Analysis of Free Malonaldehyde Formed in Lipid Peroxidation Systems *via* a Pyrimidine Derivative

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A high-performance liquid chromatographic (HPLC) method for the determination of malonaldehyde (MA) in foods and biological samples was developed. MA was derivatized by reaction with urea under acidic conditions to form 2-hydroxypyrimidine, which was subsequently measured by HPLC. The highest yield (98%) of the product was obtained when 100 nmol of MA was reacted with 60 mmol of urea for 60 min at 100°C. Arachidonic acid, linolenic acid, linoleic acid and oleic acid were oxidized by a $FeCl_2/H_2O_2$ reagent in aqueous solution. MA formed was determined as 2-hydroxypyrimidine by HPLC. Arachidonic acid produced the highest level of MA (60 nmol/mg fatty acid), whereas oleic acid did not produce any. The formation levels of MA in microsomes upon enzymatic and nonenzymatic oxidation were 34 nmol/mL and 45 nmol/mL, respectively. Antioxidative activity of α-tocopherol was also monitored successfully by this HPLC method.

KEY WORDS: HPLC, hydroxypyrimidine, lipid peroxidation, malonaldehyde.

Malonaldehyde (MA) forms from lipids enzymatically or nonenzymatically upon oxidation (1). MA has been used as a diagnostic compound for the freshness of lipid-rich foods because it forms from unsaturated fatty acids by action of sunlight (2), metal ions (3) and lipoxygenases (4). MA can also be used as a biomarker of hepatotoxicity of CCl_4 (5). MA is reportedly implicated in various biological phenomena, including aging (6), mutagenesis (7), and carcinogenesis (8). Therefore, interest in measurement of MA stems both from its usefulness as an indicator of peroxidative processes and from its potential toxicity to biological systems.

Determination of MA is difficult, and its direct analysis has never succeeded because it is extremely unstable and reactive. The thiobarbituric acid assay (TBA) is the method most widely used to monitor lipid peroxidation. This assay involves spectrophotometric measurement of adducts from TBA and lipid peroxidation products (9). However, this method is not specific to MA. High-performance liquid chromatography (HPLC) has been used to separate and quantitate an MA-TBA adduct (10). The MA-TBA adduct reportedly has two tautomeric structures (11), which is not ideal for quantitative analysis. Investigation of an appropriate derivative of MA is the first avenue in developing an HPLC method for MA. In the present study, MA was reacted with urea and the product, 2-hydroxypyrimide, was then analyzed by HPLC.

EXPERIMENTAL PROCEDURES

Chemicals. MA was prepared from 1,1,3,3-tetra-ethoxypropane (TEP), purchased from Aldrich Chemical Co. (Milwaukee, WI), by acid hydrolysis. Urea, arachidonic acid, linolenic acid, linoleic acid, oleic acid, and authentic 2-hydroxypyrimidine were purchased from Sigma Chemical Co. (St. Louis, MO).

Rat liver microsomes. Rat liver microsomes were prepared from a male Sprague-Dawley rat (250–280 g, two months old) according to the procedure of Pederson and Aust (12).

Reaction of MA and urea. TEP (220 mg) was dissolved in 100 mL of 1% H_2SO_4 solution, and the solution was allowed to stand for 1 h at room temperature to obtain free MA (10 mM). A stock solution of MA (100 nmol/mL) was prepared by diluting 0.5 mL of this hydrolyzed solution to 50 mL with deionized water. This solution (1 mL) was reacted with 0.1 mL of different concentrations of urea (0–12 mmol/mL) in an aqueous 1.2 N HCl solution at 100°C for 1 h.

Oxidation of fatty acids and rat liver microsomes. An aqueous solution (5 mL) containing arachidonic acid, linolenic acid, linoleic acid or oleic acid (2 mg/mL), 30 mM Tris-HCl buffer (pH 7.4), and the surfactant sodium dodecyl sulfate (0.2%) was stirred with 0.2 μM $FeCl_2$ and 0.1 μM H_2O_2 at 37°C for 16 h (13). The oxidation was terminated by the addition of 1.2 mg butyrated hydroxy toluene (BHT). Enzymatic oxidation of microsomes was achieved by addition of 6 μmol ADP, 0.06 μmol $FeSO_4$, and 9 μmol NADPH to a microsome solution (5 mL) containing the same ingredients as above according to the method reported by Minotti and Aust (14). The oxidation was terminated by the addition of 1.2 mg BHT.

Analysis of MA as 3-hydroxypyrimidine. An oxidized sample was heated at 100°C for 1 h with 0.1 mL of urea (120 mmol/mL) and 0.1 mL of 1.2 N HCl. The reaction solution was cleaned by passing through a C-18 cartridge (Bond ELUT, Analytichem. International, Harbor City, CA) and washing with 1.5 mL of distilled water prior to HPLC. The final volume of the sample was adjusted to 3 mL with distilled water, and 10 μL of each sample was injected into an HPLC (Waters, Millipore Corp., S. San Francisco, CA) equipped with a 250 mm × 4.6 mm i.d. Develosil ODS-5 reverse-partition column (Nomura Chemical Co., Ltd., Aichi, Japan) and a Waters Model 481 variable-wavelength ultraviolet (UV) detector, for 2-hydroxypyrimidine analysis. Distilled water was used as an eluting solvent.

