Ozonized Unsaturated Triglycerides as Precursors of Urinary Dicarboxylic Acids

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Abstract

Oral administration of ozonized sunflower oil to Wistar rats has produced changes in the urinary content of dicarboxylic acids. Heptanedioic acid (Pimelic acid) and nonanedioic acid (Azelaic acid) were the major increased dicarboxylic acids founded. The aim of this work is the study of the urinary dicarboxylic acid profiles of Wistar rats, orally treated with ozonized standard triglycerides. The dicarboxylic acids were extracted and derivatized before the analysis by Gas Chromatography - Mass Spectrometry. The urinary dicarboxylic acid profiles of the rats that received ozonized triolein, only showed the increasing of heptanedioic and nonanedioic acids. However, when ozonized trilinolein was applied, the profile is similar to that obtained, when ozonized sunflower oil was administered. A biochemical mechanism to explain the formation of dicarboxylic acids from ozonated unsaturated triglycerides was proposed.

Introduction

Ozonized Sunflower Oil (OLEOZON®) is a registered drug that has shown antimicrobial effects against virus, bacteria and fungi (Cajigas et al., 1990; Lezcano et al., 1996; Lezcano et al., 1998). On the other hand, toxicological studies of OLEOZON® have demonstrated that this product is not mutagenic or genotoxic and has not secondary reactions in human patients (Llerena et al., 1995; Martinez et al., 1995; Remigio et al., 1998). Since 1994 it has being studied the use of ozonated sunflower oil in the treatment of giardiasis in animal models and in humans (Menéndez et al., 1995; Hernández, 1999).
Oral administration of ozonized sunflower oil to Wistar rats has produced changes in the urinary content of dicarboxylic organic acids (Jardines et al., 1998). An increment in urine of heptanedioic, octanedioic, octenedioic, nonanedioic, decenedioic and dodecenedioic acids has been observed. Heptanedioic acid (Pimelic acid) and nonanedioic acid (Azelaic acid) were the major increased dicarboxylic acids founded.

It is supposed that these increased acids are produced because of the unsaturated fatty acid metabolism and a possible mechanism to explain it, was previously proposed (Fig. 1) (Jardines et al., 1998). Linoleic and oleic acids are the main unsaturated fatty acids present in the sunflower oil (Vajda and Saenz, 1976). These fatty acids have two and one double bonds respectively in their structure and that’s why they quickly react with the ozone.

The reaction between ozone and unsaturated triglycerides occurs by the well-known Criegee mechanism (Bailey, 1978). Taking into consideration the unsaturated fatty acid composition of sunflower oil and the ozone-olefin reaction mechanism, during the ozonation of unsaturated triglycerides, aldehydes and carboxylic acids with three, six and nine carbon atoms are expected to be obtained. In this reaction hydroperoxides, ozonides and some others peroxidic or polyperoxidic species could also be obtained (Lede O. et al, 1998). The peroxidic and hydroperoxidic species partially decompose forming again aldehydes and carboxylic acids with different carbon lengths in their structures (Lede O. et al, 2001).

All the ozonation triglycerides products are metabolized in the organism by the action of different enzymes (lipase, aldehyde dehydrogenase, peroxidase and glutation-S-transferase) and they are converted to different dicarboxylic acids (final excretion products). One of the carboxylic groups is obtained by the action of the lipase and the other by the combination of ozonation and some enzyme action (aldehyde dehydrogenase, peroxidase and glutation-S-transferase).

The aim of this work was the study of the urinary dicarboxylic acid profiles of Wistar rats, orally treated with ozonized standard triglycerides (Triolein and Trilinolein) using a combination of a liquid - liquid extraction method with the Gas Chromatography / Mass Spectrometry techniques.
**Figure 1.** Proposed mechanism for urinary dicarboxylic acid formation in Wistar rats after oral administration of ozonated sunflower oil.

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Materials and Methods

Solvents and Reagents

Analytical grade diethyl ether, ethyl acetate, methanol, anhydrous sodium sulfate, sodium chloride, potassium hydroxide, and hydrochloric acid were obtained from BDH (England). N-nitro-N-metil-p-toluensulfonamide, trilinolein (99%), and triolein (99%) were purchased from Sigma (USA). All the reagents were used without previous purification.

