ELSEVIER

Journal of Chromatography B, 731 (1999) 223–229

JOURNAL OF
CHROMATOGRAPHY B

High-performance liquid chromatographic assay of hydroperoxide levels in oxidatively modified lipoproteins

Yu Yamaguchi*, Satomi Kagota, Masaru Kunitomo, Jun Haginaka

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya, Hyogo 663-8179, Japan

Received 6 April 1999; received in revised form 21 May 1999; accepted 26 May 1999

Abstract

An anion-exchange HPLC method has been developed for the chemiluminescence (CL) assay of hydroperoxide (HPO) levels in native and oxidized low density lipoproteins (N- and Ox-LDLs, respectively) of Watanabe heritable hyperlipidemic (WHHL) rabbits. The method involves anion-exchange HPLC separation in N- and Ox-LDLs using a DEAE–glucomannan gel, and direct CL detection of HPOs in them without extraction of the lipids following postcolumn reaction with isoluminol, microperoxidase and Triton X-100. Addition of Triton X-100, which could solubilize lipids, was essential for the detection of HPOs in N- and Ox-LDLs. With an increase in the degree of oxidation, Ox-LDL was more retained on the DEAE–glucomannan gel with a concomitant increase in the CL intensity. The proposed method could analyze the HPO levels in N- and Ox-LDLs of WHHL rabbits without extraction of the lipids. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Lipid peroxidation; Atherosclerosis; Hydroperoxide; Lipoproteins; Oxidized low density lipoprotein

1. Introduction

Oxidized low density lipoprotein (Ox-LDL) has been believed to be a notable factor in the development of atherosclerosis since Palinski et al. [1] demonstrated that it was present in atherosclerotic plaques. Recently, several reports have shown that Ox-LDL is also present in blood in patients with atherosclerosis and related diseases [2–7]. Enzyme-linked immunosorbent assays were mainly used for detection of Ox-LDL [2,3] and for detection of autoantibodies to Ox-LDL [4,5]. Unfortunately, those required a long time for analysis and the preparation of specific antibodies. Furthermore, thin-layer chromatography [6] and HPLC [7] were utilized for detection of Ox-LDL. However, the former required a long time for analysis and the latter showed poor resolution.

On the other hand, a major part of lipid peroxides produced in Ox-LDL is lipid hydroperoxides (HPOs) [8]. If we can determine the HPO levels in Ox-LDL or plasma lipoproteins without extraction of the lipids, we will be able to know the degree of oxidation of Ox-LDL or plasma lipoproteins. Thus, it is important to develop a simple, fast, sensitive method for the assay of lipid HPO levels in Ox-LDL or plasma lipoproteins. Yamamoto et al. [9] reported an HPLC method based on the chemiluminescence

*Corresponding author. Tel.: +81-798-471-212; fax: +81-798-412-792.
E-mail address: yusan@mwu.mukogawa-u.ac.jp (Y. Yamaguchi)

0378-4347/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00242-X

(CL) detection of lipid HPOs using isoluminol and microperoxidase. Miyazawa et al. [10] described the HPLC–CL detection of lipid HPOs using luminol and cytochrome *c*, and found increased lipid HPO levels in plasma LDL on atherosclerotic and hyperlipidemic patients. However, both methods required extraction of lipids from plasma samples. Thus, the lipid HPO levels in native (N)- and Ox-LDLs or each plasma lipoprotein could not be analyzed by the methods described above.

Previously, we reported a fast and precise anion-exchange HPLC method for the separation of N-LDL and Ox-LDLs of Watanabe heritable hyperlipidemic (WHHL) rabbits with a DEAE–glucomannan gel by stepwise elution and with fluorometric (FL) detection of the total cholesterol (TC) level [11]. Moreover, we found that the N-LDL of WHHL rabbits seemed to be mildly oxidized. In this study, we tried to analyze the HPO levels in N- and Ox-LDLs of WHHL rabbits without extraction of the lipids, based on the CL detection following postcolumn reaction with isoluminol, microperoxidase and Triton X-100.

