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Journal of Chromatography B, 688 (1997) 197–203

JOURNAL OF CHROMATOGRAPHY B

Quantitative analysis of histidine and *cis* and *trans* isomers of urocanic acid by high-performance liquid chromatography: a new assay method and its application

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Received 5 March 1996; revised 8 July 1996; accepted 9 July 1996

Abstract

To elucidate the factors involved in dry skin and the skin damage caused by UV light, it is necessary to analyze small amounts of stratum corneum to determine amino acid contents. A new assay method for this purpose is described. Dabsylated amino acids including histidine and the *cis* and *trans* isomers of urocanic acid were analyzed quantitatively by high-performance liquid chromatography (HPLC), using a reversed-phase column. Histidine and the isomers of urocanic acid were separated from 36 other amino acids thought to be present in the extract of stratum corneum. In the presence of the 36 amino acids, standard calibration curves were obtained from 0.25 to 2.5 pmol/μl, for histidine and for both isomers of urocanic acid. The coefficients of variation for the reproducibility of the analysis at 1.0 pmol/μl were 3.8%, 2.9% and 2.5% for the *cis* and *trans* isomers of urocanic acid and for histidine, respectively. Amounts of 2 to 50 pmol of *cis* and *trans* isomers of urocanic acid and histidine in the stratum corneum were detected. The ratio of the *cis* to the *trans* isomer of urocanic acid in sunburned stratum corneum was more than three times that in normal stratum corneum. This method appears to be useful for the determination of small amounts of histidine and of the *cis* and *trans* isomers of urocanic acid in the stratum corneum.

Keywords: Histidine; Urocanic acid

1. Introduction

Ultraviolet (UV) radiation is implicated in various types of skin damage, such as sunburn, photoaging, actinic keratosis and skin cancers [1]. The skin contains various agents that absorb UV light, e.g., melanin and urocanic acid [2]. Epidermal urocanic acid, particularly its *cis* isomer, appears to have an immunoregulatory function [3,4]. Urocanic acid is biosynthesized from histidine by the enzymatic action of the enzyme histidase [5]. In normal epidermis, urocanic acid is present predominantly as the *trans* isomer, but following exposure of the skin to UV, a mixture of the *cis* and *trans* isomers is formed [2,6]. Urocanic acid acts as a sunscreen [7] or as a photoreceptor in the epidermis [8]; however, the relative amounts of the *cis* and *trans* isomers in sunburned stratum corneum have not yet been determined.

To elucidate the influence of sunburn by UV light on the skin, it is of interest to determine the amounts of the two urocanic acid isomers in the stratum

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PII S0378-4347(96)00316-7

corneum. Previous measurements of urocanic acid, done by its absorbance at 267 nm [9], have not been sufficiently sensitive and large samples were necessary. It was therefore impossible to determine the amount of urocanic acid in the small samples obtained from the stratum corneum. In this study, we demonstrate a method by which the amounts of not only the *cis* and *trans* isomers of urocanic acid but also the amount of histidine (the amino acid precursor of urocanic acid) can be assayed in small samples of the stratum corneum. We used high-performance liquid chromatography (HPLC) with a reversed-phase column separating prelabeled amino acids with dabsyl chloride (dabsyl–Cl) [10].

2. Experimental

2.1. Chemicals

Urocanic acid was obtained from the Aldrich, (Milwaukee, WI, USA). The reversed-phase column (Wakosil 5C18) and AN and B type standard amino acid mixtures were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dabsyl chloride was obtained from Pierce (Rockford, IL, USA) and was recrystallized from acetone solution. The reversed-phase column (TSK-GEL, ODS-120 T) was purchased from Tosoh (Tokyo, Japan), for collection of *cis* isomer of urocanic acid. Other chemicals, all of analytical grade, were obtained from various commercial sources.

