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Identification of acrolein from the ozone oxidation of unsaturated fatty acids

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By-products of lipoperoxidation reactions may be associated with the genesis or the progression of several diseases as arteriosclerosis, diabetes and cancer, among many others. Acrolein, at first a widely distributed environmental pollutant, is currently known as a compound capable of being generated as a result of metabolic reactions within biological systems, highly toxic and the most electrophilic of the α, β-unsaturated aldehydes formed during lipoperoxidation. In the present study:

1 **The separation of acrolein and malondialdehyde was achieved at alkaline pH with the use of high voltage capillary electrophoresis in uncoated fused-silica capillaries.**
2 **It was demonstrated how the oxidation of fatty acids (arachidonic/linoleic) with ozone generates, in dose-dependent form, acrolein as one of the by-products of the lipoperoxidation process. The oxidation of open human erythrocyte membranes with ozone also generated acrolein.**
3 **After aldolic condensation, aldol-acrolein derivative has a positive reaction with 2-thiobarbituric acid (TBA) and shows a maximum absorption at 498 nm. This novel characteristic is used in its identification after the separation of the by-products.**
4 **It is possible to suggest that in the classic reaction of the denominated thiobarbituric acid reactive substances (TBARS), when used as an indicator of the degree of peroxidation in biological systems, a portion of acrolein could be present but dwarfed by the TBA-MDA adduct.**

Keywords: acrolein; ozone; malondialdehyde; lipoperoxidation; capillary electrophoresis

Introduction

For several years it has been known that the oxidation of lipids or lipo-peroxidation generates a series of by-products with notable reactivity. Among these compounds are the aldehydes derived from peroxidation. Acrolein, 2-propenal or acrylaldehyde is the most reactive and cytotoxic aldehyde identified in peroxidation processes.[1] In the past, it was known as a component of environmental pollution and recognised for its irritating power and its ability for depressing the respiratory immune response.[2] It is now known as an important lipoperoxidation product. Immunoenzymatic assays used for detecting acrolein have revealed that a considerable amount of the aldehyde is freed during the oxidation of low density lipoproteins (LDL) when exposed to Cu^{2+} ions.[3] It was recently shown that during the respiratory burst of activated neutrophils, the hydrogen-chloride peroxide myeloperoxidase system can convert hydroxi-amino acids into unsaturated α, β aldehydes, among them acrolein, in large amounts.[4] Acrolein could be implicated in the etiopathology of arteriosclerosis. Its highly electrophilic character allows it to avidly react with several amino acids, in such a way that it can form Schiff bases with amino groups, β-substituted alkenals and addition products, as is the case of the acrolein-lysine adduct (N^{α}-acetyl-N^{ε}-(3-formyl-3,4 dihydropiperidine) lysine (EDP-lysine), a compound identified through immuno-histochemical analysis from fatty streak lesions of arterial tissue in humans.[5] Acrolein also induces a response to stress in alveolar macrophages, apoptosis and necrosis.[6] In the past it was known that acrolein could be formed as a metabolic product from polyamines, from the cytostatic compound cyclophosphamide, constituting a metabolite of allylic compounds such as methylamine.[7] It has been postulated that the cardiovascular damage observed in experimental animals and produced by allylamine is mediated by the formation of acrolein through enzymatic deamination catalyzed by the enzyme semicarbazide sensitive amino-oxidase (SSAO).[8–10] The assessment of acrolein in atmospheric air is generally verified using gas chromatography.[11] Given the instability, in the case of endogenous acrolein, it is best to identify the adducts formed in its interaction with amino acids and its separation using reverse-phase HPLC[5] such as has been done with other derived aldehydes, as is the case of 4-hydroxy-2-nonenal.[12] The identification and measuring of aldehydes and their metabolites are good indicators of lipidic peroxidation. The most widely used lipoperoxidation index is without

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a doubt, the formation of malondialdehyde (MDA) where this aldehyde reacts with 2-thiobarbituric acid (TBA) at a low pH and high temperature for a coloured reaction with a strong absorption of approximately 532 nm.[13]

Methods

The following reagents used were purchased from Sigma Chemical Co., USA, and were of the maximum available purity: 1, 1, 3, 3-tetramethoxypropane (malondialdehyde bis (dimethyl acetal), acrolein 90% (GC), 9, 12-octadecadienoic acid (linoleic acid) and 5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid). The capillary regenerator solution and the capillaries used (75 μm $\times$ 57 cm) were purchased from Beckman Instruments, Inc. (Fullerton, CA, USA). The HPLC grade water was purchased from Mallinckrodt Baker, S.A. de C.V. (Xalostoc, Mexico).

