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Determination of malondialdehyde in breath condensate by high-performance liquid chromatography with fluorescence detection

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Abstract

An automated and rapid method for quantifying malondialdehyde (MDA) in breath condensate was developed and validated. The method is based on derivatisation with thiobarbituric acid, HPLC separation and fluorescence detection and is optimised for determination of MDA in breath condensate. Sample collection is non-invasive and simple. The detection limit (4.1 n*M*) is low, precision is good and the analysis time is short. The response is linear in the concentration range of 0.020 to 1.0 μ*M*. Samples could be stored for 1 month at −20°C and for 3 months at −80°C without losses. Using this method, there was no statistically significant difference between patients with asthma and patients without asthma. However, among females, subjects with asthma had higher MDA levels as compared to females without asthma (0.17 vs. 0.12 pmol/s, $p=0.04$). The use of the method when studying airway inflammation has to be further evaluated.

Keywords: Malondialdehyde

1. Introduction

Lipid peroxidation (LPO) is a free-radical-mediated degradative process, where polyunsaturated fatty acids in cell membranes are preferentially affected, leading to the formation of lipid hydroperoxides. The accompanying decomposition of the hydroperoxides gives rise to a wide variety of carbonyl secondary oxidation products, which can be monitored to assess LPO [1,2]. Malondialdehyde (MDA), one of the major products, has been found to be elevated under various conditions of oxidative stress, where reactive oxygen and nitrogen species (ROS/RNS) are considered to play important roles [3,4].

There are several methods for determination of MDA and the most widely employed techniques are based on spectrophotometric or spectrofluorometric determination of the pink condensation product formed from MDA and 2-thiobarbituric acid (TBA) [5], usually referred to as the TBARS (thiobarbituric acid reactive substances) test. The test is known for its lack of specificity because of the presence of

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interfering chromogens, but with high-performance liquid chromatography (HPLC) techniques the specificity is improved [6–14]. It is known that identical MDA–TBA adducts can be yielded from other compounds, depending on the reaction conditions [2,15,16]. However, according to Esterbauer and Cheeseman [17], the yields of the condensation product from other TBA-positive compounds are much lower than that from MDA. Since breath condensate is a simpler matrix than, for example, plasma, which most MDA determinations are based on, this would imply a reduction of interferents. There are also other possibilities to determine MDA, for example by HPLC as the 2,4-dinitrophenylhydrazine derivative [18,19], but the detection limits are not low enough to determine breath condensate MDA in a reliable way. These methods also require more sample preparations and larger sample volumes, and likewise for the selective gas chromatography–mass spectrometry (GC–MS) methods reported [20–22]. MS detectors are also relatively expensive.

Antczak et al. have shown increased levels of TBARS in breath condensate in subjects with asthma [23]. Hence, there are indications that MDA in breath condensate may be used as a marker of airway inflammation – a key feature of asthma. Breath condensate should also reflect airway inflammation more specifically compared to plasma or urine.

Here, we report on a method developed to determine malondialdehyde in breath condensate. Our aim was to develop a non-invasive, simple, rapid and sensitive method, demanding a minimum of effort from both the test subjects and the testing personnel. Breath condensate is a simpler matrix compared to, for example, plasma or urine, hence improving on selectivity. The method was tested in a small group of patients with and without asthma, attending consecutively at the Section of Allergology at the university hospital.

2. Experimental

2.1. Chemicals and reagents

TBA and BHT (butylated hydroxytoluene) were purchased from Sigma (St. Louis, MO, USA). MDA (malondialdehyde tetrabutylammonium salt, purity >98%) and ortho-phosphoric acid, 85% was from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, UK). The following reference substances were used: acetone and acrolein (Merck, Darmstadt, Germany), formaldehyde and propanal (Sigma) and acetic aldehyde (Fluka). All chemicals were of the highest grade available. Water was purified using a Millipore Alpha-Q system (Millipore, Bedford, MA, USA).

