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Thermal stability of 7-trimethylsilylperoxy-cholest-5-ene 3β-acetates at high temperature

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RESUMEN

Estabilidad térmica del 7-trimetilsilil-peroxy colestan-5eno 3β-acetato a elevadas temperaturas.

La degradación térmica de los dos hidroperóxidos isómeros (7α- y 7β-hidroperóxidos) del colestan-5-eno 3β-acetato (CA) produce varios productos, de los cuales la mayoría han sido ya identificados; los dos 7-hidroxi-CA α- y β-isómeros se encuentran entre estos compuestos. En el caso de la degradación del 7α-hidroperoxi-CA, el isómero 7α-hidroxi-CA se encuentra presente en mayor proporción, mientras que el isómero 7β- se genera principalmente a través del 7β-hidroperoxi-CA. La degradación térmica de los derivados del trimetilsilil (TMS) 7-hidroperoxi-CA da lugar a muchos productos, de los cuales varios compuestos son los mismos que se obtienen de los correspondientes isómeros 7-hidroperoxi-CA no silanizados. Inesperadamente se hallaron elevadas cantidades de derivados TMS del 7-hidroperoxi-CA (7α- o 7β-).

También se encontraron trazas de 7-hidroperoxi-CA libre, formado a través de la degradación térmica de cada 7-hidroperoxi-CA (isómeros α y β).

PALABRAS-CLAVE: Acetato de colesterilo - Cromatografía de gases - Espectrometría de masas - Estabilidad térmica - Oxidación.

SUMMARY

Thermal stability of 7-trimethylsilylperoxy-cholest-5-ene 3β-acetates at high temperature.

The thermal degradation of the two hydroperoxy isomers (7α- and 7β-hydroperoxides) of cholest-5-ene3β-acetate (CA) generates many products, of which most have already been identified; the two 7-hydroxy-CA α- and β-isomers are among these compounds. In the case of the 7α-hydroperoxy-CA degradation, the isomer 7α-hydroxy-CA is present in a high proportion, while the 7β-isomer is generated in a larger amount from the 7β-hydroperoxy-CA. The thermal degradation of the trimethylsilyl derivatives (TMS) of the 7-hydroperoxy-CA gives rise to many products, of which several components are the same as those obtained from the corresponding non silylated 7-hydroperoxy-CA isomer. We have found an unexpectedly high amount of the TMS derivative of the 7-hydroperoxy-CA (7α- or 7β-isomers).

Traces of free 7α-hydroperoxy-CA, from thermal degradation of each 7-hydroperoxide-CA TMS (7α- and 7β- isomers), were also found.

KEY-WORDS: Cholesteryl acetate - Gas chromatography - Mass spectrometry - Oxidation - Thermal stability.

1. INTRODUCTION

Studies of cholest-5-en 3β-acetate (CA) oxidation can lead to a better understanding of the reaction mechanisms of the main derivatives of cholesterol oxidation (oxysterols). Since a simplified model system with an esterified hydroxyl group in position 3 of the cholesterol molecule is employed, analytical conditions of the oxysterol study are more simplified and quite similar to these of methyl oleate oxidation products (Smith, 1981; Bortolomeazzi *et al.*, 1994a; Bortolomeazzi *et al.*, 1994b; Lercker *et al.*, 1996; 1999).

The first oxidation products of an unsaturated molecule are the hydroperoxides, which are very reactive and thus very unstable. In order to stabilize the hydroperoxide structures, it is therefore necessary to prepare their corresponding hydroxy derivatives by chemical reduction. The peroxidation mixture that is used for the hydroperoxide synthesis always contains pre-formed hydroxy derivatives with a similar structure to that of the hydroperoxides. To avoid these hydroxy derivatives being taken for the products of the hydroperoxide reduction, the reduction must be carried out after the hydroperoxide collection from the peroxidation mixture (Fig. 1).

Several cholesterol oxidation products (COPs) have great biological significance because of their claimed toxicity (Maerker, 1987; Gallina Toschi *et al.*, 1993; Bösinger *et al.*, 1993; Smith, 1996).

