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Note

High-performance liquid chromatographic determination of (*Z*)- and (*E*)-urocanic acid in human skin

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Urocanic acid [3-(1H-imidazol-4-yl)-2-propenoic acid] (UCA), a constituent of mammalian stratum corneum, is believed to act as a natural protective agent against sunburn [1—6]. Several high-performance liquid chromatographic (HPLC) procedures for the determination of UCA have been reported in recent years [7—12]. Especially the methods of Morrison et al. [11] and Caron et al. [12] seem to be very attractive at first glance for separation of both possible geometric isomers of UCA. In agreement with Caron et al. we found the method of Morrison et al. to be unreliable, because the described elution conditions caused rapid deterioration of the stationary phases. Caron et al. determined (*Z*)- and (*E*)-UCA in human plantar callus. In our opinion their determination methods are less attractive for the following reasons: (1) The use of a relatively high amount of biological material (50 mg) and the location (plantar callus) from which the sample was obtained. (2) Study of the effect of light on the *E* to *Z* isomerization of UCA is not possible

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under normal circumstances of daily routine using plantar callus. (3) Polar phases of the amino type show poor reproducibility and stability after prolonged use. (4) The mechanical homogenization of plantar callus, by means of perchloric acid, is a very tricky and time-consuming procedure and a significant decomposition of UCA may occur.

In our experiments, we used the reversed-phase mode, which is in principle capable of separating *Z* and *E* isomers [13].

A new HPLC procedure for the determination of (*Z*)- and (*E*)-UCA in 1—2 mg of the horny layer of human skin has been developed, which is sensitive enough to measure concentrations of UCA down to 150 ng/mg wet weight of human stratum corneum. This makes the method particularly attractive for paediatric work, especially in the diagnosis of histidinaemia, in which a decreased concentration of UCA in the stratum corneum has been reported [14—16].

A disturbance of the epidermal UCA content may be one of the etiological factors in hydroa vacciniformia, a very rare skin disease [17].

EXPERIMENTAL

All chemicals used were of the highest purity available. The mobile phase was filtered through a 0.45-μm filter and degassed by ultrasonication prior to use.

Procedure

With an ordinary fingernail clipper a small amount of stratum corneum (1—2 mg) was removed from the fingertips, weighed and transferred to a centrifuge tube. Then 100 μl of potassium hydroxide solution (1 mol/l) were added and the tube was vortexed briefly. After 10 min, the tube was centrifuged for 10 min at 6000 *g* and 4°C. Subsequently the supernatant was transferred to a 5-ml volumetric flask. This step was repeated. The residue was washed with 200 μl of water followed by 10 min centrifugation. The combined supernatants were acidified with 200 μl of orthophosphoric acid solution (2/3 mol/l) and made up to 5 ml with mobile phase; 50 μl were injected on the column.

Standard aqueous solutions were prepared using authentic UCA (anhydrous, stored desiccated in the dark at 4°C; Sigma, Munich, F.R.G.), under protection against light, in the range 20 μg per 0.5 ml to 0.31 μg per 0.5 ml and stored at —20°C. Samples were stable for at least two months.

Liquid chromatography

The analyses were performed using a liquid chromatograph consisting of a Beckman 112 solvent delivery module, a Beckman 420 controller, and a Beckman 340 organizer fitted with an Altex 210 inject system with 100-μl sample loop. Two columns, an Ultrasphere TM ODS, 25 cm × 4.6 mm I.D., 5 μm (Altex), and a MPLC®-microbore separation cartridge with an RP-18 3 cm × 2.1 mm, 5-μm guard column and an RP-18 22 cm × 2.1 mm, 5-μm main column (Kontron, Eching, F.R.G.), were tested. A Uvikon 720 LC spectrophotometer (Kontron) combined with a plotting integrator (C-R2AX, Shimadzu, Düsseldorf, F.R.G.) was used as detector; this was operated at 277

nm. The mobile phase was a mixture of potassium dihydrogen phosphate (0.02 mol/l) pH 3.7 with 1 g/l heptanesulphonic acid (sodium salt) and acetonitrile 93:7 (v/v). The flow-rate was 0.8 ml/min.

Mass spectrometry

Mass spectra were recorded on a double-focussing mass spectrometer (Varian Mat 311 A, Varian, Bremen, F.R.G.) in the electron impact mode with an electron energy of 70 eV.

RESULTS AND DISCUSSION

During the experiments the Ultrasphere ODS column showed slight tailing. Thus for quantitative analyses the MPLC-system was used. The accuracy of the method was tested with a standard sample containing 5 μg per 0.5 ml of water irradiated for 2 h with sunlight. The total amount of UCA was 5.02 ± 0.14 (S.D.) μg per 0.5 ml.

The coefficient of variation (C.V.) was 2.8% (n = 10, within-assay). The corresponding data for the between-assay were: 5.50 ± 0.19 μg per 0.5 ml, C.V. = 3.8%. Recovery was determined by adding 10, 20 and 30 μg of UCA to three preextracted stratum corneum samples of 1.2, 1.3 and 1.9 mg wet weight. A mean recovery of 96.0 ± 2.4%, C.V. = 2.5%, was found.