2.2. Preparation of cis isomer of urocanic acid

The *cis* isomer of urocanic acid was prepared by the method of Morrison et al. [11], with some modifications. Briefly, commercial urocanic acid (the *trans* isomer) was dissolved in 0.01% HCl solution and irradiated by UV light, the wavelength of which was 300 to 400 nm and the strength of which was 0.61 kJ/m^2 per min, for one day at room temperature, to produce *cis* urocanic acid. This solution was subjected to HPLC using an analytical reversed-phase column (TSK-GEL, ODS-120 T) with 0.025% HCl as an eluant (1.0 ml/min), the *cis* urocanic acid fraction was collected, and this collecting procedure was repeated. The prepared *cis* isomer of urocanic acid (1.2 mg, recovery: 40%) was identified by UV and nuclear magnetic resonance (NMR) imaging. The ϵ value of the prepared *cis* form (λ_{max} 270 nm, ϵ=8280) was smaller than that of the *trans* form (λ_{max} 268 nm, ϵ=20 100) [12]. The NMR data of the prepared urocanic acid (*cis* form) was as following; ^{1}H NMR (DMSO-6d, TMS), δ 8.98(s, 1H), 8.14(s, 1H), 6.98(d, J=12.7 Hz, 1H), 6.07(d, J=12.7 Hz, 1H). In contrast, the NMR data of authentic urocanic acid (*trans* form) was as following; ^{1}H NMR (DMSO-6d, TMS), δ 7.77(s, 1H), 7.49(s, 1H), 7.47(d, J=16.1 Hz, 1H), 6.28(d, J=15.6 Hz, 1H). These results suggest that the prepared urocanic acid was a *cis* form [13].

2.3. Reaction of amino acids and dabsyl–Cl

The amino acids were reacted with dabsyl–Cl by the method of Chang et al. [14], with slight modifications. Because urocanic acid and dabsyl–Cl do not readily react, a suitable preincubation time was determined. To obtain optimal reaction conditions for the preparation of dabsylated urocanic acid, the reaction mixture was preincubated for 0, 0.5, 1 or 2 h at room temperature before the dabsylation proceeded at 70°C. The amino acids, in 50 m*M* sodium carbonate buffer (pH 9.0), were reacted with an acetonitrile solution of dabsyl–Cl (200 nmol) at room temperature while the reaction vessel was rotated, and then further reaction was carried out for 10 min at 70°C. The solution was evaporated to dryness under vacuum conditions at 60°C, and the product was dissolved in 250 μl of 70% ethanol.

2.4. Analysis of dabsylated amino acids by high-performance liquid chromatography

An HPLC system equipped with a 510 Model pump, 680 gradient controller and U6K injector from Waters Associates (Milford, MA, USA) was used. Various amounts of dabsylated amino acids (0.25, 0.5, 1.0 or 2.5 pmol/μl) were subjected to HPLC with a reversed-phase column. The amino acids were eluted with 20 m*M* acetate buffer (pH 6.5) and the same buffer containing 70% acetonitrile in gradient conditions; solvent A was acetate buffer (20 m*M*, pH 6.5) and solvent B was solvent A containing 70% acetonitrile. The gradient was 25–45% solvent B in

0–38 min, 45–82% solvent B in 38–65 min, and 82–100% solvent B in 65–66 min, kept at 100% solvent B for 66–76 min, then 100–25% solvent B in 76–77 min. The flow-rate was 1 ml/min. The dabsylated amino acids were detected at 463 nm using an LC spectrophotometer (Lambda-Max Model 481, Waters Associates). Peak responses were measured by peak-area integration using a Model 740 data module (Waters).

The standard calibration curves of histidine and of the *cis* and *trans* isomers of urocanic acid were obtained at 0.25 to 2.5 pmol/μl in the presence of the other 36 amino acids, when 200 nmol of dabsyl–Cl was used, as shown in Fig. 1.

2.5. Analysis of histidine and the cis and trans isomers of urocanic acid in the stratum corneum

Normal stratum corneum and peeled stratum corneum sheet after sunburn were obtained from the lower leg of volunteers. Consent was obtained before getting the stratum corneum. The sample (100 μg) was extracted in 200 μl of 1 m*M* phosphate buffer (pH 7.4). To determine the optimal time for the extraction of the *cis* and *trans* isomers of urocanic acid and histidine, the homogenized stratum corneum was stirred for 0, 15, 30 or 60 min. After centrifugation at 18 000 *g* for 10 min, proteins in 25 μl of the supernatant were precipitated by the addition of 75 μl of acetonitrile (1:3), the mixture was centrifuged at 18 000 *g* for 10 min, and 80 μl of the supernatant was evaporated under vacuum conditions at 60°C. Dabsylation was performed by the same method as that described above. After the reacted sample solution was evaporated, the product was dissolved in 250 μl of 70% ethanol; 20 μl of this ethanol solution was then injected into the HPLC column. The significant difference in the amounts of *cis* and *trans* isomers of urocanic acid, histidine and *cis*/*trans* (*c*/*t*) ratio between normal and sunburned stratum corneum was determined by Student's *t*-test. Probability values less than 5% were considered significant.