System and separation parameters
The samples were analyzed using the capillary electrophoresis system P/ACE TM series 5000 from Beckman Instruments, Inc. (Fullerton, CA, USA) online with a Diode Arrangement Detector for spectral analysis at a wavelength range of 190 to 600 nm (512 diodes). In all cases, the free capillary columns measuring 57 cm long (50 cm to the detection window) and 75 μm of internal diameter, built within the P/ACE cartridge for capillary cooling, were used. The samples were pressure-injected at 0.5 p.s.i. for 5 s. The optimum separation voltage used in all cases was 25 kV (normal polarity) and the treatment between capillary runs was: 4 min of capillary washing with the running buffer; 4 min with the 1.0% NaOH regenerating solution; 6 min washing with water and 6 min of draining previous to the injection of the sample with the running buffer (high pressure: 15 p.s.i.). In all cases, the running buffer used was 0.1 M borate pH 8.27. For the analysis, the Beckman system Gold software © 1991 was used.

Fractions obtention
The total estimated capillary volume at 27°C and 0.798×10^{-2} pois was 2544 nl. Taking into account that the first separated compound appeared at approximately 3 min after having started the run, the system was programmed to stop the separation after 3 min of the run. The system was ordered to inject 312.4 nl of the mixture at low grade pressure (0.5 p.s.i.) for 1 min. This sequence is repeated 50 times and corresponds to the first fraction.

Every five runs, the apparatus was programmed to carry out another four consecutive injections (in their corresponding tubes) equivalent to additional four fractions containing 312.4 nl each. The main rationale behind this was that the first fraction would contain the first compound given that it includes the volume equivalent to the distance to the window detector, that is to say 7 cm. Given that the concentration of the first compound was expected to be 9.5 times lower, five injections of the same fraction were done for each. The volumes were adjusted at the end. The fraction was collected in micro-tubes containing 0.375% TBA solution.

Fatty acid vesicles (ARLI)
Precisely 40 mg of linoleic acid and 0.40 mg of arachidonic acid were mixed in 4 ml of Tris/HCl at pH 8.0. A suspension was formed by intense agitation for a period of 30 min. Later on, the mixture was taken to a final concentration of 7.2 mM using the same buffer.[14] Vesicles were always freshly prepared. Immediately before the assays, 200 μl of the ARLI solution were taken and the volume adjusted to 1000 μl with HPLC water for a final concentration of 1.44 μM. The resulting solution was suctioned into a plastic syringe where the oxygen-ozone mixture was used for verifying the reaction. The final intense agitation for 20 min insures the complete exhaustion of the ozone oxidising capacity. The polar phase containing the aldehydes is obtained after the lipid extraction with chloroform-methanol (2 : 1), agitated for 10 min and centrifuging the sample at 2500 *g* for 15 min.

Erythrocyte ghosts
Open erythrocyte membranes were prepared by hypotonic lysis obtained from complete blood samples from adult volunteers, collected in sterile test tubes containing heparin as an anticoagulant, as originally described by Steck.[15] The entire process was verified at 4°C and on the same day the experiment was performed. Finally, the membrane's protein concentration was 3.5 μg/μl on the average. The oxidation takes place using a volume equivalent to 70 μg and adjusting exactly to 1000 μl with HPLC water. The same procedure as described for ARLI including the extraction with the chloroform-methanol 2 : 1 mixture was followed.

