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Note

Determination of free malonaldehyde by gas chromatography with an electron-capture detector

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Malonaldehyde (MA) is of interest primarily as a product formed during lipid peroxidation and as an index of tissue damage induced by oxygen free radicals. The most widely used method for the determination of MA levels is based on its reaction with thiobarbituric acid (TBA test). The TBA test, however, is not specific for MA, as many other substances that exist in biological samples react positively with TBA. Therefore, several workers have proposed that TBA reaction products be separated chromatographically to improve the specificity of this method [1–3]. In addition, the reaction factors employed in the TBA test, strongly acidic conditions and an elevated reaction temperature, also raise the possibility of generating MA as an artifact. Hence methods other than the TBA test have recently been proposed [4,5]. The reaction between MA and phenylhydrazines proceeds readily under mild conditions. A high-performance liquid chromatographic (HPLC) technique using 2,4-dinitrophenylhydrazine (DNPH) has been developed for MA in urine [6,7] and serum [8]. There are many papers on the determination of MA, but little work has been done on its determination by gas chromatography (GC).

In this study, we attempted to determine MA levels in aqueous solutions and in urine as a pentafluorophenylhydrazine (PFPH) derivative using GC with

0378-4347/90/$03 50

electron-capture detection (ECD), as PFPH is more sensitive than other phenylhydrazines to ECD. This technique was simple and sensitive for the measurement of free MA in aqueous solutions.

EXPERIMENTAL

Reagents

Pentafluorophenylhydrazine (PFPH), *p*-dibromobenzene and 1,1,3,3-tetraethoxypropane (TEP) were obtained from Tokyo Kasei (Tokyo, Japan). All other reagents were of analytical-reagent grade.

Preparation of MA standard solution

Hydrolysis of TEP to produce MA was accomplished by the method of Csallany et al. [9]. TEP (1 mmol) was dissolved in 100 ml of 0.01 *M* hydrochloric acid and the mixture was heated in a water-bath at 50°C for 60 min. The MA stock solution was kept in the dark at 4°C until used. The absorbance at 267 nm was used to determine the MA content of the standard solution. A working solution was prepared by diluting the stock solution.

Apparatus and conditions

A Shimadzu GC-7A gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector was used. GC separation was performed on a 2-m glass column packed with 2% OV-17 on 60–80 mesh Chromosorb W AW DMCS (Gaskuro Kogyo, Tokyo, Japan) maintained at 110°C. The injector and detector temperatures were both 190°C. The nitrogen flow-rate was 40 ml/min. The peak areas were recorded by a Shimadzu Chromatopac C-R1A.

For the GC–mass spectrometric (GC–MS) analysis, a Hitachi GC–MS instrument (Hitachi M-80B) equipped with a computer-controlled data analysis system (Hitachi M-1010) was used. The MS conditions were electron energy 70 eV, separator temperature 190°C, acceleration voltage 3 kV and ionization current 100 μA.

Standard procedure

In a 10-ml capped glass tube, 0.5 ml of MA solution, 0.5 ml of PFPH solution (50 μg/ml) and 0.2 ml of 0.1 *M* NaH_2PO_4 were placed and the mixture was maintained at room temperature for 60 min. Two drops of 9 *M* sulphuric acid, followed by 0.5 ml of *n*-hexane containing an appropriate amount of *p*-dibromobenzene as an internal standard were added to extract the resulting MA–PFPH. After shaking vigorously for 30 s, the hexane layer was removed and dried over anhydrous sodium sulphate. A 1–2 μl aliquot of the hexane layer was injected onto the GC column. For the measurement of MA added to human urine, we used spiked urine in place of MA aqueous solution. The mixtures after shaking were centrifuged at 800 *g* for 10 min when necessary.

RESULTS AND DISCUSSION

We applied PFPH to the derivatization of MA as the possibility existed that a small amount of free MA was determined under milder conditions. As expected, the condensation reaction between MA and PFPH proceeded readily. As shown in Fig. 1, a cyclic product with M^+ at m/z 234 was indicated by MS, which was the same as that of the DNPH derivative reported previously [6,8].

