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UVB photolysis of hydrocortisone 21-acetate

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ABSTRACT

Hydrocortisone 21-acetate (HCA) in methanol solution undergoes photodegradation under UVB light, as monitored by HPLC. Five main photoproducts have been isolated and characterized by means of NMR and mass spectroscopy. One of them derives from a Norrish I photoreaction which cleaves the C17–C20 bond of the steroid yielding the andro-derivative, a second product comes from a Yang-type photorearrangement which links C18 to C20 yielding a cyclobutane adduct. The former photoproduct, in turn, undergoes further photolysis giving rise to various photoproducts, of which three have been characterized. The first is a stereoisomer of the andro-derivative, the others arise from the opening of the five-membered ring. HCA also proved photounstable in the solid state and in a commercial formulation for topical use, thus confirming the requirements of the Pharmacopeias for light protection of this drug.

Indeed, experiments on LPS-stimulated THP-1 cells demonstrated the loss of anti-inflammatory activity when HCA was UVB-photodegraded. The radical mechanism involved in HCA photolysis seems also responsible for the *in vitro* photohemolytic effect and lipid peroxidation induced by HCA in combination with UVB light.

1. Introduction

Hydrocortisone acetate (11β,17α-dihydroxypregna-4-ene-3,20-dione-21-acetate) is a glucocorticoid used in therapy for the treatment of different rheumatoid and allergic pathologies and dermatitis, both by systemic and topical way [1].

The presence of the acetate group makes the drug more stable in solution and its time of action longer. The keto group in position 3, the double bond between carbons 4 and 5, the hydroxyl in 11 and the β ketolic chain in 17 are crucial for its therapeutic activity [2].

Corticosteroids are sensitive to UV radiation. They are a typical example of bichromophoric moiety: all possess an aliphatic ketone in the side chain linked to position 17 of the D ring, which absorbs UVB light. Ring A bears a keto group that is conjugated with either one or two double bonds, depending on the specific drug. In the former case (hydrocortisone, flurandrenolide, alcinonide, etc.) the chromophore is mainly sensitive to UVC. In the latter (betamethasone, fluocinolone, triamcinolone, etc.) both UVA and UVC effectively induce photolysis [3].

For some of these, the photolysis has been studied in different conditions and environments (for a review, see [4]), and the UVB photostability and phototoxicity in aqueous medium have been recently reported for triamcinolone [5] and fluocinolone [6].

As the photostability of HCA has been assessed only in the solid state [7], the aim of this study was to investigate the photodegradation of the compound, including isolation and identification of its main photoproducts both in solution and in a commercial formulation.

In order to obtain preliminary information about the biological consequences of HCA photolysis, the anti-inflammatory activity before and after irradiation was tested on THP-1 cells. Moreover, the photoreactivity towards biological substrates in terms of photohemolysis and lipid photoperoxidation was investigated.

2. Experimental

2.1. Chemicals

Hydrocortisone 21-acetate (HCA) was purchased from ICN Biomedicals Inc., Aurora, OH, USA. Cortaid™ dermatological cream was from Carlo Erba, Milano, Italy. Linoleic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,6-di-*tert*-butylhydroxyanisole (BHA), Dulbecco's Modified Eagle's

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Medium (DMEM), fetal calf serum (FCS), and trypsin were obtained from Sigma–Aldrich (Milano, Italy).

2.2. Irradiation procedures

HCA was dissolved (10^{-5} M) in methanol or PBS and irradiated, in quartz cuvettes, by means of two Philips PL-S 9 W/12 lamps mainly emitting at 312 nm.

Light doses were measured with a Model 97503 radiometer (Cole Palmer, Niles, IL, USA) equipped with a CX-312 sensor.

2.3. Analytical methods

The irradiated solutions were analysed by HPLC (PerkinElmer Series 200 instrument equipped with a diode array detector set at 245 nm and a Merck LiChroCART Purospher STAR RP-18 column (5 μm, 250 mm × 4 mm), eluted with acetonitrile–water, 30:70, containing 0.05% trifluoroacetic acid for 3 min, then linearly increasing acetonitrile up to 60% within 15 min, at a flow rate of 1 mL/min.

The irradiated methanol solution was also concentrated and submitted to TLC (silica gel plates, 0.25-mm thickness, E. Merck, Darmstadt, D, eluted with chloroform–methanol, 95:5, v/v).

HCA was also irradiated at the solid state and in a commercial cream (both as 0.5-mm layers between two optical glasses whose transmittance was higher than 95%). For the HPLC analysis, the solid was dissolved in methanol, the cream in a tetrahydrofuran–water mixture (9:1, v/v). In these cases, elution was performed isocratically (water–acetonitrile 52:48, v/v containing 0.05% trifluoroacetic acid).

Mass spectrometric measurements were carried out on a API-TOF Mariner spectrometer (PerSeptive Biosystems, Stratford, TX, USA), and the injection of the samples was achieved with a micrometric syringe pump (Harvard Apparatus, Holliston, MA, USA).

NMR measurements – standard ^{1}H and ^{13}C spectra, homocorrelated (COSY and NOESY), and heterocorrelated (HMBC and HMQC) experiments – were carried out with a Bruker AMX300 spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C). Samples were dissolved in $CDCl_3$; J is given in Hz.