Exposure to ozone
The ozone used in the experiments was generated from a high voltage electric arch (Biozon, Fagor; Bask Tec, Mexico), connected to an ultrapure oxygen tank. The ozone mixture was deposited in a container where the mixture circulated constantly. The mixture volumes were extracted using a needle and a syringe, through a rubber stopper adhered to the container. The syringes were sealed and all the reactions were carried out inside, agitating the mixture for 30 min using a magnetic stirrer. Ozone concentration curves were carried out within the plastic syringes each time an experiment was carried out measuring the iodine

concentration (ε=25 200/350 nm) as a product of the stechiometric reaction of ozone with the Kl.[16,17] The ozone concentration estimated was 1 μg per ml of the gas mixture taken from the container.

Preparation of standards

A malondialdehyde (MDA) stock solution was prepared from the acid hydrolysis of the compound, previously distilled malondialdehyde-bisdimethylacetal.[18] Approximately 164.2 mg of the compound were removed and 100 ml of 1% sulphuric acid (v/v) were added. After letting rest at room temperature for 2 h, 1 ml of the solution was taken and adjusted to a 100 ml volume with more 1% sulphuric acid (v/v). The stock solution has an approximate concentration of 10 mM, while the final solution for 0.1 mM spectrophotometric assessment, verified using an ε=13 700 and ε=153 000 for the MDA-TBA adduct, should have a variation of no more than 2%. Approximately 100 μl of this solution was taken and the same volume of 1% NaOH added. The solution was allowed to rest in the dark for 30 min. The formation of the NaMDA form is verified at 266–267 nm.[19] The volume is adjusted to 1 ml using a 0.01 mM pH 8.27 borate buffer. The final concentration of the solution was 10 μM. In acrolein's case, at 2°C, 20 μl of the bottle were removed (90%, d=0.849) and dissolved in 20 ml of water (HPLC grade). From this solution, 100 μl were taken to which an equal volume of 1% NaOH was added. The aldolic condensation was monitored at 266 nm. The final acrolein solution was 1.6 mM. Under these conditions, additional spectral readings and electrophoretic runs were conducted. The uv/vis scannings were carried out with a spectrophotometer Beckman DU-650, Beckman Instruments, Inc. (Fullerton, CA, USA).

The values reported of the migration times are expressed as mean ± standard deviation, while the percentage dispersion values correspond to variation coefficients.

Results

In the present study, we observed that the product obtained from the aldolic condensation of an acrolein standard presents a positive reaction with TBA and shows a maximum absorption at 498 nm. Under the same conditions, a malonaldehyde standard presents the maximum characteristic of the MDA:TBA conjugate 1 : 2 at 532 nm.[20] However, the ultraviolet absorption of both compounds shows the same maximum at 266 nm at pH 8.2. An acrolein and malonaldehyde mixture at an alkaline pH was separated by capillary zone electrophoresis (free solution capillary electrophoresis). The ozone oxidation of pure fatty acids (arachidonic/linoleic) in a suspension gives as a result, the freeing in the aqueous phase of a mixture that shows the same absorption maximum at 266 nm. The electrophoretic separation reveals the formation of two peaks with the same migration times as acrolein and MDA. Finally, when separating the fractions, it is shown that their reaction with TBA corresponds to the absorbencies of both acrolein (498 nm) and MDA (532 nm) standards in its reaction with TBA, demonstrating that the ozone oxidation of unsaturated fatty acids can generate acrolein as a peroxidation by-product.

The oxidation of unsaturated fatty acids (arachidonic/linoleic) by ozone generates a hydrosoluble mixture with strong absorption at 266 nm at an alkaline pH (Figure 1). The absorption of the mixture obtained is proportional to the degree of oxidation, that is, to the concentration of ozone. In the inserted graph, the same maximum absorbency at 266 nm is shown after oxidising the open human erythrocyte membranes with ozone. The mixture of components obtained after the separation of lipids with organic solvents shows a positive reaction with TBA, with the characteristic coloration of what are known as TBARS. The separation with high voltage, alkaline pH and in an open capillary of 75 μm of internal diameter of the mixture beforementioned, reveals two peaks with retention times equivalent to 2.7 ± 0.25 and 5.8 ± 0.23 min (Figure 2). As a result of the analysis of several aldehydes, it was proven that the retention (or migration) times correspond to acrolein and MDA respectively, as was proven when testing the standards of these aldehydes under the same separation conditions