The peak eluting at 4.4 min was identified as 2-hydroxypyrimidine by comparing its spectral data [mass spectrometry (MS), UV, and nuclear magnetic resonance (NMR)] to those of the authentic compound. Quantitative analysis of the product was conducted by comparing a peak height of 2-hydroxypyrimidine in a standard solution to that of the product. An excellent linear relationship between an HPLC peak height and a concentration of 2-hydroxypyrimidine was obtained from an experiment with standard 2-hydroxypyrimidine solutions.

Recovery efficiency testing on MA from sample mixtures. The testing samples were prepared by spiking 10,

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50, or 100 nmol/mL MA into a 5-mL aqueous solution containing exactly the same ingredients as the above solution, except oxidizing agents. The spiked samples were stirred for 1 h with 120 mmol/mL urea and then analyzed for 2-hydroxypyrimidine by the HPLC method described above.

Addition of α-tocopherol to the microsome samples with $FeCl_2/H_2O_2$ or ADP/$FeSO_4$/NADPH. Different amounts of α-tocopherol were added to the microsome solutions prepared by the method described above. Each solution was heated at 37°C for 1 h with $FeCl_2/H_2O_2$ or with ADP/$FeSO_4$/NADPH. The level of MA formed in each sample was measured as 2-hydroxypyrimidine by HPLC.

RESULTS AND DISCUSSION

Formation of 2-hydroxypyrimidine from a reaction of MA and urea was first reported in 1959 (15). Later, this reaction was applied to measure levels of MA as 2-hydroxypyrimidine in biological systems by UV spectrometry at 309 nm (16). In our preliminary experiment, measurement of UV absorbed by 2-hydroxypyrimidine in a microsome sample was extremely difficult because many constituents interfered with UV absorption. Cleaning a sample with gel or ion-exchange chromatography did not reduce the interference significantly. Therefore, separation of 2-hydroxypyrimidine derived from MA in a microsome sample was conducted by HPLC.

Figure 1 shows yields of 2-hydroxypyrimidine from the reaction of 100 nmol MA and different amounts of urea (0–240 mmol). The results indicate that the yield of 2-hydroxypyrimidine reached almost 100% when 60 mmol of urea was used. Figure 2 shows yields of 2-hydroxypyrimidine from the reaction of 100 nmol MA and 60 mmol of urea over different reaction times. The results

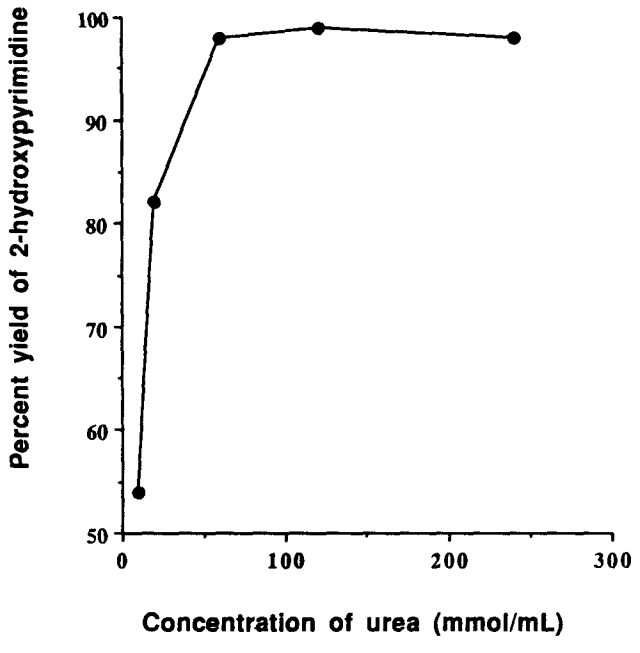

FIG. 1. Yields of 2-hydroxypyrimidine from the reaction of 100 nmol MA and different amounts of urea.

