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Development and Application of Organic Reagents for Analysis. V.[1] High-Performance Liquid Chromatographic Determination of Lipoperoxides in Biological Fluids with 1,3-Diphenyl-2-thiobarbituric Acid[2]

KENICHIRO NAKASHIMA, TAIJI ANDO, TAKAKO NAKAMIZO, and SHUZO AKIYAMA*

Faculty of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852, Japan

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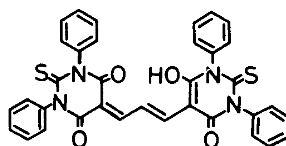
A rapid, sensitive, and selective high-performance liquid chromatographic method has been developed for the quantification of lipoperoxides (malondialdehyde, MDA) in biological fluids (rat plasma and human serum) with visible detection of 1,3-diphenyl-2-thiobarbituric acid (DPTBA) condensate. The assay was linear up to 500 pmol per assay tube of MDA. The lower limit of detection of MDA was 10 pmol per tube ($S/N > 2$), and the coefficient of variation at the 50 pmol/tube level was 2.6% ($n = 5$). A linear relationship was obtained between the peak height and the amount of rat plasma or human serum (10—100 μl). A comparison of the DPTBA-high-performance liquid chromatographic method with the thiobarbituric acid-colorimetric one was made for normal human sera. The correlation coefficient (r) was 0.954 with 18 samples. The method is simple and useful for routine assay of trace amounts of lipoperoxides in body fluids.

Keywords——HPLC; lipoperoxide; malondialdehyde; 1,3-diphenyl-2-thiobarbituric acid; rat plasma; human serum; visible detection

The well-known thiobarbituric acid (TBA) assay method consists of measuring spectrophotometrically the red-colored reaction product of malondialdehyde (MDA) or malonaldehyde-like substances derived from peroxidized polyunsaturated lipids, with TBA.[3,4] However, the reactivity of barbituric acid (BA) and its derivatives with MDA and the absorption spectral properties of the resulting condensates have not been systematically studied.[5,6] Thus, thirteen kinds of BA derivatives were prepared to examine the reactivity of these compounds with MDA, and the resulting condensate products (pigments) were isolated to measure their ultraviolet (UV) spectra.[7] Among them, 1,3-diphenyl-2-thiobarbituric acid



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(DPTBA) condensate (I) was found to have the largest molar absorption coefficient in ethanol. Then, a convenient method for sensitive colorimetric determination of MDA with DPTBA was developed and it was applied for the determination of lipoperoxides in rat liver and rat plasma.[7] In recent years it has been found that in the assay of bioproducts the known determination methods are subjected to several limitations due to interference by bilirubin, sugars, aldehydes, or sialic acids.[3,4,7-10] Therefore, to improve both selectivity and sensitivity we have developed an appropriate analytical procedure based on high-performance liquid

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chromatography (HPLC).[2]

The present paper describes an HPLC method utilizing visible detection for the assay of lipoperoxides in rat plasma and human serum following derivatization to DPTBA condensate.

Experimental

Apparatus——A Hitachi model 635A liquid chromatography pump was used together with a JASCO model 100—IV LC spectrophotometric detector and a Hitachi model 561 recorder. A stainless-steel column (150×4 mm i.d.) was packed with LiChrosorb RP-18 (5 μm, Merck) by the slurry technique.[11]

Chemicals and Reagents——1,1,3,3-Tetraethoxypropane (TEP), which generates MDA in acidic aqueous solution, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acetonitrile was of HPLC grade (Wako Pure Chemicals, Osaka, Japan) and water was deionized and further glass-distilled. DPTBA was prepared according to the literature[12] and recrystallized from methanol. The standard solution of bilirubin (Wako) was used as such. (−)-*N*-Acetylneuramic acid (NANA, Merck, West Germany) was dissolved in water (1 mg/ml) and then used as a solution of so-called sialic acids. Control serum (Precinorm® U) was purchased from Boehringer Mannheim GmbH (Mannheim, West Germany). All other chemicals used were of analytical grade.