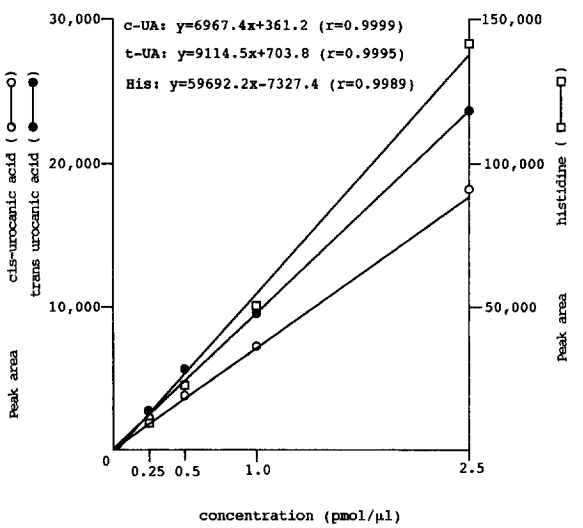

Fig. 1. Calibration curves of standard dabsylated histidine and the *cis* and *trans* isomers of urocanic acid. Open and closed circles indicate *cis* and *trans* isomers of urocanic acid, respectively, and squares indicate histidine. Abbreviations: *c*, *cis*; *t*, *trans*; UA, urocanic acid.

3. Results

The sensitivity of urocanic acid and histidine was highest when the reaction mixture was preincubated for 1 h. The elution profile of dabsylated standard amino acids, including the *cis* and *trans* isomers of urocanic acid and histidine, is shown in Fig. 2. Histidine and the two isomers of urocanic acid were completely separated from the other amino acids. The limits of detection in the analysis of the standard two isomers of urocanic acid and histidine were 0.1 and 0.02 pmol/μl, respectively. The coefficient of variation for the analytical reproducibility of each amino acid is shown in Table 1. Based on the above findings, histidine and the *cis* and *trans* isomers of urocanic acid in the human stratum corneum were assayed. When the extracted time was examined, they were extracted thoroughly by homogenizing only the stratum corneum sample, as shown in Fig. 3. A typical elution profile using 0.8 μg (dry weight) of the stratum corneum is shown in Fig. 4. The limit of quantification of urocanic acid isomers was 0.3 μg. The amounts of the two isomers of urocanic acid and of histidine, which were calculated based on the calibration curves (Fig. 1), are shown in Table 2. Since urocanic acid absorbs UV light and is a very important substance for the UV protection of fair-skinned people [7] and the *cis* isomer increases after

