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Determination of malonaldehyde in oxidized biological materials by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was used to determine the level of malonaldehyde (MA) in materials containing unsaturated fatty acids and rat liver microsomes peroxidized *in vitro*. The detection limit was 8.3 pmol for fatty acid samples and 25 pmol for microsomal samples. The method was specific to MA and the relative standard deviation was 4.34–5.14%. The recovery of MA was about 100%. In general, the MA values in oxidized materials obtained by the proposed HPLC method were lower than those obtained by the thiobarbituric acid method, although similar results were obtained with both methods for microsomal samples oxidized by NADPH. The effect of temperature on the HPLC results was investigated and it was found that the MA values obtained by derivatization at 25°C, followed by separation using HPLC, reflected the situation of the peroxidation more accurately.

INTRODUCTION

Lipid peroxidation, the oxidative deterioration of polyunsaturated lipids, has been implicated in diverse pathological conditions including drug toxicities, various liver disorders, cardiac ischaemia, thermal injury and ageing. The most widely used method for the determination of the level of malonaldehyde (MA), which is a degradation product of peroxidized lipids, is based on its reaction with thiobarbituric acid (TBA test). The TBA test, however, is not specific for MA, as many other substances which are also formed in the peroxidation process give positive reactions with TBA[1–4]. The reaction factors employed in the TBA test also raise the possibility of generating MA as an artifact[5,6]. Recently, we[7] and others[8] developed an HPLC method using 2,4-dinitrophenylhydrazine (DNPH) as a derivatizing reagent. The reaction was specific to MA and proceeded readily at room temperature.

In this study, we attempted to determine MA as MA–DNPH in various

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peroxidized samples containing unsaturated fatty acids and rat liver microsomes. The values obtained by the conventional TBA and the high-performance liquid chromatographic (HPLC) methods were compared and the effect of the temperature employed in the reaction of MA and DNPH for the HPLC method was examined.

EXPERIMENTAL

Reagents and materials

Arachidonic acid, linolenic acid, thiobarbituric acid (TBA) and *tert.*-butyl hydroperoxide (*t*-BOOH) were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was purchased from Nacalai Tesque (Kyoto, Japan). 2,4-Dinitrophenylhydrazine (DNPH), 1,1,3,3-tetraethoxypropane and 2-nitroresorcinol were obtained from Tokyo Kasei (Tokyo, Japan). Microsomes were prepared from rat liver homogenates made in iced 1.15% KCl solution by differential centrifugation[9]. Protein was determined by the method of Lowry *et al.*[10]. All other reagents were of analytical-reagent grade.

Lipid peroxidation

The ascorbate-induced system for the fatty acids contained *ca.* 70 μmol of each fatty acid, 100 μ*M* $FeSO_4$, 1 m*M* KH_2PO_4, 0.4 m*M* ascorbate and 0.5% Triton X-100 in 2.0 ml of reaction mixture. The mixtures were incubated at 56°C for 2 h. For microsomes, 200-μl samples, 50 μ*M* $FeSO_4$, 1 m*M* KCl and 0.4 m*M* ascorbate in 1.0 ml of air-saturated 0.1 *M* phosphate buffer (pH 7.5) were incubated at 56°C for 2 h. The *t*-BOOH-induced system contained *ca.* 70 μmol of each fatty acid, 100 μ*M* $FeSO_4$, 1 m*M* *t*-BOOH and 0.5% Triton X-100 in 2.0 ml of reaction mixture. The mixtures were incubated at 56°C for 1 h. The NADPH-induced system for microsomes contained 100-μl samples, 50 μ*M* $FeCl_3$, 4 m*M* ADP, 1 m*M* KCl and 0.4 m*M* NADPH in 1.0 ml of the above buffer, and the mixtures were incubated at 37°C for 1 h.

