Urocanic acid in photodermatology
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CHAPTER 6

Oxidative breakdown and conversion of urocanic acid isomers by hydroxyl radical generating systems

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Abstract

\textit{Cis}-urocanic acid (\textit{cis}-UCA), formed from \textit{trans}-urocanic acid (\textit{trans}-UCA) by photoisomerization, has been shown to mimic suppressive effects of UV on the immune system. It is our hypothesis that UCA oxidation products in the skin play a role in the process of immunosuppression. Recently, both UCA isomers were found to be good hydroxyl radical scavengers and in this context we investigated the formation of products resulting from the interaction of hydroxyl radicals with UCA. Hydroxyl radicals were generated by 1. UV/H\textsubscript{2}O\textsubscript{2} (photooxidation), 2. ferrous ions/H\textsubscript{2}O\textsubscript{2} (Fenton oxidation) and 3. cupric ions/ascorbic acid. Oxidation products were identified by spectrometric methods and assessed by reversed phase HPLC analysis. The photooxidation of UCA was induced by UV-B and UV-C, but not by UV-A radiation. Photooxidation and Fenton oxidation of \textit{trans}-UCA, as well as of \textit{cis}-UCA yielded comparable chromatographic patterns of UCA oxidation products. Several of the formed products were identified. The formation of three identified imidazoles was shown in UV-B exposed corneal layer samples, derived from human skin.

Part II: Oxidation of UCA by hydroxyl radicals
Chapter 6

1. Introduction

Trans-uurocanic acid (trans-UCA) is a major ultraviolet (UV)-absorbing component of the human epidermis. Absorption of UV radiation from the UV-C region (200-290 nm) into the UV-AI region (340-400 nm) causes photoisomerization of epidermal trans-UCA into cis-UCA in vivo as well as in vitro (1-3). Cis-UCA has been shown to mimic some, though not all, of the effects of UV on immunity, suggesting that this compound is an important mediator of UV-induced immunosuppression (4). Although there is experimental evidence for the immunosuppressive potential of cis-UCA (4-9), the failure to produce this effect in several models in vitro is puzzling (10-13). To seek an explanation for the seemingly contradictory results of in vivo and in vitro experiments, we suggest the involvement of products formed from UCA isomers upon interaction with reactive oxygen species (ROS). Until now, most attention has been focussed on the photoisomerization of UCA and only some studies refer to other photochemical aspects (14). Formation of cis-UCA from trans-UCA may be only part of the initiation of immunosuppression: similar levels of cis-UCA can be induced by UV-A and UV-B, whereas only UV-B induces immunosuppression (15). The hypothesis of a possible involvement of ROS is supported by the observations that antioxidants have an abrogatory effect on the cis-UCA induced immunosuppression (16,17). There is strong evidence for the generation of hydroxyl radicals in the epidermis. Hydroxyl radicals can be generated under physiological conditions from hydrogen peroxide upon UV irradiation or from hydrogen peroxide with transition metal ions, predominantly ferrous (Fe$^{2+}$; Fenton reaction) and cuprous (Cu$^+$) ions (18,19). Normal human skin contains approximately 200 µM iron, predominantly complexed to ferritin (20,21). The release of free ferrous ions by UV irradiation (22), the presence of cellular hydrogen peroxide (23,24) and UV itself in combination with hydrogen peroxide (25) are the prerequisites for the generation of hydroxyl radicals. Under conditions of oxidative stress, generated hydroxyl radicals may react with the UCA isomers, which were earlier recognized as good hydroxyl radical scavengers (26). In this paper oxidation products under conditions of photooxidation by UV-B irradiation and through Fenton oxidation. Several of the formed products have been identified and could also be demonstrated in UV-B exposed corneal layer samples of human skin.

2. Materials & methods

2.1. Chemicals

Trans-UCA, L-ascorbic acid, imidazole-4-carboxylic acid, imidazole-4-carboxaldehyde, imidazole-4-acetic acid (sodium salt), 4-(hydroxymethyl)
imidazole-HCl and tetrabutylammonium hydroxide were supplied by Sigma-Aldrich/Fluka Chemie BV (Zwijndrecht, The Netherlands). Cis-UCA was kindly supplied by dr. W.M.P.B. Menge of the Free University, Department of Pharmacology, Amsterdam, The Netherlands. Ferrous ammonium sulphate hexahydrate was obtained from Brocades-ACF (Maarssen, The Netherlands) and copper(II) sulphate pentahydrate from BDH Chemicals Ltd. (Poole, England). These salts served as sources for ferrous ions \( \text{Fe}^{2+} \) and cupric ions \( \text{Cu}^{2+} \). Fe\(^{2+}\) solutions were freshly prepared each time and were argon purged. The remaining chemicals were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was used throughout.