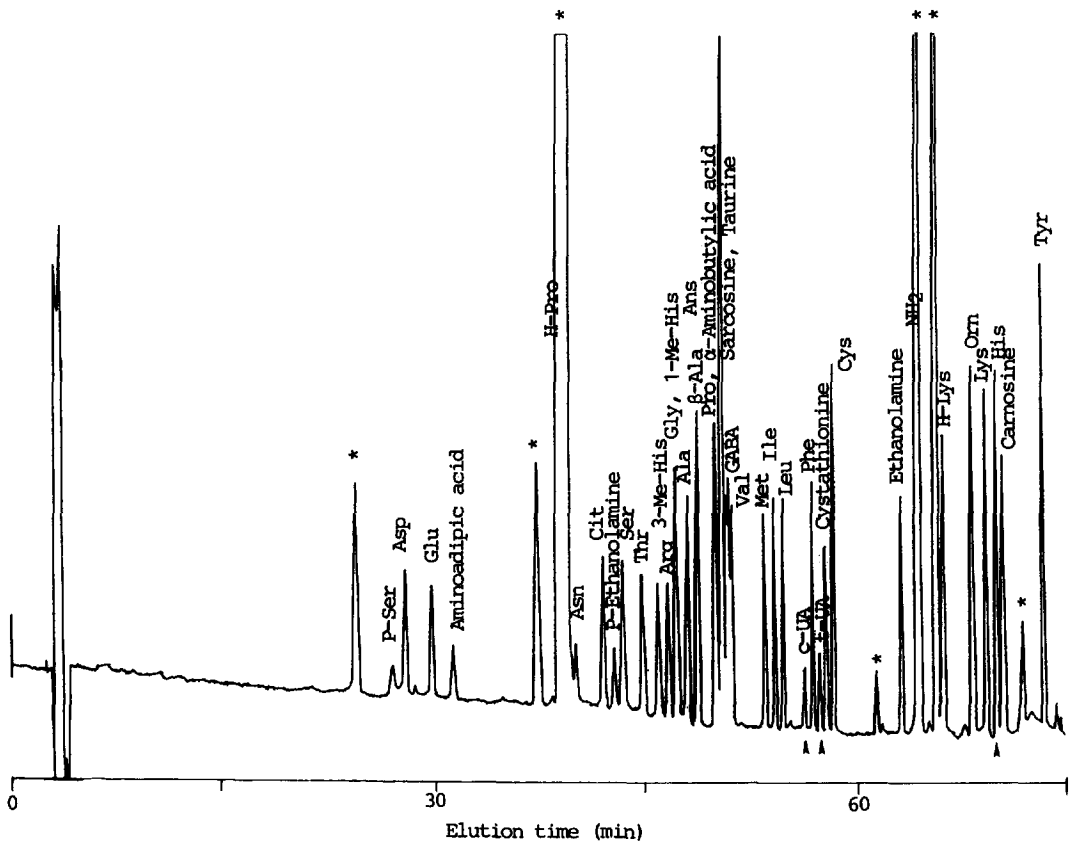

Fig. 2. Elution profile of standard dabsylated amino acids. The concentration of each amino acid was 1.0 pmol/μl. Arrowheads indicate histidine and the *cis* and *trans* isomers of urocanic acid. Asterisks indicate reagent peaks. Abbreviations: P-, phospho-; H-, hydroxy-; Me-, methyl-; *c*-, *cis*-; *t*-, *trans*-; UA, urocanic acid.

UV irradiation [6], the ratio of the *cis* and *trans* isomers of urocanic acid in sunburned stratum corneum was measured. A significant difference between normal and sunburned stratum corneum was observed in the *c/t* ratio ($P<0.005$), but not in the *cis* or *trans* isomers of urocanic acid or histidine.

4. Discussion

In this study, we demonstrated a method for the quantitative analysis, at the picomole level, of histidine and the *cis* and *trans* isomers of urocanic acid in the stratum corneum. Since urocanic acid and

Table 1
Reproducibility of standard dabsylated histidine and the *cis* and *trans* isomers of urocanic acid

	Urocanic acid		Histidine
	cis	*trans*	
Peak area	8495.8±319.7	10333.6±312.0	51702.6±1297.5
C.V. (%)	3.8	2.9	2.5
Elution time (min)	55.041±0.342	56.839±0.047	69.566±0.035
C.V. (%)	0.62	0.082	0.050

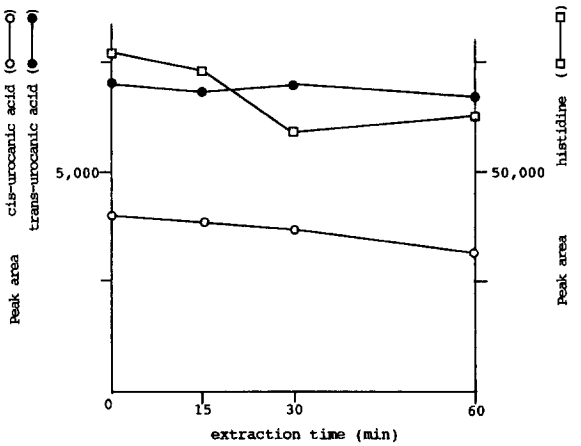

Fig. 3. Optimal time for extraction of the stratum corneum. Open and closed circles indicate *cis* and *trans* isomers of urocanic acid, respectively, and squares indicate histidine.