Measurement of MA

The MA formed was determined by the TBA method of Uchiyama and Mihara[11] and by the HPLC procedure. In the TBA method, extraction was carried out with *n*-butanol and then the absorbance of the organic phase was measured at 532 nm. Calibration graphs were prepared using MA obtained by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. The HPLC method was as follows; for the fatty acid peroxidation systems, a 0.1-ml volume of peroxidized sample was reacted with 0.5 ml of DNPH solution (0.5 mg/ml in 1 *M* HCl) containing an appropriate amount of 2-nitroresorcinol as internal standard (IS) at room temperature (25°C) for 1 h or in boiling water-bath (100°C) for 10 min. An aliquot of the reaction mixture was injected into the HPLC column. For microsomal peroxidation systems, a 0.3-ml volume of peroxidized sample was reacted with 0.3 ml of DNPH solution (2.5 mg/ml in 1 *M* HCl) containing an appropriate amount of 2-nitroresorcinol at 25°C for 1 h. After centrifugation (5000 *g* for 10 min), an aliquot of the supernatant was injected. MA was calculated from an independently prepared calibration graph.

HPLC

A Model 6A high-performance liquid chromatograph (Shimadzu, Kyoto,

Japan) equipped with a UV spectrophotometric detector set at 310 nm was used for quantification of MA. The HPLC separations were performed on a Cosmosil 5 C_{18} packed column (250 × 4.6 mm I.D.; Nacalai Tesque) with a mobile phase consisting of acetonitrile–0.01 *M* hydrochloric acid (45:55, v/v) at a flow-rate of 1.5 ml/min at room temperature.

RESULTS AND DISCUSSION

We have previously described an HPLC method for determining MA in serum[7]. The technique, which uses DNPH as a derivatizing reagent, is simple, sensitive and specific. Therefore, we used this method to determine the MA levels in materials containing unsaturated fatty acids and microsomes, but without alkali treatment. The effect of protein on the recovery of MA added to the various concentrations of bovine serum albumin solution is shown in Fig. 1. As the concentration in *in vitro* systems was below 0.5%, the recovery was almost 100% without alkali hydrolysis. In the materials containing fatty acids, the MA–DNPH formed is possible soluble in coexisting fatty acids. For this reason, we studied the recovery of MA using an authentic synthesized MA–DNPH (Fig. 2). In the absence of Triton X-100, the peaks corresponding to both MA–DNPH and IS decreased and the recovery of MA–DNPH calculated from the peak height was about 40%. However, on adding Triton X-100 to the reaction mixture, about 100% of the MA–DNPH was recovered. Under these conditions, the