2. Experimental

2.1. Materials and chemicals

Microperoxidase (MP-11) from equine heart cytochrome *c* was purchased from Sigma (St. Louis, MO, USA.). Cholesterol oxidase (Type A) from *Streptomyces* sp., peroxidase (Type III) from horseradish and cholesteryl ester hydrolase (Type A) from *Pseudomonas* sp. were from Toyobo (Osaka, Japan). Homovanillic acid and isoluminol were from Tokyo Kasei (Tokyo, Japan), Triton X-100 and EDTA from Nacalai Tesque (Kyoto, Japan) and Dulbecco's phosphate buffered saline (PBS) from Nissui Seiyaku (Tokyo, Japan). Lipid Calibrator from Wako (Osaka, Japan) was used for the standard solution of TC. Other chemicals of an analytical-reagent grade were used without further purification. Water purified with a MilliQ Jr. system (Millipore, Tokyo) was used for the preparation of the eluents and samples.

2.2. Chromatography

The experimental setup used in this study is as reported previously [11,12]. Briefly, DEAE–glucomannan gel [13,14] kindly donated by Kurita (Tokyo) was packed into a 50×4.6 mm I.D. column using water as the slurry and packing solvent, and used for the separation of N- and Ox-LDLs of WHHL rabbits. The eluents used were as follows: eluent A, 20 m*M* sodium phosphate buffer (pH 7.0); eluent B, 1 *M* sodium chloride. N- and Ox-LDLs were separated by stepwise elution using eluents A and B (0% of eluent B, 0–5 min; 20% of eluent B, 5–10 min; 40% of eluent B, 10–15 min; 100% of eluent B, 15–25 min). The flow-rate was maintained at 1.0 ml/min. The separation was carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan).

After postcolumn reaction with a CL reagent, isoluminol, microperoxidase and Triton X-100, in 100 m*M* borate buffer (pH 10.0), HPO levels of N- and Ox-LDLs were detected by a CLD-10A CL detector (Shimadzu, Kyoto, Japan) set at 40°C. The CL reagent was delivered at a flow-rate of 1.0 ml/min.

On the other hand, the determination of TC levels of N- and Ox-LDLs was carried out according to the method reported previously [12]. Briefly, the separation conditions were the same as described above except that eluents A and B included 10^{-3} *M* EDTA. With regard to postcolumn reaction conditions, a cholesterol reagent, cholesteryl ester hydrolase (5 μg/ml, 0.7 U/ml), cholesterol oxidase (20 μg/ml, 0.4 U/ml), peroxidase (50 μg/ml, 7 U/ml) and homovanillic acid (500 mg/ml), were dissolved in 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100, and delivered at a flow-rate of 0.5 ml/min. Further, a 0.1 *M* sodium hydroxide solution was delivered at a flow-rate of 0.5 ml/min to alkalize the effluent. After the postcolumn reaction with these reagents, N- and Ox-LDLs were detected with an excitation wavelength of 325 nm and an emission wavelength of 420 nm using a RF-535 spectrofluorometer (Shimadzu).

2.3. Preparation of blood samples and LDL

A 100 ml aliquot of blood of WHHL rabbits was drawn into tubes containing 100 mg of EDTA for anticoagulation and protection of autoxidation of N-LDL. The plasma was separated by centrifugation (1500 *g* for 10 min) from the blood, stored at 4°C and used within 2 days. N-LDL ($d = 1.019$–1.063

g/ml) was sequentially isolated by ultracentrifugation [15] from the plasma samples. A Beckman TL-100E ultracentrifuge (Palo Alto, CA, USA) was used for the separation of N-LDL with a TLA 110.3 fixed-angle rotor at 40 000 *g* for 330 min and at 4°C. The isolated N-LDL was then extensively dialyzed against PBS and immediately used for each experiment.

2.4. Preparation of Ox-LDLs

An aliquot of N-LDL was oxidized by incubation with 5 μ*M* $CuCl_2$ at 37°C for 3 or 24 h. After incubation, Ox-LDLs were dialyzed with PBS at 4°C, and used as experimental samples.