dabsyl–Cl do not react readily, preincubation at room temperature for 1 h was necessary. The reactive intensity of dabsylated histidine was about five times as high as that of dabsylated urocanic acid. This difference may arise from their differing molecular forms. The calibration curves and the reproducibility of the analysis of histidine and of the two standard isomers of urocanic acid indicate that this method would be useful for the practical analysis of the small sample amounts of the stratum corneum, since the calibration curves for the two isomers of urocanic acid and of histidine were obtained from 0.25 to 2.5 pmol/μl, and the coefficient of variation for each amino acid was less than 5%. Previously reported analyses of urocanic acid by HPLC required large amounts of the stratum corneum [15–17], and the application of these methods was limited to large samples. Small amounts of urocanic acid and his-

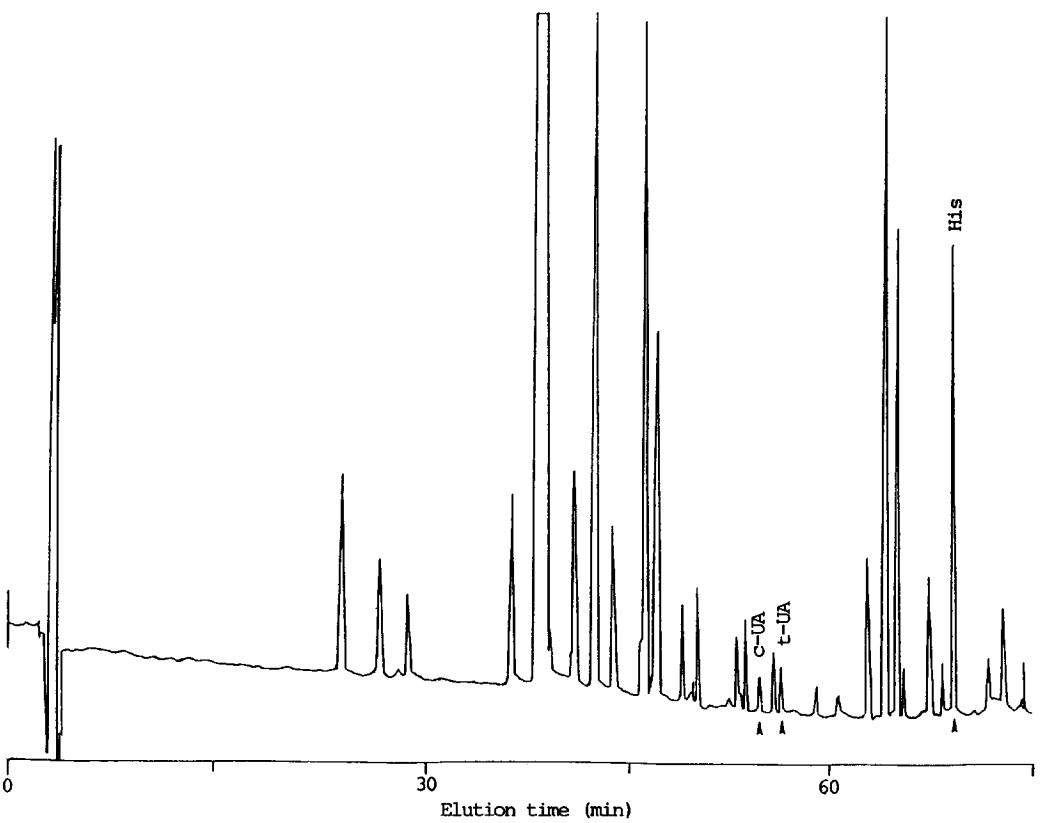

Fig. 4. Elution profile of dabsylated amino acids in the stratum corneum. Arrowheads indicate histidine and the *cis* and *trans* isomers of urocanic acid. Abbreviations are the same as those in Fig. 1.