Standard Solution——A stock standard solution of MDA was prepared by dissolving TEP in water (5 μmol/ml), and was stable for two weeks at 4 °C. A working solution ($\leq$10 nmol/ml) was prepared by dilution of the stock solution.

The DPTBA stock solution was made with DPTBA (0.296 g, 1 mmol) and 0.2 M disodium hydrogen phosphate (50 ml), the pH was adjusted with 4% (v/v) phosphoric acid to 3.0 and then the whole was made up to 100 ml with water. It was prepared just before use.

Analytical Procedures

Preparation and Separation by HPLC of MDA Condensates with TBA Derivatives——An HCl–sodium acetate buffer (pH 2, 2.5 ml) and one of the derivatives in DMSO (0.12 M, 0.5 ml) were added to a solution of TEP (5 μmol/ml, 1 ml). The mixture was heated at 98 °C for 30 min, chilled for 5 min, and then pyridine (1.0 ml) was added to dissolve the resulting precipitates. One of six kinds of prepared pigments solution (1 ml) was mixed thoroughly, and then a 50 μl aliquot was injected into the high-performance liquid chromatograph (see Fig. 1).

Assay Procedure——DPTBA solution (1 ml) and water (50 μl) were added to rat plasma or human serum (50 μl) in a 3 ml-vial. The well-mixed solution was incubated at 95 °C for 40 min in a heating bath, and chilled for 5 min in tap water, then acetonitrile–pyridine (4:1, v/v, 0.5 ml) was added to dissolve the resulting precipitates. After 1 min of vortex mixing, the mixture was centrifuged at 3000 rpm for 10 min. An aliquot (50 μl) of the supernatant obtained was subjected to HPLC.

Calibration Graph——A calibration graph was prepared with a standard aqueous solution of MDA. Evaluation of chromatograms was based on the peak height of MDA condensate. The calibration graph was linear up to 500 pmol/tube and the lower limit of detection was 10 pmol/tube ($S/N>2$). The coefficient of variation at 50 pmol/tube of MDA was 2.6% ($n=5$).

Results and Discussion

Chromatography of MDA Condensates with TBA Derivatives

In the preliminary experiments with six kinds of 2-thio-barbituric acid derivatives, various factors affecting the retention and separation of MDA condensate were examined with HPLC. Figure 1 showed a typical chromatogram, in which two peaks arising from DPTBA- and 1,3-diethyl-TBA-condensates with MDA show better separation than the others. In the present work, DPTBA was chosen as a reagent for the quantification of MDA.

Chromatography of MDA Condensate with DPTBA in the Standard System and in Body Fluids

Before establishment of the subsequent procedures the effects of DPTBA concentration, reaction time, temperature, and pH were examined. The effect of DPTBA concentration on the reaction with MDA was examined in the range of 2.0—12.0 μmol/tube. Constant peak heights were obtained at more than 8 μmol/tube of DPTBA (Fig. 2). Figure 3 shows the effects of the reaction time and temperature. Peak heights were constant at more than 30 min at 95 °C. The effect of the pH of DPTBA solution on the reaction with MDA was examined. DPTBA solutions of various pH values were prepared with 0.2 M sodium hydrogen phosphate

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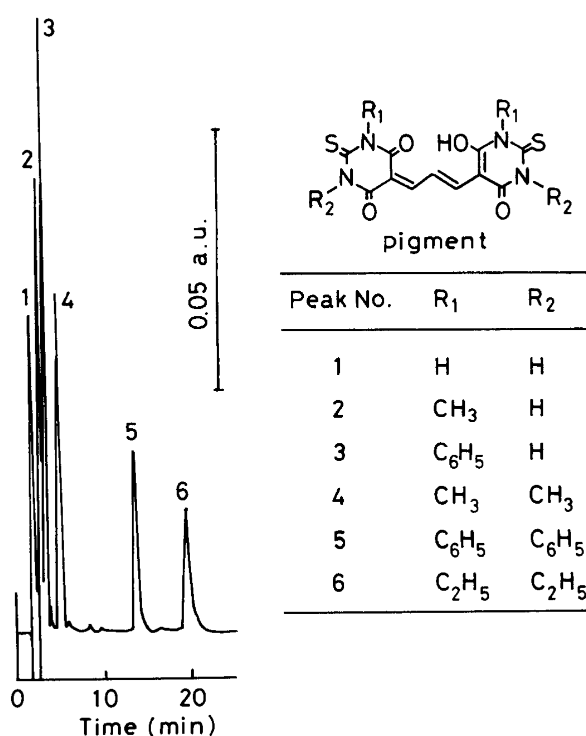

