Journal of Chromatography, 495 (1989) 235–238
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4894

Note

High-performance liquid chromatographic determination of malonaldehyde in serum

SATOSHI KAWAI* and KAZUTO KASASHIMA

Gifu Pharmaceutical University, Mitahora, Gifu 502 (Japan)

and

MASAFUMI TOMITA

Department of Legal Medicine, Kawasaki Medical School, Matsushima, Kurashiki 701-01 (Japan)

(First received March 14th, 1989, revised manuscript received May 27th, 1989)

Malonaldehyde (MA) is useful as an indicator of lipid peroxidation, cellular ageing and free radical damage, and has been determined by high-performance liquid chromatography (HPLC) in the free form [1–5] or derivatized with 2-thiobarbituric acid (TBA) [6–12], dansylhydrazine (DNSH) [13,14] or by the Hantzsch reaction [15] On an ODS column, free MA is eluted shortly after or within the void volume, and many compounds present in biological samples can therefore interfere with the MA peak On the other hand, reactions with TBA or DNSH require hot and acidic conditions and the HPLC of the reaction products requires the use of detection in the visible usage

Recently, an HPLC technique using 2,4-dinitrophenylhydrazine (DNPH) as a derivatizing reagent has been developed for the determination of MA in urine [16,17], but its application to human serum has not been reported Reaction with DNPH proceeds readily at room temperature and HPLC with UV detection can be used This paper describes a modified HPLC method that is simple, sensitive and specific for the determination of MA in serum as the DNPH derivative.

0378-4347/89/$03 50

EXPERIMENTAL

Materials

HPLC-grade acetonitrile was purchased from Wako (Osaka, Japan) and 1,1,3,3-tetraethoxypropane, DNPH and 2-nitroresorcinol from Tokyo Kasei (Tokyo, Japan)

Preparation of MA standard solution

1,1,3,3-Tetraethoxypropane (1 mmol) was dissolved in 100 ml of 0 1 *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 0 1 *M* hydrochloric acid The concentration was calculated by assuming 100% conversion to MA.

HPLC

A Model 5A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 310 nm and a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, U S.A) was used for quantification of MA The HPLC separations were performed on a Develosil ODS-5 column (250×4 6 mm I D) (Nomura Chemical, Aichi, Japan), 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

Procedure

(a) MA standard solution A 0 5-ml volume of MA standard solution was reacted with 0 2 ml of DNPH solution (0 5 mg/ml in 1 *M* hydrochloric acid) containing an appropriate amount of 2-nitroresorcinol as an internal standard (IS) at room temperature for 1 h An aliquot of the reaction mixture was injected into the HPLC column

(b) Serum To 0 4 ml of serum in a 1 5-ml centrifuge tube were added 0 1 ml of 1% sodium hydroxide solution and 0 5 ml of acetonitrile, and the well mixed solution was incubated at 60°C for 30 min in a heating bath After centrifugation at 2000 *g*, 0 1 ml of 1 *M* hydrochloric acid was added to 0 5 ml of the supernatant and the mixture was reacted with 0.2 ml of the DNPH solution containing an appropriate amount of 2-nitroresorcinol as IS at room temperature for 1 h After centrifugation, if necessary, an aliquot of the reaction mixture was injected into the HPLC column

RESULTS AND DISCUSSION

MA reacted readily with DNPH in an acidic medium to give 1-(2,4-dinitrophenyl)pyrazole and its structure was confirmed by mass spectrometry The mass spectrum agreed precisely with that reported by Ekstrom et al [16],

having M^+ at m/z 234, the base peak at m/z 158 $[M-NO,NO_2]^+$ and an ion at m/z 204 $[M-NO]^+$ The effects of the pH of the reaction mixture, reaction temperature, reaction time and DNPH concentration on the reaction yield were examined by using the ratio of the peak height to that of the IS Acidity in the reaction mixture hardly affected the yield of MA with DNPH in the pH range 0 5–2 0 At pH > 3, the yield decreased gradually The condensation reaction proceeded readily at room temperature to reach the maximum ratio in 30 min The final conditions are described in *Procedure a* above

The method was applied to the determination of MA in serum In all previous papers on the reversed-phase HPLC of MA, neutral aqueous solutions containing an organic solvent such as methanol or acetonitrile were used, whereas we used an acidic solution containing acetonitrile as the mobile phase, allowing the presence of large amounts of existing compounds in serum Decreasing the proportion of acetonitrile in the mobile phase resulted in longer MA retention times, but the resolution of MA from unknown peaks in serum was improved. Acetonitrile–0.01 *M* hydrochloric acid (45 55, v/v) was chosen as a suitable mobile phase, taking into account sufficient resolution from interfering peaks Moreover, the use of 2-nitroresorcinol as an IS made the reliable trace determination of MA possible Acetonitrile was added at a concentration up to 50% (v/v) in order to deproteinize the serum sample As reported previously [4], it has been assumed that MA undergoes reactions with biomolecules such as amino acids, proteins or nucleic acids and MA is released from the bound form of MA–biomolecule complexes by alkali treatment at 60°C for 30 min The final conditions adopted are described in *Procedure b* above.