A series of preliminary investigations were carried out in order to find suitable conditions for reaction and extraction. Fig. 2 shows the effects of the reaction time and temperature. Incubation at room temperature was preferable to incubation at 50 °C. The condensation reaction at room temperature reached a maximum at 30–120 min, then decreased gradually. The optimum pH was between 4 and 5, as shown in Fig. 3.

The resulting derivative, which was extractable from the aqueous solution with *n*-hexane, was 10^3–10^4 times more sensitive to ECD than to flame ionization detection. A typical GC separation of MA as its PFPH derivative is illustrated in Fig. 4. It required the addition of sulphuric acid to prevent interference from unknown peaks.

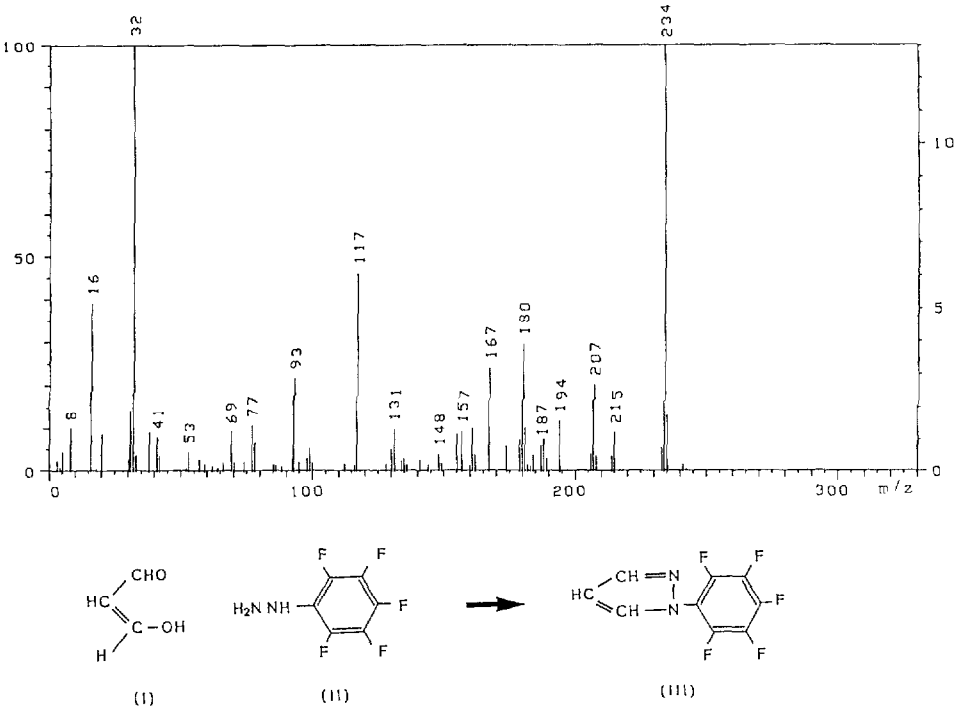

Fig 1. Electron impact mass spectrum of MA–PFPH adduct. Reaction between MA (I) and PFPH (II) gives PFPH derivative of MA (III)