TBA reagent was prepared as a 25 m*M* TBA solution in 0.30 *M* phosphoric acid–KOH buffer, pH 3.5. The solvation of TBA was accomplished by mixing in a sonicating water bath. Fresh solution was prepared daily. The stock solution of MDA (5.0 m*M*) was prepared by dissolving 40 mg of MDA tetrabutylammonium salt in 25 ml of water-ethanol (60:40, v/v). The working standards were prepared by diluting the stock solution with water. No preliminary acid hydrolysis of the MDA standard was performed prior to the TBA reaction. The stock solution was stable for 1 month at +7°C, while working standards were prepared daily.

2.2. Sample collection

The samples were collected using a breath condenser (EcoScreen; Jaeger, Würzburg, Germany). Subjects were first asked to rinse their mouth with water. While wearing a nose clip, they then had to breathe tidally through a mouthpiece and a two-way non-rebreathing valve at a normal frequency for a period of 4 min. A saliva trap was connected to the valve. The temperature in the lamellar condensor tube was −20 to −25°C. Breath condensate was obtained in a sample container attached to the lamellar condensor tube after centrifugation (1200 rpm for 5 min) and the volume was determined gravimetrically. The sample was transferred to a 1.5-ml polypropylene microtube and immediately stored at −20°C, until analysis. The samples were thawed at ambient temperature just before use. Breath condensate for validation of the method was collected from healthy subjects when needed and pooled. The pooled samples always included breath condensate from at least five subjects.

2.3. Analytical procedure

For preparation of samples, 50-μl aliquots of

breath condensate were added to 450 μl TBA reagent in 0.6-ml polypropylene vials. The vials were capped with crimp caps with silicone/PTFE septas (Scantec Lab, Gothenburg, Sweden). The samples were vortex-mixed for 1 s and derivatisation was performed in a water bath at 95°C for 60 min. After cooling in an ice bath for 5 min, the samples were allowed to adapt to room temperature for about 40 min. After vortexing the samples for 1 s HPLC analysis was performed at ambient temperature.

2.4. HPLC analysis

HPLC analysis was performed using a PU-980 HPLC pump and a FP-920 fluorescence detector (Jasco, Tokyo, Japan). The analytical column was a Nucleosil 100 C_{18}, 150×4.6 mm I.D., particle size 5 μm (Macherey-Nagel, Düren, Germany). An aliquot of 20 μl was injected into the HPLC system using a laboratory robot (Gilson, Villiers-le-Bel, France) consisting of a 233 XL sample injector equipped with a Rheodyne 7010 injection valve (Rheodyne, Berkley, CA, USA), a 402 syringe pump (5 ml syringe, 500 μl transfer tubing) and Gilson 735 software. The mobile phase was composed of acetonitrile–20 m*M* potassium phosphate buffer, pH 6.8 (20:80, v/v). The flow-rate was 1.3 ml/min and the TBA–MDA adduct was monitored by fluorescence detection, with excitation at 532 nm and emission at 553 nm and with gain ×100. Chromatograms were registered and peak areas determined using HPLC-HP Chemstation software (Hewlett-Packard).

2.5. Calibration

MDA tetrabutylammonium salt was used for calibration instead of tetraethoxypropane (TEP; bis-diethyl acetal) used by many others. The substance TEP is reported to be more stable than pure MDA [24], which participates in aldol-type self-condensation reactions to generate acid and heat labile polymers in varying molecular masses and polarities [25]. However, the stock solution of MDA could be stored for 1 month at +7°C without detectable change of the signal output. Furthermore, TEP has to be hydrolysed at a low pH to form MDA, and during this reaction other by-products are believed to be formed as well, which might interfere in fluorescence studies [26]. Using MDA tetrabutylammonium salt the acid hydrolysis step could be excluded, making this substance our choice for calibration.

