Communications to the Editor

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LIPOPEROXIDES IN RAT PLASMA FOLLOWING DERIVATIZATION TO 1,3-DIPHENYL-2-THIOBARBITURIC ACID CONDENSATE[1)]

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A sensitive method for the determination of lipoperoxides with 1,3-diphenyl-2-thiobarbituric acid is described which employs high performance liquid chromatography with visible detection. The calibration curve is linear up to 500 pmol per assay tube of malondialdehyde. The limit of detection of malondialdehyde is 10 pmol per assay tube. The method is simple and useful for routine assay of a trace amount of lipoperoxides in rat plasma. A linear relationship was obtained between the peak height and the plasma sample size (10—100 μl).

KEYWORDS ——— visible detection; lipoperoxide; 1,3-diphenyl-2-thiobarbituric acid; malondialdehyde; rat plasma; high performance liquid chromatography

A convenient method for a sensitive colorimetric determination of malondialdehyde (MDA) with 1,3-diphenyl-2-thiobarbituric acid (DPTBA) was previously developed to apply to the determination of lipoperoxides in rat liver and rat plasma.[2)]

Recently, the literature has revealed that in the assay of bioproducts the known determination methods are subject to several limitations (*e.g.*, interference by bilirubin, sugars, aldehydes, or sialic acids).[3)]

Therefore, we studied the development of an appropriate analytical procedure using high performance liquid chromatography (HPLC) to improve both selectivity and sensitivity. In preliminary experiments with six kinds of 2-thiobarbituric acid (TBA) derivatives we examined various factors affecting the retention and separation of MDA-condensates.[2)] Figure 1 shows a typical chromatogram, in which the two peaks arising from DPTBA- and 1,3-diethyl-TBA-condensates with MDA indicate better separation than the others. In this work, DPTBA was chosen and utilized as a reagent for the determination of MDA.

This communication reports the sensitive HPLC method with visible detection for the assay of lipoperoxides in rat plasma following derivatization to DPTBA-condensate.

A recommended procedure is as follows. To a standard aqueous solution of MDA (1,1,3,3-tetraethoxypropane, ≦ 10 nmol/ml, 50 μl) or rat plasma (10—100 μl) in a

10 ml vial was added a solution of DPTBA in $Na_2HPO_4-H_3PO_4$ buffer (1 ml).[4] The whole was diluted to 1.1 ml with water. The well-mixed solution was incubated at 95°C for 40 min in a heating bath, chilled for 5 min in a tap water, and then CH_3CN–pyridine (4 : 1 (v/v), 0.5 ml) was added. 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.[5]

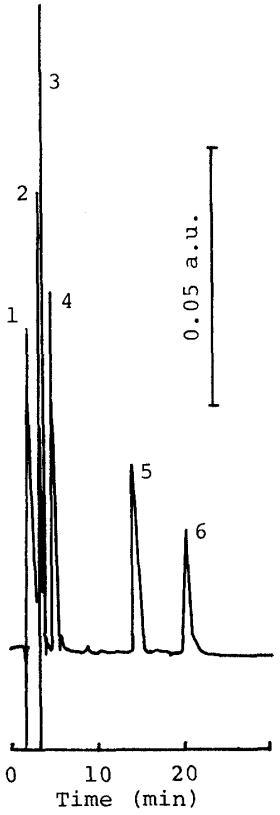
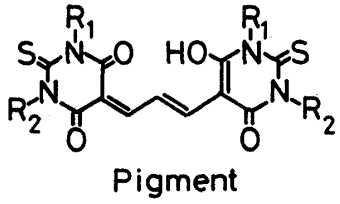


Fig. 1. Chromatogram of MDA-Condensates with TBA Derivatives (Pigments)[a]



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

a) The preparation of MDA condensate with TBA derivative[2]: To a solution of 1,1,3,3-tetraethoxypropane (50 nmol/ml, 1 ml) was added HCl–CH_3COONa buffer (pH 2, 2.5 ml) and a TBA derivative in dimethylsulfoxide (0.12 M, 1 M = 1 mol dm^{-3}, 0.5 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.
HPLC analysis of the pigment mixture: Six kinds of the prepared pigment solution (1 ml of each) were mixed thoroughly, and then 50 µl of it was injected.
HPLC conditions: stationary phase, LiChrosorb RP-18[5]; mobile phase, CH_3CN/0.1 M NaCl/H_2O (42/50/8 (v/v)); flow rate, 0.7 ml/min; detection, 537 nm.

Table 1. Recovery and Assay of MDA in Rat Plasma

	Recovery (%)		Assay
	Amount of MDA added (pmol/tube)		Amount of MDA
	75.0	25.0	
Plasma (50 µl)	101.5 (n=5)	98.6 (n=5)	1.03 nmol/ml
C.V. (%)	4.56	2.43	6.77 (n=5)

An addition of the CH_3CN–pyridine mixture effectively dissolved the resulting precipitates. Separation of the standard sample by means of HPLC and monitoring the eluate at 537 nm is shown in Fig. 2a. Note that peak 1 assigned to the MDA-condensate with DPTBA was well separated from the other peaks.[6] The height of peak 1 remained constant even after the supernatant was left standing for 10 h.

