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Journal of Chromatography B, 751 (2001) 193–197

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Influence of citrate and EDTA anticoagulants on plasma malondialdehyde concentrations estimated by high-performance liquid chromatography

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Received 1 March 2000; accepted 8 August 2000

Abstract

Estimation of lipid peroxidation through MDA formation measured by assaying thiobarbituric acid (TBA) reactive products separated by HPLC remains the method of choice to study the development of oxidative stress in blood plasma. In this report we describe the influence of citrate and EDTA anticoagulants used for blood collection on estimation of MDA concentrations using HPLC analysis of MDA-TBA adducts. We analyzed a group of 40 blood donors (21 men and 19 women), median age 27 years, range 19–48 years. The mean MDA concentration in citrate plasma was 1.43±0.51 μmol/l (range: 0.61–2.57 μmol/l) and in EDTA plasma 0.36±0.10 μmol/l (range: 0.13–0.63 μmol/l). There was a significant difference in MDA mean concentration that we attribute to different antioxidant properties of anticoagulants used for blood collection. Consistency in the choice of anticoagulant is clearly extremely important. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Citrate; EDTA; Malondialdehyde

1. Introduction

Malondialdehyde (MDA) is generally formed as a consequence of lipid peroxidation and/or of prostaglandin's metabolism [1,2]. MDA is reactive toward amino groups of proteins and nucleic acids, it has been inferred to have mutagenic and cytotoxic roles, and possibly to be a participant in the onset of atherosclerosis. When heated at acidic pH, MDA reacts with a number of nucleophiles to yield a variety of condensation/dehydration products [3]. The 2-thiobarbituric acid (TBA) yields at these conditions with MDA red fluorescent pigment (MDA-2TBA) widely used in clinical chemistry laboratories for the spectrophotometric or spectrofluorimetric measurement of MDA amounts [4].

Many factors (e.g., stimulus for and conditions of peroxidation) modulate MDA formation from a lipid [3]. TBA-test reagents and constituents have profound effects on the test response to the MDA. As pointed out by Cordova et al. [5], both the acidification and the boiling of plasma samples, essential

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PII: S0378-4347(00)00453-9

procedures in TBA method, facilitate autoxidation of polyunsaturated fatty acids (PUFA). Some lipid may still be present in samples, even after removal by precipitation with proteins. Therefore, addition of antioxidant (butylated hydroxytoluene–BHT) to the reaction mixture is essential for inhibition of lipid oxidation without affecting the formation of the MDA-2TBA chromogen [6]. The TBA test is intrinsically non-specific for MDA; non-lipid-related materials as well as fatty peroxide-derived decomposition products other than MDA are TBA positive [7] which results in overestimating of MDA levels.

HPLC techniques were proposed for determining MDA in which condensation compound of MDA and TBA is separated from interfering substances by chromatography on a column of octadecyl silica gel [8–15]. HPLC methods generally yield lower values than those based on direct measurements, and this would seem to reinforce their specificity.

Sample preparative steps constitute the other pitfalls responsible for the lack of specificity and the poor reproducibility of the technique in complex biological fluids and for the large variability of results among techniques. One of these preparative steps influencing MDA yields in the early stages of sample preparation is the choice of an anticoagulant for blood collection.

Lepage et al. [16] suggested blood collection under EDTA anticoagulant for plasma MDA (P-MDA) determination to prevent autoxidation since platelets are rich in lipoperoxides and appear to be the main source of plasma MDA. Wong et al. [13] used the same conditions for blood collection. They found that malondialdehyde concentrations were increased by 1.5 to 2 times after 1 h storage of serum or of plasma specimens collected with heparin or citrate and stored at 4°C. In addition, Knight et al. [17] compared MDA concentration in plasma from 230 male and 148 female adult blood donors. They showed that man had higher MDA concentrations in plasma when compared with women, older donors had higher values than younger, and plasma from EDTA-anticoagulated blood had significantly lower MDA concentrations than does plasma from blood treated with sodium citrate. Nielsen et al. [18] compared MDA concentrations in serum and plasma from 13 fasting volunteers (seven men and six women, ages 20–33 years). Plasma samples were collected in Venoject tubes with different anticoagulants. They used TBA derivatization of MDA in samples and HPLC analysis of MDA-2TBA. Comparable concentrations of MDA were found both in serum and plasma anticoagulated either by citrate or heparin and much lower concentrations of MDA were found in EDTA anticoagulated plasma (Table 1). In contrast, Wasowicz et al. [19] analyzed spectrofluorimetrically thiobarbituric acid-reactive substances (TBARS) in plasma collected in EDTA and estimated TBARS concentrations (in MDA equivalents) by 30% significantly higher than from serum and heparinized plasma. In this paper we examined the influence of anticoagulants used in blood collection on MDA concentrations estimated by HPLC of MDA-2TBA.