Unsaturated Triglycerides Ozonation

Fifty milliliters of the substrate was placed in a bubbling reactor, with an oxygen flow of 10 L/h. The reactor was immersed in a water bath at 25 °C. It was used an OZOMED-400 ozone generator (Cuba). The ozone dose was determined by measuring the absorbance at 256 nm, in an Ultrospec III spectrophotometer (Pharmacia). The applied ozone dose was between 50 - 55 mg ozone / g of substrate.

Animals

Twenty four female rats Wistar with a corporal weight from 200 to 225 g were placed in metabolic cages (Tecniplast, Italy) under controlled conditions of temperature and humidity, water ad libitum and appropriate standard feeding. A group of six animals were orally treated with a unique dose of 3.3 mL of ozonated triolein by kg of animal’s weight. The other six animals were treated with the same dose of ozonated trilinolein. Two controls groups of six animals each were used with the same doses of triolein and trilinolein respectively. The urine of each animal was collected during 24 hours and kept at -10 °C until they were analyzed.

Creatinine Determination

The Jaffé method was employed for creatinine determination. One milliliter of urine was diluted with 49 mL of bidestillated water. A small fraction (0.2 mL) of this sample is mixed in a glass cuvette with 2 mL of a solution containing picric acid (35 mmol/L) and sodium hydroxide (0.32 mol/L), followed by the absorbance measurement against air at 490 nanometers (Boehringer Mannheim GmbH, 1979).

Liquid - liquid Extraction of Urinary Organic Acids

Urine samples (5 mL), containing appropriate amounts of internal standard (Heptadecanoic acid) and sodium chloride, were acidulated with hydrochloric acid (pH=1) and extracted two times. Firstly with ethyl ether and later with ethyl acetate, equal volume of both solvents were used (5 mL). The organic faces were mixed and dried with anhydrous sodium sulfate. The mixture was filtered and the solvents were removed under nitrogen flow at room temperature. One milliliter of ethyl ether was used to dissolve the extracted residue and diazomethane was bubbled to obtain the methyl esters of the existing dicarboxylic acids. Nitrogen was bubbled until dryness, and 50 µL of ethyl acetate were used to dissolve the final products. The samples were kept at -20 °C until the Gas Chromatography / Mass Spectrometry analysis.
Gas Chromatography / Mass Spectrometry Analysis

An AUTOMASS GC/MS (UNICAM, England) was used. A FFAP Supelco capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness) was employed for the separation of dicarboxylic acids methyl esters. The temperature - programming columns conditions used were: 80 °C initial temperature (2 min), 8 °C/min to 220 °C, and held at 220 °C for 10 minutes. An ionization voltage of 70 eV over the mass range of 30 - 400 AMU was used to fragment the components. The interface and the ion source temperature were set at 240 °C and 250 °C respectively. Injector temperature was set at 280 °C. The helium gas carrier was maintained at a linear flow rate of 1 mL/min and the injection volume was 0.1 microliter. An automated mass spectra library was used for identifying the compounds (NIST, 1990). An internal standard method was used to quantify the analyzed compounds, taking into consideration the respective response factors.

Results and Discussions

The urinary dicarboxylic acids profile of Wistar rats (Fig. 2), after oral administration of ozonized triolein, showed an increment in the concentration of heptanedioic and nonanedioic acids (Table I) if compared with his respective control. As it could be expected, there were no appreciated increments of any other dicarboxylic acids. These dicarboxylic acids are formed taking into consideration the way I of the proposed mechanism (Fig. 1).

![Figure 2](image.png)

Figure 2. Urinary dicarboxylic acids profile of Wistar rats orally treated with ozonized triolein. Heptanedioic acid (ADC 7), nonanedioic acid (ADC 9) and heptadecanoic acid (AG 17).

