Simultaneous Determination of Urocanic Acid Isomers and Histidine in Human Stratum Corneum by High-Performance Liquid Chromatography

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Histidine ammonia-lyase (E.C.4.3.1.3) catalyzed degradation to *trans*-urocanic acid (UCA) is one of the major metabolic pathways of histidine in liver and skin. *Trans*-UCA is further metabolized in liver by urocanase (E.C.4.2.1.49) while it accumulates in skin, predominantly in the stratum corneum because no urocanase activity is present in the epidermis.^{1,2} Both *in vitro* and in the skins of experimental animals and humans, *trans*-UCA isomerizes to *cis*-UCA following exposure to ultraviolet (UV) light.^{2,4} In addition, topical application of *trans*-UCA was shown to reduce UV-induced erythema in humans.^{3,5} Therefore, *trans*-UCA has been believed to function as a natural sun-screen agent against sunburn and against UV-induced damage to DNA in the skin,^{2,3,5} though recent studies have shown that the level of an endogenous *trans*-UCA in the skin is too low to exert its photoprotective effect.⁶ In contrast to the *trans* isomer, *cis*-UCA acts as an initiator of immunosuppression which may be a critical step in UV-induced pathogenesis of skin cancer and other cutaneous and systemic diseases.^{2,4,7-9} Though a variety of factors affect UCA contents in the skin,^{10,11} the major ones are the level of bacterial flora capable of degrading UCA,¹¹ and the availability of free histidine produced from the histidine-rich protein of the keratohyalin granule in the epidermis.¹⁰ Therefore, the assays of UCA isomers as well as histidine are of great importance in the photodermatological study.

Geometrical isomers of UCA in the skin have been determined by high-performance liquid chromatography (HPLC) with a UV absorbance detector.^{4,12-14} However, histidine has also been measured by alternative methods^{10,15,16} such as an amino acid analyzer because no method other than a capillary gas chromatography-mass spectrometry¹⁷ was available for the simultaneous assay of the three substances. Recently, Takahashi *et al.* have reported the HPLC method using precolumn derivatization with dabsyl chloride for the assays of UCA isomers and histidine in the skin.¹⁸ However, the reagent is reactive with all other amino acids and amines in biological samples. Thus, the method requires precise chromatographic separations of UCA isomers and histidine from the interferences and thus becomes time-consuming. On the other hand, we have developed a specific HPLC method for the

assay of serum histidine,¹⁹ which is based on the separation by a reversed-phase ion pair chromatography followed by the selective detection of histidine with postcolumn fluorescence derivatization using *o*-phthalaldehyde (OPA). Since its separation mode has also been used for the HPLC assay of UCA isomers,^{13,14} we attempted to develop a reversed-phase ion pair chromatography with the sequential detections of UCA isomers at 264 nm and histidine with the postcolumn derivatization. This HPLC method was applicable to the simultaneous assay of UCA isomers and histidine in human stratum corneum in combination with the tape-stripping method.^{14,20,21}

Experimental

Chemicals

Histidine monohydrochloride, agmatine sulfate, spermidine phosphate, putrescine dihydrochloride, glutathione, sodium heptanesulfonate and OPA were obtained from Wako Pure Chemical Industries Ltd. (Osaka). 1-Methylhistidine and 3-methylhistidine were purchased from Hoechst Japan Ltd. (Tokyo). Amino acids were from Ajinomoto Company (Tokyo). Pyroglutamic acid and histamine dihydrochloride were obtained from Tokyo Kasei Organic Chemicals (Tokyo). *Trans*-UCA was obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade acetonitrile was from Kanto Chemical (Tokyo). All other reagents used were of analytical grade. Milli-Q (Nihon Millipore Kogyo, Yonezawa) water was used in the preparations of an eluent and reagent solutions. Stock standard solutions of *trans*-UCA (10 mM) and histidine (20 mM) were prepared in 0.02 M HCl and stored in a refrigerator. Working standard solutions of *trans*-UCA and histidine were prepared by diluting their stock solutions with 0.02 M HCl before use. A standard solution of a mixture of *cis*- and *trans*-UCA was prepared by UV irradiation of 0.2 mM *trans*-UCA in 0.02 M HCl according to the method of Shibata *et al.*¹⁴ OPA solution was freshly prepared by dissolving in HPLC-grade acetonitrile.

