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Simultaneous HPLC Analysis of Ceramide and Dihydroceramide in Human Hairs

Youn-Sun Lee, Kyeong-Mi Choi, Mi-Hwa Choi, So-Young Ji, Jae-Myung Yoo, Yong-Moon Lee, Jin-Tae Hong, Yeo-Pyo Yun, and Hwan-Soo Yoo

College of Pharmacy and CBITRC, Chungbuk National University, Cheongju 361-763, Korea

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Ceramide, a major class of hair lipid, can help determine the physicochemical properties of human hairs such as the chemical diffusion barrier and water retention. In this study, we developed a quantitation method for ceramide and dihydroceramide, a saturated form of ceramide, in human hairs. Lipids were extracted with ethanol from human hairs spiked with *N*-oleoyl-D-*erythro*-C_{17} sphingosine, an internal standard. Ceramide and dihydroceramide were resolved by TLC and deacylated by sphingolipid-ceramide deacylase to release sphingosine and dihydrosphingosine, respectively. The hair content of ceramide was measured by HPLC following derivatization with *o*-phthalaldehyde. The limits of detection and quantification for ceramide extracted from hair fibers were 0.1 and 1 pmol, respectively. The linear range of hair weight for determining ceramide and dihydroceramide contents was 1 to 50 mg, with R^2 values of 0.9695 and 0.9898, respectively. The recoveries of ceramide and dihydroceramide from intra-day and inter-day assays were 99.55% to 98.53%, respectively. Concentrations of dihydroceramide from the hair roots to distal tip ends ranged from 10.42 ± 2.19 to 1.20 ± 0.11 nmol/g hair, while those of ceramide ranged from 2.27 ± 0.22 to 1.47 ± 0.15 nmol/g hair. The present analytical method provides a simultaneous and reproducible quantification of ceramide and dihydroceramide, and may be used as a potential biomarker for lipid abnormality-related diseases.

Key words: Ceramide, Dihydroceramide, Sphingolipid, Hairs, HPLC

INTRODUCTION

Hair fibers are composed of an outer layer of cuticle cells and an inner core of cortical and medullary cells. Medullary cells are located in the central part of hair fibers and surrounded by spindle-shaped cortical cells that are protected by cuticle cells (Jones and Rivett, 1997). The physicochemical properties of human hair such as the barrier against exogenous chemicals and the water retention are maintained by the lipid membrane complex between the cuticle and cortical cells (Hilterhaus-Bong and Zahn, 1989; Gniadecka et al., 1998; Masukawa et al., 2005a). Hair lipids include ceramides, free fatty acids, triglycerides, cholesterol, cholesterol sulfates, squalene and wax esters (Brosche et al., 1994), and can be useful as biomarkers for lipid abnormality-related diseases (Brosche et al., 2001; Mogos et al., 2002; Caprara et al., 2006).

Ceramides consist of sphingoid bases covalently bound to a fatty acid by an amide linkage (Fig. 1) and are key intermediates in the biosynthesis of all complex sphingolipids (Fig. 2). Ceramides are involved in the regulation of diverse cellular functions such as cell growth, differentiation, senescence, apoptosis and immune responses (Spiegel and Merrill, 1996). Ceramide is a lipid mediator of cellular stress responses (Pettus et al., 2002) and is related to the differentiation of keratinocytes where apoptosis occurs (Hamanaka et al., 2005). Human hair also contains ceramides and dihydroceramides in trace amounts (Hussler et al., 1995a; Masukawa et al., 2006). Ceramides play a major role in the water-retaining property and barrier function of hair as well as skin (Masukawa and Tsujimura, 2006; Masukawa et al., 2006). Dihydroceramide is structurally similar to ceramide except

Correspondence to: Hwan-Soo Yoo, College of Pharmacy and CBITRC, Chungbuk National University, Cheongju 361-763, Korea
Tel: 82-43-261-3215, Fax: 82-43-268-2732
E-mail: yoohs@chungbuk.ac.kr