2.2. High Performance Liquid Chromatography (HPLC)

Trans-UCA and cis-UCA were separated from each other and from UCA oxidation products on 4.6 x 250 mm reversed-phase columns with a flow of 0.8 ml/min, delivered by P-3500 HPLC-pumps (Pharmacia, Uppsala, Sweden). Isocratic elution was performed with either 10 mM sodium phosphate, containing 1-2 mM tetrabutylammonium hydrogen sulphate and 0-2% acetonitrile (pH 7.2) on an Alltima C\(_{18}\) column (Alltech, Deerfield, IL) or with 10 mM ammonium formate buffer, containing 0.4 mM tetrabutylammonium hydroxide and 4% acetonitrile (pH 7.2-7.5) on a Luna C\(_{18}\) column (Phenomenex, Torrence, CA). pH was adjusted with 0.1 M formic acid. Samples of 20 to 200 \( \mu \)L were injected by a Promis II autosampler (Spark Holland, Emmen, The Netherlands) and chromatographic data of experiments in vitro were recorded on an SP 4270 integrator (Spectra Physics, San Jose, CA) and the data of corneal layer samples were recorded on a computer with software for chromatography (Jasco-Borwin, JMBS Developpements, Le Fontanil, France). Peak area data from samples were only processed under identical HPLC circumstances. A UV-detector (Applied Biosystems, model 759A, Foster City, CA) was set for either 210, 226 and 257 nm detection, depending on the application.

2.3. Perchloric acid extraction of corneal layer of UV-exposed skin

Scrapings of corneal layer were collected from the sole of the foot, micronized with a dismembrator to obtain a powder, vacuum-dried and kept at -20 °C until use. Before UV-irradiation, an amount (~10 mg) of micronized corneal layer powder was moistened with 20 \( \mu \)L water and sandwiched between quartz plates. For the extraction of UCA oxidation products from irradiated corneal layer material, the samples were first shaken with pure diethylether to remove lipid material. The residue was dried in a mild flow of argon gas. Then, 400
ul of perchloric acid solution (0.6 M) was added and the samples were allowed to stand for 5 minutes with occasional agitation. The perchloric acid solutions were neutralized with approximately 60 ul potassium hydrox­ide solution (4 M). Potassium perchlorate was allowed to precipitate, fol­lowed by centrifugation (3000 rpm for 5 min.). The supernatant was cooled in ice to obtain an additional precipitation. Finally, the sample was cen­trifuged with higher speed (13000 rpm for 10 min.) and the supernatant was passed through a 0.22 μm membrane filter prior to HPLC analysis.

2.4. UV-irradiations for the induction of UCA photooxidation

Photooxidation in vitro was performed in a 1-cm quartz cuvette, filled with 2.0 ml sample, which was placed in the parallel beam of a filtered 1000 W xenon arc lamp (Oriel, Stratford, CT). The solutions were magnetically stirred during irradiation. To minimize infrared (heat) radiation, the beam was passed through a water filter (7 cm), reflected by a dichroic mirror and filtered through a 1-mm UG11 filter. Short-wave cut off was achieved by passing the beam through WG280, WG305 or WG335 filters with 3 mm thickness each (Schott-Jena, Mainz, Germany). Xenon lamp emission filtered through WG280 included UV-C, UV-B and UV-A; through WG305 included UV-B and UV-A and through WG335 only UV-A was present. Two narrow bands in the UV-B and UV-A spectral regions were selected to monitor the xenon-arc emission. The probe of a calibrated EG&G 550 radiometer (Salem, MA, USA) was equipped with a neutral density filter, aligned with a narrow band filter type UV-M-IL (Schott-Jena, Mainz, Germany) with a transmission maximum of 21% at 303 nm and a half-width of 11.5 nm to monitor UV-B output. To monitor UV-A output, a fil­ter type UV-PIL (Schott-Jena) with a transmission maximum of 44% at 363 nm and a half-width of 18 nm was used. Transmission spectra of the optical filters were checked on a Perkin Elmer Lambda 40 UV/VIS spectrome­ter (Norwalk, CT, USA).

Corneal layer samples were UV-exposed with the use of a liquid light guide added to the solar simulator equipment. A positive quartz lens was placed behind the UG11 filter in such a way that the xenon-arc beam was focussed into the entrance aperture of the liquid light guide. The UV-B output in the UG11-transmitted UV-spectrum with a spectral distribution of approximately 280-400 nm was monitored as described above and a UV-B dose of 9.6 kJ/m² was used to induce photooxidation in the corneal layer sample to enable reliable HPLC-determination of the imidazolic UCA oxidation products. A control sample was similarly treated with the excep­tion that the short wave part of the UV-spectrum (< 360 nm) was cut off with a KV 370 filter (Schott-Jena).
Additional UV-irradiations were performed with fluorescent tubes TL12, used as a UV-B source, and with TL10R, used as a UV-A source (Philips, Eindhoven, The Netherlands) on samples in vitro that were magnetically stirred in small Petri dishes. The UV-B output was measured with an IL 443 phototherapy radiometer, fitted with a SEE 1240 silicon detector probe and the UV-A output with an IL 442A phototherapy radiometer with a SEE 115 detector probe (International Light, Newburyport, MA, USA).