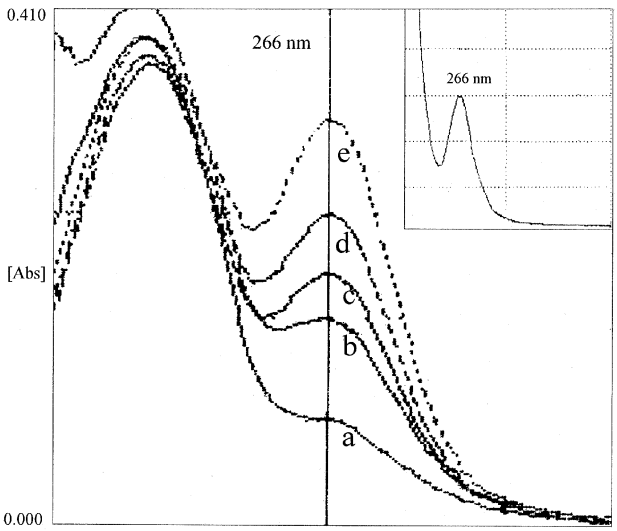

Figure 1 Increase in the absorption at 266 nm of a polar fraction extracted from the mixture of fatty acids (arachidonic/linoleic) or ARLI, exposed to 1.37 (**a**), 2.75 (**b**), 4.13 (**c**), 5.51 (**d**), 6.89 (**e**) μg of ozone/nmol of fatty acids. The exposure took place within the plastic syringes from which the ozone aliquots were taken as described in the Methods section. In the inserted graph, the polar phase of a sample of open erythrocyte membranes oxidised with 3.8 g of ozone/μg of membrane protein is shown

(Figure 3). The inserted figure shows how the independent reactions from TBA with acrolein and MDA presented close, but yet different absorption peaks (acrolein at 498 nm and MDA at 532 nm). However, it is clear that only 5% (calculated area %) of acrolein is generated from the total mixture reactive to TBA.

In Figure 4, the electropherograms corresponding to the product of the oxidation of erythrocyte membranes (discontinuous line) and an aldol-acrolein standard (continuous line) to 266 nm are shown. An identical migration time of 2.7 min is shown. The variation between two consecutive runs was less than 1.5%. When the capillary conditions are optimum, the migration is not considerably affected after 20 min. Once there is capillary instability, it may be re-established with the following treatment: water for 30 min; HCl 1 M for 5 min; water for another 30 min and capillary regeneration with NaOH 1% for 5 min. The max-

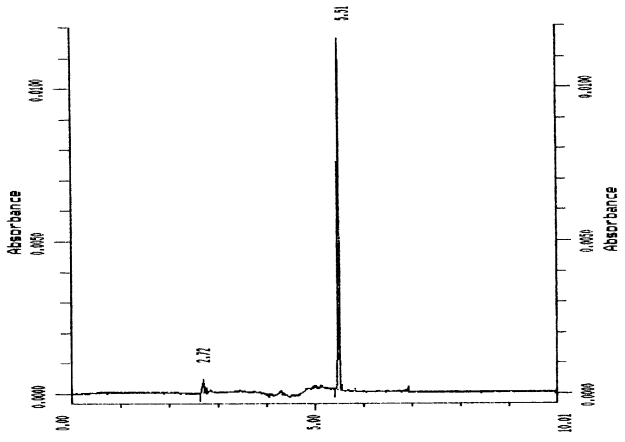

Figure 2 Electropherogram of the oxidation products from fatty acids (araquidonic/linoleic) after the extraction with organic solvents. The software analysis of the area % reveals two peaks correspondent to 5 and 95% approximately. The separation conditions were the same as those described for the standards in Figure 3. The ozone concentration was 4.2 μg/nmol of free fatty acids. The maximum variation estimated for the migration times of ten samples was 9.3 and 4.1% for acrolein and malondialdehyde, respectively

