Journal of Chromatography, 514 (1990) 29–35
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 470

High-performance liquid chromatographic determination of malonaldehyde using *p*-nitrophenylhydrazine as a derivatizing reagent

SATOSHI KAWAI*, TETSURO FUCHIWAKI and TAKEFUMI HIGASHI
Gifu Pharmaceutical University, 5–6–1, Mitahora-higashi, Gifu 502 (Japan)
and
MASAFUMI TOMITA
Department of Legal Medicine, Kawasaki Medical School, Matsushima, Kurashiki 701-01 (Japan)
(First received August 8th, 1989; revised manuscript received March 9th, 1990)

ABSTRACT

A high-performance liquid chromatographic procedure for the specific determination of free malonaldehyde (MA) is described. MA solution was mixed with 1 *M* acetate buffer (pH 3.76) and was reacted with *p*-nitrophenylhydrazine (NPH) hydrochloride solution in ethanol. An aliquot of the reaction mixture was injected into a C_{18}-5 column with a mobile phase consisting of acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v) and detection at 315 nm. MA reacted readily with NPH in a weakly acidic medium at room temperature, giving 1-(*p*-nitrophenyl)pyrazole. The method is specific for free MA. The response was linear in the range 36–720 ng/ml of MA and the detection limit was 6 ng/ml with a 20-μl injection.

INTRODUCTION

Lipid peroxidation has been considered to be a basic reaction involved in membrane damage. Malonaldehyde (MA) is a by-product of lipid peroxidation and its measurement has been suggested as being useful as an indicator of cellular ageing and free radical damage.

There are several high-performance liquid chromatographic (HPLC) methods for monitoring MA derivatized with 2-thiobarbituric acid (TBA)[1,2], dansylhydrazine (DNSH)[3] or 2,4-dinitrophenylhydrazine (DNPH)[4,5] or by the Hantzsch reaction[6]. Reactions of MA with TBA or DNSH require hot and acidic conditions, and the HPLC of the reaction products requires the employment of visible detection. A major drawback of these methods from a biochemical point of view is the artifactual generation of MA from the biological materials that can be induced under the strongly acidic conditions and elevated temperatures required for the formation of the derivatives. In a previous paper[5], we reported an HPLC method using DNPH for the

0021-9673/90/$03.50 © 1990 Elsevier Science Publishers B.V.

determination of MA; where the reaction proceeded readily at room temperature and HPLC with UV detection could be used.

Recently, we found *p*-nitrophenylhydrazine (NPH) to have different advantages to DNPH as a derivatizing reagent for MA: (1) DNPH reacts with MA in a strongly acidic medium, whereas NPH reacts in a weakly acidic medium at room temperature, i.e., under milder conditions, which means that the procedure is specific for free MA; and (2) MA-NPH standard has higher molar absorptivity than that of MA-DNPH at their respective λ_{max}, 315 and 305 nm, and therefore the use of NPH is more suitable for the determination of trace amounts of MA. In this paper, we propose a specific and sensitive HPLC method for the determination of free MA.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and isopropanol were purchased from Wako (Osaka, Japan), and 1,1,3,3-tetraethoxypropane (TEP), NPH hydrochloride (NPH · HCl) and 2-nitroresorcinol from Tokyo Kasei (Tokyo, Japan).

Preparation of MA standard solution

TEP (1 mmol) was dissolved in 100 ml of 0.01 *M* hydrochloric acid and the mixture was heated at 50°C for 1 h. This MA stock solution was kept at 4°C in the dark and the appropriate standard solution was made by dilution with water.

Preparation of MA-NPH standard

A standard of the derivative (MA-NPH) obtained by reaction of MA with NPH was prepared by heating at 50°C for 10 min a mixture of 20 ml of MA in 0.01*M* hydrochloric acid (720 μg/ml) and 15 ml of NPH · HCl in 0.01*M* hydrochloric acid (2 mg/ml). The mixture was extracted with 60 ml of ethyl acetate, the extract was washed with 10 ml of 0.01 *M* hydrochloric acid and three 10-ml volumes of water, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was purified by sublimation under reduced pressure: yellow powder, m.p. 124–126°C; analysis, calculated for $C_9H_7O_2N_3$, C 57.14, H 3.73, N 22.21, found C 57.28, H 3.80, N 22.17%; λ_{max}, 315 nm; ε, 13 400 in the mobile phase.