Preparation of human stratum corneum extract

According to the reported methods^{14,20,21} with slight modification, the stratum corneum was stripped by applying a piece of cellophane adhesive tape (20 mm × 18 mm wide; Nichiban, Tokyo) to the skin in the region of the inner forearm,

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the lower abdomen or the back of the hand for 10 s. UCA and histidine on the tape were extracted by immersing the tape in 1.5 ml of 0.1 M KOH in a 2.0 ml screw-capped polypropylene tube for 30 min with occasional vortexing. The extract was neutralized with 0.5 ml of 0.38 M HCl and filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). A portion of the filtrate (20 μl) was injected onto the HPLC column. The 20 successive tape-stripping experiments were performed on one of the authors and the single tape-stripping experiments were done on the backs of the hands of 10 young female students (range 20–22 years) as volunteers after their informed consents were obtained.

Chromatographic system and conditions

HPLC system consisted of an L-6000 pump (Hitachi, Tokyo) and an LC-9A pump (Shimadzu, Kyoto) for deliveries of an eluant and OPA reagent, a DGU-12A degasser (Shimadzu), a CTO-10A column oven (Shimadzu), a Rheodyne Model 7725i sample injector (Rheodyne, Cotati, CA, USA), a UV-4200 absorbance detector equipped with a 17.7 μl flow cell (Hitachi), an F-1050 fluorescence detector equipped with a 12 μl flow cell, and two D-2500 data processors (Hitachi). Separation was performed at 40°C by using a 100 $\times$ 4.6 mm i.d. Develosil ODS UG-3 column with a particle size of 3 μm (Nomura Chemical, Seto) as an analytical column which was protected by a guard-pack cartridge column (a 10 $\times$ 4.0 mm i.d. Develosil ODS UG-5, particle size of 5 μm), and a 7:93 (v/v) mixture of acetonitrile and 20 mM potassium phosphate buffer (pH 3.7) containing 5 mM sodium heptanesulfonate as an eluent. OPA reagent used for the postcolumn detection of histidine was a 15:1 (v/v) mixture of 50 mM potassium phosphate buffer (pH 8.0) and 50 mM OPA in acetonitrile. Both the eluent and OPA reagent were filtered through the membrane filter before use. The eluent was delivered to the column at a flow-rate of 0.5 ml min⁻¹ through a preheater tube (stainless-steel tube, 10 m $\times$ 0.8 mm i.d.) and the sample solution (20 μl) was introduced to the column. The eluate from the column was passed through a UV detector and was then added with OPA reagent delivered at a flow-rate of 0.5 ml min⁻¹ to a mixing T-joint through a preheater tube (stainless-steel tube, 10 m $\times$ 0.8 mm i.d.) and a resistor polytetrafluoroethylene (PTFE) tube (20 m $\times$ 0.25 mm i.d.). The mixture was passed through a reactor tube (coiled PTFE tube, 2.5 m $\times$ 0.5 mm i.d., coil diameter of 20 mm) to generate fluorescence. All the columns, preheater-, resistor- and reactor tubes were placed in the column oven maintained at 40°C. UCA isomers were detected at 264 nm, whereas histidine was measured by the fluorescence at 435 nm with an excitation wavelength of 365 nm (uncorrected). Elutions of other standard biological substances undetected by either of the above methods were monitored at 220 nm, except for polyamines such as agmatine, putrescine and spermidine which were detected by a postcolumn derivatization with a mixture of OPA and 2-mercaptoethanol, a fluorogenic reagent system for primary amines.²²

Results and Discussion

HPLC method for the simultaneous determinations of UCA isomers and histidine

Among a number of HPLC conditions available for the separation of UCA isomers,^{4,12–14} we chose a reversed-phase ion pair chromatography because its separation mode was also successfully used in our HPLC method for the assay of histidine.¹⁹ As a result, separation of UCA isomers and

Fig. 1 Chromatograms monitored with UV- and fluorescence detectors of a mixture of *cis*-urocanic acid (*cis*-UCA), *trans*-urocanic acid (*trans*-UCA), 20 amino acids, glutathione (GSH) and pyroglutamic acid. Injected amount of each was 20 pmol per 20 μl . His, histidine; Phe, phenylalanine; Trp, tryptophane. *Solvent peak. Ex, excitation wavelength; Em, emission wavelength.

histidine was achieved at 40°C on a C18 reversed phase column with a 7:93 (v/v) mixture of acetonitrile and 20 mM phosphate buffer (pH 3.7) containing 5 mM sodium heptanesulfonate at a flow rate of 0.5 ml min⁻¹. After the separation, UV (264 nm) detector and the postcolumn derivatization with OPA were used for the sequential detections of UCA isomers and histidine. The postcolumn derivatization was carried out under the same conditions as our original ones¹⁹ except that acetonitrile was used instead of methanol as a solvent for OPA.