2.5. Electrophoresis

Agarose gel electrophoresis of N-LDL and the prepared Ox-LDLs was done as described by Nobel [16] using commercial kits, Gel Universal/8 (Corning, Chiba, Japan). Aliquots of N- and Ox-LDLs were subjected to 1% agarose gel electrophoresis and stained with fat red 7B. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the N-LDL and prepared Ox-LDLs was done as described by Laemmli [17]. Aliquots of the N- and Ox-LDLs were loaded on SDS-PAGE and stained with Coomassie Brilliant Blue.

2.6. Lipid and protein analysis

The TC levels in the N- and Ox-LDLs were determined by a fluoroenzymatic method as described previously [18]. The thiobarbituric acid reactive substances (TBARS) levels were estimated according to the method described by Yagi [19]. Protein contents in N- and Ox-LDLs were measured by the method of Lowry et al. [20].

2.7. Method validation

The repeatability and intermediate precision data based on three replicates were obtained with the assay of the N-LDL and Ox-LDL (24 h) samples. The samples were stored at 4°C until assay.

The calibration graphs were constructed by plotting the peak area (CL intensity) to the TC level, and analyzed using a least-squares regression program (Stat View software package, Abaxus Concepts, Calabasas, CA, USA).

3. Results and discussion

3.1. Postcolumn reaction conditions for the assay of HPO levels in N- and Ox-LDLs

In this study, we separated N-LDL and Ox-LDL by an anion-exchange HPLC using a DEAE–glucomannan gel and chemiluminescently detected HPOs

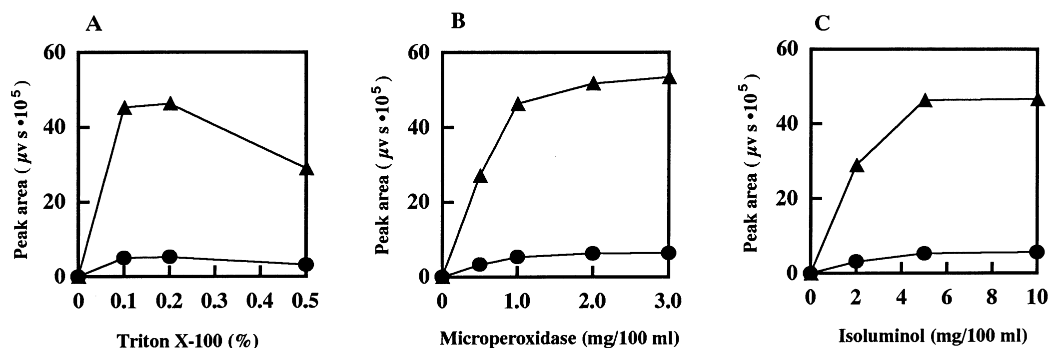

Fig. 1. Effects of concentrations of Triton X-100 (A), microperoxidase (B) and isoluminol (C) on the CL intensity of N- and Ox-LDLs of WHHL rabbits. A 20-μl aliquot of each sample was injected and the peak area was measured. The concentrations of N- and Ox-LDLs were 10 μg/ml, respectively, as the TC level. Other conditions are as follows: Triton X-100, 0.2% (B and C); microperoxidase, 1 mg/100 ml (A and C); isoluminol, 5 mg/100 ml (A and B). ●, N-LDL; ▲, Ox-LDL (24 h).

Table 1
Repeatability data for the assay of HPO levels in N-LDL and Ox-LDL (24 h) of WHHL rabbits[a]

LDL	TC level (μg/ml)	Mean peak area (μv s·10^5)	RSD[b] (%)
N-LDL	5	2.70	0.29
	10	5.36	0.81
	50	28.35	0.72
	100	72.22	0.22
	250	188.07	0.27
Ox-LDL (24 h)	0.1	3.27	0.42
	0.5	4.87	0.14
	1	6.27	1.93
	5	18.25	0.47
	10	46.26	0.33
	50	296.96	0.85

[a] Experimental conditions are given in the text.
[b] Relative standard deviation of five replicates.

in them without extraction of the lipids following postcolumn reaction with isoluminol, microperoxidase and Triton X-100.