HPLC

A Model 5A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 315 nm and a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.) were used for quantification of MA. The HPLC separations were performed on a Develosil C_{18}-5 column (250 × 4.6 mm I.D.) (Nomura Chemical, Aichi, Japan) with a mobile phase consisting of acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v) at a flow-rate of 1.0 ml/min at room temperature.

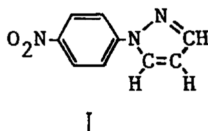
Procedure

A 1-ml volume of MA standard solution was mixed with 0.6 ml of 1 *M* acetate buffer solution (pH 3.76) and was reacted with 0.2 ml of NPH · HCl solution (0.5

mg/ml in ethanol) containing an appropriate amount of 2-nitroresorcinol as an internal standard (I.S.) at room temperature for 1 h. An aliquot of the reaction mixture was injected into the HPLC column. The concentration was calculated from a calibration graph obtained using mixtures of known amounts of MA-NPH standard and I.S.

RESULTS AND DISCUSSION

A typical separation of MA-NPH is illustrated in Fig. 1 using 2-nitroresorcinol as an I.S. Optimum HPLC conditions were established on the basis of a series of preliminary investigations. MA reacted readily with NPH in a weakly acidic medium giving 1-(*p*-nitrophenyl)pyrazole (MA-NPH), and the structure (I) was confirmed by mass spectrometry having M^+ (base peak) at *m/z* 189, and elemental analysis of the standard.



MA standard solution was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane (TEP). The amount of MA in a freshly prepared solution was measured by using the reaction with NPH in comparison with the MA-NPH standard

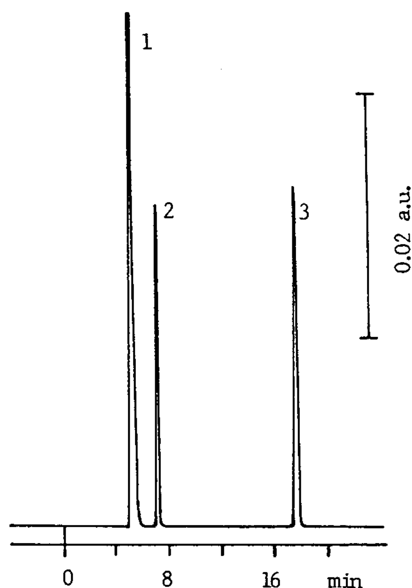


Fig. 1. Elution profile of MA (7.2 μg/ml) with a 10-μl injection after treatment with NPH reagent containing 16 μg of I.S. Peaks: 1 = NPH; 2 = I.S. (2-nitroresorcinol); 3 = MA-NPH.

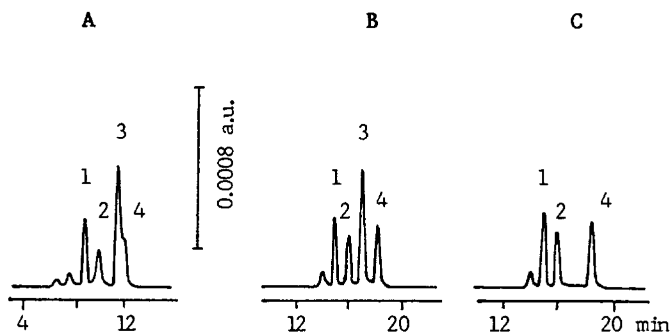


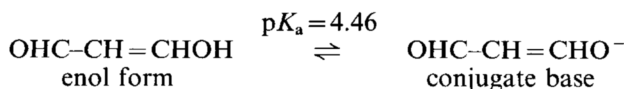
Fig. 2. Elution profile of MA (A and B, 100 ng/ml; C, blank) with a 20-μl injection after treatment with NPH reagent. Peaks: 1, 2, 4 = interfering peaks produced from NPH reagent; 3 = MA-NPH. Mobile phase: (A) acetonitrile–0.01 *M* hydrochloric acid (40:60, v/v), 1.5 ml/min; (B and C) acetonitrile–isopropanol–0.01 *M* sodium dihydrogenphosphate (30:10:60, v/v/v), 1.0 ml/min.

and the value corresponded to about 98% of that calculated from TEP taken assuming 100% conversion to MA. MA was relatively stable, but could not be stored for a long time in the pure state. It was found that the content of MA in the stock solution decreased to 85% after 2 months in a refrigerator and to 72% in the laboratory at room temperature. Therefore, it is advisable to prepare MA stock solution freshly once a week or to check the content of MA by comparison with MA-NPH standard.