By other hand, the urinary dicarboxylic acids profile (Fig. 3) of Wistar rats, after oral administration of ozonized trilinolein was very similar to that obtained when ozonized sunflower oil was administered (Jardines et al., 1998). All the dicarboxylic acids were incremented. A possible explanation of this fact can is given taking into consideration the way II of the mechanism presented in Fig. 1.
### Table I. Rats urinary dicarboxylic acids concentration after oral administration of ozonized unsaturated compounds

<table>
<thead>
<tr>
<th>Dicarboxylic acid</th>
<th>Ozonized Triolein (N=6)</th>
<th>Ozonized Trilinolein (N=6)</th>
<th>Ozonized Sunflower Oil (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptanedioic (Pimelic acid)</td>
<td>32.6 ± 1</td>
<td>23.4 ± 0.9</td>
<td>9.6 ± 0.8</td>
</tr>
<tr>
<td>Octanedioic (Suberic acid)</td>
<td>-</td>
<td>0.27 ± 0.05</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Octenedioic</td>
<td>-</td>
<td>1.9 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Nonanedioic (Azelai acid)</td>
<td>5 ± 1</td>
<td>79 ± 4</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Decenedioic</td>
<td>-</td>
<td>0.040 ± 0.007</td>
<td>0.047 ± 0.005</td>
</tr>
<tr>
<td>Dodecenedioic</td>
<td>-</td>
<td>0.020 ± 0.005</td>
<td>0.043 ± 0.005</td>
</tr>
</tbody>
</table>

**Legend:**
- The concentration is expressed as micrograms of dicarboxylic acid by milligrams of creatinine.
- The ozonized sunflower oil data are taken from Jardines et al. 1998.

![Figure 3](image-url). Urinary dicarboxylic acids profile of Wistar rats orally treated with ozonized trilinolein. NI-No identified, heptanedioic acid (ADC 7), octenedioic acid (ADC 8:1), nonanedioic acid (ADC 9), decenedioic acid (ADC 10:1), dodecenedioic acid (ADC 12:1), heptadecanoic acid (AG 17).
Dicarboxylic acid concentrations obtained in this study were compared with those obtained when ozonated sunflower oil was administered (Table I). With ozonized triglycerides a higher increment in the urinary dicarboxylic acid concentration was observed. The triglycerides model compounds (Triolein and trilinolein) and the sunflower oil studied were ozonized in similar conditions. Nevertheless, the concentrations of some urinary dicarboxylic acids are significantly different from one substance to another.

The differences could be explained taking into consideration the next facts:
1. The differences in the unsaturated fatty acids composition of triglycerides and the Sunflower oil. The triolein it is only formed by oleic acid, this fatty acid has one unsaturation (C₉ - C₁₀) and the ozone only react by this position. After the ozone - triolein reaction the nonanedioic acid is produced and later in the rat organism, could be β-oxidized to produce heptanedioic acid, as it is presented in Fig. 1. Nevertheless the trilinolein has two double bonds in its structure and there are two possibilities for ozone attack and a higher variety of dicarboxylic acids could be obtained.

   The sunflower oil is formed by triglycerides with a 60-65 % of linoleic acid and 20-30 % of oleic acid in its composition. Although the linoleic acid react with ozone 1.6 times faster than oleic acid, both compounds react quickly with ozone (k= \(10^5 - 10^6\) L mol⁻¹ s⁻¹) at the concentration range they are in the Sunflower oil (Giamalva et al., 1986).

2. The presence of natural antioxidants in vegetable oils that react with ozone, which although in a small concentration, reduce the ozone - unsaturated fatty acid in the same proportion. These natural antioxidants are not present in the unsaturated triglycerides used in this work.

3. The unsaturated fatty acids distribution in the sunflower oil triglycerides. There are some saturated fatty acids forming part of this triglycerides and it affect in some extension the ozone - unsaturated fatty acid reaction. It means that there is a different grade of unsaturated fatty acid accessibility if we ozonized single unsaturated triglycerides or mixture triglycerides containing saturated and unsaturated triglycerides.

Conclusions

The study of the urinary dicarboxylic acid profile of Wistar rats, previously orally treated with ozonized unsaturated triglycerides has demonstrated the origin of the increment of these acids when ozonated sunflower oil was orally administered. The results obtained confirm the proposed mechanism for ozonized sunflower oil metabolism in rats. The differences observed were explained taking into account chemical considerations between ozonized sunflower oil and the unsaturated triglycerides used as models. The urinary dicarboxylic acid profile depends on the composition of ozonized substance orally administered.
References


• Vajda O. and Saenz T., Química de los alimentos, Tomo 1, (Editorial Científico - Técnica, La Habana, 1976), p. 230.