Figure 1 shows chromatograms of a standard mixture of 20 amino acids, *cis*- and *trans*-UCA, glutathione and pyroglutamic acid, the last one is known to present in the skin. In the chromatogram monitored at 264 nm, *cis*- and *trans*-UCA were observed at 4.9 and 6.4 min, respectively, which were well separated from all detectable amino acids. Histidine was shown to be eluted at 5.5 min by monitoring at 220 nm but it was not detected at 264 nm. On the other hand, histidine was detected at 6.2 min with a fluorescence detector. The retention time (Rt) was slightly longer than that (5.5 min) described above because the reaction coil was inserted in the line. Though glutathione was also detected at 3.7 min, the other 19 amino acids (Rt < 19 min), pyroglutamic acid (Rt = 2.3 min), ornithine (Rt = 5.4 min), 1-methylhistidine (Rt = 5.5 min), lysine (Rt = 5.6 min) and 3-methylhistidine (Rt = 5.8 min) gave no peak and thus did not interfere with the quantitative detection of histidine. No interference was also observed from histamine (Rt = 39.7 min) and polyamines (Rt > 32 min).

The present HPLC method gave linear calibration curves for *cis*-UCA ($r = 0.999$) and *trans*-UCA ($r = 0.999$) over the range of 0.5–1500 pmol per injection (20 μl) with the relative standard deviation (RSD) of 0.58% and 0.31% at 10 pmol ($n = 10$), and with the detection limits ($S/N = 5$) of 40 fmol and 70 fmol, respectively. For histidine, a linear calibration curve ($r = 0.999$) was obtained over the range of 0.1–1000 pmol per

Fig. 2 Chromatograms monitored with UV- and fluorescence detectors of the extract of human stratum corneum stripped from the skin on the back of the hand (A) and the blank extract from a piece of cellophane adhesive tape (B). Detected amounts were 9.7, 7.0 and 11.7 pmol per injection (20 μ l) for *cis*-urocanic acid (*cis*-UCA), *trans*-urocanic acid (*trans*-UCA) and histidine (His), respectively. *Solvent peak. Ex, excitation wavelength; Em, emission wavelength.

injection (20 μ l) with RSD of 0.9% at 10 pmol ($n = 10$) and with the detection limit ($S/N = 5$) of 10 fmol.

In order to apply the HPLC method to the simultaneous assays of UCA isomers and histidine in human stratum corneum, we chose the tape-stripping method as a simple and non-invasive sampling procedure.^{14,20,21} Figure 2A shows the typical chromatograms of an extract from the stratum corneum of the back of the hand. Both the chromatograms monitored with UV- and fluorescence detectors were very simple and UCA isomers and histidine were clearly detected. In the chromatogram monitored at 264 nm, however, a small peak due to tryptophane at 18.8 min was observed in the several stratum corneum extracts. Thus, at least 20 min is required for one run of HPLC analysis. Figure 2B shows the chromatograms of blank extract from the tape, where neither UCA isomers nor histidine were detected. Though several unknown substances (not amino acids) were observed in the chromatogram monitored at 264 nm, they did not interfere with the assay of UCA isomers.

Extraction efficiencies of UCA isomers and histidine with 0.1 M KOH from the stripped stratum corneum on the cellophane tape were confirmed by repeating the extraction procedure on the previously extracted tape. The mean percentages ($n = 20$) of the second extracted amount in the sum of the first and second extracted amounts were 1.8% for a total of UCA and 1.3% for histidine, indicating that both substances were recovered quantitatively from the tape with the first extraction.