2.5. Fenton oxidation

Cis-UCA and trans-UCA isomers (40-250 μM) were oxidized with a hydroxyl-radical-generating system that consisted of various concentrations of ferrous ammonium sulphate (25-400 μM) with or without EDTA (500 μM) and a fixed hydrogen peroxide concentration of 500 μM (the Fenton reagent), either in sodium phosphate (20 mM) of pH 7.2, or in water. In addition, two hydroxyl radical-generating systems with copper ions (Cu²⁺) were used, consisting of 50 μM copper(II)sulphate with either 500 μM hydrogen peroxide or 500 μM ascorbic acid. Metal ions were added as a final addition and this was considered to be the start of the oxidation reaction. Fe²⁺ and EDTA were premixed before addition. The reaction volume was most often 2 ml and after the usual reaction time of 10 min. the sample was immediately frozen (-18 °C) until use. Prior to HPLC-analysis the sample was thawed and filtered through 0.22 μm syringe filter.

2.6. UCA photooxidation on a preparative scale

Trans-UCA (8 mM) was photooxidized with hydrogen peroxide (40 mM) in a Petri dish with UV-radiation (λ > 270 nm) from Philips TL-12 fluorescent tubes. Irradiation was stopped when HPLC-analysis revealed an approximate of 97% breakdown of total-UCA (~16 hours). The final mixture was vacuum dried in a DNA Speedvac® device (Savant Instruments Inc., Farmingdale, NY) to remove volatile components, e.g. excess of hydrogen peroxide. The light-yellow residue was dissolved in water (approximately 1.0 g/l) for chromatographic separation or for other purposes and was referred to as PO-mix (photooxidation mix). PO-mix solutions were injected into the HPLC system and collected fractions were vacuum dried overnight. The removal of tetrabutylammonium ions was required for mass analysis and was performed by solid phase extraction (SPE) on C₁₈ silica. The collected fraction was acidified with formic acid up to a concentration of 100 mM. Then, the sample (1-2 ml) was slowly aspirated through the reconstituted SPE column, followed by a wash step with 0.5 ml 100 mM formic acid. After collection, the solution was vacuum dried overnight.

Part II: Oxidation of UCA by hydroxyl radicals
2.7. Spectrometric analyses

UV-spectra were scanned on the spectrophotometer, used for optical filter measurement (see above). Mass spectra were obtained on a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) by the electrospray technique. Capillary voltage was set at 3.5 kV and the cone voltage on 25 V. Samples, dissolved in methanol/water (80/20) were introduced by direct injection. Positive ion scans were made in the mass range of m/z 50-250. 1H NMR (400 MHz) spectra were recorded on a ARX 400 spectrometer (Bruker, Karlsruhe, Germany), using a dual probe.

3. Results

3.1. UCA isomers and photooxidation

The oxygen-oxygen bond of hydrogen peroxide can be cleaved by UV radiation to yield two hydroxyl radicals (25). Upon scavenging these radicals, UCA will be degraded and/or converted into oxidation products. The ability of simulated solar UV radiation to convert trans-UCA in the presence of hydrogen peroxide into photooxidation products was tested in vitro. The results, obtained by reversed-phase HPLC analysis, are shown in Fig.1 a-d. Under the conditions used, hydrogen peroxide eluted close to void volume and trans-UCA and cis-UCA eluted with markedly different elution times of 20 and 64 minutes, respectively. The unirradiated control sample showed that no interaction occurred between trans-UCA and hydrogen peroxide (Fig.1a). The exposure of 80 μM trans-UCA in the absence of hydrogen peroxide at pH 7.2 to WG280-filtered xenon-arc emission (including UV-C and UV-B) resulted only in the formation of cis-UCA via the process of photoisomerization (Fig.1b). However, when trans-UCA was irradiated in the presence of 500 μM hydrogen peroxide under identical conditions, several additional peaks appeared in the chromatograms and both trans-UCA and cis-UCA peaks were strongly reduced (Fig.1c). The degree of oxidative breakdown was comparable for both cis-UCA and trans-UCA, which finding is in accordance with recently published second order rate constants of both UCA isomers for hydroxyl radical scavenging (26).

In contrast, when exposures were performed with simulated solar radiation with UV-C and UV-B blocked out by a WG335 filter, only photoisomerization was seen with virtually no formation of photooxidation products (Fig.1d). Table 1 summarizes the irradiation conditions (condition 1-5) related to the extent of photooxidation, photoisomerization and breakdown of urocanic acid. The most extensive breakdown of UCA was obtained with 'full' UV (UV-C, -B and -A), and showed lowest amount of UCA that was left over. In addition, 'full' UV yielded the largest amount of photooxidation products (condition 1). The virtual absence of photooxidation...
Fig. 1. Chromatograms of 80 μM trans-urocanic acid in 20 mM phosphate buffer pH 7.2. The initial concentration of hydrogen peroxide was 500 μM. Injection volume was 80 μL.

