Journal of Chromatography, 621 (1993) 77–81
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7094

Short Communication

Highly sensitive high-performance liquid chromatography for the measurement of malondialdehyde in biological samples

Kenji Fukunaga, Tetsuya Suzuki and Kozo Takama*

Department of Food Science and Technology, Faculty of Fisheries, Hokkaido University, Hakodate, Hokkaido 041 (Japan)

(First received May 5th, 1993; revised manuscript received August 20th, 1993)

ABSTRACT

A highly sensitive method for the measurement of malondialdehyde as thiobarbituric acid–malondialdehyde complex by reversed-phase high-performance liquid chromatography with fluorescence detection in biological samples is described. As samples, 20 μl of rat plasma or 10% (w/v) liver homogenate mixed with 2.0% thiobarbituric acid in 2 *M* sodium acetate buffer containing 0.05% butyl hydroxytoluene (pH 3.5) were heated at 95°C for 45 min to give the complex. The complex, extracted with *n*-butanol, was chromatographed on a system equipped with a reversed-phase column, and the eluted peak was monitored with a fluorescence detector (excitation wavelength 515 nm, emission wavelength 553 nm). The mobile phase was a acetonitrile–water (2:8, v/v) under isocratic conditions at ambient temperature, and a single analysis was done in *ca.* 4 min. The minimum detection level for malondialdehyde was as low as 0.05 pmol. The *n*-butanol extract was stable at least for 3 days. The simple mobile phase, the extremely sensitive detection limit, and the stability of the complex make this system applicable to routine clinical analysis with a small amount of tissue or biopsy sample.

INTRODUCTION

Lipid peroxidation is considered to be an important pathophysiological factor in ageing [1] and several clinical significant diseases, such as cancer [2], cardiovascular [3], or hepatic diseases [4]. For this reason, many methods have been employed to measure the degree of lipid peroxidation. Many investigators have attempted to determine lipid hydroperoxides or related substances in biological samples [5–8]. Since one of the major secondary oxidation products of polyunsaturated fatty acid is malondialdehyde (MA), measurement of MA has been taken to reflect the level of lipid hydroperoxide [9].

Several methods have been employed to measure the MA in biological samples [10–13]. Because of its procedural simplicity and sensitivity, the thiobarbuturic acid (TBA) test has been frequently and commonly used to determine MA. But TBA is not specific for MA, so several other compounds may gave coloured products that absorb at or close to the maximum absorbance of the TBA–MA complex at 532 nm. Simple measurement at 532 nm after reaction with TBA could include coloured substances derived from

* Corresponding author.

0378-4347/93/$06.00

contaminants such as amino acids, bile pigments, sugars, haemoglobin, etc. Fluorescence detection can distinguish the TBA–MA complex from these other products.

This paper describes a highly sensitive method for the measurement of MA using simple sample preparation and high-performance liquid chromatography (HPLC). This procedure is particularly adaptable to a small amount of tissue or a biopsy sample, and a large number of samples can be analysed under sensitive and selective conditions without any difficulty.

EXPERIMENTAL

Animals

Male Wistar rats (body weight 180–230 g) were kept in wire-bottomed cages and allowed free access to standard chow and water. They were starved for 24 h before the experiment. Blood was collected from the heart by syringe under diethyl ether anaesthesia, and transferred to a tube containing sodium heparin. The plasma was then removed by centrifugation at 750 *g* for 15 min at 4°C. After blood collection, the liver was removed and perfused with 0.9% NaCl via the portal vein before homogenization. The liver homogenate was prepared in a ratio of 1 g of wet liver to 9 ml of 0.9% NaCl by using a glass–PTFE homogenizer.

Chemicals

HPLC-grade acetonitrile and TBA were obtained from Nacalai Tesque (Kyoto, Japan), and 1,1,3,3-tetraethoxypropane (TEP) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of the highest commercial grade available.