The reproducibility of the present method was also examined by using the pooled extract from the tape-stripped stratum corneum on the back of the hand. The RSD of within assay were 2.3, 0.6 and 0.6% ($n = 10$) and those of day-to-day assay were 2.0, 1.1 and 1.6% ($n = 6$) for *cis*-UCA, *trans*-UCA and histidine, respectively. Norval *et al.* claimed poor reproducibility of a reversed-phase ion pair chromatography for the assays of UCA isomers.²³ However, our HPLC method with the same separation mode showed good reproducibilities in their retention times and peak heights. These improvements are probably due to precise temperature control (40°C) of the eluent by insertion of a preheater coil in the line, as described in Experimental, such a coil is inherently required for the

Table 1 Contents of urocanic acid isomers and histidine in the single tape-stripped human stratum corneum from the back of the hand measured in December

Volunteer	Content/nmol cm ⁻²				<i>Cis</i> ^b , %	UCA ^a /His ^a
	UCA ^a			His ^a		
	<i>Cis</i>	<i>Trans</i>	Total			
A	0.097	1.062	1.159	1.275	8.4	0.91
B	0.051	0.211	0.262	0.280	19.5	0.93
C	0.011	0.061	0.072	0.078	15.3	0.92
D	0.028	0.135	0.163	0.616	17.2	0.26
E	0.195	0.270	0.465	0.325	41.9	1.43
F	0.041	0.163	0.204	0.234	20.1	0.87
G	0.123	0.333	0.456	0.638	27.0	0.71
H	0.027	0.191	0.218	0.160	12.4	1.36
I	0.089	0.341	0.430	0.418	20.7	1.03
J	0.067	0.118	0.185	0.258	36.2	0.72
Mean	0.073	0.289	0.361	0.428	21.9	0.91
SD	0.055	0.287	0.311	0.348	10.4	0.33

a. UCA and His represent urocanic acid and histidine, respectively.

b. The percentage of UCA presents as *cis*-isomer.

reproducible postcolumn detection of histidine with OPA.¹⁹

Distribution of UCA isomers and histidine in human stratum corneum

Since the present method enabled the sensitive and reliable assays of UCA isomers and histidine in the stratum corneum, their distribution patterns were investigated in the stratum corneum of several body sites.

Table 1 shows the concentrations of UCA isomers and histidine in the single tape-stripped stratum corneums from the back of the hand of 10 healthy female volunteers. The inter-individual variations were large in the levels of UCA isomers and histidine and in the percentage of *cis*-UCA, whereas the variation was less in the ratio of total UCA to histidine.

Figure 3 shows the results of the successive tape-stripping experiments where twenty strips were successively taken from the same location of the inner forearm, the back of the hand and the lower abdomen of the same subject. The levels of UCA isomers varied depending on the number of strips as well as body site. The level of histidine also changed with the number of strips, however, its change closely paralleled that of a total UCA in all cases. Schwarz *et al.*¹⁵ who measured histidine by amino acid analyzer, briefly mentioned the similar tendency between UCA and histidine on the skin of the left scapula.

Figure 4 shows the percentage of UCA present as *cis*-isomer. The percentages measured in October were in the order of the back of the hand > the inner forearm > the lower abdomen. This order is reasonable because the *trans*-*cis* isomerization of UCA occurs more easily in a sunlight-exposed site (the back of the hand) than in a less exposed site (the inner forearm) or in a shielded site (the lower abdomen).^{6,14,24} In the case of the inner forearm, the *cis* percentages were higher in July than in October and gradually decreased with the increase in number of strips. The former is explained as seasonal variation in UV radiation dose from the sun²⁵ and the latter is interpreted by the suggestion that the effect of UV radiation gradually decreases from the upper layer to a deeper layer in the skin.²¹

In combination with the tape-stripping method, the present HPLC method is applicable to the simultaneous assays of UCA isomers and histidine in the stratum corneum on the skin of any body site. It has several advantages over the reported

Fig. 3 Distribution patterns of total urocanic acid (●) and histidine (○) in different layers of 20 successive tape-stripped human stratum corneums from skin on the back of the hand (A), the lower abdomen (B) and the inner forearm (D) measured in October, and from the inner forearm (C) measured in July.

Fig. 4 The percentage of urocanic acid present as *cis*-isomer in different layers of 20 successive tape-stripped human stratum corneums from skin on the back of the hand (■), the lower abdomen (●) and the inner forearm (●) measured in October, and from the inner forearm (○) measured in July.

methods^{17,18} such as selectivity, simplicity, rapidity, high sensitivity and reproducibility. The present method is also useful in the diagnosis of histidinaemia, one of hereditary metabolic disorders characterized by a virtual deficiency of histidine ammonia-lyase, in which UCA decreases²⁶ and histidine probably increases in the stratum corneum.

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