N- and Ox-LDLs were separated with stepwise elution as reported previously [11], except that EDTA was omitted from the eluents used because the addition of EDTA to the eluents interfered with the enzymatic reaction of microperoxidase.

Optimization of the postcolumn reaction conditions for the CL detection of N- and Ox-LDLs was performed with respect to the concentrations of CL reagent, Triton X-100, microperoxidase and isoluminol, affecting the CL intensity. The flow injection system was used for the optimization of the postcolumn reaction conditions. Fig. 1 (parts A, B and C) illustrates the effects of the concentrations of Triton X-100, microperoxidase and isoluminol on the CL intensities of N- and Ox-LDLs. Addition of Triton X-100 was essential for the detection of HPOs in N- and Ox-LDLs without extraction of the lipids as shown in Fig. 1 (part A). Perhaps, the role of Triton X-100 could be solubilization of the lipids. The addition of 0.2% Triton X-100 was sufficient to obtain the maximal response. With regard to the microperoxidase concentration, both LDL samples required microperoxidase >1 mg/100 ml, as shown in Fig. 1 (part B). With an increase in the isoluminol concentration, the CL intensities of N- and Ox-LDLs were increased, as shown in Fig. 1 (part C). At an isoluminol concentration >5 mg/100 ml, the constant response was obtained. Thus, the optimal postcolumn reaction conditions obtained are as fol-

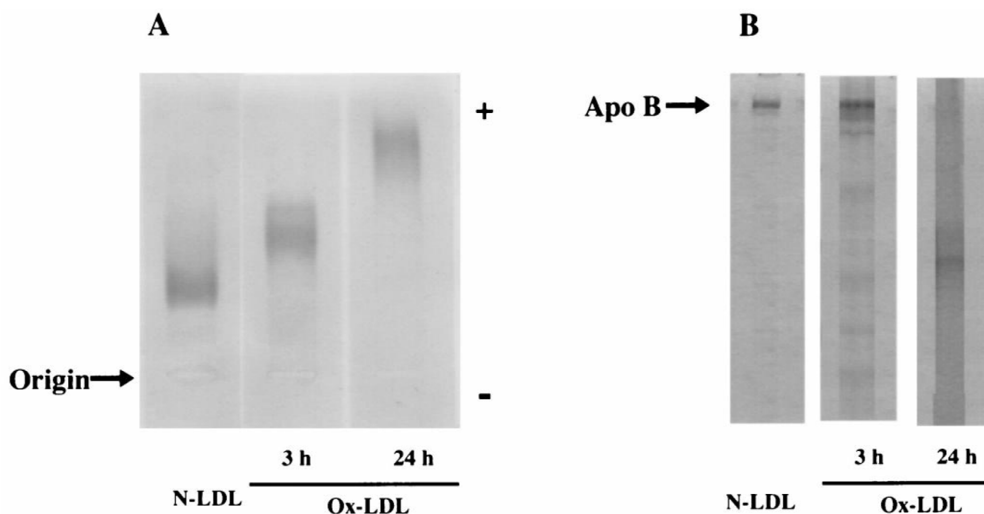

Fig. 2. Agarose gel electrophoresis (A) and SDS-PAGE (B) of N- and Ox-LDLs of WHHL rabbits. Aliquots of LDLs were subjected to 1% agarose gel electrophoresis and stained with fat red 7B. Aliquots of LDLs were loaded on SDS-PAGE gel electrophoresis and stained with Coomassie Brilliant Blue. Position of apolipoprotein B is indicated by arrow.

lows: microperoxidase, 1 mg/100 ml; isoluminol, 5 mg/100 ml; and Triton X-100, 0.2%.

