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Journal of Chromatography B, 749 (2000) 41–47

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of histidine and urocanic acid isomers in the human skin by high-performance capillary electrophoresis

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Received 12 November 1999; received in revised form 13 April 2000; accepted 23 June 2000

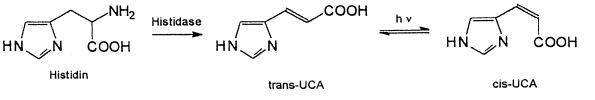
Abstract

Histidine was baseline separated from histamine, 1-methylhistamine and *cis*- and *trans*-urocanic acid using high-performance capillary electrophoresis (HPCE) on a fused-silica column (50 cm×75 μm) with 0.05 *M* NaH_2PO_4 buffer, pH 5.0, and 12 kV. The detection limit of histidine, *trans*- and *cis*-urocanic acid was 10^{-6} *M* at a wavelength of 214 nm. The detection limit of the urocanic acid isomers was slightly enhanced to $5 \cdot 10^{-7}$ *M* at 267 nm. The transformation of the *trans*-urocanic acid standard in vitro into the *cis*-isomer was dependent on the time of exposure and the energy of the light source. UVB light induced a significantly faster conversion than UVA light. The HPCE method was used for the characterization and measurement of histidine and urocanic acid in human skin eluates. The concentrations of histidine, *trans*- or *cis*-urocanic acid in ethanol washes from the skin of healthy, non-allergic volunteers were $2.22 \pm 0.40 \cdot 10^{-5}$, $0.96 \pm 0.26 \cdot 10^{-5}$ and $1.04 \pm 0.30 \cdot 10^{-5}$ *M*, respectively, (mean±SEM, $n=8$). The results obtained by HPCE correlated well with data obtained by HPLC. Correlation coefficients of $r^2=0.981$, $r^2=0.814$ and $r^2=0.956$ were found for histidine, *trans*- and *cis*-urocanic acid, respectively.

Keywords: Histidine; Urocanic acid

1. Introduction

Urocanic acid (UCA) is an imidazole-acrylic acid derivative that is synthesized in vivo from its precursor amino acid histidine (Hid) by the enzyme histidase, a histidine ammonia-lyase [1]. It is present in the human skin in two iso-forms, with highest concentrations being in the uppermost layer of the epidermis, the stratum comeum [2]. UV irradiation of the *trans*-isomer (*t*UCA) in vitro or in vivo in the human skin results in a dose-dependant photoisomerization into the *cis*-isomer (*c*UCA) [3].



UCA exhibits distinct biological functions. It is a natural sunscreen with photoprotecting activity against UV damage to DNA by its function as a photoreceptor capable of absorbing UVB photons [4,5]. Likewise, *c*UCA has been linked to UV

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00376-5

induced immunological alterations with systemic immune suppression that may play a critical role in skin cancer development [6,7]. Several studies have shown that skin cancer has increased over the last few years. Exaggerating sun bathing, the increasing use of artificial UV irradiation in sun tanning beds and the reduction of the ozone layer with the subsequent augmented terrestrial UV irradiation are responsible for the increasing skin tumor incidence [8–10]. Therefore, the measurement of UCA in the human skin as a marker for the degree of UV irradiation might be of clinical importance. Thus far, UCA has been determined in biological samples by paper chromatography or high-performance liquid chromatography (HPLC) [11–14]. The objective of this study was the feasible use of high-performance capillary electrophoresis (HPCE) for the separation of the UCA isomers, its precursor Hid and other metabolites of Hid such as histamine (His), 1-methylhistamine (MH). The method was used for the characterization and measurement of Hid, *t*UCA and *c*UCA in human skin eluates and the results were compared to data obtained by HPLC.