Table 1
Literature data on HPLC analysis of MDA in blood plasma

Anticoagulant	Mean P-MDA±SD (μmol/l)	Literature
EDTA	2.51±0.75	[8]
Not described	1.27±0.98	[9]
Citrate	1.08±0.61	[10]
EDTA	0.86±0.18	[11]
EDTA or heparin	0.456±0.066	[12]
EDTA	0.45±0.18	[13]
Citrate	0.37±0.25	[14]
EDTA	0.23±0.04	[15]
EDTA	0.58±0.17	[17]
Citrate	0.88±0.19	[17]
Heparin	1.27	[18]
Citrate	0.89	[18]
EDTA	0.26	[18]

2. Material and methods

2.1. Chemicals and reagents

Methanol (LiChrosolv grade) was supplied by Merck (Darmstadt, Germany). Water was double glass-distilled and deionized on Milli-Q50 (Millipore, Vienna, Austria), 1,1,3,3-Tetraethoxypropane (TEP), and butylated hydroxytoluene (BHT) were supplied by Sigma–Aldrich (Prague, Czech Republic). All other chemicals were of analytical grade.

2.2. Sample preparation

Blood was collected in parallel by venipuncture into polypropylene tubes, first to EDTA, and subsequently to citrate anticoagulant. Final citrate concentration in citrate anticoagulated blood (final volume of 2 ml) was 0.76% (26 mmol/l), final EDTA concentration in EDTA anticoagulated blood (final volume 2 ml) was 0.18% (4 mmol/l). All individuals tested agreed to this study at the time of blood collection. All samples were obtained in accordance with the Ethical Committee regulations of our Institute. The samples were centrifuged within 30 min of collection, plasma supernatant was transferred to polypropylene Eppendorf tubes in 200 μl aliquots, frozen in dry-ice and stored at −70°C until used.

To 200 μl of either EDTA or citrate plasma in a polypropylene Eppendorf tube (1.5 ml) were added 50 μl of 50 mmol/l EDTA in 1% sodium hydroxide solution and 250 μl of 10 mmol/l butylated hydroxytoluene (BHT) in acetonitrile within 5 min from the start of the thawing of stored plasma. After thorough mixing the solution was incubated at 60°C for 30 min in a water bath [14]. The samples cooled at room temperature were centrifuged at 40 000×*g* for 30 min. A total of 150 μl of the supernatant was transferred to polypropylene Eppendorf tube (1.5 ml), 600 μl of 25 mmol/l TBA in an acetate buffer (2.0 mol/l, pH 3.0) was added, well mixed, and heated in a boiling water bath for 60 min. After cooling in ice-water bath, the samples were centrifuged at 40 000 *g* for 30 min and 40 μl of supernatant was injected without any prepurification step into the HPLC [8,10]. The standard solutions of MDA (10.0, 5.0, 2.0,1.0, 0.5, 0.2, 0.1 and 0.0 μmol/l) prepared from TEP by acidic hydrolysis as described previously [14] were processed in the same way.

2.3. Influence of anticoagulants on the calibration curve

Calibration curves were constructed according to the hitherto described procedure. The standard solutions of MDA were prepared either in water or in water solutions of 26 mmol/l of citrate or of 4 mmol/l of EDTA.

2.4. Apparatus and chromatographic conditions

Both EDTA and citrate samples from an individual were processed together and were included in the same analytical run. Separations were carried out according to Suttnar [14] using a LKB 2150 HPLC pump (LKB-Produkter AB, Bromma, Sweden) fitted with a Rheodyne 7125 sample injector, a LDC Spectromonitor 4100 (LDC Analytical, Riviera Beach, USA; UV-VIS detector fitted by second order visible filter) operating at 532 nm, a membrane degasser (LDC Analytical) and a data station (Watrex, Prague, Czech Republic). The column was NUCLEOSIL 100 C18, 125×3 mm, 5 μm (Macherey-Nagel, Duren, Germany).

Elution of MDA-2TBA was performed isocratically with a mixture of methanol and a buffer consisting of 100 mmol/l H_3PO_4, pH 6.5, adjusted with NaOH (40:60, v/v) at a flow-rate of 0.5 ml/min at room temperature.

3. Results and discussion

The analytical conditions used for alkaline hydrolysis of bound MDA and for chromatographic analysis were the same for both EDTA and citrate plasma. Enhanced buffer concentration (100 mmol/l) used in mobile phase facilitated the column equilibration of sample containing 1.6 mol/l acetate buffer, pH 3 (final values). The chromatograms of MDA-2TBA obtained from MDA standard in water and either citrate or EDTA anticoagulated plasma are shown in Fig. 1. The limit of the MDA detection was estimated to the 0.07 μmol/l. BHT and EDTA were added to prevent oxidation during alkaline hydrolysis and during TBA reaction. The calibration curve prepared by plotting the peak areas of the TEP standards was linear within a range of 0 to 10 μmol/l with average $r^2=0.997$. The pooled standard deviation for replicate assays ($n=5$) using an identical plasma sample spiked with 0.5, 1.5 and 3 μmol/l MDA was 0.10 μmol/l in within-day runs and 0.15 μmol/l in between-day runs. Plasma samples were analyzed without delay for within-run test, and stored at −70°C for between-run test. We tested MDA concentration estimated in paired plasma samples analyzed both immediately (after the first

