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Short communication

Improvement in the high-performance liquid chromatography malondialdehyde level determination in normal human plasma

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Abstract

We report a very rapid and simple isocratic reversed-phase HPLC separation of malondialdehyde (MDA) in normal human plasma without previous purification of the MDA–2-thiobarbituric acid (TBA) complex. The separation of MDA–TBA complex was performed using a 250×4.6 mm Nucleosil-5C18 column with a mobile phase composed of 35% methanol and 65% 50 m*M* sodium phosphate buffer, pH 7.0. Samples of 50 μl (composed of 100 μl plasma mixed with 1.0 ml of 0.2% 2-thiobarbituric acid in 2 *M* sodium acetate buffer containing 1 m*M* diethylenetriaminepentaacetic acid, pH 3.5, and 10 μl of 5% 2,6-di-*tert*.-butyl-4-methylphenol in 96% ethanol, incubated at 95°C for 45 min [K. Fukunaga, K. Takama and T. Suzuki, Anal. Biochem., 230 (1995) 20] were injected into the column. The MDA–TBA complex was eluted at a flow-rate of 1 ml/min and monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm. Analysis of groups of normal male and female volunteers gave plasma levels of MDA of 1.076 nmol/ml with a coefficient of variation of about 58%. No significant statistical differences were found between male and female groups, and no correlation was discovered on the age.

Keywords: Malondialdehyde

1. Introduction

Lipid peroxidation is considered to be important to the development of atherosclerosis [1] and to be involved with ageing [2], and several clinically significant disorders, such as cancer [3], cardiovascular [4] and liver diseases [5]. For these reasons, many methods have been developed to measure the degree of lipid peroxidation. Malondialdehyde (MDA), one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids, has been shown to be of biological significance. MDA is speculated to be formed from fatty acids containing at least three double bonds [6]. Since MDA has been found elevated in various diseases purportedly related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [7,8].

MDA determination is based on spectrophotometric or spectrofluorimetric measurement of the condensation product formed from MDA and 2-thiobarbituric acid (TBA) [9]. HPLC techniques are useful for determining MDA in which the condensation compound of MDA and TBA is separated from interfering substances [10–15]. A very sensitive, rapid and simple HPLC determination of MDA level

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without solvent extraction procedure has been reported [15]. However, the authors determined MDA plasma level in male Wistar rats. We found the application of this techniques to the determination of MDA plasma in normal human subjects unsatisfactory due to interference by several other compounds (see Fig. 1). Here, we report a modification of the HPLC method published by Fukunaga et al. [15] and its application to the determination of plasma MDA in human volunteers.

2. Materials and methods

Human healthy volunteers are blood donors at the Laboratory of Chemistry and Clinical Analysis of the San Agostino Hospital, Modena. Blood samples were collected from healthy human volunteers with CPDA-1 (composed of citric acid, sodium citrate, sodium phosphate monobasic, dextrose and adenine) as anticoagulant. Plasma from different healthy volunteers was obtained by centrifuging whole blood and then stored at −80°C until used.

TBA was from BDH. Diethylenetriaminepentaacetic acid (DTPA) and 2,6-di-*tert*.-butyl-4-methylphenol (BHT) were from Sigma. MDA (malondialdehyde tetrabutylammonium salt, purity >98%) was from Fluka. All chemicals used were HPLC grade and of the highest guaranteed chemical grade.

For preparation of samples, an aliquot of 100 μl plasma was mixed with 1.0 ml of 0.2% TBA in 2 *M* sodium acetate buffer containing 1 m*M* DTPA, pH 3.5, and 10 μl of 5% BHT in 96% ethanol. The sample mixture was incubated at 95°C for 45 min [15,16]. After cooling at room temperature, the samples were centrifuged at 10 000 *g* for 5 min and the supernatant used for HPLC analysis.

HPLC equipment was from Jasco: pump, Model 880 PU; system controller, Model 801 SC; ternary gradient unit, Model 880-02; injector, Rheodyne equipped with a 100-μl loop; FP-210 fluorescence detector. The MDA–TBA complex was separated using a 250×4.6 mm stainless-steel column Nucleosil-5C18 (HPLC Technology Ltd., UK). The mobile phase was composed of 35% methanol and 65% 50 m*M* sodium phosphate buffer, pH 7.0, passed through a 0.45-μm Millipore filter before use. 50 μl samples were injected onto the column. The MDA–TBA complex was eluted at a flow-rate of 1 ml/min and monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm. After the daily work, the column was washed with 50% HPLC-grade water and 50% methanol.