3.2. Method validation

As shown in Table 1, the repeatability of the proposed method was checked at TC levels of 5–500 μg/ml and 0.1–100 μg/ml in N- and Ox-LDLs, respectively, and the relative standard deviations (RSDs) were highly reproducible, at less than 2%. Additionally, the RSDs of the intermediate precision of N- and Ox-LDLs assays were 0.6% and 0.8%, respectively, at a concentration of 10 μg/ml as the TC level. A linear correlation was found between the CL intensity and TC levels for the range 5–250 μg/ml and 0.1–10 μg/ml in N-LDL and Ox-LDL (24 h), respectively. The regression equations obtained were $y = 7.64 \cdot 10^4 x - 4.11 \cdot 10^5$ with a correlation coefficient of 0.998 for N-LDL and $y = 5.95 \cdot 10^4 x - 3.52 \cdot 10^5$ with a correlation coefficient of 0.996 for Ox-LDL (24 h), where y is a peak area of CL intensity and x is a TC level. The quantitation limit was 5 μg/ml as the TC level in the case of N-LDL and 0.1 μg/ml in the case of Ox-LDL (24 h) with a 20-μl injection, less than 2% in RSD.

3.3. Application to determination of HPO levels in N- and Ox-LDLs

Ox-LDLs were prepared by incubation of N-LDL with 5 μM $CuCl_2$ for 3 or 24 h. The degree of modification of Ox-LDLs was estimated by the electrophoresis, and TBARS and HPO levels. More oxidized LDL migrated faster in the anodic direction on agarose gel electrophoresis (Fig. 2, part A). As shown in Fig. 2 (part B), N-LDL showed a discrete protein band at the position of apolipoprotein B on SDS-PAGE, and Ox-LDL (3 h) showed the broad band of the apolipoprotein B. On the other hand, the apolipoprotein B band disappeared from Ox-LDL (24 h). For the separation of N- and Ox-LDLs, we employed an anion-exchange HPLC method using a DEAE–glucomannan gel column with stepwise elution. When 1 M sodium chloride as eluent B was used, Ox-LDL (3 h) and Ox-LDL (24 h) could be separated from N-LDL and fluorometrically detected as the TC level (Fig. 3, part A). The more oxidative Ox-LDL was eluted by a higher concentration of