Analytical procedure

An aliquot of 20 μl of plasma, 10% (w/v) liver homogenate, or MA external standard (TEP solution) was mixed with 2.0 ml of 0.2% TBA in 2 *M* sodium acetate buffer (pH 3.5) and 20 μl of 5% butyl hydroxytoluene (BHT) (Nacalai Tesque) in 99% ethanol. The sample mixture was heated in a 95°C oil-bath for 45 min. After cooling with tap water, the TBA–MA complex was extracted with *n*-butanol. Then 2 ml of *n*-butanol were added and mixed vigorously, and the mixture was centrifuged at 1500 *g* for 10 min. The *n*-butanol extract was then passed through a 0.2-μm Millipore filter (Bedford, MA, USA). To check the recovery, different amounts of TBA–MA complex were added to the plasma and liver after heating.

HPLC analysis

Reversed-phase HPLC was performed isocratically using a Hitachi Model L-6200 pump (Tokyo, Japan) with a Rheodyne 7125 injector (Berkeley, CA, USA) and Hitachi Model F-1050 fluorescence detector [22]. The separation was carried out with a Nucleosil C_{18} (10-μm) stainless-steel column (250 mm × 4.6 mm I.D.) (GL Sciences, Tokyo, Japan). The analytical column was protected by a guard column (10 mm × 4.0 mm I.D.) packed with the same material (GL Sciences). The mobile phase, acetonitrile–water (2:8, v/v), was passed through a 0.45-μm Millipore filter and degassed under vacuum for 10 min before use. The TBA–MA complex was monitored by fluorescence detection, with excitation at 515 nm and emission at 553 nm [9]. The injection volume was 20 μl, and the flow-rate was 1.0 ml/min at ambient temperature. The data were processed with a Hitachi model D-2500.

Direct spectrophotometric analysis

To determine the recovery of a known amount of TBA–MA complex added to the plasma and liver homogenate, it was extracted from the homogenate with *n*-butanol, then measured spectrophotometrically. The absorbance was measured at 532 nm using a Hitachi Model U-2000 spectrophotometer. The MA content of plasma and liver was calculated from the standard curve prepared using different amounts of TBA–MA complex. Values were expressed as nmol MA per ml of plasma or g of liver.

RESULTS AND DISCUSSION

The TBA test has been performed in various

TABLE I

RECOVERY OF TBA–MA COMPLEX ADDED TO PLASMA AND LIVER SAMPLES (n = 8)

To the plasma and liver homogenate after reaction with TBA, 5.0 or 10.0 pmol of TBA–MA complex were added, then samples were extracted with n-butanol and analysed by HPLC. The recovery was estimated by dividing the amount of TBA–MA complex obtained from the HPLC system by the original amount of TBA–MA complex added to the sample.

Sample	Standard TBA–MA complex added (pmol)	Recovery (%)
Plasma	5.0	98.2
	10.0	99.6
Liver	5.0	101.1
	10.0	99.4

TABLE II

REPRODUCIBILITY FOR TBA–MA COMPLEX IN STANDARDS MEASURED BY HPLC

MA (pmol)	Relative fluorescence intensity (peak area)[a]	Coefficient of variation (%)
0.05	3515 ± 124	6.3
0.1	6980 ± 209	3.0
0.5	34 826 ± 731	2.1
1.0	71 077 ± 645	0.9
5.0	345 448 ± 2793	0.8
10.0	689 427 ± 3379	0.5

[a] Each value represents the mean ± S.D. (n = 10).

ways [6,9,13], but previous methods are more complicated than ours. The samples were merely mixed with 0.2% TBA solution.

Several mobile phases, acetonitrile–water or methanol–water in various proportions, were examined with the aims of short analysis time, good separation, and minimum use of organic solvent. With the ODS column using acetonitrile–water (2:8, v/v) as the mobile phase, a good separation and high reproducibility resulted. TEP was used as the external standard, and the level of lipid peroxide was expressed as nanomoles of MA. The peak area of the TBA–MA complex, monitored by the fluorescence detector, was linear up to 20 pmol of MA, and the correlation coefficient was 0.9999. The minimum detectable level for MA was 0.05 pmol.

The results of the recovery study and reproducibility (represented by coefficient of variation, C.V.) for the TBA–MA complex are shown in Tables I and II, respectively. The recovery was in excess of 98%, and the reproducibility was very high at all levels.