The determination of MDA in samples was performed with a two-point calibration curve based on three water blank measurements and three measurements at a higher concentration level (0.50 μ*M*, corresponding to 10 pmol/20 μl sample injected). It is shown that for samples with low analyte concentrations evaluated by the calibration curve technique, the optimum precision and accuracy could be obtained by using a minimum number of calibration points and performing multiple measurements on these [27]. The linearity was of course checked before this calibration routine was implemented.

In samples from test subjects the production of MDA, expressed as pmol/s (concentration in the sample×sample volume/collection time; μ*M*×μl/s) was determined. Production instead of concentration was used because consideration to total sample volume after 4 min of collection was taken.

2.6. Study population

Test subjects ($n=44$) were randomly selected among patients attending consecutively at the Section of Allergology, Sahlgrenska University Hospital, during a 2-week period. Breath condensates were collected from the patients and, in addition, they also completed a questionnaire, comprising of questions about their asthma condition and current respiratory symptoms and smoking habits. Atopy was defined as a positive skin prick test against common allergens. They also performed a lung-function test (spirometry). The group comprised of 14 males (mean age of 36 years, 21% current smokers) and 30 females (mean age of 38 years, 17% current smokers).

2.7. Statistical evaluation

The Student's *t*-test was used for the statistical analysis of the validation results. SAS software package (SAS Institute, Cary, NC, USA) was used for the statistical evaluation of the study population. Comparisons between groups were based on non-parametric tests (Kruskal–Wallis, Spearman). Mean values have been presented and *p*-values have been written out.

3. Results and discussion

3.1. Derivatisation conditions

The optimal pH for analysis was assessed by using TBA reagents with different pH values, ranging from 2.0 to 4.0. The maximum peak area of both the pooled sample and the standard (0.50 μ*M*) was obtained at pH 3.5 (Fig. 1), which is consistent with other reports [13,28]. The effect of the reaction time in the water bath was also examined. It was found that the peak area of the pooled sample increased somewhat with time (Fig. 2). The standard (0.50 μ*M*), however, did not increase after 60 min. Therefore, as well as to keep the total analysis time down, a reaction time of 60 min was chosen since the sample peak areas at this time were large enough and stable enough to make reliable determinations on. Vials with crimp caps had to be used during derivatisation because ordinary screw-capped vials were not tight enough, giving variable results. The proposed procedure for derivatisation is simple and rapid and does not include centrifugation, solvent extraction or sample neutralisation, which is used by many others.

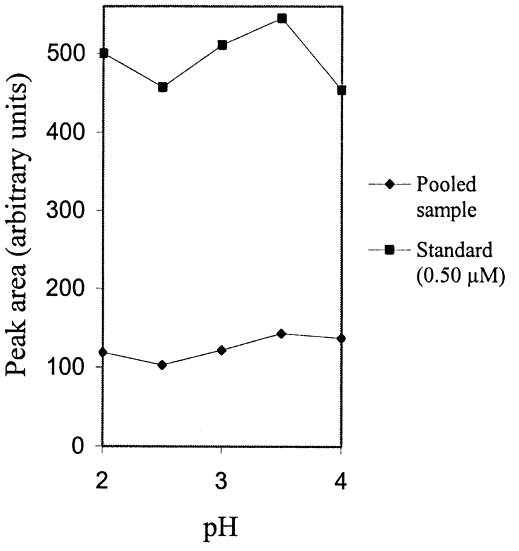

Fig. 1. Effect of pH on the assay of MDA. TBA reagents of varying pH values were used for assaying pooled sample and MDA standard. The changes in peak area were tracked.