The plasma MDA level was calculated from the calibration curve (Fig. 3) prepared by using pure MDA as the calibration standard. Values were expressed as nmol of MDA/ml plasma.

3. Results and discussion

Fukunaga et al. reported a highly sensitive and simple HPLC technique for measuring MDA in plasma of rats [15]. This method separates the MDA–TBA complex, formed after incubating 10 μl plasma and TBA at 95°C in the presence of DTPA and BHT, on reversed-phase HPLC using acetonitrile–water (7:3) as mobile phase, and fluorimetric detection. However, the application of this HPLC technique to MDA measurement in normal human plasma was unsatisfactory due to the interference by several compounds (Fig. 1). Modification of the acetonitrile–water ratio or column (μBondapak-C_{18}, 300×3.9 mm, from Waters, Division of Millipore, CA, USA; Hyperil-5ODS, 250×4.6 mm, HPLC Technology LTD, UK) was ineffective (not shown).

To improve the HPLC MDA–TBA complex separation in normal human plasma, several mobile phases were proved, and an isocratic elution consisted of 35% methanol and 65% 50 m*M* sodium phosphate buffer, pH 7.0, was found satisfactory (Fig. 2). As illustrated in the chromatographic profile of Fig. 2, the MDA–TBA complex is resolved from the other unknown peaks detected in the normal human plasma. Yang et al. [12] separated MDA produced from lipid peroxidation in the brain cortex of rats by a reversed-phase HPLC method utilizing a binary gradient elution in which mobile phase A consisted of 35% methanol and 65% 50 m*M* sodium phosphate buffer, pH 7.0. The mobile phase A reported by Yang et al. [12] was found optimal by us for isocratic determination of the MDA–TBA complex in normal human plasma.

Fig. 3 shows a calibration curve of MDA constructed by several separations of increasing amounts

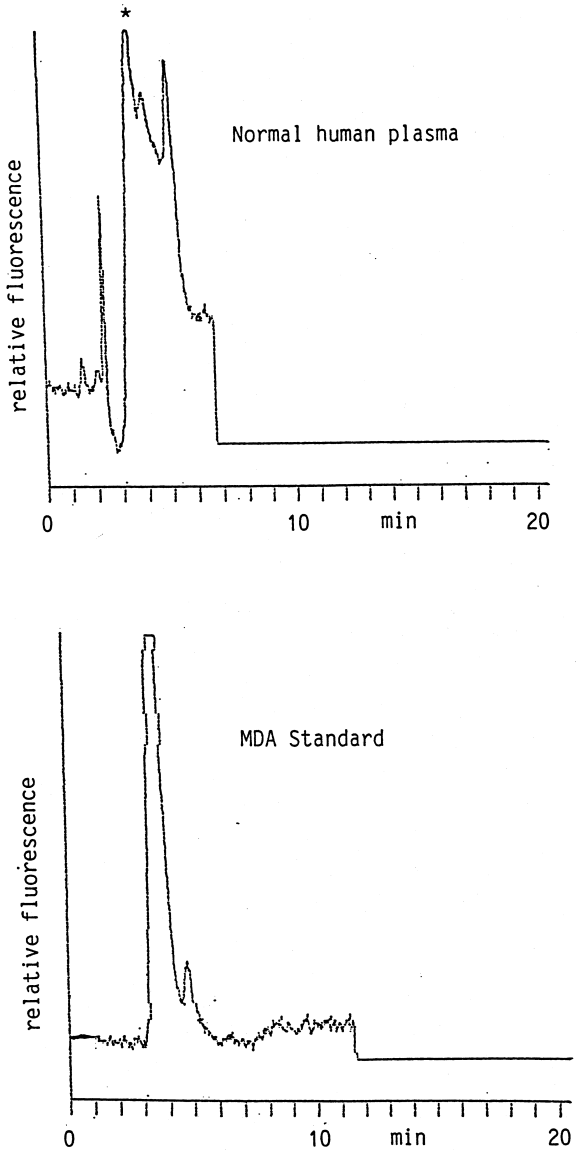

Fig. 1. HPLC separation of MDA standard (15 pmol) and in normal human plasma (100 μl) according to the method of Fukunaga et al. [15]. The asterisk indicates the MDA–TBA peak.