Peak No.	R_1	R_2
1	H	H
2	CH_3	H
3	C_6H_5	H
4	CH_3	CH_3
5	C_6H_5	C_6H_5
6	C_2H_5	C_2H_5

Fig. 1. Chromatograms of MDA-Condensates with TBA Derivatives (Pigments)

HPLC conditions: column, LiChrosorb RP-18 (5 μm, 15 cm × 4 mm, i.d.); mobile phase, CH_3CN–0.1 M $NaCl$–H_2O (42 : 50 : 8, v/v); flow rate, 0.7 ml/min; detection, 537 nm.

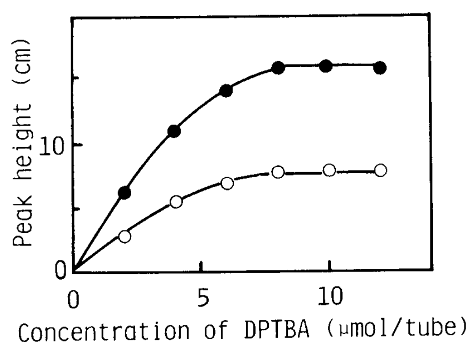

Fig. 2. Effect of DPTBA Concentration

—○—, MDA: 0.25 nmol; —●—, MDA: 0.5 nmol.

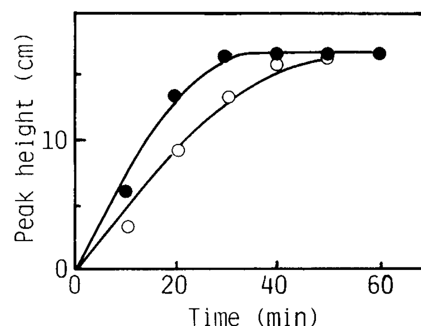

Fig. 3. Effects of Reaction Time and Temperature

—○—, at 80 °C; —●—, at 90 °C.

and 4% phosphoric acid. Though the heighest peak was obtained at pH 2.8, the DPTBA solution was unstable at this pH. Thus pH 3.0 was selected as the optimum.

Figure 4a shows the chromatogram of the standard sample, in which peak 1 (assigned to the condensate of DPTBA with MDA) is well separated from the other peaks. The standard method showed that peak 1 was not subject to interference from peak 2, bilirubin, which was added as a preliminary check of the feasibility of assay of body fluids, while no peak appeared in the reaction of NANA with DPTBA. The quantitation of lipoperoxides in plasma of Wistar male rats (7 weeks old) and control human serum was successfully carried out by this method. Figure 4b shows a chromatogram of rat plasma with no MDA added. A typical chromatogram of control human serum is shown in Fig. 4c.

The height of the peak arising from the MDA condensate with DPTBA remained constant even after the supernatant had been left standing for 8—10 h in all cases.

Next, the effects of bilirubin and NANA were examined. The addition of bilirubin ($\leqq$5.0 μg/tube) and NANA ($<$50 μg/tube) to the standard system had no influence on the recovery of MDA (0.25 nmol/tube, 18.0 ng/tube).

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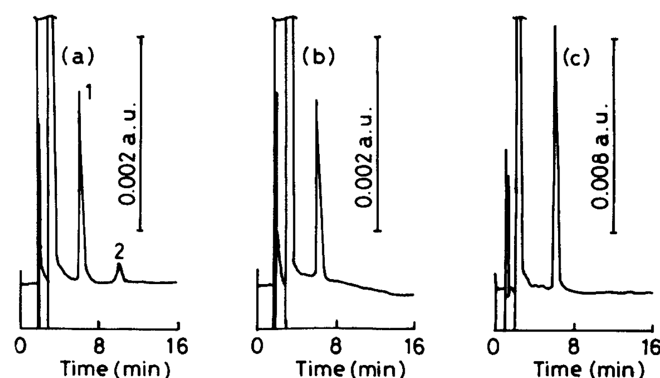