a. with hydrogen peroxide; not irradiated,
b. without hydrogen peroxide; irradiated with a WG280 filtered xenon-arc lamp,
c. with hydrogen peroxide and irradiated as 1b,
d. with hydrogen peroxide and irradiated with a WG335 filtered xenon-arc lamp. Peaks assigned with A-H correspond with photooxidation products. Separation was performed on an Alltima C_{18} column with UV detection at 210 nm. The eluent consisted of 10 mM sodium phosphate pH 7.2 with 1.0 mM tetrabutylammonium hydrogen sulphate. Further experimental conditions are described in the text.
**RESULTS**

<table>
<thead>
<tr>
<th>Source</th>
<th>Irradiation</th>
<th>Emission</th>
<th>Dose</th>
<th>Range</th>
<th>Specific</th>
<th>Special</th>
<th>Optical</th>
<th>Ultraviolet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. X-arc</td>
<td>WGe280</td>
<td>14.0 - 405 mm</td>
<td>32</td>
<td>32</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. X-arc</td>
<td>WGC305</td>
<td>29.7 - 405 mm</td>
<td>18</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. X-arc</td>
<td>WGC335</td>
<td>31.8 - 405 mm</td>
<td>11</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. TL-12 (4)</td>
<td>none</td>
<td>280 - 350 mm</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. TL 10R (4)</td>
<td>none</td>
<td>318 - 440 mm</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**PHOTOOXIDATION CONDITIONS**

Initial concentrations: 40 µM trans-UCa and 500 µM hydrogen peroxide in 20 mM sodium phosphate pH 7.2.

**Table 1**. The formation of urocnic acid (UCa) (photooxidation products is dependent on the spectral distribution of UV.

CO N - O E

O en cp

85 o

CD 3 3 CO 2

O en

CD =f

2

CD O' 

O 3 c

CO C

CD O 3 3

3 c

S C Baum

2 O 3

CD CQ

O 3.

CD CD

M

Cl Cl

S C

CO E

•85
Oxidative breakdown and conversion of UCA products with UV-C and UV-B cut off (condition 3), is also found after using a UV-A fluorescent lamp (condition 5), which delivers an even larger dose of UV-A. Blocking out UV-C by the use of the WG305 filter (condition 2) showed intermediate results, between irradiation with 'full' UV and with UV-A alone. This irradiation condition (nr. 2) provided the closest simulation with the spectral UV distribution of terrestrial solar radiation produced by an overhead sun on a bright day (27). From the data in Table 1 it is clear that irradiation with UV shorter than approximately 320 nm and in the presence of hydrogen peroxide can initiate photooxidative processes.

3.2. Identification of UCA oxidation product.

To obtain sufficient photooxidation product for identification, trans-UCA was photooxidized with hydrogen peroxide at millimolar concentrations as described in Materials & Methods. After reconstitution of the PO-mix in water, HPLC- and spectrometric analyses were performed in order to elucidate the structures of the UCA oxidation products.

Peak B (Fig. 2) was identified as imidazole-4-carboxaldehyde (ImCHO). Its UV-spectrum ($\lambda_{\text{max}}$ 257 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak B authentic ImCHO resulted in a single chromatographic peak. An 6-fold increase in peak B height resulted, when running the HPLC-system with the absorbance detector set at 257 nm, in stead of 226 nm, according the shape of the UV-spectrum of ImCHO at neutral pH. After derivatization with 2,4-dinitrophenylhydrazine, a procedure based on that for allantoin (28), the hydrazone of ImCHO showed identical chromatographic behaviour as the hydrazone of an authentic sample.

Peak C (Fig. 2) was identified as imidazole-4-acetic acid (ImAc). Its UV-spectrum ($\lambda_{\text{max}}$ 213 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak C with authentic ImAc resulted in a single chromatographic peak. The dry sample was treated with methanol/HCl and n-butanol/HCl before mass spectrometric analysis. Peak masses [M + H]$^+$ of 141 and 183 were obtained for the methyl- and butylesters of imidazole-4-acetic acid. Mass spectra were identical to those obtained with the methyl- and butylesters of authentic imidazole-4-acetic acid.

Peak D (Fig. 2) was identified as imidazole-4-carboxylic acid (ImCOOH). Its UV-spectrum ($\lambda_{\text{max}}$ 226 nm at pH values > 5, otherwise $\lambda_{\text{max}}$ 212 nm) was identical to an authentic sample, that was commercially available. Co-injection of peak D with authentic ImCOOH resulted in a single chromatographic peak. Proton resonance (1H-NMR) analysis was done in D$_2$O, showing vinylic
Fig. 2. Comparable chromatographic patterns in the formation of UCA oxidation products from 80 μM trans-UCA and 500 μM hydrogen peroxide in water (no buffer). Left: after Fenton oxidation with 250 μM Fe²⁺ and right: after photooxidation with 'full' UV, containing a UV-B dose of 32 kJ/m². The cis-UCA peak is missing after Fenton oxidation, due to the absence of photoisomerization. Peak assignation (A-G) was done as in Figure 1c. Peak A is hydrogen peroxide. Peaks B, C and D refer to imidazole-4-carboxaldehyde, imidazole-4-acetic acid and imidazole-4-carboxylic acid, respectively. Chromatographic conditions were identical to those applied in Figure 1, except the column was renewed.

protons in a ratio 1:1 with shifts of 7.76 and 7.53 ppm, similar to an authentic sample. Mass spectra of the methyl- and butylesters showed [M + H]+ peaks at mass 127 and 169. Similar mass spectra were obtained from an authentic sample of ImCOOH. Checks on co-elution behaviour were carried out with various eluent compositions, to ensure the proposed identities.