2. Material and methods

2.1. Preparation of standards

His, MH, Hid and *t*UCA were dissolved in 0.01 *M* HCl at a concentration of 10^{-2} *M* in 1.5-ml Eppendorf tubes. These stock solutions were diluted with 0.001 *M* HCl to 10^{-4} *M* for the qualitative analysis. The 10^{-2} *M* *t*UCA stock solution had to be slightly heated in order to get the *t*UCA into solution. A 10^{-4} *M* solution of *t*UCA dissolved with 0.001 *M* HCl in 1.5-ml Eppendorf tubes was used to obtain *c*UCA. The samples were exposed to UVB or UVA radiation for 0, 1, 3, 8, 18, 38 and 58 min within 20 cm distance of the light source representing 0.084 J/cm^2/min or 0.276 J/cm^2/min, respectively. A Waldmann UV 800 K instrument was used as a light source with a UVB spectrum ranging from 280 to 360 nm (maximum at 305 nm) and a UVA spectrum from 310 to 410 nm (maximum 355 nm). The tubes were removed from the UV light source at the different times and covered with an aluminum foil and kept frozen at −25°C prior to HPCE analysis. Special care was taken with the UCA standards and skin samples to avoid environmental UV light by wrapping the tubes with aluminum foil.

2.2. Calibration curves

The Hid and *t*UCA stock solutions were further diluted with 0.001 *M* HCl to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, $5\cdot10^{-7}$ and 10^{-7} *M* for the calibration curves. One set of 0.3 ml *t*UCA standards in 1.5-ml Eppendorf tubes ranging from 10^{-3} to 10^{-7} *M* was analyzed using HPCE. Another set of 0.3 ml *t*UCA standards in 1.5-ml Eppendorf tubes ranging from 10^{-3} to 10^{-7} *M* was irradiated with a sun simulator (Dermalight Vario II, Dr. Hoenle, Martinsried, Germany) to generate *c*UCA. The samples were exposed to the light source providing UVB, UVA and visible light at a distance of 120 cm for 60 min representing 0.012 J/cm^2/min UVB and 0.102 J/cm^2/min UVA. Standards were run in duplicates and unknowns were calculated from the calibration curve.

2.3. Preparation of skin extracts

Human skin samples were taken in summertime from eight healthy Caucasian volunteers (two males and six females, mean age 33±6 years) without any differences in skin coloration. UCA was extracted from the skin with the elution method described by Stäb et al. [15]. A glass funnel with a surface area of 14.52 cm^2 was tightly pressed on to the skin of the inner side of the left forearm. The funnel was filled with 3.0 ml of ethanol–water (1:1, v/v) and the extraction was carried out for 3 min by gently agitating the solvent in rotary movements. The extracts were collected in 15-ml plastic tubes and dried in a vacuum centrifuge. The dried samples were reconstituted in 0.5 ml 0.001 *M* HCl, spun for 3 min at room temperature and 13 000 *g* prior to injection. Results are expressed as mean+SEM in 10^{-5} *M*.

2.4. High-performance capillary electrophoresis

HPCE separations were performed on a Beckman PACE instrument equipped with a photodiode array detection system and an autosampler (Beckman, München, Germany). Separations were carried out at

25°C on a 50 cm×75 μm fused-silica column with 0.05 *M* NaH_2PO_4 buffer, pH 5.0, and 12 KV. Dual wavelength detection at 214 and 267 nm was used to monitor the separations. Synthetic standards or samples were injected under pressure for 15 s.

2.5. Correlation of HPCE with HPLC

The same eight skin samples that were run on HPCE were also separated on HPLC and the concentrations of Hid, *t*UCA and *c*UCA were compared to the results obtained by HPCE. The HPLC separations were performed isocratically on a reversed-phase C_8 column, 250×4.6 mm (Nucleosil 5 μm Macherey and Nagel, Dassel, Germany). The mobile phase was 0.01 *M* aqueous triethylammonium phosphate (TEAP), pH 3.0, with 0.005 *M* 1-octansulfuric acid sodium salt as an ion pairing reagent and acetonitrile at a ratio of 90:10. The separations were performed at room temperature with a flow-rate of 1.0 ml/min and were monitored at 210 nm. The injected sample volume was 0.05 ml [16].

2.6. Chemicals

All chemicals used were of analytical grade. Hid, His and *t*UCA were purchased from Sigma (Deisenhofen, Germany). MH was obtained from Calbiochem (Frankfurt, Germany) and concentrated phosphoric acid came from Fluka (Germany). All other chemicals used were of HPLC gradient grade quality and obtained by Merck (Darmstadt, Germany).