Fig. 4. Chromatograms of MDA-Condensate with DPTBA in Rat Plasma and Human Serum

(a) standard 1, MDA (60 pmol/tube); 2, bilirubin (5 μg/tube).
(b) rat plasma (50 μl/tube).
(c) human serum (50 μl/tube).
HPLC conditions: column, LiChrosorb RP-18 (5 μm, 15 cm × 4 mm i.d.); mobile phase, CH_3CN–0.1 M NaCl (1 : 1, v/v); flow rate, 0.7 ml/min; detection, 537 nm.

Table I. Recovery Test and Lipoperoxide (MDA) Levels in Rat Plasma and Human Serum

Sample	Recovery (%)		Assay
	Amount of MDA added (pmol/tube)		Lipoperoxide (MDA) level (nmol/ml)
	75.0	25.0	
Rat plasma (50 μl)	101.5	98.6	1.03
(C.V.[a] (%))	(4.6)	(2.4)	(6.8)
	Amount of MDA added (pmol/tube)		Lipoperoxide (MDA) level (nmol/ml)
	500.0	200.0	
Control human serum (50 μl)	95.9	106.7	8.60
(C.V.[a] (%))	(3.8)	(1.8)	(2.9)

a) Coefficient of variation, ($n=5$).

Good linear relationships between the peak height and the sample size (rat plasma and control human serum) were obtained through the range of 10 to 100 μl.

Table I shows the assay results and the recovery of MDA when it was added to rat plasma and control human serum. Recovery tests were made by addition of standard MDA to rat plasma and control human serum. The recovery was estimated from the calibration graph, which was based on the increases in peak height caused by addition of known amounts of MDA. The results are shown in Table I. Concentrations of lipoperoxides (MDA) in rat plasma and control human serum were assayed by using 50 μl of each sample. Mean lipoperoxide levels of rat plasma and control human serum were found to be 1.03 and 8.60 nmol/ml, respectively (Table I).

Comparison of DPTBA-HPLC and TBA-Colorimetric Methods

Figure 5 summarizes the results of determination of MDA in normal human serum (18 persons, 21—54 ages) by the DPTBA-HPLC and conventional TBA methods, and shows the relationship found by linear regression analysis. The obtained equation was $y=0.49x+0.30$, where y=concentration obtained by the present HPLC analysis and x=concentration obtained by the TBA method; the correlation coefficient, r, was 0.954 ($n=18$). The amount of MDA determined by the HPLC method was found to be less than that by the TBA method. This result suggests that the proposed method can sensitively determine the MDA-condensate separated by HPLC (Fig. 1), whereas the TBA-colorimetric method tends to overestimate the quantity of MDA-condensate since it responds to other analogous pigments such as TBA condensate with sugar, bilirubin, and aldehydes.

In conclusion, the present HPLC method has several advantages, *i.e.*, the assay system is

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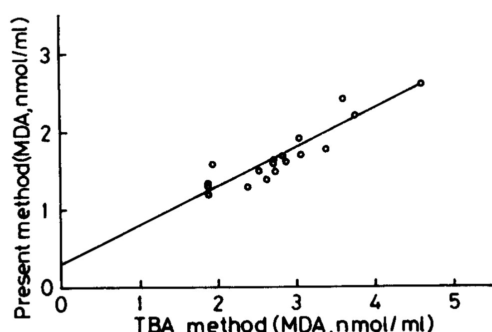

Fig. 5. Correlation between the Results of the DPTBA-HPLC and TBA-Colorimetric Methods

$y=0.49x+0.30$ ($r=0.954$, $n=18$).

simple (extraction and deproteinization processes can be omitted), and the selectivity and sensitivity are superior to those of the conventional TBA method (peak separation is good and the sample size can be minimized). Since the serum concentration of lipoperoxides can be determined easily and accurately, this method should be useful as a tool for the diagnosis and monitoring of geriatric diseases.

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