Typical chromatograms of TBA–MA in standard solution, plasma and liver are shown in Fig. 1. The retention time for the TBA–MA complex is 3.7 min. The variations in the retention time for different samples were negligible.

When determining the level of TBA–MA by HPLC (Table II), the extraction step is necessary to avoid interferences and to extend the life of the column by separating protein or particles from the incubation mixture. Comparison of the results of the spectrophotometric and HPLC methods for the standard TBA–MA complex con-

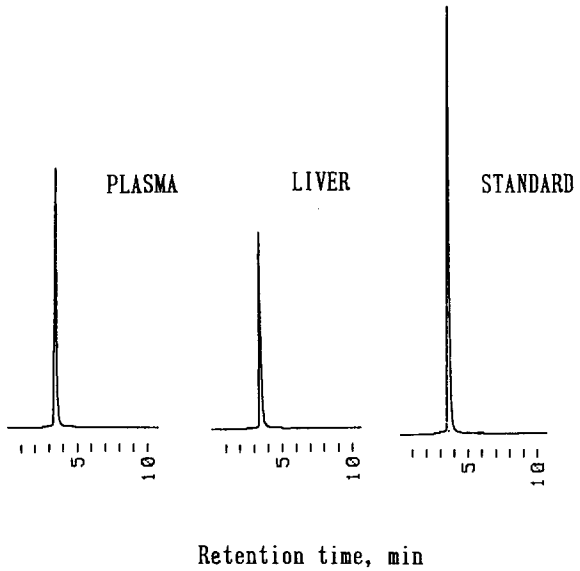

Fig. 1. Typical chromatograms of plasma, liver sample and MA standard solution. Chromatographic conditions and method of sample preparation are as given in Experimental. A 10-μl volume of the n-butanol extract was injected into HPLC system. The TBA–MA complex was monitored by fluorescence detection, with excitation at 515 nm and emission at 553 nm. The chromatograms represent the equivalent of 0.1 μl of plasma, 10 μg of liver and 0.2 pmol of MA standard, respectively.

TABLE III

COMPARISON OF THE MA LEVEL DETERMINED BY HPLC AND SPECTROPHOTOMETRIC METHODS

Spectrophotometric method[a]	HPLC method[a]	
	With *n*-butanol extraction	Without *n*-butanol extraction
Plasma (nmol/ml)		
2.39 ± 0.41	1.42 ± 0.12	1.27 ± 0.09
Liver (nmol/g)		
20.62 ± 4.32	13.19 ± 1.48	13.42 ± 1.83

[a] Each value represents the mean ± S.D. (n = 8).

firmed that, when the complex was added to the plasma and liver homogenate after colour development by heating, the recovery (Table I) and the reproducibility (Table II) were sufficient. The *n*-butanol extraction step produced no significant difference in the level of TBA–MA complex. Comparing MA levels determined by the HPLC and spectrophotometric methods, it was found that the HPLC method was more sensitive to smaller variations (Table III). The HPLC data clearly indicate an increased specificity and selectivity for MA.

The stability of the TBA–MA complex in standard solution, plasma and liver was studied. The *n*-butanol extracts were stored at room temperature without a light shield for 3 days. The initial concentration of TBA–MA was defined as 100%, and the percentage of TBA–MA remaining was calculated. The concentration did not change during the storage period (Table IV), indicating that the TBA–MA complex is highly stable. Thus the accepted idea that the absorption must be read within a few hours after development of colour in the TBA test would appear to be erroneous.

Many methods have been designed and employed to estimate the degree of lipid peroxidation [5–8]. One of the major secondary oxidation products of polyunsaturated fatty acids is MA, and measurement of MA has been regarded as measurement of lipid hydroperoxide [9]. Several methods have been used to measure the MA level in biological samples [10–13], the most frequently and commonly used being the TBA test [6,9,14,15]. However, it has been pointed out that the TBA test is not specific for MA [16,17], although it is simple, sensitive and a useful indicator of ageing and several diseases, as mentioned above. It has also been reported that the contribution of certain kinds of aldehydes to red pigment formation [18] and lipid peroxidation produces many complex compounds [19].