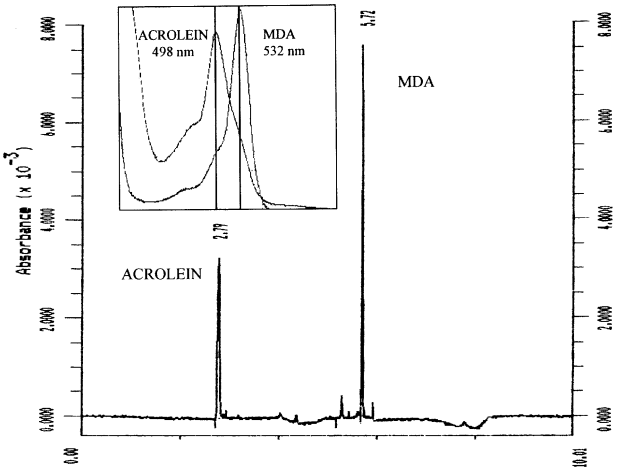

Figure 3 Electropherograms of the acrolein and malondialdehyde standards. Conditions: open capillary of fused-silica, 75 μm of internal diameter × 50 cm of distance from the detector; potential applied of 20 Kv/40 μA; borate buffer 0.1 M pH 8.27 at a stable temperature of 27°C. The variation between the two consecutive runs was less than 1.5%. In the inserted graph, spectrum runs of the products of the reactions of the acrolein and MDA standards with the TBA

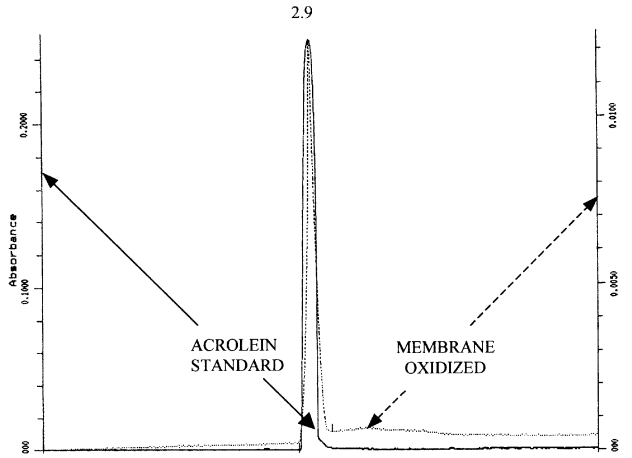

Figure 4 Superpositioning of two electropherograms corresponding to an acrolein standard and the peak corresponding to the product of the oxidation of erythrocyte membranes with ozone. The migration time of the two peaks was equal or very proximate between the two successive assays. A selected electropherogram is shown

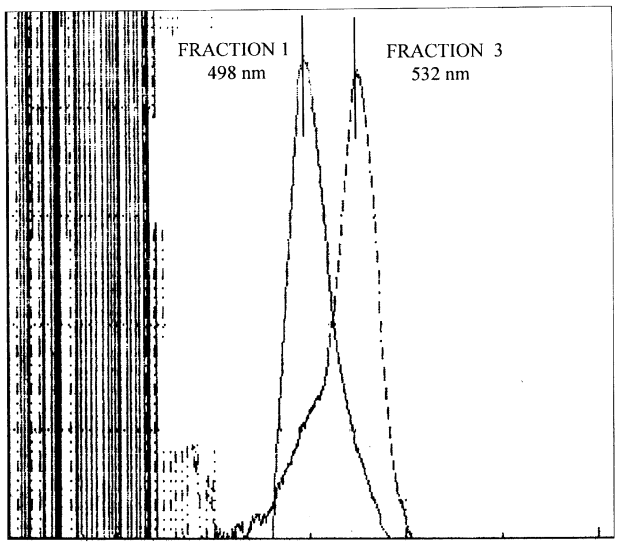

Figure 5 Spectral scan of the fractions separated from a hydrosoluble phase of erythrocyte membranes after being oxidised with ozone. The TBA reaction took place and a sample was taken without being submitted to the same conditions. The maximums of the fractions correspond to acrolein (498 nm) and malondialdehyde (532 nm). The absorbencies were found to be within the limits of the spectrophotometer. The ozone concentration in this case was 10 cm^3, equivalent to 9 μg of ozone/nmol of free fatty acids. The separation procedure is described in the Methods section