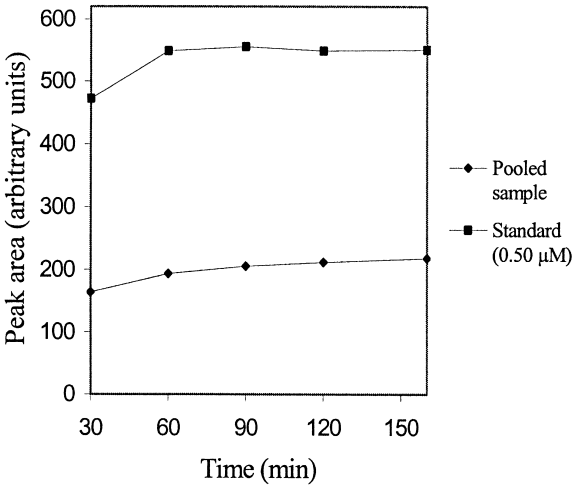

Fig. 2. Effect of incubation time on the assay of MDA. Varying incubation times in the water bath were used for assaying pooled sample and MDA standard. The changes in peak area were tracked.

3.2. Chromatography and detection

Many different mobile phases were tested and the one giving the best chromatography performance in terms of peak shape and retention time was selected. A buffer reagent had to be used, otherwise tailing became too prominent. Acetonitrile, instead of methanol, which is often used by others, gave a lower backpressure in the HPLC system. Therefore, a higher flow-rate could be used and a shorter retention time was obtained. To prove homogeneity of the peak, lower acetonitrile concentration (12%) of the mobile phase was used to allow separation of possible interferents. After standard addition to a pooled sample (6.2 pmol added), the shape of the peak, with a retention time of 9.8 min, was not changed. This indicated that no other substances were present in the peak. Analysis without a column together with a lower flow-rate was also tested, but the peak shape was not satisfactory, resulting in lower repeatability (RSD: 16%, $n=5$) when a pooled sample was analysed. Absence of the column also meant that no separation was performed, which would have resulted in lower selectivity.

We used an excitation wavelength of 532 nm, also used by others [10,11,29,30] since the signal output

was enhanced, instead of 515 nm, preferred by many others. However, a partial overlap between excitation at 532 nm and emission at 553 nm has been reported [31]. To investigate this further, calibration and determinations of sample and spiked sample (5.8 pmol added) were performed using both ex 515 nm/em 553 nm and ex 532 nm/em 553 nm. The sample and spiked sample concentrations were found to be 0.086 vs. 0.084 μ*M* and 0.20 vs. 0.20 μ*M*, respectively, i.e., no difference between excitation wavelengths was found. The signal output was enhanced by 31% using excitation wavelength 532 nm instead of 515 nm.

Typical chromatograms from a pooled sample and the standard are shown in Fig. 3. The MDA adduct was identified by adding MDA standard to a sample prior to derivatisation, which resulted in an increased sample peak area that was proportional to the added amount. Blank samples had relatively large, but stable, peak areas (RSD: 2.2%, $n=10$). They varied from approx. 10% up to approx. 70% of the sample peak areas, depending on sample concentration. The average retention time was 2.3 min and a new analysis was performed every 3.5 min.

3.3. Validation

3.3.1. Selectivity

Aldehydes and ketones in human breath have been determined by Lin et al. [32]. Since carbonyls have been proposed as possible interferents in the MDA–TBA reaction, this was further evaluated. Based on the report by Lin et al., the concentrations of possible interferents – water soluble, small carbonyls such as formaldehyde, acetaldehyde, propanal and acetone – in breath condensate were estimated. Acrolein was also included due to another study, reporting on acrolein as a possible interferent in MDA assays [33]. The calculations were based on the maximum concentration reported for each substance. The concentrations in breath condensate were estimated by dividing the amount of each substance in breath by the theoretical water content in breath, provided that the substances were completely condensed in the breath condenser during collection. In this way a concentration value of each substance was obtained, which we used in our tests, subsequently. Since all the tested substances were more volatile than water and since the condensation efficiency in the con-