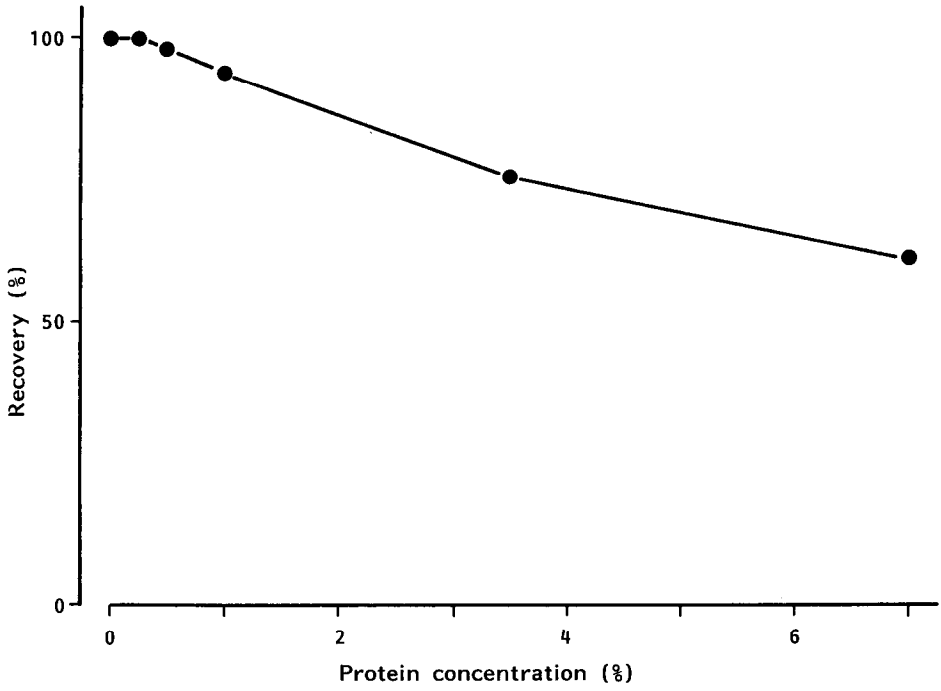

Fig. 1. Effect of protein concentration on the recovery of added MA. Samples containing 3.6 μg/ml of MA with various concentrations of bovine serum albumin were incubated with the same volume of DNPH aqueous solution at 25°C for 1 h.

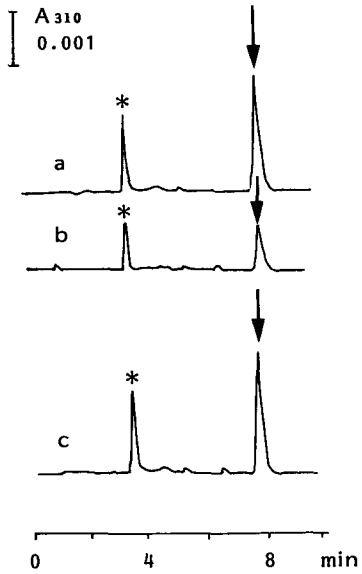

Fig. 2. Recovery of MA-DNPH (arrowed peaks) in aqueous solutions containing linolenic acid. (a) MA-DNPH aqueous solution (6 μg/ml) with 1.6 μg of 2-nitroresorcinol as IS; (b) as (a) with 0.2% linolenic acid added; (c) as (b) with 0.1% Triton X-100 added. Asterisks indicate IS. The volume of all samples was the same.

calibration graphs for both fatty acids and microsomes were linear up to 18 μg/ml of MA. The detection limits with a 20-μl injection were 8.3 and 25 pmol for fatty acids and microsomes, respectively. The relative standard deviations from the 1:5 incubation samples containing 1.44 and 3.6 μg/ml of MA were 4.73% and 5.14% (n = 10), respectively, and that from the 1:1 incubation samples containing 1.44 μg/ml of MA was 4.34% (n = 10). Incubation at 100°C increased the peak height for MA–DNPH to about 1.2 times that at 25°C. HPLC profiles for the materials containing arachidonic acid or microsomes peroxidized *in vitro* are shown in Fig. 3. The retention times for IS and MA–DNPH were *ca.* 3.5 and 7.8 min, respectively. The peak at *ca* 4.5 min represented unreacted DNPH. The peak at *ca.* 7.8 min was specific to MA, as shown in Fig. 3.

Table I shows the formation of MA from the unsaturated fatty acids in the oxidation system containing ascorbate. In this system, the MA values determined by the HPLC method were 20–25% of those obtained by the TBA method in the control assay and 60% in the oxidation system. The TBA method is not specific to MA[1–4]. In addition, it is well known that MA is produced at high temperatures and under acidic conditions[6]. Therefore, using the HPLC method, we investigated the effect of heat on the production of MA. As shown in Table I, no difference was found between the MA values obtained at 25 and at 100°C for either of the fatty acid samples. Hence it is obvious that TBA reacts with substances other than MA in fairly large amounts. The MA values in the peroxidized samples obtained by the TBA method increased to 2–3 times those in non-oxidized samples. The increase obtained by the HPLC method was 5–7 times greater. Hence the effect of peroxidation observed with the HPLC method was greater than that measured by the TBA method. Similar results were obtained with