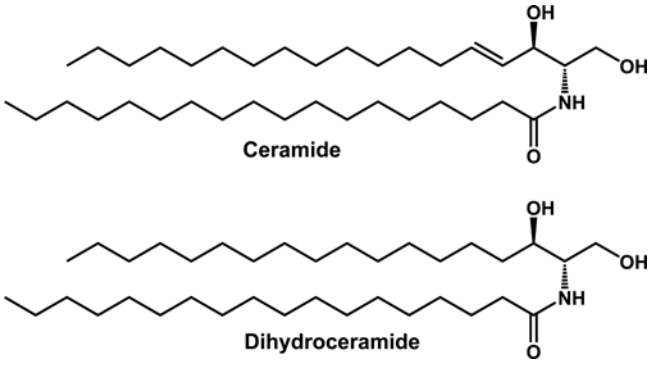

Fig. 1. Chemical structures of ceramide and dihydroceramide

that it does not have a double bond at the 4,5 position (Fig. 1) and occurs in the *de novo* sphingolipid biosynthetic pathway (Fig. 2). Dihydroceramide blocks channel formation in mitochondria, leading to the inhibition of apoptosis (Siskind and Colombini, 2000; Stiban et al., 2006).

The endogenous nature of ceramide makes it important in understanding its physiological role in the maintenance of human hair (Brosche et al., 1994). TLC/densitometry methods using silica gel plates are conventionally used to determine diverse lipids such as squalene, wax esters, triglycerides, and free fatty acids in human hairs (Masukawa et al., 2005a). However, ceramides in human hairs are difficult to analyze using TLC. The ceramide band in the TLC plate can be contaminated with surfactants from shampoo used in hair care. Mass spectrometry is also used for determining the amounts and classes of ceramides in hairs (Adachi et al., 2004; Masukawa and Tsujimura, 2006). However, these expensive machines and sophisticated techniques are not available to most laboratories. HPLC methods with fluorescence detection could provide sensitive quantitation of hair ceramides.

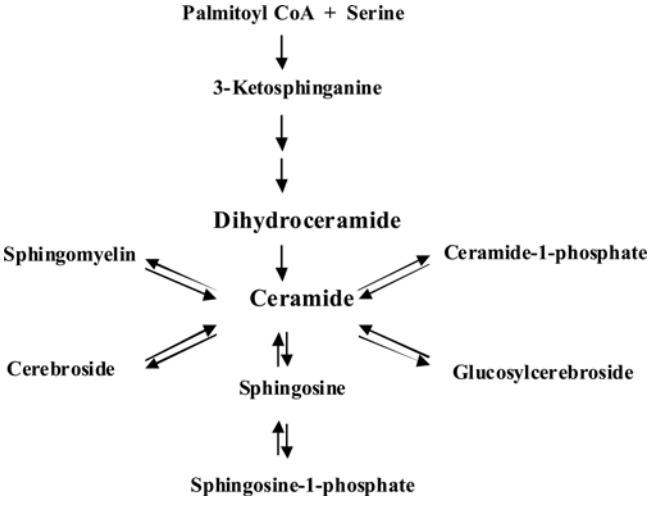

Fig. 2. Sphingolipid metabolism pathway. Ceramide can be synthesized from either the condensation of palmitoyl CoA and serine or the turnover of sphingomyeline, glycosphingolipids and sphingosine. Dihydroceramide occurs in the *de novo* sphingolipid pathway in mammalian cells.

The purpose of this study was to develop a sensitive and specific method to determine trace amounts of ceramides in hairs based on an internal standard with HPLC following TLC purification and enzymatic deacylation. We applied this method to the comparison of ceramide and dihydroceramide contents along the distance from the proximal root end to the distal tip end of human hairs for different ages in both genders.

MATERIALS AND METHODS

Materials

N-oleoyl-D-*erythro*-sphingosine (C_{17} base), ceramide (C_{18} base) and dihydroceramide (C_{18} base) were purchased from Avanti Polar Lipids, Inc. (Alabaster). Sphingolipid ceramide *N*-deacylase (SCDase) was from Takara Bio. Inc. Pyridine and diisopropylether were from Sigma. HPLC-grade methanol was purchased from Merck KBaA. o-Phthalaldehyde (OPA) was obtained from Molecular Probes, Inc. Other reagents were of the highest purity available.