Additionally, the formation of glyoxylic acid (GLX) could be demonstrated after derivatization with 2,4-dinitrophenylhydrazine. GLX was not seen in
Table 2. Trans-urocanic acid left over in hydroxyl radical generating systems [1]

<table>
<thead>
<tr>
<th>% (error) [2]</th>
<th>% (error) [2]</th>
</tr>
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<tbody>
<tr>
<td>in phosphate buffer pH 7.2</td>
<td>in water</td>
</tr>
<tr>
<td>Fe²⁺ (µM) - EDTA + EDTA³⁻ [3]</td>
<td>initial pH ~ 5.3</td>
</tr>
<tr>
<td>0</td>
<td>100 (1)</td>
</tr>
<tr>
<td>50</td>
<td>95 (1)</td>
</tr>
<tr>
<td>100</td>
<td>92 (6)</td>
</tr>
<tr>
<td>200</td>
<td>78 (3)</td>
</tr>
<tr>
<td>400</td>
<td>60 (12)</td>
</tr>
</tbody>
</table>

[1] All solutions were air saturated, except the iron(II) solution. Initial [hydrogen peroxide] = 500 µM and initial [trans-UCA] = 40 µM in all cases.
[2] Difference between duplicate measurements. Their mean value is presented.
[3] Na₂EDTA solution was premixed with iron(II) solution, immediately before the Fenton oxidation.

the chromatogram due to its low UV absorption, but it could be identified by HPLC technique as the 2,4-dinitrophenylhydrazone, based on a procedure for allantoin [28]. GLX was detected in all photo- and Fenton oxidized UCA samples, that were derivatized with 2,4-dinitrophenylhydrazine. Amino acid analysis with ninhydine derivatization, carried out on the PO-mix, could demonstrate the formation of aspartic acid and glycine. These amino acids were also found on prolonged UV irradiation of UCA without the presence of hydrogen peroxide [29].

### 3.3. UCA isomers and Fenton oxidation

Next, we studied the Fenton oxidation, another natural oxidation process. Trans-UCA and cis-UCA were oxidized in vitro without UV exposure but in the presence of a transition metal ion and hydrogen peroxide. The initial hydrogen peroxide concentration was fixed at 500 µM in all experiments and the ferrous ion concentration was varied from 0 to 400 µM. Four sets of conditions were compared: 1. Fe²⁺ in phosphate buffer pH 7.2, 2. Fe²⁺ in phosphate buffer plus EDTA, 3. Fe²⁺ without buffer with a initial pH of 5.5-5.3 and 4. Cu²⁺ in phosphate buffer plus ascorbate. Table 2 shows oxidative breakdown of trans-UCA with hydrogen peroxide in increasing order: condition 1 < 2 < 4 < 3.
Phosphate buffer of neutral pH was often used in studies that report transition metal driven oxidations of the Fenton type and the buffer was included here. However, the addition of Fe$^{2+}$ at final concentrations of 100-400 μM to phosphate caused a turbid solution of insoluble iron phosphate. Under this condition the smallest degree of breakdown was obtained, probably due to a reduction of free Fe$^{2+}$ in solution. On the other hand, complexation of Fe$^{2+}$ to EDTA prior to addition of phosphate, resulted in a clear solution and a larger breakdown was found (Table 2). The largest breakdown was seen in the absence of phosphate buffer, dependent on the UCA concentration (here: 40, 100 or 250 μM) and the pH value was less defined (5.5-5.3). After the start of the Fenton reaction in water medium, there was a rapid fall of the pH value from 5.1 to 3.4, when 250 μM UCA was used. We attribute this effect to the formation of relatively strong acids, such as glyoxylic acid (GLX).

In another hydroxyl-radical-generating system, based on copper ions (Cu$^{2+}$), the combination of Cu$^{2+}$/ascorbic acid/hydrogen peroxide caused a large breakdown of trans-UCA (Table 2) and a moderate yield of UCA oxidation products, in favor of ImCOOH. Without ascorbic acid, the system with Cu$^{2+}$ (50 μM) and hydrogen peroxide (500 μM) showed little breakdown (88% trans-UCA left; data not shown). For the situation in vivo, one must remember that the epidermal copper content is lower than iron [20].