2.4. In vitro assays

2.4.1. Detection of anti-inflammatory activity

THP-1 cells (Human acute monocytic leukemia cell line) were seeded (10^5 cells/well) in 96-well culture plates (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany) in RPMI medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1% v/v antibiotic solution, 0.05 mM β-mercaptoethanol, 10% v/v heat-inactivated fetal bovine serum (FBS) (all provided by Gibco, Milan, Italy). To achieve the macrophage differentiation THP-1 cultures were supplemented with 50 nM phorbol-12-myristate-13-acetate (Calbiochem, Milano, Italy) for 48 h. Medium was then changed and cell monolayers were pretreated in the dark with HCA 50 or 25 μM in 10% v/v methanol previously exposed to 30 J/cm^2 UVB irradiation, which induced almost total photolysis. Thirty minutes later, 10 μg/mL lipopolysaccharide (LPS, from *Salmonella enteritidis*, Sigma–Aldrich, Milano, Italy) was added to cells and incubated for 30 h.

Conditioned medium was then collected and subjected to IL-1β quantification using a commercially-available ELISA kit (Biosource, Milano, Italy). Optical densities were measured using an ELISA plate-reader (Spectra I Tecan, Graz, Austria) at a wavelength of 450 nm. IL-1β levels were expressed as pg/mL. Values were determined from replicates of 3–5 wells from three independent experiments.

To determine a possible toxic effect of irradiated drug and to ensure equal cell amount, cell monolayers were subjected to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay (Sigma). Briefly, at the end of each experiment, cells were incubated with 5 mg/mL MTT in PBS at 37 °C for 5 h and then 100 μL of 0.01 N HCl in 10% w/v sodium dodecylsulfate were added. Culture plates were incubated at 37 °C for further 18 h. Absorbance was measured at 550 nm. Survival ratios were expressed in percentages with respect to untreated cells.

2.4.2. Photohemolysis

Whole blood, collected from untreated albino mouse and treated with heparin as the anticoagulant, was washed with PBS (0.01 M phosphate buffer, 0.135 M NaCl, pH 7.2), centrifuged (2500 rpm for 15 min at room temperature) and the supernatant and the buffy coat discarded. The procedure was repeated until the supernatant was colorless. Red blood cells (RBC) were resuspended in PBS in order to have A_{650} between 0.6 and 0.7 and were used within 48 h. The RBC suspension, in the presence of hydrocortisone acetate (made 50 μM with a concentrated stock solution in MeOH), was incubated at 37 °C for 15 min in the dark and then divided into two fractions: one was kept in the dark, the other was irradiated with increasing light doses. Hemolysis percentage was determined by spectrophotometric measurements at 650 nm where intact RBCs absorb [8]. A RBC suspension added with methanol and irradiated was used as a further control. Hemolysis was determined both in the presence and in the absence of oxygen, the latter obtained by argon flushing.

2.4.3. Linoleic acid peroxidation

Linoleic acid solutions (10^{-3} M) in PBS containing 0.05% Tween 20 and 10 μM HCA were irradiated with increasing UVA or UVB doses, detecting the absorbance at 233 nm (hydroperoxide acid absorbance). Lipid peroxidation was also measured in the presence of 10 μM of the free radical scavenger BHA. Control samples were a linoleic acid solution irradiated without HCA and a linoleic acid solution with HCA kept in the dark.

3. Results and discussion

3.1. HCA photolysis

The light sensitivity of HCA was studied by means of spectrophotometric analysis, followed by TLC and HPLC separation of the photoproducts formed.

The evolution of HCA with increasing irradiation UVB doses (up to 30 J/cm^2) was followed by UV/Vis absorption spectroscopy in methanol (10^{-5} M solutions, Fig. 1). The UV absorption spectrum of HCA is characterized by a strong band around 255 nm with a small shoulder between 290 and 320 nm. By increasing UVB irradiation (up to 30 J/cm^2), a strong decrease in spectral intensity was detected, without appearance of new spectral features.

Conversely, the irradiation of the HCA solution with UVA did not induce significant modifications in the UV absorption spectrum, even under high light doses (up to 30 J/cm^2).

The photolysis was also followed by HPLC: the chromatogram (Fig. 2) shows the decrease of the peak corresponding to unmodified HCA (t_R = 9.7 min) while the UVB irradiation dose increased, as well as the appearance of several new peaks.

UVB induced about 30, 60, and 80% degradation of HCA in methanol solution after 5, 15, and 30 J/cm^2, respectively, while UVA caused disappearance of about 1, 3, and 8% of the drug at the same doses. The greater efficiency of UVB is clearly due to the higher absorption of the compound in the UVB region ($\varepsilon \approx 80\ M^{-1}\ cm^{-1}$) with respect to UVA ($\varepsilon \approx 4\ M^{-1}\ cm^{-1}$), while at the wavelength of

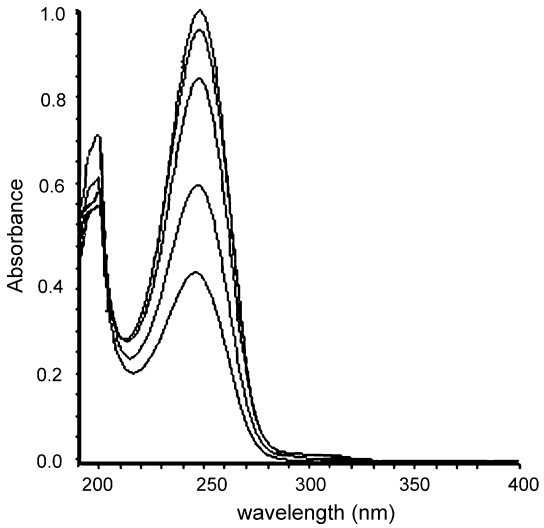


Fig. 1. Photodegradation of hydrocortisone acetate (HCA), 10^{-5} M in methanol, by UVB light (up to 30 J/cm^2).

the maximum absorption (242 nm) ε is 15,500 M^{-1} cm^{-1}. The photolysis in PBS was even higher, reaching almost 90% after 30 J/cm^2 UVB.