MA-NPH standard was very easy to prepare and was used to construct calibration graphs. The yellow powder in the mobile phase showed λ_{max} at 315 nm, which was favourable for preventing interferences from various coexisting substances present in biological samples as well as MA-DNPH (λ_{max} = 305 nm), and the molar absorptivity (ε = 13 400) at 315 nm was higher than that of MA-DNPH at 305 nm (ε = 10 000), which was helpful for the trace determination of MA.

An NPH · HCl solution in ethanol was unstable and when a mixture of acetonitrile and 0.01 *M* hydrochloric acid (40:60, v/v) was used as the mobile phase some small peaks unfortunately appeared at a position close to the retention time of MA-NPH, as shown in Fig. 2A. In order to solve this problem in trace analysis, the effect of the composition of the mobile phase on the separation of MA-NPH from the interfering peaks was examined. The use of sodium dihydrogenphosphate which was better than hydrochloric acid for maintenance of the column, did not affect the separation, but addition of 10% isopropanol was found to improve the separation considerably (Fig. 2B). Fig. 2C shows the blank obtained from a solution without MA.

The effect of the pH of the reaction mixture on the reaction yield of MA and NPH was examined by using the ratio of the peak height to that of the I.S. and the results are shown in Fig. 3. MA is a weak acid (pK_a = 4.46)[7], which means that it exist dominantly as its conjugate base at >4.46:



$$\underset{\text{enol form}}{\text{OHC–CH=CHOH}} \underset{}{\overset{\text{p}K_a=4.46}{\rightleftharpoons}} \underset{\text{conjugate base}}{\text{OHC–CH=CHO}^-}$$

The conjugate base is considered to be much less reactive than is MA toward NPH (nucleophile). The effect of pH at after 30 min of reaction (solid symbols)

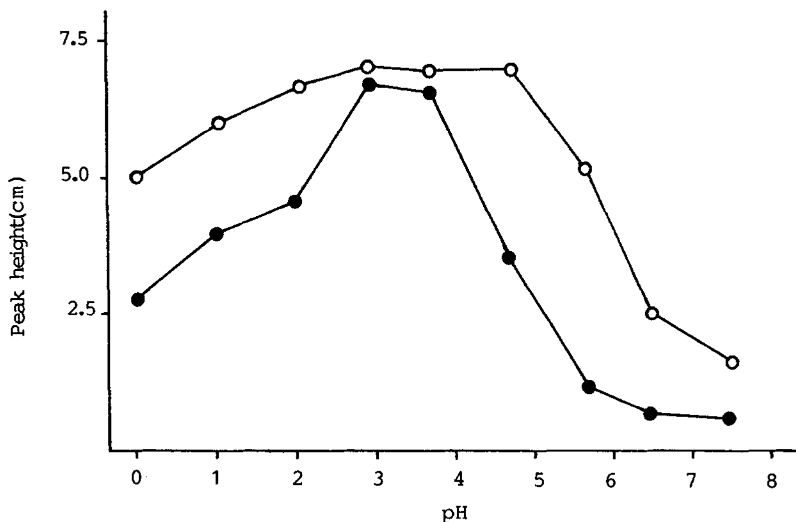


Fig. 3. Effect of pH on reaction yield of MA and NPH. A 1-ml volume of MA (7.2 μg/ml) was treated with 0.2 ml of NPH · HCl solution (0.5 mg/ml in ethanol) at various pH. Complex formation was determined by HPLC. (●) For 30 min at 25°C; (○) for 120 min at 25°C.

supports the above mechanism. However, the acidity of the reaction mixture hardly affected the reaction yield of MA and NPH at 120 min (open symbols) in the pH range 2.0–4.76. It is considered that the conjugate base which is present in equilibrium is converted to MA as the reaction of NPH and MA proceeds. MA reacted with DNPH in strongly acidic medium (pH <1)[5], whereas reaction with NPH decreased under these conditions. Reaction of MA with NPH involves nucleophilic attack by the basic nitrogen atom on the carbonyl carbon. Protonation of the carbonyl oxygen makes the carbonyl carbon more susceptible to nucleophilic attack. However, the nitrogen atom can also undergo protonation, removing unshared electrons, and is then no longer nucleophilic. The optimum conditions depend on the basicity of the reagent and on the reactivity of the carbonyl compound. The condensation reaction proceeded readily in a weakly acidic medium (pH 3.0–4.5) at room temperature to reach the maximum ratio in about 60 min. The effect of NPH concentration was also examined until there was no further increase in the amount of MA-NPH generated from the reaction. The final conditions adopted are those described under *Procedure*.