An UV trace from the HPLC analysis of serum after treatment with DNPH reagent is shown in Fig 1A Fig 1B shows the separation of MA standard solution and Fig. 1C shows a chromatogram of the same serum sample as in A, but spiked with the MA standard

The recovery of MA was measured by using various known amounts (1440, 144 and 72 ng/ml) of MA added to serum prior to deproteinization with acetonitrile and carrying them through the entire procedure The recovery was 86 0–90 0% (compare Fig 1B and C) The calibration graph was linear up to 18 ng/ml MA in serum and the detection limit at a signal-to-noise ratio of 3 was 10 ng/ml in serum with a 20-μl injection The mean relative standard deviation for replicate assays ($n=5$) using an identical serum sample spiked with 72 ng/ml MA was 4 6% Fig 1A shows that there is no detectable free or bound MA in normal human serum.

Another advantage of the present method over that of Ekstrom et al [17] is its simplicity They extracted the derivative with an organic solvent prior to injection into the HPLC system and employed gradient elution, whereas in our method the reaction mixture is injected directly and isocratic elution is used Therefore, the method is suited to routine clinical use and appears to be prom-

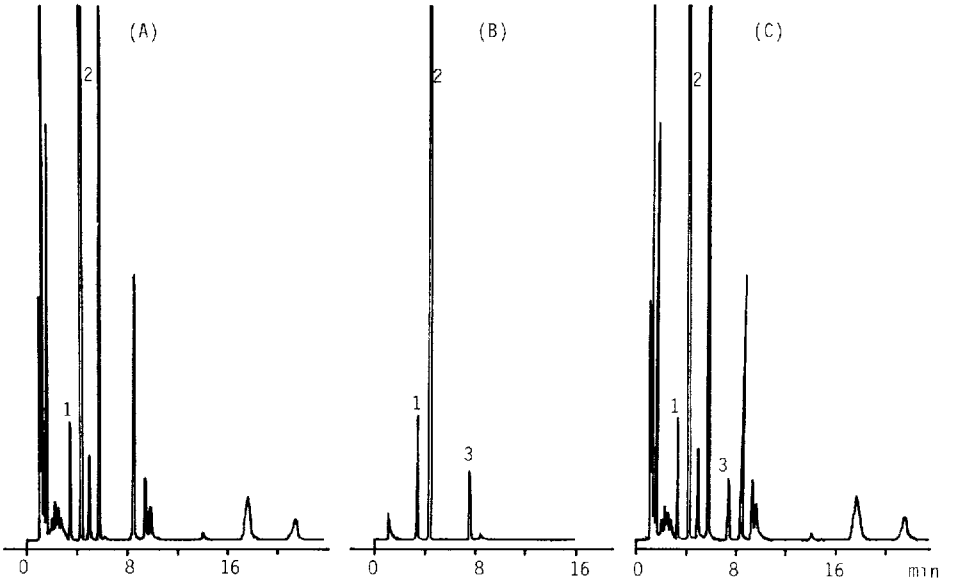

Fig 1 Elution profile of MA after treatment with DNPH reagent (A) Control human serum, (B) MA standard solution (144 ng/ml) with 250 ng of 2-nitroresorcinol as IS, (C) as A, but with added MA standard (final MA concentration in serum, 144 ng/ml) Peaks 1 = IS, 2 = DNPH, 3 = MA For HPLC conditions, see Experimental

using for the determination of MA in biological materials with respect to sensitivity, specificity and reproducibility

REFERENCES

1 H Esterbauer, J Lang, S Zadravec and T F Slater, Methods Enzymol , 105 (1984) 319
2 A S Csallany, M D Guan, J D Manwaring and P B Addis, Anal Biochem , 142 (1984) 277
3 A W Bull and L J Marnett, Anal Biochem , 149 (1985) 284
4 H -S Lee and A S Csallany, Lipids, 22 (1987) 104
5 C Largillière and S B Mélancon, Anal Biochem , 170 (1988) 123
6 R P Bird, S S O Hung, M Hadley and H H Draper, Anal Biochem , 128 (1983) 240
7 H H Draper, L Polensek, M Hadley and L G McGirr, Lipids, 19 (1984) 836
8 K Nakashima, T Ando and S Akiyama, Chem Pharm Bull , 32 (1984) 1654
9 K Nakashima, T Ando, T Nakamizo and S Akiyama, Chem Pharm Bull , 33 (1985) 5380
10 L W Yu, L Latriano, S Duncan, R A Hartwick and G Witz, Anal Biochem , 156 (1986) 326
11 H Seto, Eisei Kagaku, 33 (1987) 436
12 J Therasse and F Lemonnier, J Chromatogr , 413 (1987) 237
13 T Hirayama, N Yamada, M Nohara and S Fukui, J Assoc Off Anal Chem , 66 (1983) 304
14 T Hirayama, N Yamada, M Nohara and S Fukui, J Sci Food Agric , 35 (1984) 338
15 K Kikugawa, T Kato and A Iwata, Anal Biochem , 174 (1988) 512
16 T Ekstrom, P Garberg, B Egestad and J Hogberg, Chem -Biol Interact , 66 (1988) 177
17 T Ekstrom, M Warholm, T Kronevi and J Hogberg, Chem -Biol Interact , 67 (1988) 25