For preparative purposes, larger volumes of more concentrated (10^{-3} M) solutions in methanol were irradiated with 30 J/cm^2, the solvent removed, and the mixture resolved by TLC. This procedure allowed five photoproducts to be isolated in sufficient amount for structure elucidation.

For comparison, the analytical data for HCA (see Scheme 1) relevant for assignment of the structure of photoproducts are reported here: TLC, R_F = 0.49. HPLC, t_R = 9.7. ^{1}H NMR, δ 5.68 (1 H, br s, 4-H); 5.05 and 4.84 (1 H each, two d, 21-H, J^2 12.6); 2.8-0.98 (series of multiplets); 2.18 (3 H, s, 23-Me); 1.44 (3 H, s, 19-Me); 0.98 (3 H, s, 18-Me). ^{13}C NMR, δ 205 (20-C), 198 (3-C), 173 (22-C), 172 (5-C), 121 (4-C). MS, m/z 405 $[M+H]^+$.

3.2. Characterisation of the photoproducts

Photoproduct **1**. TLC, R_F = 0.12. HPLC, t_R = 4.1 min. ^{1}H NMR, δ 5.69 (1 H, br s, 4-H); 4.51 (1 H, m, 11-H); 4.37 and 4.13 (1 H each, two d, 21-H, J^2 11.9); 2.5-0.8 (series of multiplets); 2.10 (s, 3H, Me-23); 1.39 (s, 3H, Me-19). ^{13}C NMR, δ 199 (3-C), 173 (22-C), 171 (5-C), 122 (4-C), 67 (18-C). MS, m/z 405 $[M+H]^+$.

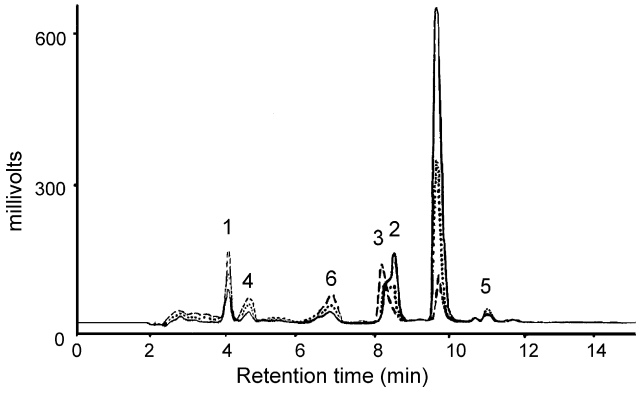


Fig. 2. HPLC profiles of a methanol solution of HCA irradiated with increasing doses of UVB: (—) 5 J/cm^2, (●) 15 J/cm^2, and (–) 30 J/cm^2, respectively. Peaks are labelled according to the numbering of the photoproducts described in the text.