A linear relationship was obtained between the peak height of the MDA-condensate and the concentration of standard MDA ($\leqq$ 0.5 nmol/tube[7]). The limit of detection is 10 pmol/tube of MDA.[8] The coefficient of variation for the five replicated experiments was 2.64% (50 pmol/tube).

The standard method was successfully applied to the determination of lipoperoxides in plasma of Wistar male rats (7 weeks old). Figure 2b shows the chromatogram of rat plasma with no MDA added. The recovery of MDA when it was added to rat plasma was fairly good as shown in Table 1. The linear relationship between the peak height and the rat plasma sample size is indicated in Fig. 3.

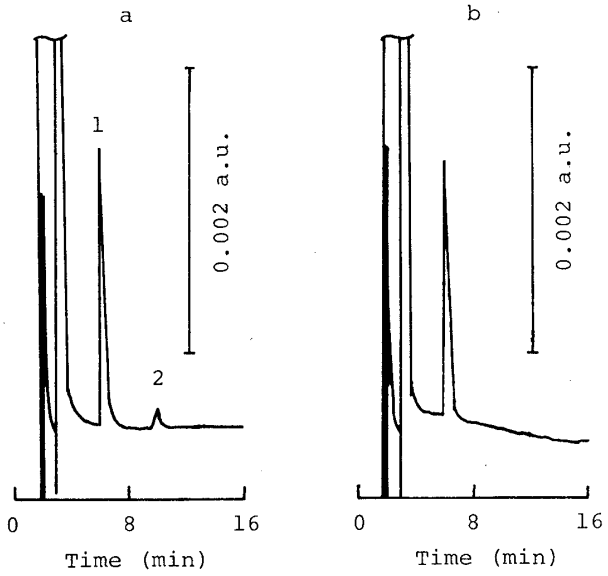


Fig. 2. Chromatograms of Reaction Products of MDA, Bilirubin, and Rat Plasma

a: standard
1: MDA (60 pmol/tube)
2: bilirubin (5 μg/tube)
b: rat plasma (50 μl/tube)

HPLC conditions:
column,[5] LiChrosorb RP-18; mobile phase, CH_3CN/0.1 M NaCl (1 : 1, v/v); flow rate, 0.7 ml/min; detection, 537 nm.

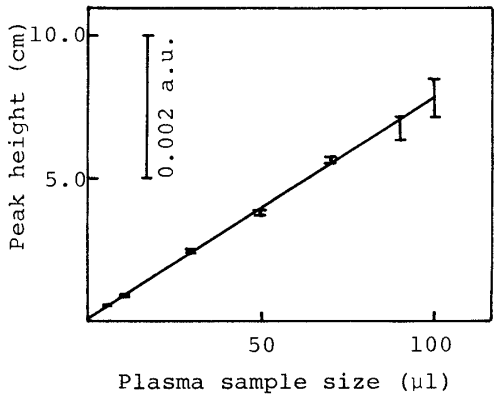


Fig. 3. Relationship between Plasma Sample Size and Peak Height of the Reaction Product of Plasma Lipoperoxides with DPTBA

On the basis of these experiments the quantities of MDA estimated from the calibration curve is also listed in Table 1.

In conclusion, the presented method has several remarkable features.

1. The assay system is simple: Both the extraction and deproteinization processes can be omitted.
2. The selectivity and the sensitivity are superior to the conventional methods[3]: A peak separation is good and the sample size can be minimized.

Studies on HPLC analysis of lipoperoxides in biological samples such as animal tissues or human serum according to this method are currently under way and will be reported in a following paper.

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4) The preparation of the reagent solution: DPTBA[2] (0.296g, 1 mmol) was dissolved in 0.2 M (1 M = 1 mol dm^{-3}) Na_2HPO_4 (50 ml). This solution was adjusted to pH 3 by adding 4%(v/v) H_3PO_4 and then the whole was arranged finally to 100 ml with H_2O. The subsequent condensation reaction proceeded quantitatively.

5) A stainless steel column (15 cm x 4 mm i.d.) was packed with ODS chemically bonded silica gel (LiChrosorb RP-18, 5 μm, Merck).

6) The preliminary experiments for the assay of human serum was tried simultaneously. As Fig. 2a shows, we found that the peak attributed to MDA-condensate with DPTBA was not subject to interference from that of bilirubin. Retention times were 6 and 10 min for MDA and bilirubin.

7) In this paper, a tube means *ca.* 1.6 ml which is the total volume of the reaction mixture.

8) When the sensitivity of the detector was increased (0.04 aufs), a tiny impurity peak was observed and overlapped unfavorably with the peak derived from MDA. However, the limit of detection was 10 pmol/tube ($S/N \leq 2$).

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