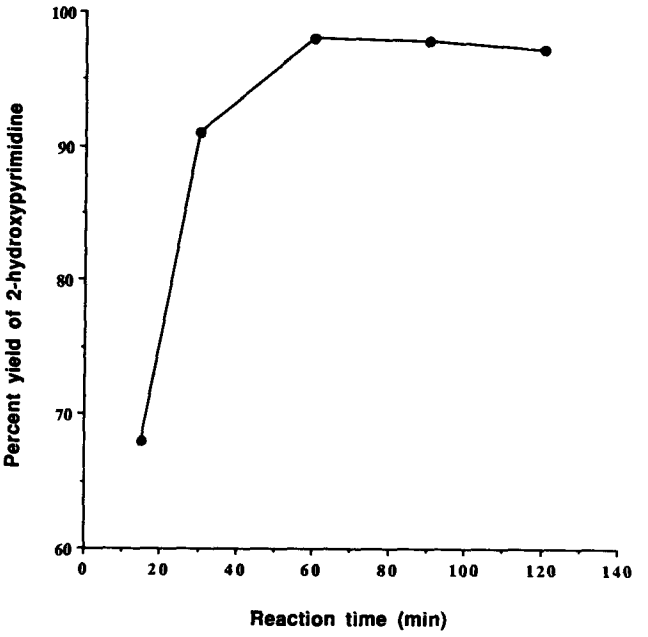

FIG. 2. Yields of 2-hydroxypyrimidine from the reaction of 100 nmol MA and 60 mmol urea over different reaction times.

show that the yield reaches maximum (approximately 98%) after 60 min and then declines slightly.

The percent recoveries of MA from a linoleic acid sample were 99.1, 99.7, and 99.3 when the samples were spiked with 10 nmol/mL, 50 nmol/mL, and 100 nmol/mL of MA, respectively. The percent recoveries of MA from a microsome sample were 86.0, 91.0, and 90.0 when the samples were spiked with 10 nmol/mL, 50 nmol/mL, and 100 nmol/mL of MA, respectively. The lower recoveries from microsome samples may be due to bonding with active sites of proteins (17).

Table 1 shows results of the determination of MA formed from fatty acids and microsomes upon oxidation by a method developed in the present study. Figure 3 shows a typical HPLC of a microsome sample with ADP/$FeSO_4$/NADPH. Arachidonic acid produced the highest level of MA, whereas oleic acid did not produce any. These results are consistent with a previous report (18).

The formation levels of MA in microsomes upon enzymatic (ADP/$FeSO_4$/NADPH) or nonenzymatic ($FeCl_2/H_2O_2$) oxidation were determined satisfactorily by

TABLE 1

Results of MA Determination from Oxidized Fatty Acids and Rat Liver Microsomes

Sample	Oxidizing agent	Amount of MA[a]
Arachidonic acid	$FeCl_2/H_2O_2$	60.5 ± 1.9
Linolenic acid	$FeCl_2/H_2O_2$	33.4 ± 0.9
Linoleic acid	$FeCl_2/H_2O_2$	33.9 ± 1.9
Oleic acid	$FeCl_2/H_2O_2$	0
Microsome	$FeCl_2/H_2O_2$	44.6 ± 1.0
Microsome	ADP/$FeSO_4$/NADPH	34.0 ± 0.5

[a]Values are mean ± standard deviation (n = 3) in nmole/mg fatty acid or mL microsome.

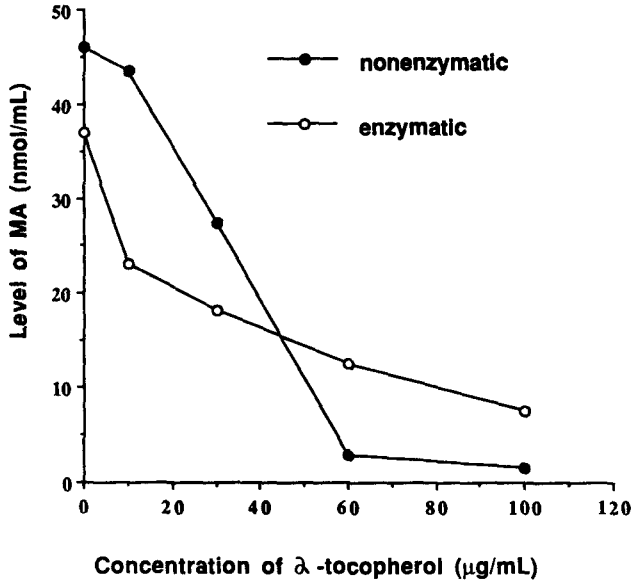

FIG. 3. Antioxidative activity of α-tocopherol in a microsome system oxidized enzymatically (ADP/$FeSO_4$/NADPH) or nonenzymatically ($FeCl_2$/H_2O_2).

the newly developed HPLC method used in this study. Antioxidative activity of α-tocopherol was also monitored successfully by this HPLC method.

The widely accepted mechanisms for the formation of MA from lipids (17) require at least three double bonds. However, our results with ethyl linoleate (two double bonds) generated a large quantity of MA, which is consistent with our previous work (12). Other studies on the formation of MA from precursors with fewer than three double bonds have also been reported (19,20). There must be additional mechanisms for MA formation.

The method developed proved to be useful for determination of MA levels in a biological sample such as microsomes. The major advantage of this method is that the process does not require a solvent extraction and is reasonably sensitive. Specific quantitation of MA is also possible, in contrast to other methods such as the thiobarbituric acid assay (TBA).

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