The aim of this study is the identification of the main thermo-degradation products of each cholesteryl acetate 7-hydroperoxide isomer (CAHPs), as peroxyl group TMS derivatized.

2. EXPERIMENTAL

2.1. Materials

Cholest-5ene 3β-acetate (cholesteryl acetate, CA) (>99%), 7α-hydroxy-cholest-5-ene 3β-ol (7α-OHC) and 7β-hydroxy-cholest-5-ene 3β-ol (7β-OHC) were supplied by Steraloids (Wilton, NH,

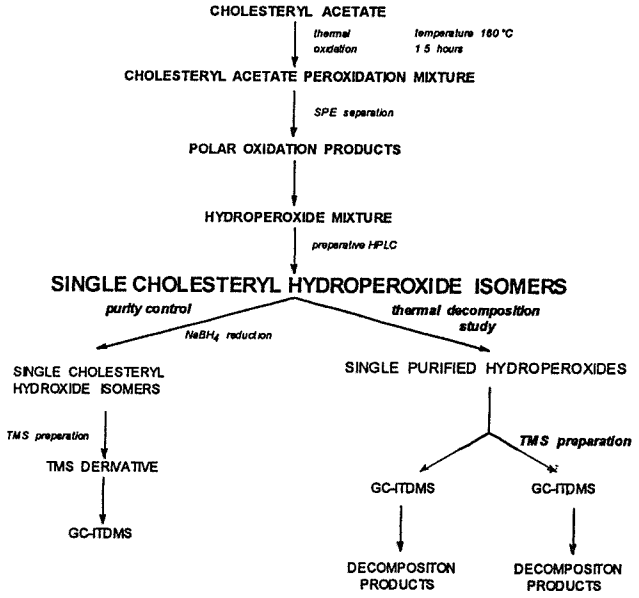

Figure 1
Flow diagram of the preparation, purification and thermal degradation of cholest-5-ene 3β-acetate hydroperoxides.

USA). The acetyl derivatives of standard with hydroxyl group in position 3 were synthesized as described in the methodology. The reagents and solvents (analytical or HPLC grade) were supplied by Carlo Erba (Milan, Italy). The solid-phase extraction (SPE) columns (Bond Elut, Analytichem International, Varian, CA, USA) were packed with 500 mg silica.

2.2. CA thermal peroxidation

CA (0.5 g) was oven-heated at 160°C for 90 min in a 20 ml sealed screw cap container.

2.3. Cholesteryl acetate hydroperoxides (CAHP) isolation

After CAHPs preparation by CA thermal peroxidation, SPE fractionation was carried out to recover the more polar products. CAHPs were then collected from this polar fraction by a subsequent HPLC purification (Fig. 1).

The peroxidized cholesteryl acetate was dissolved in 2 ml of n-hexane and loaded onto a SPE column preconditioned with 5 ml of n-hexane. The column was then eluted with 4 ml of n-hexane-diethyl ether (95:5, v/v), 5 ml of chloroform-methanol (1:1, v/v) and 5 ml of methanol.

The second fraction which contained the CA thermal oxidation products was then fractionated onto a TLC silica plate developed with n-hexane- diethyl ether (1:1, v/v). Two hydroperoxides were formed under these conditions, both in position 7 and with α and β configuration. The TLC band corresponding to the CAHPs, visualized by spraying with 0.2% 2,7-dichlorofluorescein (sodium salt)-ethanol solution and detected by UV light (254 nm), was scraped off and the single CAHP was isolated by HPLC.

The two purified hydroperoxides were then subjected to the following analysis: HPLC of the CAHP, GC of the corresponding hydroxy derivative (CAH) and GC-ITDMS of the trimethylsilyl derivative (TMS) of the CAHs.

2.4. CAHP reduction

Each CAHP was reduced in methanol with $NaBH_4$ to corresponding cholesteryl acetate hydroxides (CAHs) (Bortolomeazzi *et al.*, 1994a).

2.5. 3β-Acetate derivative preparations of 7- hydroxy-cholest-5-ene 3β-ol (7α- and 7β -isomers)

The 3β-OH acetylation of cholesterol hydroxide reference compounds (7α-OHC and 7β-OHC) was carried out via treatment of 1 mg of each diol with 0.1 ml of a mixture pyridine-acetic anhydride (1:1, v/v) for 12 h at room temperature. Preparative HPLC and GC-ITDMS were carried out for purification and identification of each 3β-acetyl derivative.