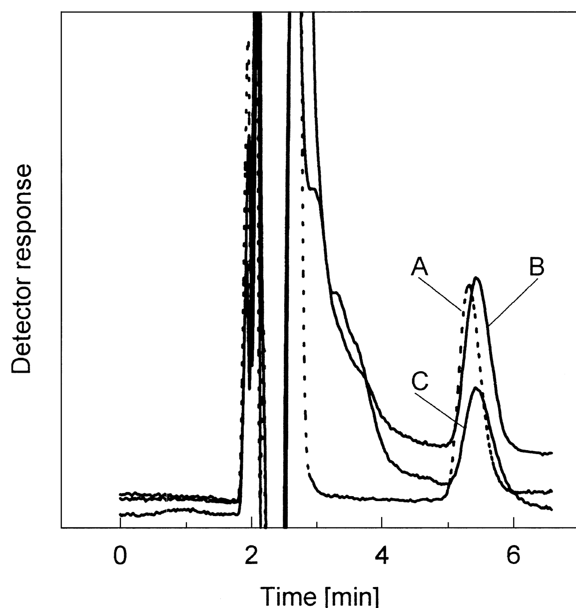

Fig. 1. Isocratic HPLC separation of MDA-2TBA obtained from MDA standard (A) (dotted line), citrate anticoagulated plasma (B), and EDTA anticoagulated plasma (C). Column, NUCLEOSIL 100 C18, 125×3 mm, 5 μm (Macherey-Nagel, Duren, Germany); mobile phase, a mixture of methanol and a buffer consisting of 100 mmol/l H_3PO_4, pH 6.5, adjusted with NaOH (40:60, v/v); flow-rate, 0.5 ml/min; detector, UV–Vis, 532 nm.

centrifugation) and after the storage for 21 days at −70°C. Additionally, we analyzed paired plasma samples both with and without addition of BHT in ethanol (2.8 mmol/l, final concentration) which were subsequently stored 21 days at −70°C. We did not observe significant differences in MDA concentrations for the both sample handlings.

The literature data on HPLC analysis of MDA in normal donor's plasma show differences in both averages and standard deviations (Table 1). In a discipline where published reference values of MDA using non chromatographic techniques can vary 100-fold [20], these results, independently of the used anticoagulants, are in relative concordance with recent reference intervals for P-MDA, established by HPLC of MDA-TBA [18] in EDTA-anticoagulated plasma (0.36 μmol/l and 1.24 μmol/l for 0.025 and 0.975 fractals).

For estimation of both EDTA and citrate anticoagulant suitability for MDA determination, we analyzed a group of 40 blood donors (21 men and 19 women), median age 27 years, range 19–48 years. The results are shown in Table 2. There was a significant difference of MDA mean concentration ($P<0.001$, two-sided Student's paired *t*-test, Instat v.2.03, GraphPad Software) that we attribute to different anticoagulants used for blood collection. In a hydrolysing step, BHT and EDTA were added to the all plasma samples prepared from either EDTA or citrate anticoagulated blood. We tested the influence of the anticoagulants present in samples on the calibration curve. We found no differences in calibration curves prepared from MDA standards in water ($y=20.671x+1.233$, $r^2=0.995$), citrate ($y=20.979x+0.943$, $r^2=0.998$), and EDTA solution ($y=20.726x+1.411$, $r^2=0.999$). The observed findings therefore reflect the influence of anticoagulants during the blood sampling procedure and exclude a possible influence of anticoagulants on the HPLC separation. Lower P-MDA concentrations in EDTA anticoagulated plasma compared with citrate anticoagulated plasma are in agreement with the findings of Knight [17] and Nielsen [18] (Table 1).

Table 2
The results comparing the effect of EDTA and citrate anticoagulants on blood plasma MDA Levels

Anticoagulant	Mean P-MDA±SD (μmol/l)	Range (μmol/l)
Citrate	1.43±0.51	0.61–2.57
EDTA	0.36±0.10	0.13–0.63

We suggest that EDTA chelates traces of transition metals (Fe, Cu) in blood much more efficiently as compared with the citrate anticoagulant. Moreover, EDTA may deplete intraplatelet calcium stores [21] and thus prevent possible additional MDA formation via possible platelet activation during blood processing. Therefore, EDTA can effectively prevent oxidation of lipids by trace metals and activation of platelets in samples during the processing of blood when blood cells are present in a system with different anticoagulants. No significant gender specific differences in MDA concentration were found and no correlation was discovered depending on the age of subjects. Our results indicate the importance of the choice of anticoagulant when MDA is measured in blood plasma as a marker of oxidative stress.

Acknowledgements

This work was supported by grants 4809-3 and 4642-3 from IGA MZ (Prague, Czech Republic).

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