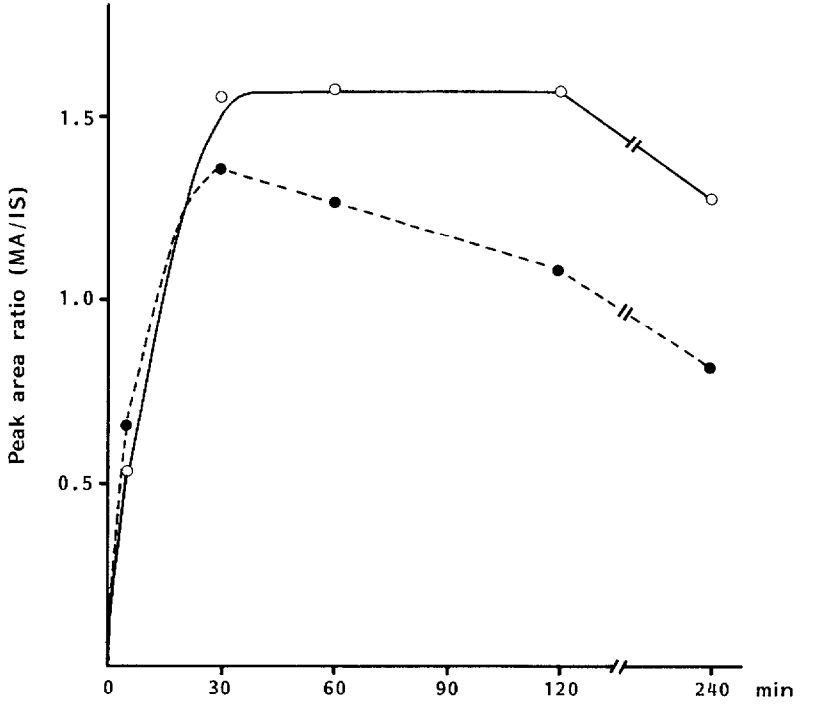

Fig. 2. Effects of reaction temperature and reaction period on the condensation reaction of MA with PFPH in aqueous solution. ○ = Room temperature; ● = 50°C.

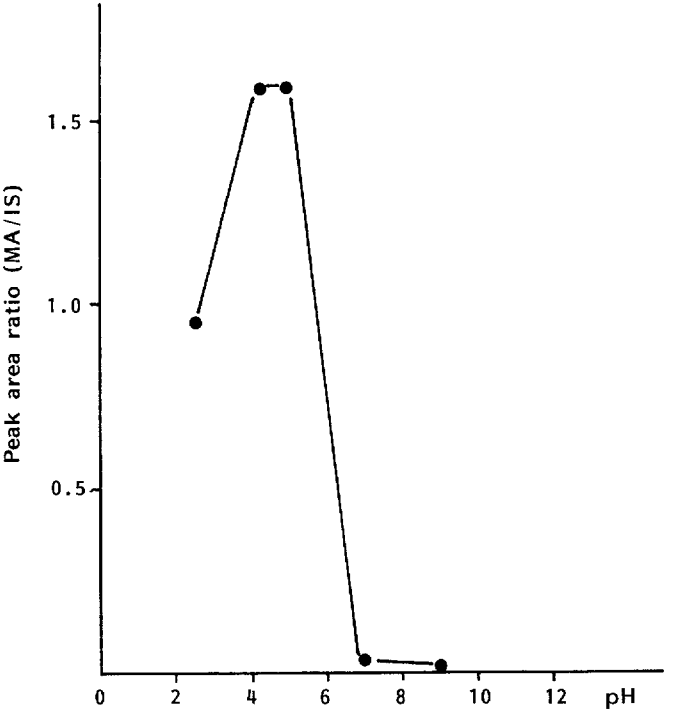

Fig 3 Effect of pH on the condensation reaction of MA with PFPH in aqueous solution.