2.6. Trimethylsilyl ether (TMS) preparation

Samples were silylated with about 0.1 ml of pyridine: hexamethyldisilazane: trimethylchlorosilane mixture in a 5:2:1 (v/v) ratio (Sweeley *et al,* 1967), for 30 min at room temperature in a desiccator. After drying in a hot water bath (about 70°C) by evaporation under nitrogen flow, samples were redissolved in 30-50 μl of benzene.

2.7. High-performance liquid chromatography (HPLC)

The liquid chromatograph was a Knauer 64 pump (Knauer, Berlin, Germany) equipped with a Rheodyne, 7125 injector (Cotati, CA, USA), an ACS 750/14 light-scattering detector (Applied Chromatographic System, Macclesfield, UK) and a 3 μm Spherisorb CN column (15 cm x 4.6 mm i.d.) (Phase Sep, Deeside, UK). Preparative and analytical HPLC separations for purification and analysis of the 7-hydroperoxide isomers were performed under isocratic conditions at 1 ml/min flow rate, using 0.5% anhydrous ethanol in n-hexane as the mobile phase and a splitter device between HPLC column and detector.

2.8. Gas chromatography-ion trap detector mass spectrometry (GC-ITDMS)

The capillary gas chromatograph (GC) was a Varian 3400 with on-line coupling to a Varian Saturn ion-trap detector (ITDMS) (Walnut Creek, CA, USA). The column was a DB-5 fused silica type (5% phenylmethyl) (J&W, Folsom, CA, USA), with 30 m x 0.255 mm i.d. and 0.25 μm film thickness. Oven temperature was programmed from 220 to 300°C, with a rate of 5°C/min. Injection was in the split mode (1:50, v/v, ratio) at 1 ml/min flow rate with helium as the carrier gas; the temperatures of the injector, the transfer line and the manifold were 300, 300 and 220°C, respectively. The filament emission current was 10 μA and an electron beam of 70 eV was used for electron impact ionization (EI).

2.9. Thermal degradation of CAHPs

Thermal degradation of the individual hydroperoxides, as TMS derivative, was performed by injection into the glass injection port of the GC-ITDMS instrument. Four different conditions were tested: two solvents of different polarity (n-hexane and 0.5% methanol) to dissolve each hydroperoxide and injection at two temperatures (308°C and 200°C).

3. RESULTS AND DISCUSSION

Each isomer of CAHPs (Fig. 1) was separately thermo-degraded in a GC injector port. It was observed that the sylanized hydroperoxide molecule (Fig. 2, peaks 5 and 14) survived the thermal treatment to which it was subjected in the instrument's injector. The main products obtained by thermal degradation of 7α- and 7β-hydroperoxy-choles-5-ene 3β-acetate (Lercker *et al.*, 1996; 1999) were cholest 5-en-7α-ol 3β-acetate (peak 15), cholest-5-en7β- ol 3β-acetate (peak 16), 7α-hydroperoxy-cholest-5-ene 3β-acetate (peak 20) and cholest-5-en-7-one 3β-acetate (peak 21). Instead, he main products obtained by thermal degradation of 7α- and 7β-trimethylsilyl-peroxy-cholest-5-ene 3β-acetate were 7α-trimethylsilyloxy-cholest-5-ene 3β-acetate (peak 2), cholest-5-ene 3β-acetate (peak 4), 7α-trimethylsilylperoxy-cholest- 5-ene 3β-acetate (peak 5), 5,6α-epoxy-7α- trimethylsilyloxy-cholestane 3β-acetate (peak 7), 7β-trimethylsilyloxy-cholest-5-ene 3β-acetate (peak 8), 5,6β-epoxy-7β-trimethylsilyloxy-cholestane 3β-acetate (peak 12), 7β-trimethylsilylperoxy-cholest-5-ene 3β-acetate (peak 14), cholest-5-en-7α-ol 3β-acetate (peak 15), cholest-5-en-7β-ol 3β-acetate (peak 16), 7α-hydroperoxy-cholest-5-en 3β-acetate (peak 20) and cholest-5-en-7-one 3β-acetate (peak 21).