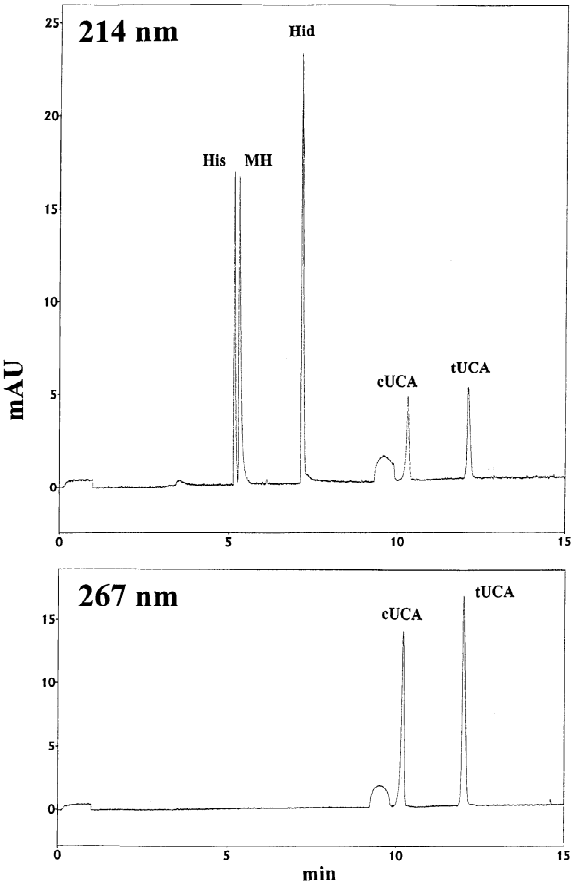

Fig. 1. HPCE separation of His, MH, His, *t*UCA and *c*UCA standards on a fused-silica capillary. All four compounds were baseline separated in less than 15 min. Top panel: separation at a wavelength of 214 nm. Bottom panel: separation at a wavelength of 267 nm. Note the enhanced detector signal at 267 nm as compared to the signal obtained at 214 nm.

3. Results

3.1. HPCE separation of standards

The amino acid Hid was completely separated from its metabolites His, MH and *t*UCA in a single run within 15 min. Likewise, *t*UCA was baseline separated from its *cis*-isomer *c*UCA (Fig. 1). The detection signal of *t*UCA and *c*UCA at 214 nm (Fig. 1, upper panel) was significantly improved by a factor of 2 when the separation was monitored at 267 nm (Fig. 1, lower panel). The sequence of elution was His, MH, Hid, *c*UCA and *t*UCA with migration times of 5.19±0.08, 5.70±0.35, 7.40±0.17, 9.74±0.41 and 12.72±0.05 min ($n=3$), respectively.

The calibration curves of Hid and *t*UCA at 214 nm were linear between 10^{-3} and 10^{-6} *M*, respectively, with a detection limit of 10^{-6} *M*. The correlation coefficient was $r^2=0.998$ for both compounds. However, the detection limit of *t*UCA at 267 nm was slightly improved to $5\cdot10^{-7}$ *M* with a linear correlation coefficient of $r^2=0.997$ (Fig. 2).