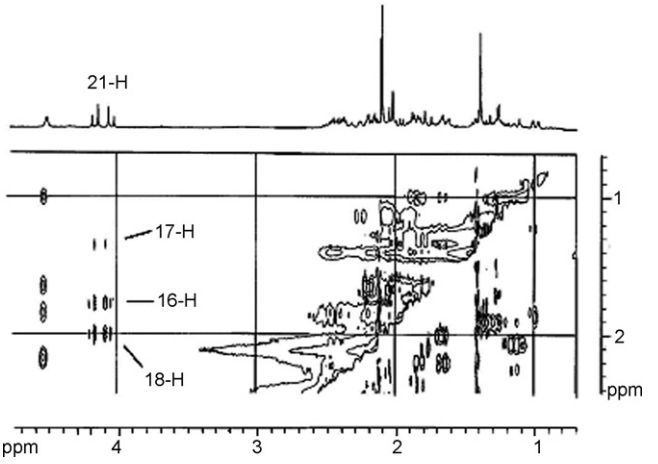


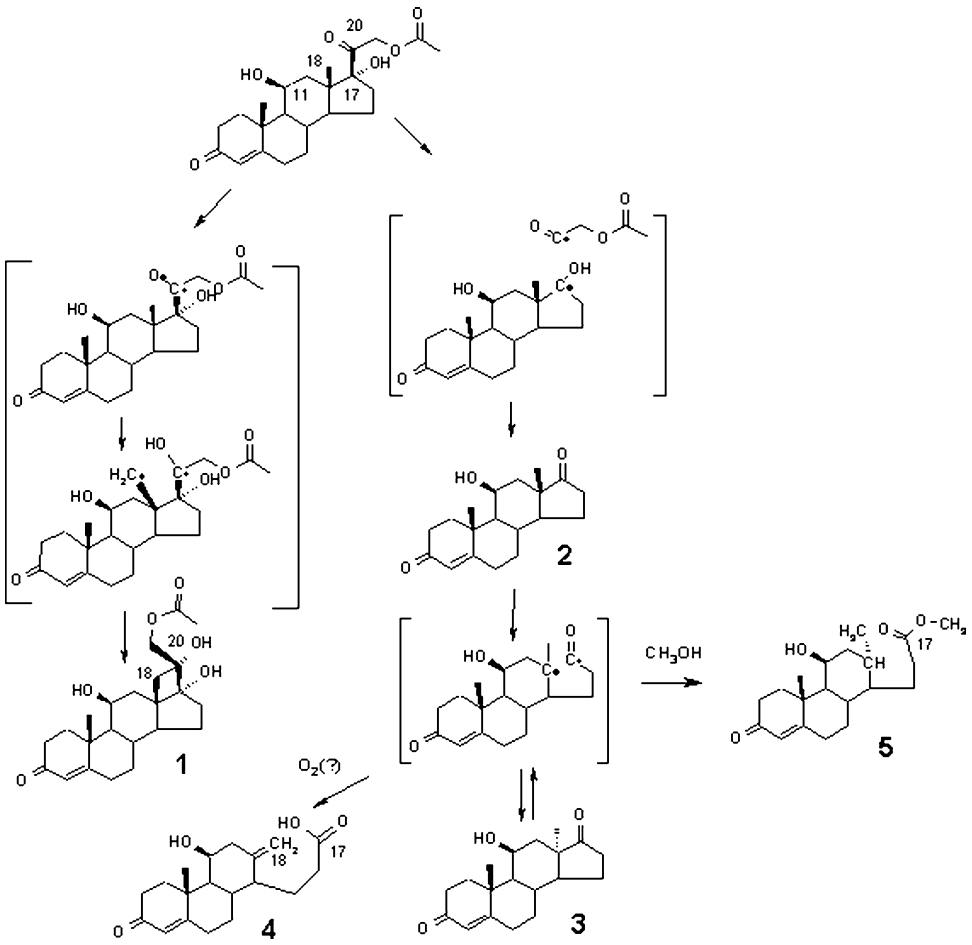
Fig. 3. NOESY spectrum of photoproduct **1**. The relevant interactions described in the text are reported.

Compound **1** only forms by UVB irradiation of HCA, thus being a primary photoproduct. Its mass spectrum indicates that it has the same molecular weight as HCA, *i.e.*, 404 Da. The ^{1}H NMR spectrum shows disappearance of 18-Me and upfield shift of 21-CH_2. Moreover, the HMBC spectrum shows long-range interactions of H-21 with carbons at 47 (18-C), 102 (20-C), 81 (17-C), and 173 ppm (22-C), instead of two (173 ppm, 22-C, and 205 ppm, 20-C) as in HCA. These interactions, in particular the 21-H/18-C one, suggest that a Yang-type photoprocess occurred, favored by the β position of both 18-Me and the side chain in 17, yielding a cyclobutane ring involving carbons 15, 18, 20 and 17 (see Scheme 1). 20-C now became a chiral center, due to the fact that the intermediate radical is planar. Only one of the possible diastereoisomers has been identified in the photoreaction mixture, however. To clarify the configuration at 20-C, the NOESY spectrum was helpful (Fig. 3). Both 21-H show three interactions with signals at 2.36, 1.72, and 1.33 ppm. The first signal belongs to one of the 18-H, as assigned by the interaction with 18-C in the HMQC spectrum. The other two signals have been assigned to one of the 16-H and one of the 17-H, respectively, on the basis of their mutual correlation in the COSY spectrum. These interactions are possible only if the chain is in the *cis* position with respect to the cyclopentane ring (*R* configuration at 20-C) and the two hydroxyls are *cis* to each other. The *S* configuration would allow 21-H to be spatially close only to one of the 18-H.

As expected from the reduction of 21-C=O to a secondary alcohol, **1** is stable under UVB radiation.

Photoproduct **2**. TLC, R_F = 0.62. HPLC, t_R = 8.5 min. ^{1}H NMR, δ 5.69 (1 H, br s, 4-H); 4.40 (1 H, m, 11-H); 2.5-0.8 (series of multiplets); 1.47 (3 H, s, 19-Me); 1.16 (3 H, s, 18-Me). ^{13}C NMR, δ 219 (17-C), 199 (3-C), 171 (5-C), 122 (4-C). MS, m/z 303 $[M+H]^+$.

The mass spectrum indicates for **2** a molecular weight of 302 Da, consistent with a Norrish I cleavage of the 17–20 carbon–carbon bond, already observed in various corticosteroids [9], leaving a keto group in 17. The loss of the side chain was confirmed by the lack of the methylene 21 and of the methyl 23 protons in the ^{1}H NMR spectrum (in HCA they resonate as two doublets at 5.03 and 4.84 ppm (J^2 17.4 Hz), and at 2.18 ppm, respectively). Compound **2** is therefore the 4-androstene-11β-ol-3,17-dione. As seen in Fig. 2, the amount of **2** is highest at low irradiation doses, while it gradually disappears on further irradiation. This behaviour is consistent with the fact that **2** still contains a carbonyl group in 17 which absorbs UVB. In fact, UVB irradiation of isolated **2** yielded photocompounds **3**, **4**, and **5**.



Scheme 1. Molecular structure of HCA and its photoproducts **1**–**5**, along with the proposed radical intermediates.