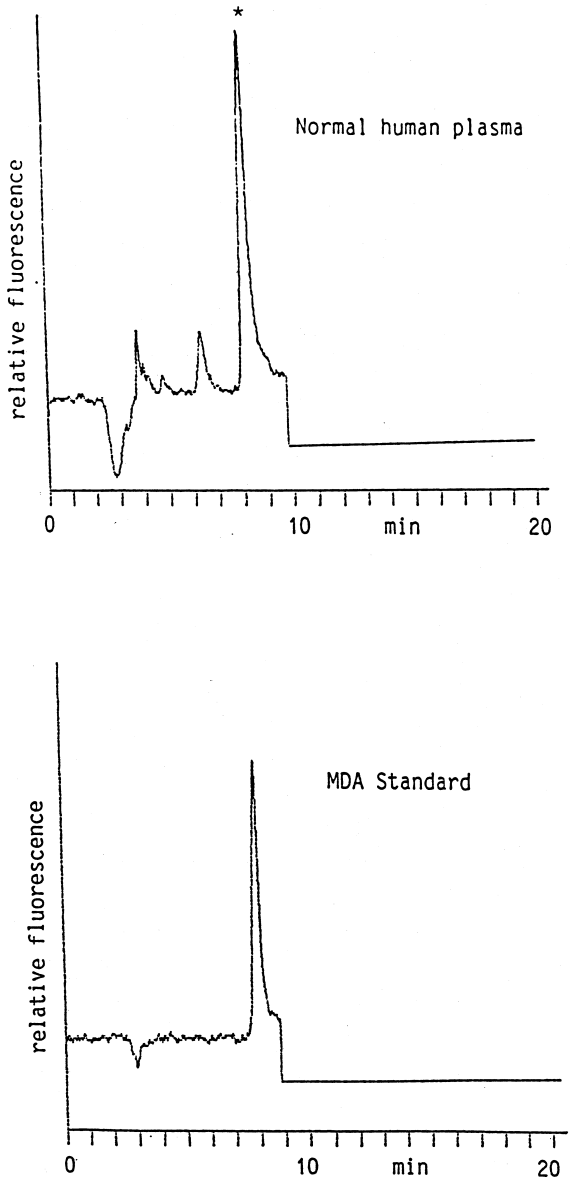

Fig. 2. Isocratic HPLC separation of MDA standard (5 pmol) and in normal human plasma (100 μl) using the mobile phase formed by 35% methanol and 65% 50 m*M* sodium phospate buffer, pH 7.0. The asterisk indicates the MDA–TBA peak.

of the MDA–TBA complex, and the reproducibility represented by the coefficient of variation for the MDA–TBA complex is shown in Table 1. The coefficient of variation was lower than 10% also for values of 15–20 pmol of MDA. Under experimental conditions, the minimum detection level for MDA was 0.5 pmol, a value greater than the 0.01 pmol reported by Fukunaga et al. [15]. Rapid separation and highly reproducible data were obtained, and neither changes in retention time nor diminution of peak resolution were observed even after 200 analyses.

MDA was measured in 100 μl of normal human

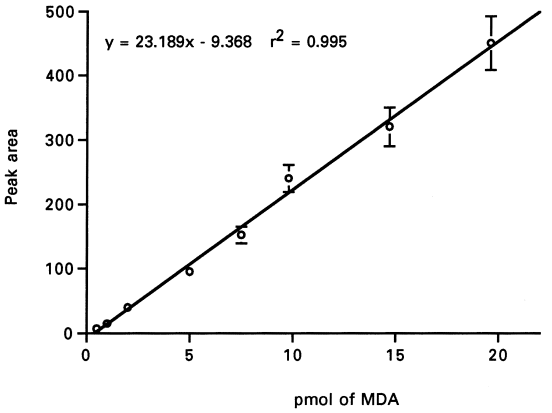

Fig. 3. Calibration curve of increasing amounts of MDA. The standard deviation values determined by at least six repetitions and the equation of the curve with the coefficient of correlation are reported.

Table 1
Reproducibility of HPLC data for the MDA–TBA complex expressed as the peak area±standard deviation (S.D.) and coefficient of variation (C.V.)