Table 2
Amounts of histidine and the *cis* and *trans* isomers of urocanic acid in the stratum corneum, and molecular ratios of *cis* and *trans* isomers of urocanic acid (*c/t*)

Normal stratum corneum							Sunburned stratum corneum						
No.	Age	Sex	Urocanic acid (pmol/μg)			Histidine (pmol/μg)	No.	Age	Sex	Urocanic acid (pmol/μg)			Histidine
			cis	*trans*	*c/t*					*cis*	*trans*	*c/t*	
1	31	m	4.1	5.0	0.82	11	1	15	m	3.7	1.7	2.16	6.8
2	32	m	5.3	27.0	0.20	5.0	2	23	f	12.1	5.0	2.40	29.0
3	34	m	12.1	13.1	0.92	27.8	2	34	m	9.2	6.4	1.44	27.4
4	38	f	2.5	9.1	0.27	12.9	4	43	m	16.0	15.4	1.04	31.0
5	44	f	8.1	8.5	0.95	5.3							
6	48	f	7.8	20.4	0.38	17.5							
Mean			6.65	13.85	0.59	13.25				10.25	7.13	1.76	23.55
S.D.			3.43	8.31	0.34	8.56				5.18	5.86	0.63	11.26

tidine have been determined by isotopically labeling amino acids using mass spectrometry [18]. Enzyme-linked immunosorbent assay (ELISA) using an anti-*cis* urocanic acid monoclonal antibody, has also been performed, directly detecting the *cis* isomer of urocanic acid in serum [19].

Histidine, a precursor of urocanic acid, is produced from filaggrin in the stratum corneum [20]. Filaggrin is derived from keratohyalin granules as profilaggrin in the stratum granulosum, [21–23] and is degraded to amino acids in the stratum corneum by various proteinases [20,24]. A flow-chart of profilaggrin to urocanic acid is given in Fig. 5. Water binding and UV light-absorbing capacity are reported to be exhibited by filaggrin derivatives [25,26]. We reported recently that the reactivity of anti-filaggrin

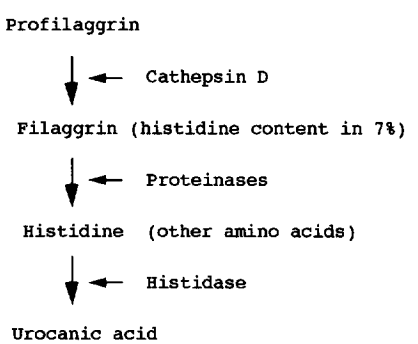

Fig. 5. Flow-chart of transformation of profilaggrin to urocanic acid during epidermal keratinization.

antibody was reduced in the skin of the lower legs of aged subjects [27] and in atopic dry skin [28], and Watanabe et al. [29] reported in psoriatic skin, in areas where the skin surface is dry and flaky because of the reduced function of skin appendages [30,31]. This finding suggests that a reduction in the content of filaggrin and of amino acids derived from filaggrin, may be a factor in the cause of dry skin conditions such as xerosis seniles or atopic dry skin.

The molecular ratio of the *cis* to the *trans* isomer of urocanic acid in sunburned stratum corneum was about three times higher than the ratio in normal stratum corneum (Table 2), indicating that the relative amount of the *cis* isomer in the stratum corneum may be increased by the action of UV light, as has been reported by Pasanen et al. [32], who showed that the content of the *cis* isomer was increased in UVB-irradiated human epidermis. Noguchi et al. [33] have addressed the question of the water-holding capacity of intercellular amino acids. We examined here the analytical conditions for only the *cis* and *trans* isomers of urocanic acid and for histidine; however, other amino acids can be analyzed at the picomole level by this method. There is not enough information about the amounts of amino acids in the stratum corneum, because it is difficult to get adequate samples of stratum corneum. However, only about 1 μg of the stratum corneum was necessary for analysis by the present method. Therefore, this method should be useful for the analysis of small amounts of stratum corneum to

elucidate the factors involved in dry skin and the skin damage caused by UV light.

Acknowledgments

We thank Dr.J. Asakura for his advice in identification of *cis* urocanic acid and Ms.E. Honda for her technical advice. This work was supported by grants from the Shiseido Foundation for Skin Aging Research and from the Japan Private School Promotion Foundation and Environmental Science Institute of Kinki University.

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