Next, a comparison was made between trans-UCA and cis-UCA in breakdown and formation of oxidation products and the results are summarized in Table 3. Comparable extents of breakdown, though slightly less pronounced, were obtained with cis-UCA. This finding is in accordance with the comparable second order rate constants of trans-UCA and cis-UCA for hydroxyl radical scavenging [26]. Hydrogen peroxide without Fe$^{2+}$ had no effect on the UCA isomers at all; however, Fe$^{2+}$ without hydrogen peroxide resulted in a slow, long-term breakdown of the UCA isomers (data not shown). The primary oxidation products formed are ImCHO and GLX. Additional experi-

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[1]. Reaction conditions: 10 minutes at room temperature. Air saturated solutions were used throughout.
[2]. ImCHO = imidazole-4-carboxaldehyde
[3]. ImCOOH = imidazole-4-carboxylic acid
[4]. GLX = glyoxylic acid
[5]. EDTA was also degraded to GLX and may have been a competitor for the oxidative breakdown and conversion of UCA.
[6]. Due to photoisomerization, the breakdown of total-UCA (trans-UCA + cis-UCA) was calculated.
<table>
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<tbody>
<tr>
<td>1. Phosphate pH 7.2</td>
<td>Trans-UCA 40</td>
<td>16 (4) 41</td>
<td>1.6 (0.4)</td>
<td>0.5 (0.1)</td>
<td>0.4 (0.0)</td>
<td></td>
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<tr>
<td>Fe²⁺ / H₂O₂ 400 / 500 μM</td>
<td>Cis-UCA 40</td>
<td>13 (0) 32</td>
<td>1.4 (0.1)</td>
<td>0.3 (0.0)</td>
<td>0.5 (0.1)</td>
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<tr>
<td>2. Phosphate pH 7.2</td>
<td>Trans-UCA 40</td>
<td>31 (8) 77</td>
<td>2.7 (0.2)</td>
<td>0.2 (0.0)</td>
<td>- [5]</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ / EDTA / H₂O₂ 400 / 500 / 500 μM</td>
<td>Cis-UCA 40</td>
<td>28 (3) 70</td>
<td>2.6 (0.4)</td>
<td>0.3 (0.0)</td>
<td>- [5]</td>
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<tr>
<td>3. No phosphate initial</td>
<td>Trans-UCA 40</td>
<td>40 (0) 100</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.3 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ / H₂O₂ 400 / 500 μM</td>
<td>Cis-UCA 40</td>
<td>97 (18) 97</td>
<td>7.9 (0.5)</td>
<td>5.1 (2.5)</td>
<td>5.0 (0.7)</td>
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<tr>
<td>3.2.</td>
<td>Trans-UCA 100</td>
<td>97 (18) 97</td>
<td>7.9 (0.5)</td>
<td>5.1 (2.5)</td>
<td>5.0 (0.7)</td>
<td></td>
</tr>
<tr>
<td>3.3.</td>
<td>Trans-UCA 250</td>
<td>195 (3) 78</td>
<td>22.2 (5.6)</td>
<td>32.2 (5.5)</td>
<td>11.2 (0.3)</td>
<td></td>
</tr>
<tr>
<td>3.4.</td>
<td>Cis-UCA 40</td>
<td>40 (0) 100</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>0.6 (0.1)</td>
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<tr>
<td>3.5.</td>
<td>Cis-UCA 100</td>
<td>96 (10) 96</td>
<td>12.5 (0.5)</td>
<td>7.4 (2.0)</td>
<td>5.7 (0.2)</td>
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<tr>
<td>3.6.</td>
<td>Cis-UCA 250</td>
<td>188 (21) 75</td>
<td>46.9 (5.5)</td>
<td>31.1 (1.2)</td>
<td>11.7 (0.4)</td>
<td></td>
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<tr>
<td>4. Phosphate pH 7.2</td>
<td>Trans-UCA 40</td>
<td>22 [6] (2) 54</td>
<td>2.4 (0.3)</td>
<td>1.5 (0.4)</td>
<td>0.2 (0.0)</td>
<td></td>
</tr>
<tr>
<td>UV-A,B,C (Table 1.1)</td>
<td>Cis-UCA 40</td>
<td>10 [6] (2) 24</td>
<td>2.8 (0.2)</td>
<td>1.0 (0.0)</td>
<td>0.4 (0.1)</td>
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<tr>
<td>5. No phosphate initial pH 5.5</td>
<td>Trans-UCA 40</td>
<td>30 [6] (5) 75</td>
<td>2.7 (0.1)</td>
<td>0.4 (0.0)</td>
<td>0.2 (0.0)</td>
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<tr>
<td>UV-A,B,C (Table 1.1)</td>
<td>Cis-UCA 40</td>
<td>22 [6] (2) 56</td>
<td>2.6 (0.5)</td>
<td>0.4 (0.0)</td>
<td>0.5 (0.0)</td>
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<td>6. No phosphate initial pH ~5.5</td>
<td>Trans-UCA 40</td>
<td>27 [6] (7) 67</td>
<td>2.3 (0.0)</td>
<td>1.2 (0.4)</td>
<td>0.1 (0.0)</td>
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<tr>
<td>UV-A,B (Table 1.2)</td>
<td>Cis-UCA 40</td>
<td>10 [6] (3) 25</td>
<td>2.2 (0.3)</td>
<td>not detectable</td>
<td>0.3 (0.0)</td>
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Table 3. Breakdown of urocanic acid (UCA) and the formation of urocanic acid (UCA) oxidation products in several hydroxyl radical generating systems. Quantities given per liter sample as mean value with its difference between duplicate measurements.
ments in which ImCHO was used as starting material, a conversion to virtually 100% ImCOOH was obtained after Fenton- or photooxidation, indicating that ImCHO can easily be oxidized into ImCOOH under the conditions used in our experiments. HPLC analysis of a POmix-sample (see Methods) revealed that ImCOOH was the major 226 nm absorbing compound, while ImCHO concentration was largely reduced.