MDA (pmol)	Peak area±S.D.	C.V. (%)
0.5	7.0±0.4	6.3
1.0	15.0±1.0	6.6
2.0	39.7±2.9	7.2
5.0	95.0±7.7	8.1
7.5	152.0±12.9	8.5
10.0	240.0±20.9	8.7
15.0	320.0±30.1	9.4
20.0	450.0±41.8	9.3

plasma from male and female volunteers. No differences in MDA separation (additional peaks) were found for blood collected with CPDA-1 or EDTA (not shown). Table 2 reports the results comparing plasma MDA values of the male and female groups.

Table 2
Statistical data of the quantitative analysis of plasma MDA in male and female volunteers

	Male	Female
Observations	15	10
Mean (nmol/ml plasma)	0.906	1.246
Median	0.697	1.067
Standard deviation	0.614	0.600
Standard error	0.159	0.190
Coefficient of variation	67.82	48.20
Significance (*t*-test)	Not significant	

No relationship was also found between MDA amounts and the age of volunteers (not shown).

Several HPLC methods have been developed for the determination of MDA in human plasma. However, these techniques generally require a long execution time [7] and prepurification of the MDA–TBA complex or elimination of interfering substances [16–18]. We report a very rapid and simple isocratic reversed-phase HPLC separation of MDA in normal human plasma without previous purification of the MDA–TBA complex. The literature data on HPLC analysis of MDA in human plasma show significant differences in the mean values and standard deviations, from 0.23 [19] to 1.27 nmol/ml [20]. We analyzed a group of normal male and female volunteers, and the mean MDA concentration in plasma was 1.076 nmol/ml, with a coefficient of variation of about 58%. This level of normal human plasma MDA is in the range published in the literature. No significant statistical differences were found between male and female groups, and no correlation was discovered depending on the age of subjects.

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References

[1] D. Steinberg, J. Witztum, J. Am. Med. Assoc. 264 (1990) 3047.
[2] C.J. Dillard, A.L. Tappel, Lipids 8 (1973) 183.
[3] R.J. Shamberger, T.L. Andreone, C.E. Willis, J. Natl. Cancer Inst. 5 (1974) 1771.
[4] T.F. Slater, B.C. Sawyer, J. Biochem. 123 (1971) 805.
[5] W. Richmond, Clin. Chem. 19 (1973) 1350.
[6] W.A. Pryor, J.P. Stanley, E. Blair, Lipids 11 (1976) 370.
[7] J. Suttnar, J. Cermak, E. Dyr, Anal. Biochem. 249 (1997) 20.
[8] M. Ohrvall, S. Tengblad, B. Ekstrand, A. Siegbahn, B. Vessby, Atherosclerosis 108 (1994) 103.
[9] H. Ohkawa, N. Ohishi, K. Yagi, Anal. Biochem. 95 (1979) 351.
[10] G.A. Cordis, D. Bagchi, N. Maulik, D.K. Das, J. Chromatogr. A 661 (1994) 181.
[11] A.S. Csallany, M.D. Guan, J.D. Manwaring, P.B. Addis, Anal. Biochem. 142 (1984) 277.

[12] C. Yang, P. Tsai, J. Wu, N. Lin, S. Chou, J. Kuo, J. Chromatogr. B 693 (1997) 257.
[13] G. Lepage, G. Munoz, J. Champagnend, B.C. Sawyer, J. Biochem. 123 (1971) 805.
[14] A.W. Bull, L.J. Marnett, Anal. Biochem. 149 (1985) 284.
[15] K. Fukunaga, K. Takama, T. Suzuki, Anal. Biochem. 230 (1995) 20.
[16] K. Fukunaga, T. Suzuki, K. Takama, J. Chromatogr. 621 (1993) 77.
[17] S. Wong, J. Knight, S. Hopfer, O. Zaharia, C. Leach, W. Sunderman, Clin. Chem. 33 (1987) 214.
[18] I.S. Young, E.R. Trimble, Ann. Clin. Biochem. 28 (1991) 504.
[19] A. Sonnérborg, G. Carlin, B. Akerlund, C. Jarstrand, Scand. J. Infect. Dis. 20 (1988) 287.
[20] J. Therasse, F. Lemonnier, J. Chromatogr. 413 (1987) 237.