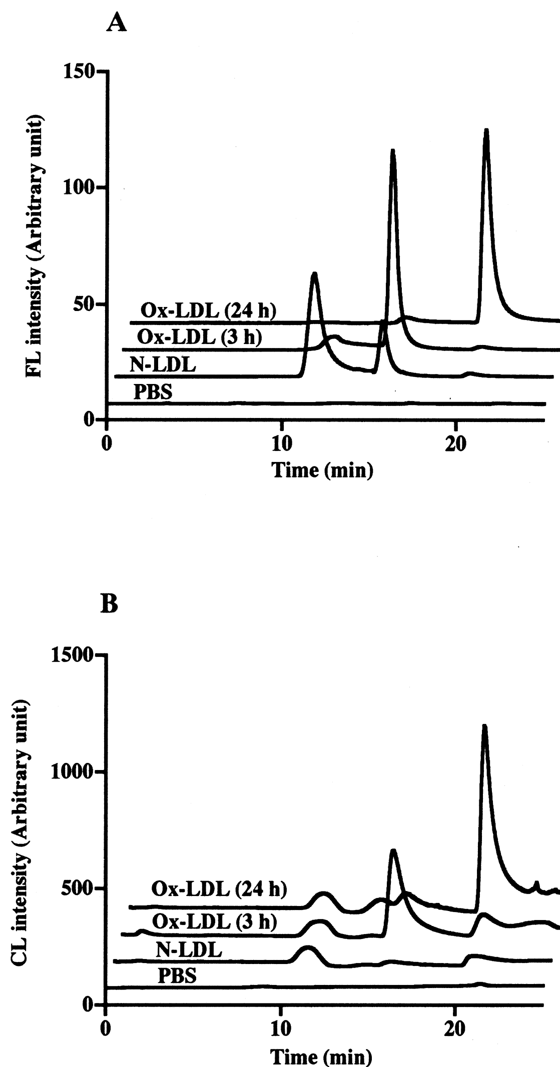

Fig. 3. Chromatograms of N- and Ox-LDLs of WHHL rabbits detected as the TC level (A) and detected as the HPO level (B). HPLC conditions: column, a 50×4.6 mm I.D. stainless column packed with a DEAE–glucomannan gel; eluents for detection as the TC level, 20 mM sodium phosphate buffer (pH 7.0) including 10^{-3} M EDTA for eluent A and 1 M sodium chloride including 10^{-3} M EDTA for eluent B; eluents for detection as the HPO level, the same eluents A and B as described above except that EDTA was omitted; stepwise elution, 0% of eluent B for 0–5 min; 20% of eluent B for 5–10 min; 40% of eluent B for 10–15 min, and 100% of eluent B for 15–25 min; flow-rate, 1.0 ml/min. N-LDL is native LDL, and Ox-LDL (3 h) and Ox-LDL (24 h) are prepared by incubation of N-LDL with 5 μM $CuCl_2$ for 3 and 24 h, respectively.

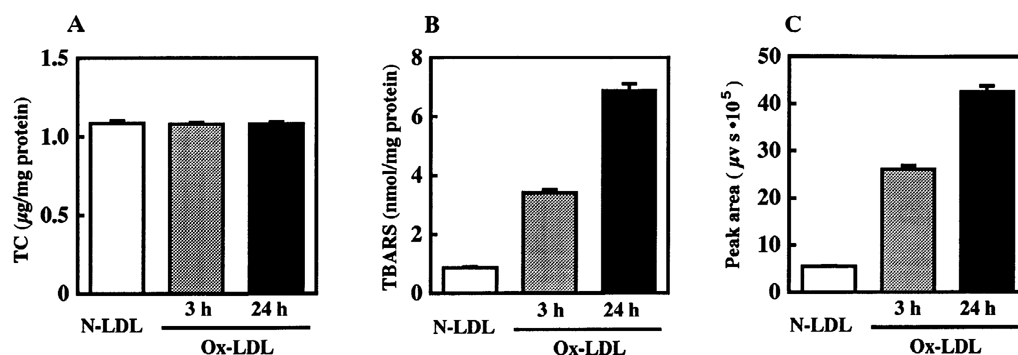

Fig. 4. TC (A), TBARS (B) and HPO (C) levels in N- and Ox-LDLs of WHHL rabbits. Ox-LDL (3 h) and Ox-LDL (24 h) are prepared as described in Fig. 3. The TC levels were estimated as described in Section 2.6., and the HPO levels were estimated from the sum of peak areas of Fig. 3 (part C).

sodium chloride. The typical chromatograms based on CL intensity of N- and Ox-LDLs detected as HPO levels are shown in Fig. 3 (part B). With an increase in the degree of oxidation of LDL, the CL intensity was increased. The FL intensities of Ox-LDLs were the same with that of N-LDL, while the CL intensities of Ox-LDLs were higher than that of N-LDL. The former are based on the TC levels in N- and Ox-LDLs, which are the same among N- and Ox-LDLs. On the other hand, the latter are based on the HPO levels, which are different in the degree of oxidation. Fig. 4 (parts A, B and C) shows the TC, TBARS and HPO levels in N- and Ox-LDLs, respectively. The HPO levels were estimated from the sum of peak areas of Fig. 3 (part C). The TBARS and HPO levels in Ox-LDLs increased with an increase in the incubation time with Cu(II), while TC levels per 1 mg protein were almost the same among N- and Ox-LDLs. The increment rate of the TBARS levels of Ox-LDLs due to the degree of oxidation was similar to that of the HPO levels. The TBARS method was based on the detection of aldehydes, which were the final product of lipid peroxidation [8]. The results obtained above reveal that there is a good correlation between the aldehyde and HPO levels in Ox-LDLs prepared by Cu(II)-promoted LDL oxidation.