Trans-UCA and cis-UCA, in relatively high concentration of 250 μM, were broken down for 78% and 75%, respectively, by the unbuffered Fenton oxidation system. Table 3 section 3 also shows that the yield of oxidation products was proportional with the initial UCA concentration. Remarkably, the yield of ImCHO from cis-UCA in this oxidation system was substantially larger than from trans-UCA. In the phosphate buffered Fenton system breakdown and yield of oxidation products were low, but comparable, across an initial UCA concentration range from 40 to 250 μM (Table 3, section 1, only results of 40 μM are shown). In the presence of EDTA, a larger breakdown and a higher yield of ImCHO resulted (Table 3, section 2). This yield was increased as higher initial UCA concentrations were used. In the unbuffered Fenton system, the breakdown of UCA and the yield of oxidation products was the largest of all systems tested, if the initial UCA concentration was high (250 μM) (Table 3, section 3).

A close resemblance was observed between the chromatographic patterns of UCA Fenton oxidation products and those of UCA photooxidation products (Fig.2). In photooxidation, the breakdown of cis-UCA was substantially reduced in comparison with the trans isomer (Table 3, section 4-6). In Fenton oxidation, this effect was less pronounced. The data of Table 3 were given for air saturated solutions. Argon-purging of the solutions, prior to Fenton-or photooxidation, enhanced UCA breakdown as well as the yield of oxidation products, both by a factor 2 to 3. Heating (to 37 °C) of argon-purged solutions slightly enhanced the yield of ImCHO.

3.4. Detection of UCA oxidation products in UV-exposed corneal layer of human skin

The UV-B induced formation of imidazole-4-carboxaldehyde (ImCHO), imidazole-4-acetic acid (ImAc) and imidazole-4-carboxylic acid (ImCOOH), the imidazolic UCA oxidation products, could be demonstrated by HPLC analysis in corneal layer samples after UV-irradiation with a monitored UV-B dose of 9.6 kJ/m².

Confirmation of their formation in skin material was based on two observations. First, by comparing the chromatographic retention times of the
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**Fig. 3.** Overlaid chromatograms showing the formation of the imidazolic UCA oxidation products by UV-B induced photooxidation in corneal layer samples. 

a. comparison of chromatographic retention times to ensure identity: the lower line was derived from a control skin sample that was exposed to UV with wavelengths > 360 nm; the middle line was derived from a skin sample that was exposed to full 'solar-like' UV (including UV-B). The formation of ImCHO, ImAc and ImCOOH can be seen as indicated by 1, 2 and 3, respectively. The upper line was derived from a reference solution, containing the imidazoles in pure form. 

b. addition of authentic compounds to ensure identity: the lower line was derived from a skin sample irradiated with 'full UV' with a UV-B dose of 9.6 kJ/cm² and the upper line was derived from a similar skin sample to which the three authentic imidazoles (ImCHO, ImAc and ImCOOH) were added. A clear increase in peak height with unaffected peak symmetry, as indicated at 1, 2 and 3, can be seen, confirming the presence of ImCHO, ImAc and ImCOOH in full UV exposed skin samples.

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skin sample peaks with those of the authentic compounds (overlaid chromatograms are shown in Fig. 3a). Second, by adding the authentic imidazoles with amounts of 1.0 nmol ImCHO, 16 nmol ImAc and 0.8 nmol ImCOOH to a 'full UV'-irradiated skin sample of 200 μl, of which 40 μl was injected into the HPLC system. The peaks of added ImCHO, ImAc and ImCOOH clearly coincides with those that were originally present after full UV exposure (Fig. 3b). Additional confirmation for ImCHO formation was obtained by running the HPLC system with a detector set at 257 nm in stead of 226 nm. This condition resulted in a dramatic enlargement of the ImCHO peak at 7.5 min. (chromatogram not shown), conform the shape of the UV-spectrum of ImCHO at neutral pH.

The amounts of imidazolic UCA oxidation product formed were 0.07, 1.4 and 0.04 μmol per gram skin for ImCHO, ImAc and ImCOOH, respectively. The control skin sample was UV-irradiated in a similar way, except that UV-B was blocked out by a KV 370 cut-off filter. The control conditions did not cause the formation of the imidazolic UCA oxidation products.