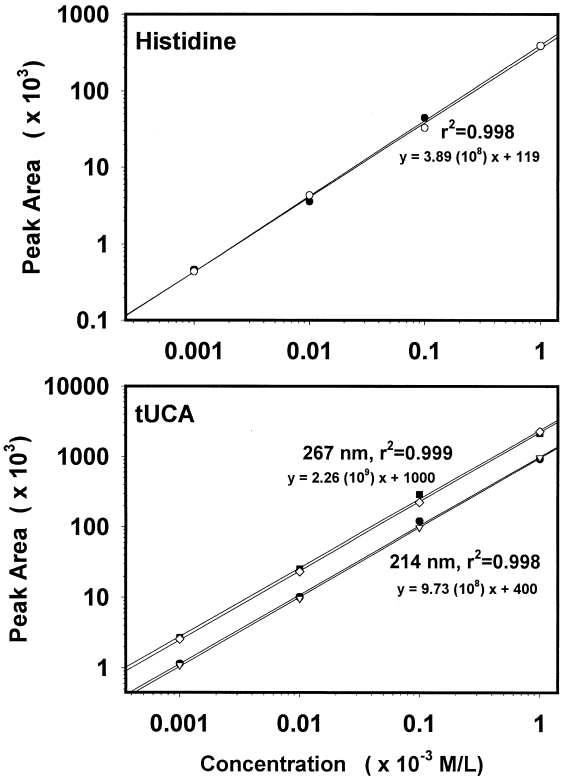

Fig. 2. Calibration curves of Hid and *t*UCA. Top panel: linear calibration curve with a correlation coefficient of $r^2=0.998$ was obtained for Hid at a wavelength of 214 nm. Bottom panel: linear calibration curve with a correlation coefficient of $r^2=0.998$ or $r^2=0.999$ was also found for *t*UCA at a wavelength of 214 or 267 nm.

3.2. Transformation of tUCA into cUCA

*t*UCA was converted to *c*UCA in vitro by irradiation with either UVB or UVA light. UVB light induced a rapid and exponential conversion of *t*UCA into *c*UCA up to 83% *c*UCA within 8–10 min. In contrast, the kinetic of the conversion of *t*UCA into *c*UCA by UVA light was significantly slower with approximately 36% conversion after 58 min (Fig. 3).

3.3. Hid and UCA isomers in human skin eluates

Hid, *t*UCA and *c*UCA but no His or MH were discovered in human skin eluates. An example is shown in Fig. 4. A sample with a low and a high concentration of *c*UCA is shown (Fig. 4, upper and

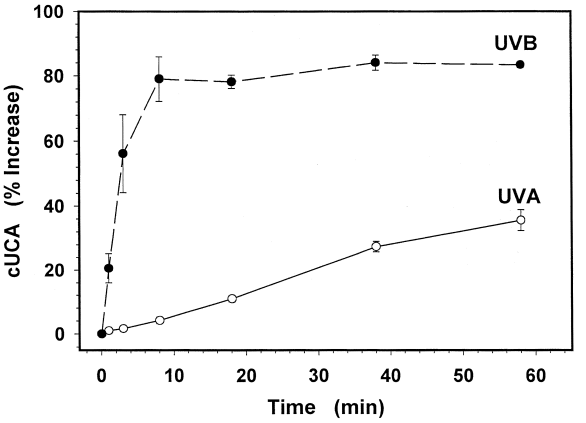

Fig. 3. Kinetic of the conversion of *t*UCA standard into *c*UCA after UVB and UVA irradiation. TUCA was rapidly converted to *c*UCA with UVB light within 8–10 min. In contrast, the conversion of *t*UCA into *c*UCA with UVA light was significantly slower.

lower panel) whereas the concentrations of Hid and *t*UCA were similar. The average concentrations of Hid, *t*UCA and *c*UCA were $2.22\pm0.40\cdot10^{-5}$,

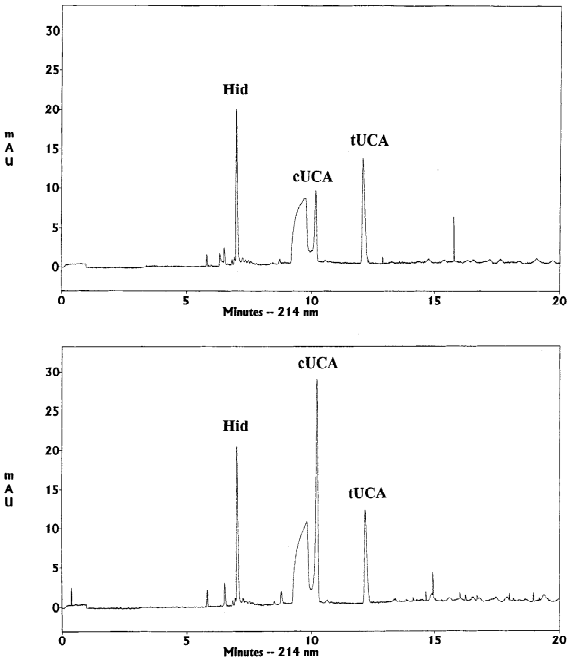

Fig. 4. HPCE separation of Hid, *t*UCA and *c*UCA in human skin samples. Top panel: skin sample with a low concentration of *c*UCA. Bottom panel: skin sample with a high concentration of *c*UCA. The concentrations of Hid or *t*UCA were similar in both samples.