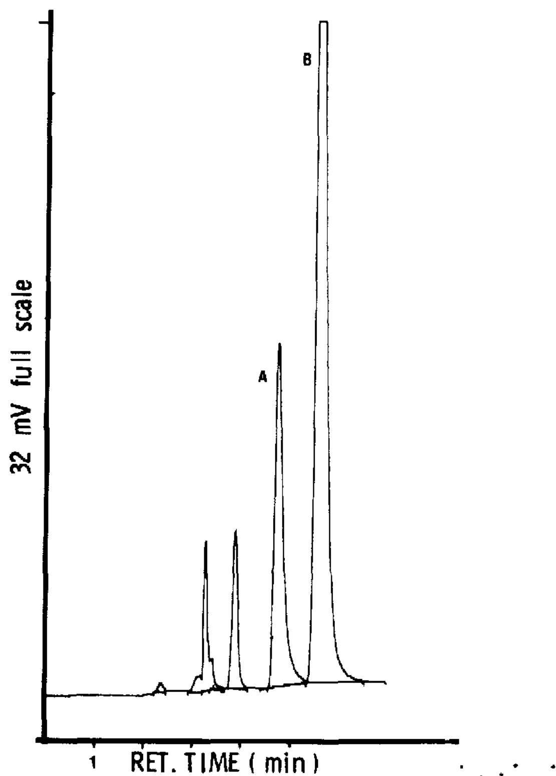

Fig. 1. Typical chromatogram of a stratum corneum extract of a 4-year-old girl. Peak A is (Z)-urocanic acid, peak B (E)-urocanic acid. For chromatographic conditions see experimental section.

The calibration was linear between 0.31 μg per 0.5 ml and 20 μg per 0.5 ml.

Solid authentic UCA and its aqueous solution protected from light had the *E* configuration. This could be proved by HPLC and infrared (IR) spectroscopy. Only one peak at 5.6 min appeared in the chromatogram. In the IR spectrum (solid material) a sharp absorption at 966 cm^{-1} is apparent, which is characteristic for the *E* configuration at a >C=C< double bond. After 2 h exposure in sunlight in a plastic vessel, the 966 cm^{-1} band decreased, coinciding with a diminution of the peak at 5.6 min. A second peak, at 4.8 min, appeared. Control samples, protected from light with aluminium foil, did not show these alterations. After irradiation, the chromatogram resembled that in Fig. 1, which is a chromatogram of a normal human stratum corneum extract.

These results provided strong evidence for *E* to *Z* isomerization due to light energy. The effluents of peaks A and B were collected separately and evaporated in the dark in a vacuum desiccator over anhydrous calcium chloride. Mass spectra of the residues were then recorded. Both spectra showed the same fragmentation pattern and were identical with authentic UCA. Residues from a second run were redissolved in 50 μl of water and reinjected. In both residues only one appropriate peak could be found. Thus, no reisomerization occurred. The conclusion of all the experimental results is that peak A (4.8 min) is (*Z*)-UCA and peak B (5.6 min) is (*E*)-UCA.

Quantification of (*Z*)- and (*E*)-UCA was made as follows: a 5 μg per 0.5 ml sample of authentic UCA (pure *E* isomer) was injected and the area under the single peak (peak B) recorded. Subsequently, the sample was irradiated and re-injected. The area of the now arising peak A was compared to standard samples of pure (*E*)-UCA. This is possible because the difference between (*Z*)- and (*E*)-UCA in terms of spectrum shape and absorption intensity is negligible within the limits of analytical error.

The described method was tested in eight children between the ages of 1.5 and 8 years (mean 4.6 ± 2.3 years) with the informed consent of their parents. Samples were taken in winter. The results are shown in Table I.

The method presented offers the opportunity to determine both geometrical isomers of UCA in human skin in one step without lengthy solvent extraction

TABLE I

UROCANIC ACID CONTENT OF STRATUM CORNEUM IN EIGHT CHILDREN

Volunteer	Age (years)	Sex*	UCA content (μg/mg wet weight)			*Z/E* ratio
			(*Z*)-UCA	(*E*)-UCA	Total UCA	
1	7	f	1.22	4.71	5.93	1:4
2	8	m	—	6.51	6.51	—
3	1.5	m	0.81	5.25	6.06	1:6
4	6	f	0.70	4.51	5.21	1:6
5	4	f	0.49	1.90	2.39	1:4
6	4	m	0.23	5.36	5.59	1:23
7	4	f	0.26	3.43	3.69	1:13
8	2	m	—	3.46	3.46	—

*m = male; f = female.

or derivatization procedures. Furthermore, seasonal variations in UCA content as well as alterations in the isomer ratio can be delineated in a very convenient manner.

Another practical aspect of this method, besides the diagnosis of histidinaemia, would be the possibility of evaluating the efficacy of sun-protective agents.

UCA determinations with the described method are currently being used to elucidate the role of UCA in hydroa vacciniformia. The results will be published at a later date.

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