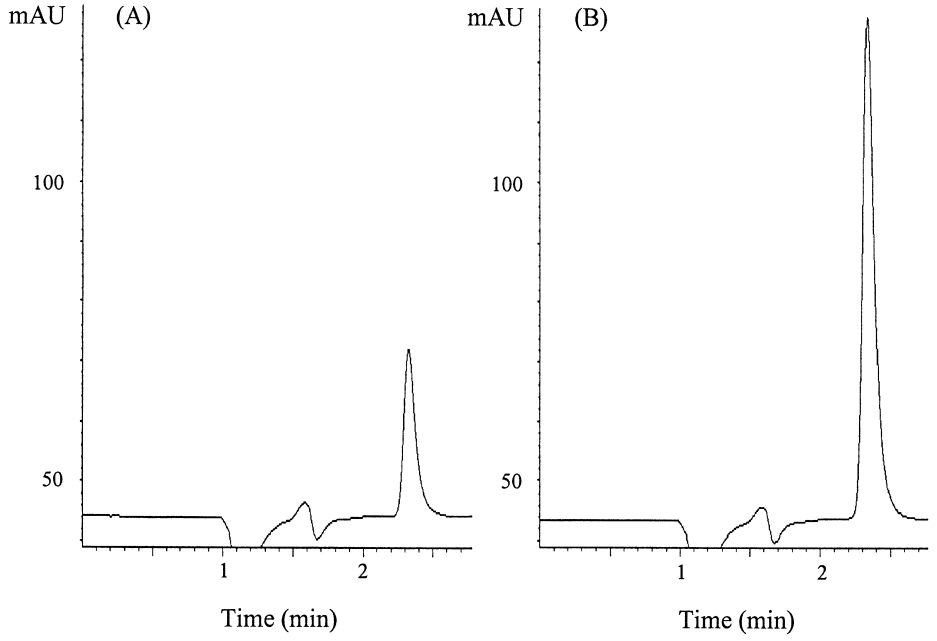

Fig. 3. Typical HPLC chromatograms of a 0.11 μ*M* breath condensate sample (A) and 0.50 μ*M* MDA standard (B).

denser was not 100%, the concentration values were probably over-estimated. Small volumes of the substances were added to the pooled samples: formaldehyde, acetaldehyde, propanal and acrolein (5 μ*M* in each 50 μl aliquot) and acetone (1 m*M* in each 50 μl aliquot). No differences in the analysed MDA concentrations could be detected, compared to the samples with no carbonyls added.

3.3.2. Effect of sample matrix

Standard addition was assessed to see if the sample matrix had any effect on MDA determination. A calibration curve (see Calibration) and a standard addition curve were prepared (both with five separate measurements at each concentration level). The standard addition curve was prepared in the following way: a pooled sample was divided into two 600 μl samples. One of the samples was spiked with a small volume of MDA standard so that the concentration in a 50-μl aliquot was 0.50 μ*M*. Samples and spiked samples were analysed and the concentration in the sample was calculated from the intercept of the calibration curve with the *x*-axis. When comparing the sample concentration calculated from the calibration curve and from the standard addition curve, the concentration was 3.1% higher for the standard addition. There was no statistical difference as the result was within the 95% confidence interval. Therefore, the matrix has no effect on the MDA determination.

3.3.3. Linearity and detection limit

Linearity was assessed by analysis of duplicate MDA standards ranging from 0.020 to 1.0 μ*M* (a total of eight concentration levels), corresponding to 0.4–20 pmol/20 μl sample injected. The linearity was very good in the concentration range studied, with a slope of 980, an intercept of −5.47 and a correlation coefficient of 0.9998. The 95% confidence interval included the origin. The limit of detection (LOD) was 4.1 n*M* (corresponding to 82 fmol/20 μl sample injected) and was calculated from $3.3SD_{bl}/S$, where SD_{bl} is the standard deviation of 10 separate blank measurements and *S* is the slope of the calibration curve [34]. The limit of quantification ($LOQ=10SD_{bl}/S$) was 12 n*M*, i.e., well below actual sample concentrations.

3.3.4. Precision

Within-assay precision was evaluated by 10 repeated, separate measurements on the same day of pooled sample and of 0.50 μ*M* standard and was found to be 3.6 and 2.2%, respectively. Between-assay precision was evaluated by five different assays, including five separate measurements of pooled sample and of 0.50 μ*M* standard over a period of 14 days and was found to be 8.9 and 4.1%, respectively. Samples and standards were stored at −20°C until analysis. Three different standard stock solutions of MDA were used for calibration during this period.