Photoproduct **3**. TLC, R_F = 0.71. HPLC, t_R = 8.2 min. ^{1}H NMR, δ 5.71 (1 H. br s, 4-H); 4.21 (1 H, m, 11-H); 2.5-0.8 (series of multiplets); 1.47 (3 H, s, 19-Me); 1.16 (3 H, s, 18-Me). ^{13}C NMR, δ 219 (17C), 199 (3-C), 171 (5-C), 122 (4-C). MS, m/z 303 $[M+H]^+$.

Compound **3** is a secondary photoproduct coming from photolysis of **2**. Like for **2**, the MS spectrum shows a formula weight of 302. The ^{1}H and ^{13}C NMR spectra are superimposable to those of **2**, as is the COSY spectrum. NOESY experiments shown Fig. 4, instead, revealed significant differences between **3** and both **2** and HCA.

In HCA, **2**, and **3**, 11-H shows dipolar interactions with the vicinal protons 12 (cross peaks indicated with a in Fig. 4) and 9 (c), and with the spatially close protons in position 1 (b). Only in **3** does 11-H also interact with both 18-Me (d) and 19-Me (e). The former interaction is possible if the junction between rings C and D is *cis*, thus leaving 18-Me in α instead of β, as in **2** and in HCA. Moreover, the *cis* configuration makes ring C in **3** more flexible than in **2**, allowing both chair and boat conformations. In the former case 11-H is spatially close to 19-Me, in the latter to 18-Me. The NOE interactions may therefore be explained by the presence of both conformers in solution. Thus, **3** comes from a Norrish I scission of the 16–17 C—C bond of **2**, leaving 16-C as a planar radical which can be re-attached from the opposite side. Indeed, UVB irradiation of isolated **2** yielded **3**. The reverse photoreaction was not observed. Instead, irradiation of **3** gave both **4** and **5**.

Photoproduct **4**. TLC, R_F = 0.15. HPLC, t_R = 4.6 min. ^{1}H NMR, δ 5.71 (1 H, br s, 4-H); 4.98 and 4.93 (1 H each, 2 br s, 18-H); 4.28 (1 H, m, 11-H); 2.5-0.8 (series of multiplets); 1.36 (3 H, s, 19-Me). ^{13}C NMR, δ 201 (3-C), 172 (5-C), 170 (17-C), 145 (13-C), 125 (4-C), 111 (18-C). MS, m/z 319 $[M+H]^+$.

The formula weight of **4** is 318 indicating, together with the NMR data, that the 17-side chain was lost and the addition of an oxygen atom occurred. 18-Me also disappeared from the ^{1}H NMR spectrum. Two poorly resolved signals are instead present at 4.98 and 4.93 ppm. The former (see the COSY spectrum in Fig. 5) shows scalar interactions (a) with the two 12-H at 2.32 ppm (which had been assigned on the basis of their cross-peaks with 11-H (b)). The latter (4.93 ppm) is coupled with 14-H at 1.7 ppm (c). Besides carbons 4 and 5, two further vinyl carbons appeared in the ^{13}C-spectrum of **4** at 145 and 111 ppm, respectively, consistent with the presence of an exocyclic methylene. Similar reactions have been observed for cyclopentanones in which proton transfer occurs from the β carbon to the C=O carbon yielding a methylene and an aldehyde [10]. In the ^{1}H spectrum of **4**, however, no signals around 10 ppm were present. Taking into account the molecular weight, a carboxyl should be formed at 17-C, most likely by the action of air, and the ^{13}C NMR spectrum shows its peak at 170 ppm.

Photoproduct **5**. TLC, R_F = 0.67. HPLC, t_R = 10.9 min. ^{1}H NMR, δ 5.69 (1 H, br s, 4-H); 4.27 (1 H, m, 11-H); 3.69 (3 H, s, OMe); 2.5–0.8 (series of multiplets); 1.38 (3 H, s, 19-Me); 0.91 (3 H, d, 18-Me, J 6.3). ^{13}C NMR, δ 200 (3-C), 173 (17-C), 171 (5-C), 123 (4-C). MS, m/z 339 $[M+H]^+$.

Compound **5** formed by UVB irradiation of HCA, but also when **2** and **3** were isolated and further irradiated. Its mass spectrum indicates a molecular weight of 338, that is, 32 Da higher than that

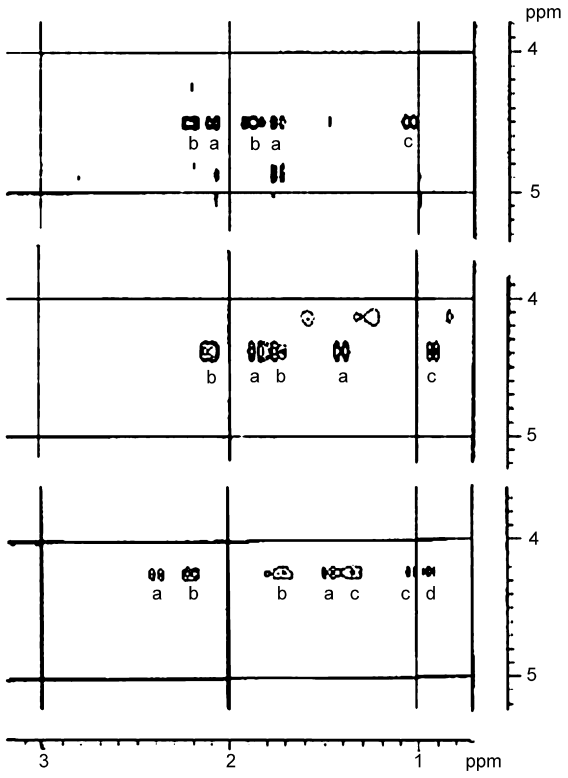


Fig. 4. Relevant sections of the NOESY spectra of HCA (upper panel), **2** (middle) and **3** (lower), showing the interactions of the proton in position 11.