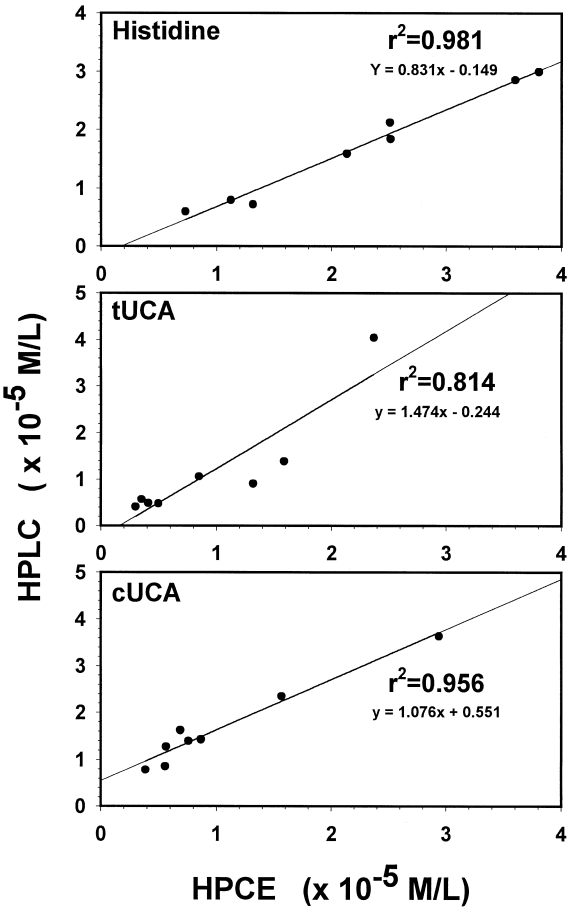

Fig. 5. Correlation between the Hid, *t*UCA and *c*UCA concentrations in human skin samples obtained by HPCE and HPLC. The concentrations of Hid, *t*UCA or *c*UCA found by HPCE correlated well with the results obtained by HPLC with a correlation coefficient of $r^2=0.981$, 0.814 or 0.956 ($n=8$).

$0.96\pm0.26\cdot10^{-5}$ and $1.04\pm0.30\cdot10^{-5}$ *M*, respectively ($n=8$).

HPLC separation of the same skin samples showed similar Hid, *t*UCA and *c*UCA concentrations. Correlation coefficients of $r^2=0.981$, $r^2$0.814 and 0.956, respectively, were obtained (Fig. 5).

4. Discussion

This study focused on the possible use of HPCE for the separation of UCA isomers from their precursor, Hid, and other metabolites of Hid such as His and MH in human skin. Several HPLC procedures have been described for the qualitative and quantitative determination of *t*UCA and *c*UCA in human skin using reversed-phase packing materials with aqueous buffers and acetonitrile or isopropanol as organic modifiers [12–14,17]. These HPLC procedures were able of separating *t*UCA from *c*UCA but none of these publications mentioned or showed the separation of Hid from *t*UCA and *c*UCA. However, the capability of measuring Hid with *t*UCA and *c*UCA is important. Since Hid is the precursor of UCA, the endogenous Hid concentrations may influence the UCA concentrations in the skin. In fact, it has been shown that the total amount of UCA in the skin is significantly higher after a Hid-rich diet as compared to a normal diet [18]. In addition, His and MH which are present in the skin and also derived from Hid but by a different metabolic pathway were included in our study. Here we report for the first time the use of HPCE zone electrophoresis for the characterization and measurement of *t*UCA, *c*UCA, Hid as well as His and MH. The separation of all five standards was accomplished in a single run in less than 15 min with UV detection at 214 nm. The determination of *t*UCA and *c*UCA was further enhanced by a factor of 2 when the separation was monitored at 267 nm. The electropherograms showed a big hump that migrated just before *c*UCA that is related to impurities of the water or the phosphate buffer. Calibration curves with Hid and *t*UCA standards at 214 nm were linear over a wide range with the lowest detectable concentration of 10^{-6} *M*, acceptable slopes and a correlation coefficient of $r^2=0.998$, respectively.