3.3.5. Stability of the MDA–TBA adduct

Stability of the TBA–MDA adduct was assessed using five aliquots of pooled sample, five blanks and five standards (0.50 μ*M*). The samples were prepared according to the procedure described and analysed immediately, as well as after 1.2, 2.5 and 3.6 h, at ambient temperature. The results showed that the samples, blanks and standards were quite stable up to 2.5 h (104, 106 and 102% recoveries, respectively). Approximately 43 samples could be analysed during this time. The recoveries after 3.6 h were 107, 108 and 105%, respectively. Therefore, it is advisable to repeat the calibration if a longer analysis time than 2.5 h is needed. This could be performed with the same blanks and standards originally used.

3.3.6. Sample stability

To assess storage stability, pooled samples were stored at different temperatures for different time periods (Table 1). Samples were stored in 1.5-ml polypropylene microtubes. The result showed that samples were stable at ambient temperature and at +7°C for 6 days, and at −20°C for 1 month, but they were not completely stable at −20°C for 3 months. According to the result, it is not advisable to store samples for periods longer than 1 month at −20°C. Samples stored at −80°C were stable for 3 months, making it the most recommended storage temperature.

It is common to add a synthetic antioxidant, e.g., BHT, to test samples such as plasma and urine, in order to suppress the formation of MDA artefacts during the analytical procedure and during storage.

Table 1
Recovery (%) of breath condensate MDA after storage

Storage temperature (°C)	*n*	Storage time			
		6 days	15 days	1 month	3 months
+25	4	100			
+7	4	104	93		
−20	4	97	100	98	71
−80[a]	4	95		103	100
−80[a,b]	4	96			

[a] Breath condensate from only one person was used.
[b] Samples were stored with BHT.

To evaluate this potential for breath condensate, 10 μl of 2 m*M* BHT in methanol was added to 1 ml of pooled sample prior to derivatisation. No difference in MDA concentration could be detected between samples with or without BHT, nor was there any difference after 6 days of storage at −80°C. The results showed that addition of BHT was not necessary when analysing and/or storing breath condensate. The reason could be that breath condensate contains less material that can be oxidised, which reduces MDA artefacts.

3.4. Breath condensate MDA in a study population

Using the described method low MDA levels could be determined in breath condensate samples with good precision. The quantification limit was well below MDA levels in the samples. There was no statistically significant difference between patients with asthma and patients without asthma (Table 2). However, among females the difference was statistically significant. There were no relations to smoking habits or atopy, and there was no correlation between the pulmonary function and MDA. This was a rather small study and in order to get a more statistically reliable result, samples from a larger study population should be used.

Table 2
The production of MDA (pmol/s) among patients with and without asthma

	All (n=44)	Females (n=30)	Males (n=14)
All patients		0.16	0.18
Patients without asthma (n=15)	0.15	0.12	0.20
Patients with asthma (n=29)	0.17	0.17[a]	0.16
With current wheezing (n=7)	0.18	0.16[b]	0.23[c]

[a] p=0.04 when compared to females without asthma.
[b] p=0.05 when compared to females without asthma.
[c] p=0.50 when compared to males without asthma.

Samples were stored for about 2 months at −20°C before analysis. Because storage losses after 3 months have been revealed at this temperature (Table 1) there could be some underestimation of MDA concentration in the samples. This should have had little impact on the comparisons between the groups studied, however, since the storage duration periods for all the samples were more or less the same.

4. Conclusions

An automated, rapid, sensitive and non-invasive method has been developed and validated in order to determine low levels of MDA in breath condensate. An experienced analyst could easily process up to 100 samples a day. The use of this method to assess airway inflammation and perhaps other oxidative conditions as well has to be further evaluated.

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