of **2** and **3**. This increase may be due to the addition of either two oxygen atoms or a methanol molecule. **2** was then irradiated in deuterated methanol. The resulting photoproduct had a molecular weight of 342, thus confirming the latter hypothesis. The ^{1}H NMR

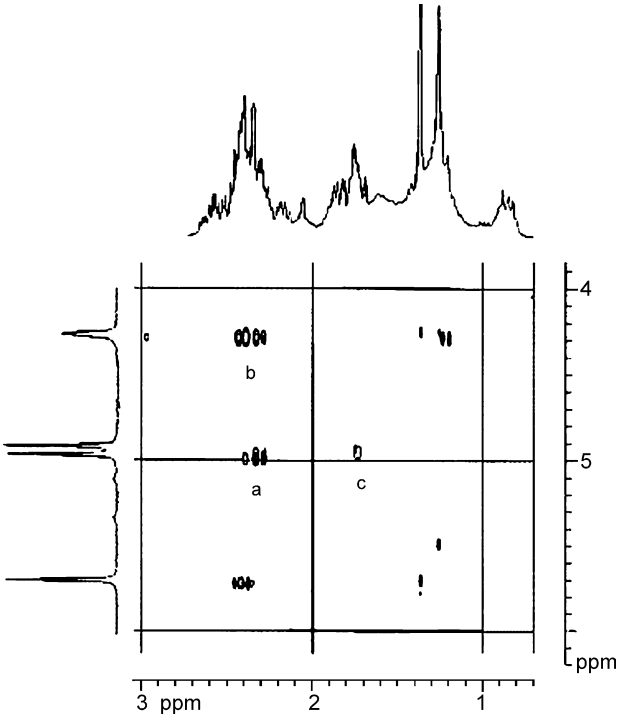


Fig. 5. COSY spectrum of photoproduct **4**, showing the J^4 interactions between the exocyclic methylene protons and 12-H and 14-H.

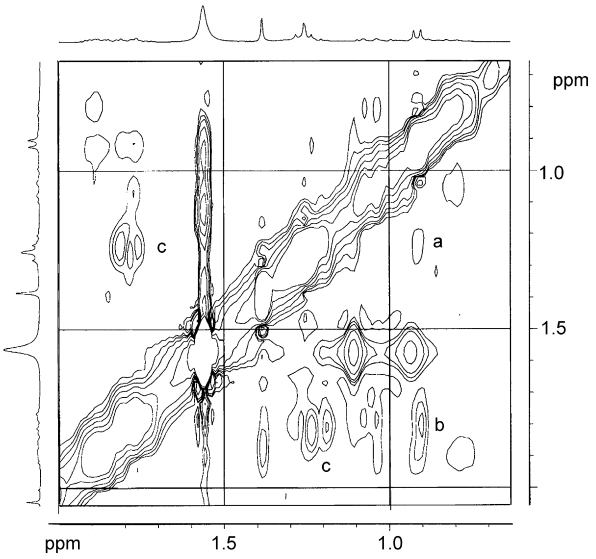


Fig. 6. NOESY spectrum of photoproduct **5**. The interactions relevant for structural assignments are described in the text.

spectrum shows the signal of the methoxy group at 3.69 ppm and that of the 19-Me at 1.38 ppm. The 18-Me is split into a doublet at 0.91 ppm, due to its coupling with the new proton in position 13. One of the two carbonyl carbons present in 1, namely the 17-C, moved upfields (219 to 173 ppm) because of its transformation into a carboxyl.

Concerning the stereochemistry at 13-C, the NOESY spectrum (Fig. 6) allowed detection of dipolar interactions between 18-Me (0.91 ppm) and two protons at 1.25 (a) and 1.80 (b) ppm, which have been identified as the two hydrogens in position 12 through their couplings with each other (c) and with 11-H (not shown). 18-Me is close to both H-12 only if it is in the α position with respect to the steroid scaffold.

A further product (**6**) is present in significant amount in the irradiated mixture of HCA, and elutes at 6.8 min in the HPLC analysis (see Fig. 2). Attempts to isolate this compound failed, because of its instability.

Irradiation in aqueous environment (PBS) expectedly resulted in the absence of the peak of **5** in the chromatogram. Instead, two very small peaks eluted at 6.1 and 9 min, but the amount recovered was not sufficient for characterization.

3.3. Photodegradation in a topical pharmaceutical formulation

The photolysis of HCA was measured in a commercial formulation for topical use (Cortaid® cream): UVB irradiation (15 J/cm^2) of a 0.5-mm layer of the cream resulted in 20% degradation of the drug (Fig. 7a). Interestingly, of the photoproducts previously characterized, only **2** was evidenced in the chromatogram. The higher stability with respect to HCA in solution may be due to the different optical properties of the cream, *e.g.*, in terms of light scattering, to the different environment in which HCA is embedded, or to the protecting effect of some excipients of the cream.