It has been postulated that MA exists in the free form, bound forms with various compounds and precursors in biological materials. A serious problem with methods for the determination of MA when applied in *in vivo* experiments is the possible formation of artificial MA during sample preparation. The amount of MA detected has been reported to be significantly influenced by the method of treatment of the sample. The procedures reported in previous papers required strongly acidic and/or hot conditions for the formation of derivatives. These methods were susceptible to artifactual generation of MA during the procedure. If the sample was treated under acidic and hot conditions, it is uncertain what proportion of the MA determined was originally present in the sample in the free state and what proportion may have been

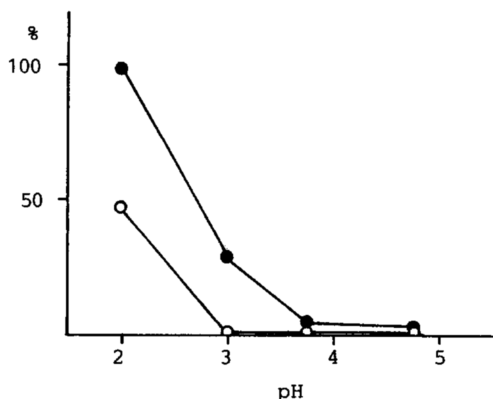


Fig. 4. Effect of pH on hydrolysis of TEP (0.1 μmol/ml): (○) at 25°C for 120 min; (●) at 50°C for 120 min. Formation of MA-NPH was determined by HPLC.

bound or may have been generated from precursors. Therefore, mild derivatization conditions are required for the reliable determination of free MA.

The reaction of MA and DNPH proceeded at room temperature, but required strongly acidic conditions (pH <1), as described previously[5], whereas the reaction with NPH proceeded under weakly acidic conditions (pH 2–5) at room temperature. In order to confirm that the present procedure is specific to free MA, 0.1 μmol/ml of TEP, a precursor of MA, was subjected to the reaction according to above procedure at pH 2, 3, 3.76 and 4.76 and 25 and 50°C, and the amounts of MA determined are shown in Fig. 4. At room temperature, about 48% of MA was liberated from TEP at pH 2, but at pH $\geqslant$ 3.76 little MA was hydrolysed from TEP even at 50°C. The results indicate that the present procedure is specific to free MA. Although the reaction of MA and NPH proceeded best at *ca.* pH 3, pH 3.76 was chosen as it is a milder condition and is also easier to adjust than pH 3.

The use of DNPH was not specific to free MA, because the conditions used were acidic enough (pH $\approx$0.5–1.0) to release MA from its precursors. Accordingly, by using the present method, the determination of free MA and that generated from its precursors is achieved from the difference between the MA levels prior to and after acid hydrolysis.

As described above, the concentration of MA in the standard solution has usually been calculated assuming 100% conversion of TEP to MA, but it is not easy to ensure an exact content of MA. Therefore, it is recommended to apply MA-NPH standard solution to construct a calibration graph for the reliable determination of MA and to standardize MA solutions.

With the reaction system used it is necessary to know the range over which MA can be determined and whether the formation of MA-NPH is linear over this range. A non-linear response indicates the possibility of a side-reaction. The response was linear in the range 36–720 ng/ml of MA in water and the correlation coefficient for the line was 0.998. The reproducibility of the method was determined by independently determining the amount of MA in six identical samples (720 ng/ml). The mean relative standard deviation was 1.4%. The detection limit at a signal-to-noise ratio of 2 was

6 ng/ml with a 20-μl injection, which was sufficient for the determination of urinary MA.

REFERENCES

1 R. P. Bird, S. S. O. Hung, M. Hadley and H. H. Draper, *Anal. Biochem.*, 128 (1983) 240.
2 J. Therasse and F. Lemonnier, *J. Chromatogr.*, 413 (1987) 237.
3 T. Hirayama, N. Yamada, M. Nohara and S. Fukui, *J. Sci. Food Agric.*, 35 (1984) 338.
4 T. Ekström, P. Garberg, B. Egestad and J. Högberg, *Chem.-Biol. Interact.*, 66 (1988) 177.
5 S. Kawai, K. Kasashima and M. Tomita, *J. Chromatogr.*, 495 (1989) 235.
6 K. Kikugawa, T. Kato and A. Iwata, *Anal. Biochem.*,174 (1988) 512.
7 L. J. Marnett and M. A. Tuttle, *Cancer Res.*, 40 (1980) 276.