4. Discussion

Imidazole compounds like histidine, histamine and carnosine are natural scavengers of hydroxyl radicals in the body and the formation of imidazolone derivatives of histidine and histamine has been demonstrated (30,31). We have shown earlier that UCA isomers are good hydroxyl radical scavengers as well, even in a more effective way than histidine. As UCA contains a reactive acrylic acid moiety the scavenging process may take a different course leading to the formation of totally different products. We hypothesized that the formation of such products may contribute to the understanding of the immunomodulating properties of the UCA isomers. HPLC analysis of photooxidation and Fenton oxidation experiments of UCA isomers showed the formation of a number of products (Fig. 1-3).

ImCOOH was the first UCA oxidation product that was identified, because it was prominently formed in vitro upon photooxidation or Fenton oxidation having the reactants on preparative scale concentrations. Subsequently it became clear that ImCOOH was a readily derived oxidation product of ImCHO upon scavenging of hydroxyl radicals. The aldehyde was directly formed from UCA, with its counterpart GLX. The formation in vitro of ImCHO is more prominent than ImCOOH when using reactant concentrations in the physiological range (Table 3). Most likely GLX may have been further oxidized to oxalic acid and to CO₂. The shift towards lower pH values in the course of UCA oxidation in water without buffer may be indicative for the formation of acidic products.
Formation of ImCHO and GLX has been observed with the irradiation of UCA in presence of a purine base (32). A suggested mechanism for this reaction involved a UCA radical cation formed either by photoionization or by electron transfer, followed by reaction with ground state oxygen. The involvement of singlet oxygen was excluded. In our case, the reaction will most likely start with addition of the hydroxyl radical to the double bond of UCA molecules as the course of the reaction seems comparable between the photooxidation and the Fenton reaction.

The formation of the identified products is only one part of the story as there is a gap between the amount of UCA converted and the formation of the three newly identified products, ImCHO, ImCOOH and GLX (Table 3). The gap may have been 'filled' with 3 compounds, identified from the POmix (see Methods), ImAc, glycine and aspartic acid. Moreover, other products are also being formed as seen with thin-layer chromatography (TLC) analysis of the PO-mix, that showed an array of overlapping fluorescent spots (TLC on silica with the eluent isopropanol/ammonia 25% 4:1). Evidence for the formation of one or more UCA dimers was obtained from mass spectral analysis, by which method a mass peak of 277 (M+H+) could be recorded from a PO-mix sample. The formation of imidazolon compounds of UCA similar to the formation of 2-oxohistidine, such as (4-imidazolon-2-yl)propenoic acid, could not be established yet.

The formation of the UCA oxidation products may not have been noticed before because most studies focussed only on the isomerization of trans-UCA and minor depletion of UCA levels may be obscured by variations in skin sampling techniques and by site-variations in epidermal UCA levels (33, 34). The finding of relative high levels of ImAc in UV-B exposed corneal layer samples (section 3.4) is in contrast with the experiments in vitro and may also be derived from other skin precursors than UCA.

As shown in this study, UV-A irradiation only results in UCA photoisomerization and virtually not in UCA photooxidation. The lack of correlation between UV-A-induced cis-UCA formation and immunosuppression (15) may suggest a possible role for UCA oxidation products in skin immunity, because it was shown here that UCA oxidation products can only be formed with UV-waves shorter than UV-A (< 320 nm). Another indication for an active role of UCA oxidation products in skin immunity is the finding that antioxidants abrogate UV- or cis-UCA-induced immunosuppression. Under these conditions the formation of UCA oxidation products may have been prevented, so that the induction of immunosuppression by UCA oxidation products would not happen.

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Preliminary tests with the mouse contact hypersensitivity model showed profound ear swelling reductions when a crude mix of UCA photooxidation products or combinations of the already identified imidazoles were applied (to be published).

For almost two decades UV-induced immunosuppression has been associated with cis-UCA. The impact of our findings is the possibility that UCA oxidation products may have a role in the phenomenon of UV-induced immunosuppression. Adopting the view of an active role for UCA oxidation products in skin immunity, we have no explanation yet for the more intense immunosuppressive action of cis-UCA over trans-UCA e.g. as observed in the mouse contact hypersensitivity model. Our future research will be focussed on the identification of other UCA oxidation products, on their formation in the skin under various conditions of oxidative stress and on testing their effects on the immune system.

In conclusion, both trans-UCA and cis-UCA can be oxidized in vitro to similar sets of oxidation products by various hydroxyl radical generating systems, which include exposure to UV (λ < 320 nm) or to the reactive ‘Fenton’ species. The primary products formed are identified as ImCHO and GLX. ImCHO can be rapidly further oxidized into ImCOOH. These findings in vitro may have relevance for the situation in vivo, because the formation of the three imidazoles ImCHO, ImAc and ImCOOH was also shown in UV-B exposed corneal layers of human skin.

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References.

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