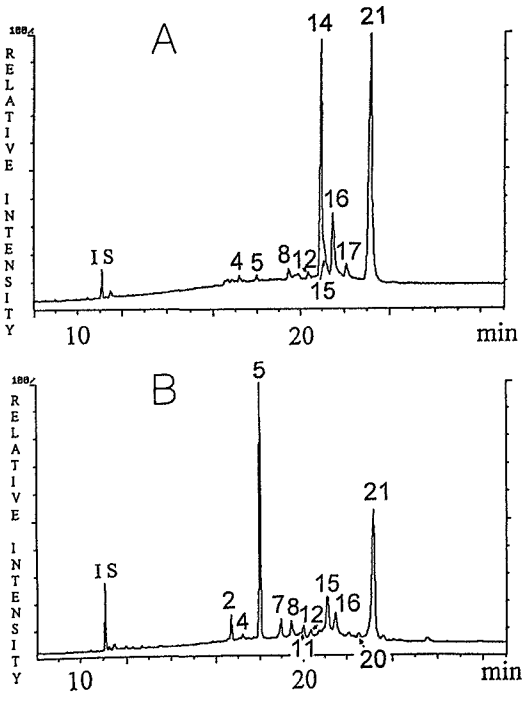

Figure 2
GC-ITDMS traces of the products from thermal degradation of the 7β-hydroperoxy-cholest-5-ene 3β-acetate (A) and 7α-hydroperoxy-cholest-5-ene 3β-acetate (B), both injected as TMS derivatives. Identification of the main peaks (Bortolomeazzi *et al.*, 1994a; Bortolomeazzi et al., 1994b; Lercker *et al.*, 1996): **IS**, internal standard (5α-cholestane); **2**, 7α-trimethylsilyloxy-cholest-5-ene 3β-acetate; **4**, cholest-5-ene 3β-acetate; **5**, 7α-trimethylsilyl-peroxy-cholest-5-ene 3β-acetate; **7**, 5,6α-epoxy-7α-trimethylsilyloxy-cholestane 3β-acetate; **8** (trace A) 7β-trimethylsilyloxy-cholest-5-ene 3β-acetate; **8** (trace B), unknown; **11**, unknown; **12**, 5,6β-epoxy-7β-trimethylsilyloxy-cholestane 3β-acetate; **14**, β-trimethylsilylperoxy-cholest-5-ene 3β-acetate; **15**, cholest-5-en7α-ol 3β-acetate; **16**, cholest-5-en-7β-ol 3β-acetate; **17**, cholest-5-en-4β-ol 3β-acetate (tentative); **20**, 7α-hydroperoxy-cholest-5-ene 3β-acetate; **21**, cholest-5-en-7-one 3β-acetate.

Fig. 3 shows the mass spectra of free 7α-hydroperoxide of CA (7α-OOHA, trace B) and its TMS derivative (A), which correspond to the peaks 20 (Fig. 2, B) and 5, respectively. The more intense fragments in both spectra are those of the molecular ion that lost 60 amu, which matches the loss of acetic acid as frequently observed in the case of the acetyl derivatives. The high stability of the fragments at m/z 400 (free peroxyl group) and 472 (with a TMS group), both fragments corresponding to the losses of 60 amu (acetic acid), confirms the stability of the hydroperoxide.

Similar results were obtained for 7β-hydroperoxide CA TMS (7β-OOHA), but the free 7β-hydroperoxide was not detected.

