydroperoxides. *Analytical Biochemistry*, **145,** 192–199.
8. B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford.
9. B. Frei, Y. Yamamoto, D. Nicolas and B.N. Ames (1988). Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Analytical Biochemistry*, **175,** 120–130.
10. B. Longhi, K. Belgmi, J.C. Nicolas and A. Crastes de Paulet (1988) Plasmatic lipid hydroperoxide assay by chemiluminescence: analytical difficulties. *Abstract of the fourth Asian Pacific congress of Clinical Biochemistry*, 441–445.
11. Y. Yamamoto, E. Niki, Y. Kamuya and H. Shimasaki (1984) Oxidation of lipids. 7. Oxidation of phosphatidylcholines in homogeneous solution and in water dispersion. *Biochim Biophys Acta*, **795,** 332–340.

12. M.J. Thomas and W.A. Pryor (1980) Singlet oxygen oxidation of methyl linoleate: Isolation and characterization of the sodium borohydride-reduced products. *Lipids*, **15,** 544–548.
13. A.E. Holley, T.F. Slater, W.J. Cox and S.N. Cox Unpublished data.
14. J.C. Coghlan, W.D. Flitter, A.E. Holley, M. Norell, A.G. Mitchell, C.D. Ilsley and T.F. Slater (1991) Detection of free radicals and lipid hydroperoxides in blood taken from the coronory sinus of man during percutaneous transluminol coronory angioplasty. *Free Radical Research Communications* In press.
15. Y. Yamamoto and E. Niki (1989) Presence of cholesterol ester in human blood plasma. *Biochemical and Biophysical Research Communications*, **165,** 988–993.
16. J. Terao, S.S. Shibata and S. Matsushita (1988) Selective quantification of arachidonic acid hydroperoxides and their hydroxy derivatives in reverse-phase high performance liquid chromatography. *Analytical Biochemistry*, **169,** 415–423.
17. V.A. Kostyuk, S. Komura and K. Yagi (1985) Reduction of various lipid hydroperoxides by rat liver homogenate. *Biochemistry International*, **11,** 803–808.
18. B. Frei, R. Stocker and B.N. Ames (1988) Antioxidant defences and lipid peroxidation in human blood plasma. *Proceedings of the Society of the National Academy of Sciences USA*, **85,** 9748–9753.

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