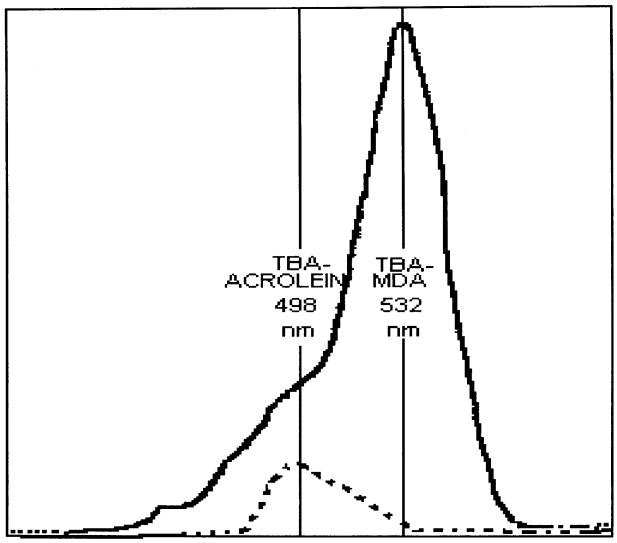

Figure 6 Explanation in the text

imum variation estimated for the migration times of ten samples was 9.3% and 4.1% for acrolein and MDA, respectively.

The separation of the fractions of an oxidised sample of erythrocyte ghosts with ozone, after reaction with TBA, shows the formation of two spectrophotometric images (Figure 5) with maximum absorbency at 498 nm and 532 nm (acroline and MDA, respectively).

Discussion

Acrolein is a highly toxic compound. Concentrations of 0.21 p.p.m. in the air are able to cause important inflammatory changes in the lungs, liver, kidneys and brain of experimental animals. In humans, it has caused deaths with concentrations under 10 p.p.m.[21,22] In addition, it seems clear that acrolein is implicated in the genesis and progression of many vascular lesions, including those associated to arteriosclerosis.[3–5] It is known that acrolein may come from different sources, among them the combustion generated from the burning of fossil combustion and fires, cigarette smoke, etc. It is also known that it can come about endogenously as a metabolite of several compounds, among them allylic alcohol, allylamine and the anti-cancer agent, cyclophosphamide.[7,8] In the current study, it was shown that the oxidation of unsaturated fatty acids and erythrocyte membranes with ozone can generate acrolein. In spite of the fact that the ozone's main toxic effect is related to its oxidising action, the information presented is direct evidence of another possible enhanced hazardous effect of ozone.

It has been said in the past that from carbonylic compounds derived from peroxidation, only malonaldehyde reacts with TBA at an acid pH.[14] In the experiments reported herein, we verify that acrolein positively reacts with TBA, which translates in a strong absorption at 498 nm. This reaction can take place after aldolic condensation. Taking into account that only a scarce amount of acrolein is generated from a mixture of fatty acids after being oxidised, it is possible that in the classical detection of malonaldehyde using the thiobarbituric acid reaction, a small fraction of TBA-acrolein could exist, eclipsed by the presence of the TBA-MDA adduct in large amounts (Figure 6). An important implication of this fact is that between α,β-unsaturated aldehydes, acrolein is the strongest electrophilic and shows the highest reactivity with sulphydryl groups of cysteine, imidazole groups of histidine and amino groups of lysine, among others, even superior to the reactivity of 4-hydroxyl-2-nonenal.[5,23]

The percentage area of the fraction analyzed by electrophoresis revealed that a portion of approximately 5% of the ozone oxidation product corresponds to acrolein, while the other 95% is MDA. Given that the formation of many other compounds during the intense oxidation of unsaturated fatty acids has been proven,[1,14] it is clear that the separation conditions and the detector used discriminate the presence of other possible peaks in the mixture. In this sense, the diode arrangement allows to establish that only a sign is seen around 266 nm with a bandwidth of 10.

Finally, the principle of the separation in the zonal capillary electrophoresis mode is the difference in the size of the solutes and its charge at a specific pH. In the present case, the condensation of aldehydes verified at an alkaline pH is a key factor that insures its detection. The presence of hydroxyl groups (that modify the molecular polarity and therefore, migration), plus an increase in the size of the molecule due to the corresponding condensation, could be the two main factors contributing to the separation, together with the resolution ability of the high voltage capillary electrophoresis system.

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