Fig. 4 shows the mass spectra of the TMS derivatives of 7α-OOHA (A) and 7β-OOHA (B), which correspond to peaks 5 and 14 (Fig. 2, A),

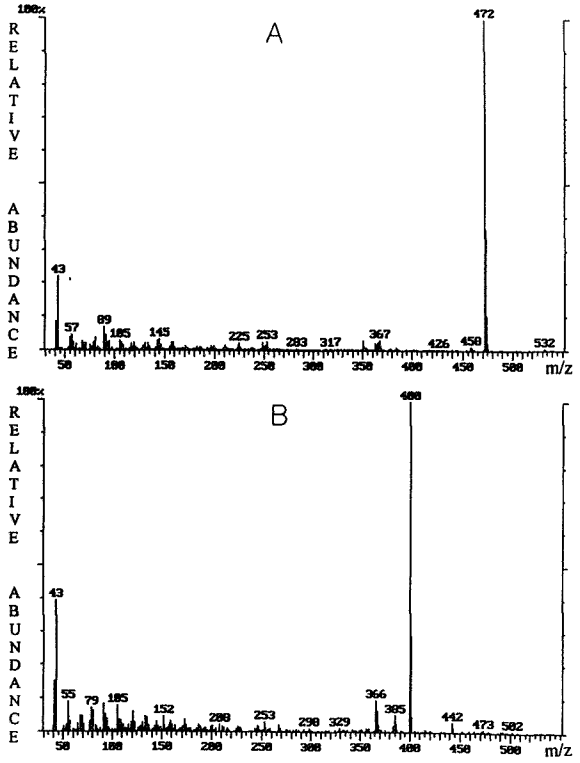

Figure 3
Mass spectra of: A, peak 20 of Fig. 2, and B, peak 5, of Fig. 2, trace B. Compounds 20 and 5 were identified as 7α-hydroperoxy-cholest-5-ene 3β-acetate and its TMS, respectively.

respectively. Little differences were found between the fragmentations of the two hydroperoxides (at m/z 208, 243, 351, 384 and 430); in fact, the spectrum A is almost identical to the spectrum B of Fig. 3, which was expected because both correspond to the TMS derivative of 7α-OOHA. These results indicate that 7β-OOHA is able to isomerize and produce 7α-OOHA, since this GC-ITDMS trace (Fig. 2, A) is the result of the 7β-OOHA degradation only. The same considerations could be done for the other hydroperoxide when it is subjected to thermal treatment (Teng *et al.*, 1973; Brill, 1984; Porter and Zuraw, 1985; Beckwith *et al.*, 1989). This is the first time that, in lipid systems, a mass spectrum of a hydroperoxide and that of its TMS derivative are recorded.

The thermal degradation of the 7-hydroperoxide by direct injection in GC-ITDMS, using two solvents with different polarity (hexane and methanol), gave very similar chromatograms; this result indicates that the thermal degradation is a monomolecular reaction mechanism. In fact, the polar solvent should show a molecular interaction with the associate molecules, given rise to a diminished bimolecular reaction extent, if present. When 7-hydroperoxides of CA

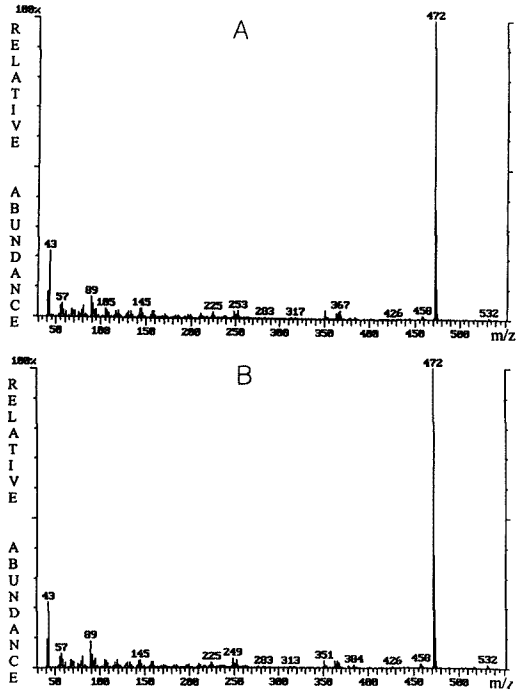

Figure 4
Mass spectra of: A peak 14 of Fig. 2 and B peak 5 of Fig. 2, trace A. Compounds 14 and 5 were identified as TMS derivatives of 7β- and 7α-hydroperoxides of cholest-en 3β-acetate, respectively.

were degraded at different temperatures of the injector port, the traces were almost identical.

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