*c*UCA is not commercially available. Therefore, the *t*UCA standard that can be purchased was used to obtain *c*UCA from *t*UCA after in vitro irradiation with UV light. The rate of conversion was closely related to the time of exposure and the energy of the light source. The energy-rich UVB light rapidly converted *t*UCA into *c*UCA whereas the conversion was markedly slower with UVA light. The transformation of *t*UCA into *c*UCA in standard or skin samples only occurs after intense UV irradiation. Marginal interior daylight or regular artificial indoor-light sources did not induce any significant conversion. In addition standards or samples with UCA isomers are stable and can be stored over a long

period of time without changes. Light-protected samples kept for 1 year did not show any changes in the ratio between *t*UCA and *c*UCA (unpublished data). Nevertheless, we decided to exclude any light-induced changes by wrapping all samples with aluminum foil.

The HPCE set-up was used to measure Hid, *t*UCA and *c*UCA in human skin eluates. Various techniques for sampling UCA from the skin are described in the literature including scraping [18] punch biopsy [19] or elution with 0.1 *M* potassium hydroxide [17]. Since these are very invasive techniques we decided to extract Hid, and the UCA isomers by the less invasive elution procedure — 50% ethanol in water — described by Stab et al. [15] The different sampling techniques have never been thoroughly investigated with respect to extraction time, extraction volume or recovery. It is nearly impossible to design and perform an accurate experiment to account for these factors. Since Hid and UCA are located in the skin, topical application or addition to the extraction medium are far from the in vivo condition. Likewise, the use of radioactive-labeled compounds for these types of experiments can be ruled out because of ethical reasons. However, the efficiency of the elution method can be assumed from elution experiments with lipids where the first elution contained 90% of the accessible lipids [20].

*t*UCA, *c*UCA and Hid were identified in the skin eluates with various concentrations and ratios of the three compounds and confirmed by spiking skin samples with Hid, *t*UCA and *c*UCA. In biological samples other compounds are also picked up with UV detection at 214 nm, especially with HPLC. No other compounds besides Hid and UCA isomers were found in the skin samples at 214 nm and we assume that with our HPCE conditions other components migrated considerably slower. His or MH were not found in any of the samples tested although they are both present in the skin and soluble in the extraction medium. The only reasonable explanation for the absence of His or MH in our samples is that they are located in deeper skin layers which are not accessible to the extraction medium. A good correlation between HPCE and HPLC [16] was found for Hid and *c*UCA whereas the results with *t*UCA were mediocre because of technical problems with the HPCE equipment and the inability to rerun some skin samples.

Even though this is a preliminary study with a very limited ($n=8$) and highly selected number of samples from healthy volunteers only, it clearly demonstrates the feasible use of HPCE for the separation of Hid and UCA isomers in human skin samples. Further experiments have to be performed in a larger number of samples to validate the procedure with respect to inter- and intra-assay variation, reproducibility and the limit of quantification in skin samples diluted to the lowest detectable concentration. Currently, experiments are being carried out in our laboratories to address all of these issues.

In summary: HPCE is a powerful tool for the separation of Hid, His, MH, *t*UCA and *c*UCA that has not been previously described. It can be used for the measurement in human skin eluates without intense or tedious purification or derivatization. The technique is fast with a high sensitivity that is equivalent to HPLC and can be automated since most of the HPCE instruments are equipped with an autosampler. The advantages are high sample throughput, the minute amounts of samples needed and the use of aqueous buffers without organic solvents for the separation.

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