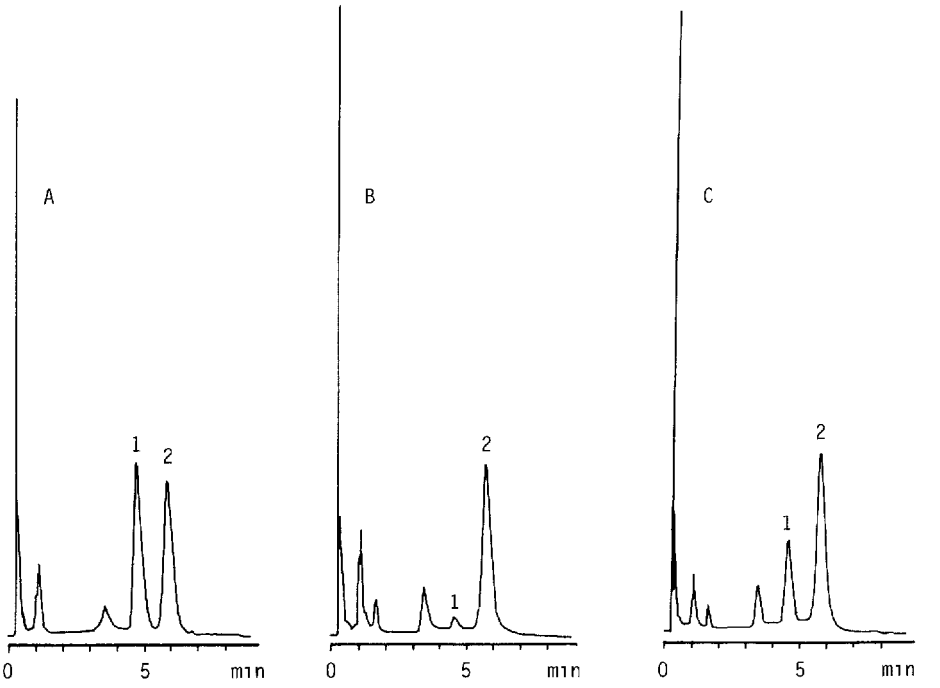

Fig 4. Typical gas chromatogram of MA-PFPH. (A) MA standard solution (2 μM); (B) normal human adult urine; (C) human urine with MA (1 μM) added Peaks: 1 = MA-PFPH, 2 = internal standard (*p*-dibromobenzene), corresponding to ca. 0.2 ng.

Within the range of concentrations studied up to 1 μM, the calibration graph for MA-PFPH exhibited good linearity ($r=0.9995$) and the detection limit was 15.6 nM. As we injected 2 μl of the hexane layer used for extraction from 0.5 ml of aqueous solution, the detection limit of MA per injection was 2.2 pg. The relative standard deviation (R.S.D.) of replicate assays ($n=8$) was 4.31% for 1 μM and 10.65% for 62.5 nM. The calibration graph using spiked urine also showed good linearity ($r=0.9729$) within the range of concentrations studied up to 2 μM. We used these concentrations of MA for construction of the calibration graph, because the level in human urine has been reported to be 0.58–2.51 μM by another GC method [10]. The R.S.D.s of replicate assays ($n=8$) using identical urine samples spiked with 2 and 0.125 μM MA were 2.50% and 7.65%, respectively. These results indicate that the method can be applied successfully to the measurement of MA in human urine. The recovery obtained was 58.1% from the experiment carried out using urine samples spiked with 2 μM MA. This result suggests that MA readily reacts with amino acids, proteins and many other compounds to form MA–biomolecule complexes as described elsewhere [11,12]. This lower recovery, however, means that our

method seems to be more specific with respect to free MA. The detection limit of MA added to urine samples was 36.3 n*M*. Thus, MA levels in normal adult human urine samples obtained by our method were found to be 0.2–0.8 μM ($n=10$). Although the study has not been completed in detail, the detection of free MA in urine would be of value if the monitoring of MA excretion could give useful information about lipid peroxidation in vivo.

Recently, GC using 2-hydrazinobenzothiazole as a derivatizing reagent has been employed for the measurement of MA in aqueous solution [10]. Although the method is specific and sensitive for MA, as with other methods, it requires hot and acidic conditions for more efficient formation of the derivative. On the other hand, our method is milder. The condensation reaction proceeds readily at room temperature to reach the maximum within 30 min under milder acidic conditions. Therefore, the free MA level in samples can be determined more specifically and accurately. There have been reports of various HPLC methods for the determination of free MA [13,14], but the detection limit is higher than that of the present method. The present results indicate that the GC–ECD method is very simple and sensitive and can be applied to the determination of free MA in biological samples.

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