In this study, a simpler, more sensitive and reliable method was developed. Its advantages are as follows. It is highly sensitive and selective, because using a conventional spectrophotometer in the visible range (532 nm) gives many peaks from contaminants, whereas fluorometric detection does not have this problem and is also more than 1000 times as sensitive. Also, the coloured substance that is believed to be the TBA–MA complex has hitherto been thought to be stable for only 4 h after colour development. However, our results show that TBA–MA can be determined without any loss of fluorescence intensity even after 3 days of reaction by heating with TBA. We believe that this finding is very important with respect to enabling the TBA test to be used for estimation of peroxidation levels *in vivo* for routine clinical investigation. Another benefit for routine clinical use is that the procedure is simple and rapid, analysis being completed within *ca.* 4 min.

TABLE IV

STABILITY OF TBA–MA COMPLEX IN *n*-BUTANOL EXTRACT STORED AT ROOM TEMPERATURE WITHOUT LIGHT SHIELD FOR THREE DAYS

Initial concentration of TBA–MA is defined as 100%.

Sample	Concentration (% of initial)			
	0 days	1 day	2 days	3 days
Plasma	100.0	98.9	99.8	99.7
Liver	100.0	99.1	99.1	100.4
Standard	100.0	99.9	100.3	100.1

The method has been successfully applied to small amounts of tissue or biopsy sample and a large number of clinical samples; *ca.* 100 samples can be analysed in one day. We wish to emphasize again that our method is more reliable than the other TBA test by HPLC [20–22].

REFERENCES

1 C. J. Dillard and A. L. Tappel, *Lipids*, 8 (1973) 183.
2 R. J. Shamberger, T. L. Andreone and C. E. Willis, *J. Natl. Cancer Inst.*, 5 (1974) 1771.
3 T. F. Slater and B. C. Sawyer, *Biochem. J.*, 123 (1971) 805.
4 W. Richmond, *Clin. Chem.*, 19 (1973) 1350.
5 H. V. Thomas, P. K. Mueller and R. L. Lyman, *Science*, 159 (1967) 532.
6 H. Ohokawa, N. Ohishi and K. Yagi, *Anal. Biochem.*, 95 (1979) 351.
7 Y. Yamamoto and E. Niki, *Biochem. Biophys. Res. Commun.*, 165 (1989) 988.
8 T. Miyazawa, K. Yasuda, K. Fujimoto and T. Kaneda, *J. Biochem.*, 103 (1988) 744.
9 K. Yagi, in K. Yagi (Editor), *Lipid Peroxides in Biology and Medicine*, Academic Press, New York, 1982, p. 223.
10 E. N. Frankel and W. E. Neff, *Biochem. Biophys. Acta*, 754 (1983) 264.
11 T. Ichinose, M. G. Miller and T. Shibamoto, *Lipids*, 24 (1989) 895.
12 A. S. Csallany, M. D. Guan, J. D. Manwaring and P. B. Addis, *Anal. Biochem.*, 142 (1984) 277.
13 W. A. Behrens and R. Madere, *Lipids*, 26 (1991) 232.
14 J. A. Buege and S. D. Aust, *Methods Enzymol.*, 52 (1978) 302.
15 M. Uchiyama and M. Mihara, *Anal. Biochem.*, 86 (1978) 271.
16 B. Halliwell and J. M. C. Gutteridge, in *Free Radicals in Biology and Medicine*, 2nd ed., Clarendon Press, Oxford, 1989, p. 228.
17 K. Kikugawa, T. Kato and A. Iwata, *Anal. Biochem.*, 174 (1988) 512.
18 R. Marcuse and L. Johansson, *J. Am. Oil Chem. Soc.*, 50 (1973) 387.
19 H. Esterbauer, K. H. Cheesaman, M. U. Dianzani, G. Poli and T. F. Slater, *Biochem. J.*, 208 (1982) 129.
20 K. Kakuda, D. W. Stanly and F. R. van de Voort, *J. Am. Oil Chem. Soc.*, 58 (1981) 773.
21 R. P. Bird, S. O. Hung, M. Hadley and H. H. Draper, *Anal. Biochem.*, 128 (1983) 240.
22 V. L. Tatum, C. Changchit and C. K. Chow, *Lipids*, 25 (1990) 226.