Therefore, a similar cream was prepared with the same excipients as the commercial sample (vaseline oil, vaseline, cetylstearylic alcohol, sodium laurylsulfate) but without the preservatives (methyl- and propyl *p*-hydroxybenzoates). Photolysis was faster, 40% with the same light dose, suggesting a partial photoprotecting effect of the two parabens, which indeed possess the phenolic chromophore able to absorb UVB light. Again, **2** was the sole photoproduct detected, suggesting that the semisolid matrix interferes

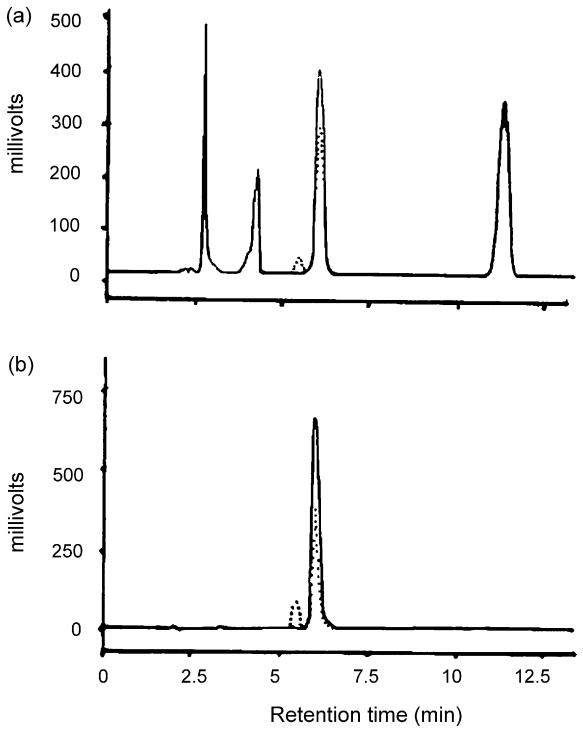


Fig. 7. HPLC profiles of a topical formulation of HCA (panel A) and of solid HCA (panel b) before (––) and after (···) 15 J/cm^2 UVB. The peaks at 2.75, 4.25, and 11.2 min in panel (a) belong to the excipients of the cream.

with the rearrangement of the molecule in its excited state and consequently with the kind of photoproducts formed.

3.4. Photodegradation in the solid state

Irradiation of HCA in the solid state with 15 J/cm^2 UVB also produced significant photolysis of the drug (about 35%), and again only **2** was found as the photoproduct (Fig. 7b). The difference with the literature data [11], which report the formation of a second photoproduct of HCA in the crystalline form, namely 4-androstene-3,11,17-trione, is most likely due to the different wavelength used (narrow band UVB vs. low pressure Hg source), as mentioned in Section 1.

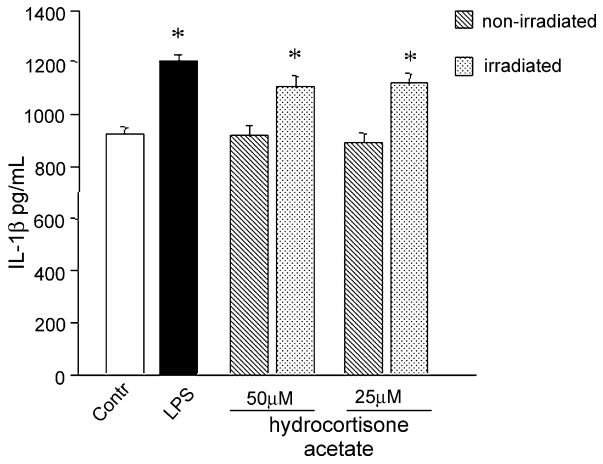


Fig. 8. *In vitro* anti-inflammatory activity. Activated THP-1 cells were pretreated for 30 min with 50 or 25 μM UVB-irradiated or non-irradiated HCA and then incubated in medium alone (control) or supplemented with LPS 10 μg/mL. Conditioned medium was collected after 30 h and IL-1β were measured by ELISA. Each experiment was performed three times with at least triplicate determinations for each condition (n = 6–9); samples were then assayed in duplicate. * denotes $p < 0.05$ vs. control.

3.5. Evaluation of the in vitro anti-inflammatory activity

To evaluate whether HCA lacks its anti-inflammatory activity following UVB irradiation, activated THP-1 cells were pretreated with irradiated and non-irradiated drug and then exposed to a well-accepted inflammatory stimulus such as LPS. As shown in Fig. 8, THP-1 exposed to 10 μg/mL LPS secreted considerable amounts of IL-1β (1204 ± 26 pg/mL) as compared to non-stimulated cells (923 ± 24 pg/mL, $p < 0.05$). The pretreatment of THP-1 with non-irradiated HCA significantly inhibited LPS-induced IL-1β release at both 50 and 25 μM. However, the UVB irradiated HCA lacks its anti-inflammatory effect on THP-1 cells. In fact, the IL-1β levels measured in the conditioned medium of cells pre-incubated with irradiated HCA, either 50 or 25 μM and then treated with LPS (1104 ± 43 and 1117 ± 36 pg/mL, respectively) are comparable to that of cells treated with LPS only. Finally, the MTT colorimetric assay performed on cell monolayers did not reveal toxic effects of the irradiated drug (data not shown).