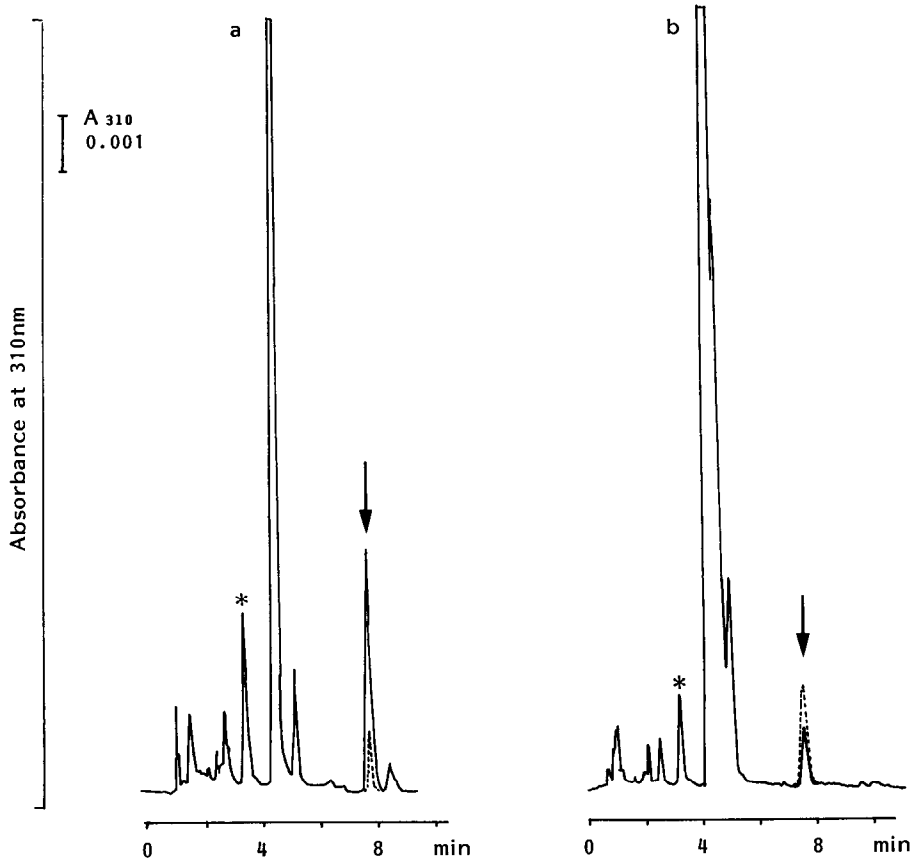

Fig. 3. Elution profile of MA-DNPH (arrowed peaks) for (a) arachidonic acid oxidized by the ascorbate system and (b) rat liver microsome oxidized by the NADPH system. The dashed line in (a) indicates the profile of the control assay and that in (b) indicates the profile of the oxidation system to which 6 nmol MA had been added. Asterisks indicate IS.

TABLE I

MA LEVELS FOUND IN ASCORBATE-INDUCED SYSTEMS CONTAINING FATTY ACIDS BY THE TBA AND HPLC METHODS

Data represent the mean values ± S.D. of three different experiments performed in duplicate. Values are expressed as nmol/ml. Samples incubated at 56°C for 2 h without ascorbate acted as the control.

Method	*Arachidonic acid*		*Linolenic acid*	
	Control	*Peroxidized*	*Control*	*Peroxidized*
TBA	76.9 ± 4.4	161.8 ± 2.5[b]	66.1 ± 7.6	154.3 ± 0.4[a]
HPLC (room temperature)	19.3 ± 1.5	95.4 ± 0.7[b]	13.1 ± 0.8	91.5 ± 3.9[b]
HPLC (boiling)	18.9 ± 1.0	100.6 ± 2.5[b]	12.8 ± 2.2	94.9 ± 4.0[b]

[a] Significantly different from control values, $p < 0.01$.
[b] Significantly different from control values, $p < 0.001$.

TABLE II

MA LEVELS FOUND IN *t*-BOOH-INDUCED SYSTEMS CONTAINING FATTY ACIDS BY THE TBA AND HPLC METHODS

Data represent the mean values ± S.D. of three different experiments performed in duplicate. Values are expressed as nmol/ml. Samples incubated at 56°C for 1 h without *t*-BOOH acted as the control.