The advantage of the proposed method is that the HPO levels in N- and Ox-LDLs can be analyzed without extraction of lipids. Moreover, the proposed method requires only 20 μg protein per sample for the assay of HPO levels in N- and Ox-LDLs. It is more sensitive and precise than the TBARS method, which requires 100 μg protein per sample.

4. Conclusion

An HPLC method has been developed for the assay of HPO levels in N- and Ox-LDLs of WHHL rabbits based on the CL detection following post-column reaction with isoluminol, microperoxidase and Triton X-100. This is the first report of the assay of HPO levels in N- and Ox-LDLs without extraction of the lipids. The proposed method could be applicable to the direct assay of HPO levels in plasma lipoproteins, and could clarify the degree of oxidation of them with real time. It would become useful for clinical and pathobiochemical research on atherosclerosis and related diseases. Further study is now in progress in our laboratory.

Acknowledgements

We wish to thank Kurita Industries (Tokyo, Japan) for the kind donation of the DEAE–glucomannan gel. This work was supported by grants from the Smoking Research Foundation (M.K.).

References

[1] W. Palinski, M.E. Rosenfeld, S. Yla-Herttuala, G.C. Gurtner, S.S. Socher, S.W. Butler, S. Parthasarathy, T.E. Carew, D. Steinbergand, J.L. Witztum, Proc. Natl. Acad. Sci. USA 86 (1989) 1372.
[2] K. Kotani, M. Maekawa, T. Kanno, A. Kondo, N. Todaand, M. Manabe, Biochim. Biopys. Acta 1215 (1994) 121.
[3] H. Itabe, H. Yamamoto, T. Imanaka, K. Shimamura, H. Uchiyama, J. Kimura, T. Sanaka, Y. Hata, T. Takano, J. Lipid Res. 37 (1996) 45.
[4] D.W. Branch, M.D. Mitchell, E. Miller, W. Palinski, J.L. Witztum, Lancet 343 (1994) 645.
[5] J.T. Wu, L.L. Wu, Clin. Lab. Med. 17 (1997) 595, and references cited therein.
[6] T. Kanazawa, T. Uemura, T. Osanai, Y. Fukushi, T. Imura, Y. Oike, K. Onodera, K. Akasaka, K. Okubo, S. Takahashi, Pathobiology 62 (1994) 269.
[7] H.N. Hodis, D.M. Kramsch, A. Pietro, G. Gittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson, A. Sevanian, J. Lipid Res. 35 (1994) 669.
[8] R.P. Patel, D. Svistunenko, M.T. Wilson, V.M. Darley-Usmar, Biochem. J. 322 (1997) 425.
[9] Y. Yamamoto, M.H. Brodsky, J.C. Baker, B.N. Ames, Anal. Biochem. 160 (1987) 7.
[10] T. Miyazawa, K. Fujimoto, S. Oikawa, Biomed. Chromatogr. 4 (1990) 131.
[11] Y. Yamaguchi, S. Kagota, M. Kunitomo, J. Haginaka, Atherosclerosis 139 (1998) 323.
[12] J. Haginaka, Y. Yamaguchi, M. Kunitomo, Anal. Biochem. 232 (1995) 163.
[13] H. Morita, A. Kitazawa, T. Tomoda, Chromatography 11 (1990) 20.
[14] J. Haginaka, H. Morita, N. Matsushita, Jpn. Kokai Tokkyo Koho JP 07 89,984 [95 98,984] (1995) (Chemical Abstract Number, 123:51725f).
[15] F.T. Hatch, R.S. Lees, Adv. Lipid Res. 6 (1968) 1.
[16] R.P. Nobel, J. Lipid Res. 9 (1968) 693.
[17] U.K. Laemmli, Nature (London) 227 (1970) 680.
[18] M. Kunitomo, Y. Yamaguchi, K. Matsushima, Y. Bando, Jpn. J. Pharmacol. 34 (1984) 153.
[19] K. Yagi, Biochem. Med. 15 (1976) 212.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.