3.6. Photodamage to biological substrates

Since the mechanism by which HCA undergoes photodegradation mainly involves radical species, it can be envisaged that biological substrates could be damaged when HCA is associated with UVB.

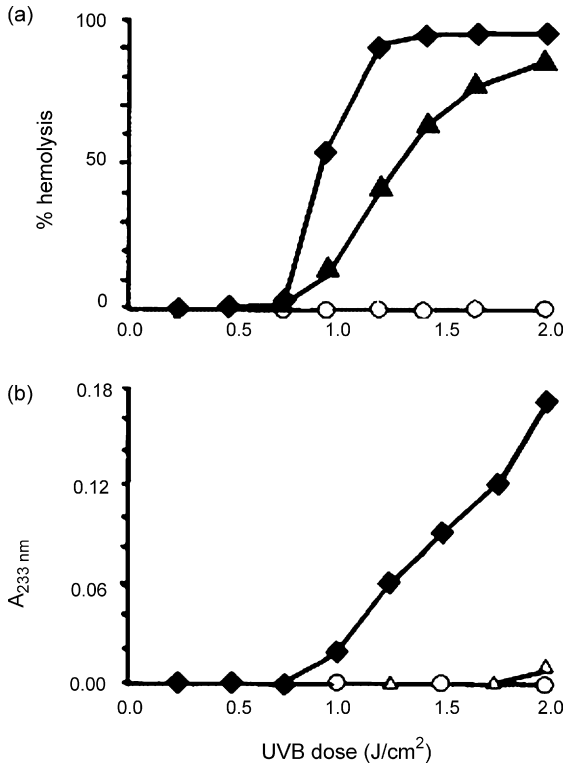


Fig. 9. Photodamage induced by HCA. (Panel a) on erythrocytes ((♦) RBC treated with HCA + UVB; (■) RBC treated with HCA + UVB in the absence on oxygen; (○) RBC treated with UVB alone). (Panel b) on linoleic acid ((♦), linoleic acid treated with HCA + UVB; (■) RBC treated with HCA + UVB in the presence of BHA; (○) linoleic acid treated with UVB alone).

Incubation of a red blood cell suspension with HCA followed by exposure to UVB light induced cell lysis (photohemolysis), determined through the reading at 650 nm of light scattering which is proportional to the concentration of intact cells (Fig. 9a). HCA induced photohemolysis in a light dose-dependent manner, thus indicating cell membrane damage in combination with light.

As expected, irradiation with UVA light, which is much less absorbed than UVB, was not effective (data not shown). At these doses, no effect was observed when RBC were irradiated without HCA, nor when they were incubated in the dark in the presence of HCA.

Photohemolysis was also evaluated in the absence of oxygen: the RBC suspension was gently flushed with argon during 15 min, carefully stopped, and irradiated as above. Fig. 9a shows that UVB-induced hemolysis was slowed down, but not inhibited, by deoxygenation.

This result suggests that the photosensitizing mechanism of HCA does not mainly involve oxygen. Indeed, photoperoxidation of linoleic acid, taken as a model of unsaturated lipids, was totally inhibited when the radical scavenger BHA was added to the solution (Fig. 9b).

4. Conclusions

HCA is highly unstable to UVB irradiation in solution, yielding several photoproducts, of which five have been characterized. Photoproduct **2** lacks the ketolic chain as a consequence of the Norrish I mechanism and 17-C is oxidized to a ketone. **2** undergoes further homolytic scission to an intermediate which photoisomerizes to give **3**, binds to the solvent to give **5**, or rearranges to give **4**. Alternatively, HCA undergoes a Yang-type reaction which yields a cyclobutane derivative (**1**) [12].

HCA also proved unstable to UVB in the solid state and in a topical formulation used for the treatment of chronic and episodic skin conditions. In both cases only photoproduct **2** was observed, showing that the photochemistry is affected by the matrix.

Besides the integrity of the four-ring scaffold, key features required for receptor binding and therefore for glucocorticoid activity include the 3-ketone, the 4,5-double bond, and the three hydroxy groups in 11, 17a, and 21. In four of the photoproducts formed these two latter hydroxyls are no longer present, in the fifth the structure undergoes distortion due to the formation of a further ring. Thus, the photoproducts should not retain the anti-inflammatory activity of the parent compound. Indeed, the experiments on LPS-stimulated THP-1 cells showed loss of anti-inflammatory activity of HCA when extensively irradiated with UVB.

No clinical occurrence of photosensitization by HCA is reported in the literature, in spite of its strong interaction with light. Nevertheless, as its photolysis is mainly mediated by radical species, some preliminary tests have been performed to ascertain whether this drug could photochemically interact with biological substrates: HCA proved to damage RBC membranes and unsaturated lipids involving mainly radical species, as shown by the small difference between the hemolytic effect in the presence and in the absence of oxygen and by the strong inhibition of lipid peroxidation induced by the radical scavenger BHA.

As UVB is a component of daylight, sun exposure of both HCA-containing pharmaceutical dosages and topically treated patients might be of clinical concern. On one side, any modification of the structure of corticosteroids, in particular the loss of the side-chain, has a profound effect on their anti-inflammatory activity. On the other side, the radical intermediates generated during photolysis of HCA could damage cell components.

Therefore, the high photolability of HCA confirms the requirements of the Pharmacopeias for light protection of this drug.

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