Method	*Arachidonic acid*		*Linolenic acid*	
	Control	*Peroxidized*	*Control*	*Peroxidized*
TBA	36.1 ± 0.4	103.3 ± 14.7[a]	55.0 ± 5.8	122.8 ± 5.0[c]
HPLC (room temperature)	5.6 ± 1.1	17.8 ± 2.6[b]	9.6 ± 0.1	33.3 ± 1.4[c]
HPLC (boiling)	9.6 ± 1.4	55.3 ± 4.0[b]	9.7 ± 1.0	51.9 ± 4.2[c]

[a] Significantly different from control values, $p < 0.05$.
[b] Significantly different from control values, $p < 0.01$.
[c] Significantly different from control values, $p < 0.001$.

the *t*-BOOH-induced peroxidation system (Table II). The MA levels obtained by the HPLC method were 15–27% of those obtained by the TBA method in the control assay and 17–54% of those in the oxidation system. In this system, a significant difference in MA levels was observed between derivatizations at 25 and at 100°C. This is probably due to the effect of *t*-BOOH under the heating conditions employed in the DNPH derivatization reaction. As shown in Tables I and II, the MA values determined by the HPLC method were lower than those obtained by the TBA method. These results suggest that the proposed method with derivatization performed at 25°C, not 100°C, can determine MA levels selectively and accurately, whereas the TBA method tends to overestimate. These results are in agreement with those of other workers[12–14]. However, many investigators have presented conflicting results for MA values in lipid peroxidation[14–17]. In this study, we found that the MA values obtained by the HPLC method increased during lipid peroxidation and that this increase could be taken as an index of lipid peroxidation.

Lipid peroxidation has been proposed as a mechanism of tissue damage. NADPH- and ascorbate-induced lipid peroxidation of microsomes have been studied

TABLE III

MA LEVELS FOUND IN NADPH- AND ASCORBATE-TREATED MICROSOMES BY THE TBA AND HPLC METHODS

Data represent the mean values ± S.D. of three different experiments performed in duplicate. Values are expressed as nmol/mg protein. Microsomes incubated without NADPH (37°C for 1 h) or ascorbate (56°C for 2 h) acted as the controls.

Method	*NADPH-induced*		*Ascorbate-induced*	
	Control	*Peroxidized*	*Control*	*Peroxidized*
TBA	0.58 ± 0.05	13.21 ± 0.32[a]	2.29 ± 0.14	7.43 ± 0.48[b]
HPLC	trace	13.10 ± 0.35[b]	1.36 ± 0.13	5.25 ± 0.38[b]

[a] Significantly different from control values, $p < 0.01$.
[b] Significantly different from control values, $p < 0.001$.

in detail[18–20], with the TBA method being the method most commonly used. We applied the proposed HPLC method to the determination of MA in microsomal samples peroxidized *in vitro* (Table III). The difference in MA values in the two control assays depends on the differences in the incubation conditions. Kikugawa *et al.*[14], using the lipid fraction from the oxidized microsomes, reported that the MA contents in oxidized lipids were too low to be taken into account. Our results, however, showed a significant increase in the MA values in both oxidation systems. The value obtained by HPLC was the same as that obtained by the TBA method in the oxidation system containing NADPH. Although similar yields of MA by the HPLC and TBA methods were not obtained in other systems, the results have been reported by Janero and Burghardt[17,21]. These data support the view that the MA values in the NADPH system represent the major TBA reactive product in the microsomal lipid oxidized. However, MA is not the only decomposition product, as a variety of molecules can be generated from lipid peroxide breakdown. The MA value obtained by HPLC was 60–70% of that measured by the TBA method in the system containing ascorbate. The correlation between MA values obtained by HPLC and the TBA method depends on the peroxidation conditions[17]. Accordingly, we are convinced that the HPLC value is a good quantitative index of lipid peroxidation and hope that this method will encourage others to carry out more detailed studies on lipid peroxidation.

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