Hair fibers and preparation

Hair fibers were collected by cutting at the proximal root end from Korean volunteers aged from 5 to 35 years. The volunteers had not treated their hairs with chemical reactions such as coloring or permanent waving. Hair samples were prepared by the modified method of Masukawa et al. (2006). Hair fibers from the root end were cut every 5 cm between the root end and the distal tip. The pieces of hair fibers were washed with distilled water and dried, and their weights were measured.

Lipid extraction and ceramide purification by TLC

Concentrations of ceramide and dihydroceramide in hair fibers were determined (Lee et al., 2007). A hair sample of 1 mg (dry weight) was spiked with 100 pmol of ceramide (C_{17} base) as an internal standard and crude lipid was extracted with 1 mL ethanol for 1 h at 37°C. The lipid extract was dissolved in 30 μL methanol spotted on a high-performance thin-layer chromatography silica-gel plate (Merck). The plate was developed in diisopropylether/methanol/29% NH_4OH (40:10:1, v/v/v). Ceramide and dihydroceramide stand-

ards were visualized by spraying with 10% sulfuric acid and drying at 150°C. The areas in the sample lane with the same Rf value as the visualized band of ceramide standard were scraped off, and both ceramide and dihydroceramide were eluted with 1 mL methanol. The eluates were transferred to polypropylene 1.5-mL tubes and dried in a Speed-Vac concentrator.

Enzymatic deacylation and HPLC analysis

The reaction buffer for deacylation of ceramide and dihydroceramide contained 25 mM Tris-HCl buffer, pH 7.5, 1% sodium cholate, 15% fatty-acid-free BSA and 150 μU SCDase. Ceramide and dihydroceramide were deacylated into sphingosine and dihydrosphingosine, respectively, by SCDase at 37°C for 12 h. BSA in the reaction buffer was precipitated by adding ethanol and removed by centrifugation, and the supernatant was dried. The sphingolipids were derivatized with OPA reagent (50 mg OPA, 1 mL ethanol, 200 μL β-mercaptoethanol, and 50 mL 3% (w/v) boric acid buffer, pH 10.5). HPLC analysis was performed using a Shimadzu Model LC-10AT pump, SIL-10AXL auto sampler system and analytical Radial-Pak cartridge (Waters Associates, Inc.) packed with Nova-Pak C_{18} reversed-phase column (4 μm, 100 mm × 8 mm). The isocratic mobile phase composition of methanol/distilled water/triethylamine (92:8:0.1, v/v/v) and a flow rate of 1 mL/min were accurately controlled by the HPLC system controller (Shimadzu SCL-10A). The Shimadzu RF-10XL fluorescence detector was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The resulting data and chromatographic profiles were evaluated using the Borwin system manager software (JMBS, France).

Statistical analysis

All values are expressed as means ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons using Sigma Stat (Jandel Co.) followed by a Newman-Keuls multiple comparison test. $*p < 0.05$ and $**p < 0.01$ were defined as statistically significant.

RESULTS

Optimization of HPLC conditions

We simultaneously measured ceramide and dihydroceramide contents using HPLC. Peaks of synthetic ceramide (C_{17} base), naturally occurring ceramide (C_{18} base) and dihydroceramide (C_{18} base) on the HPLC chromatogram appeared at retention times of 9.0, 11.3 and 15.2 min, respectively (Fig. 3). Ceramide is a major constituent of sphingolipids in most biological samples, while dihydroceramide is a minor component (Lee et al., 2007). However, the sphingolipid profiles on the HPLC chromatogram showed that the peak height of dihydroceramide was much higher than that of ceramide, suggesting that dihydroceramide may be more abundant than ceramide (Fig. 3), and important in water retention, barrier formation and cell cohesion. We used ceramide (C_{17} base) for the quantitation of

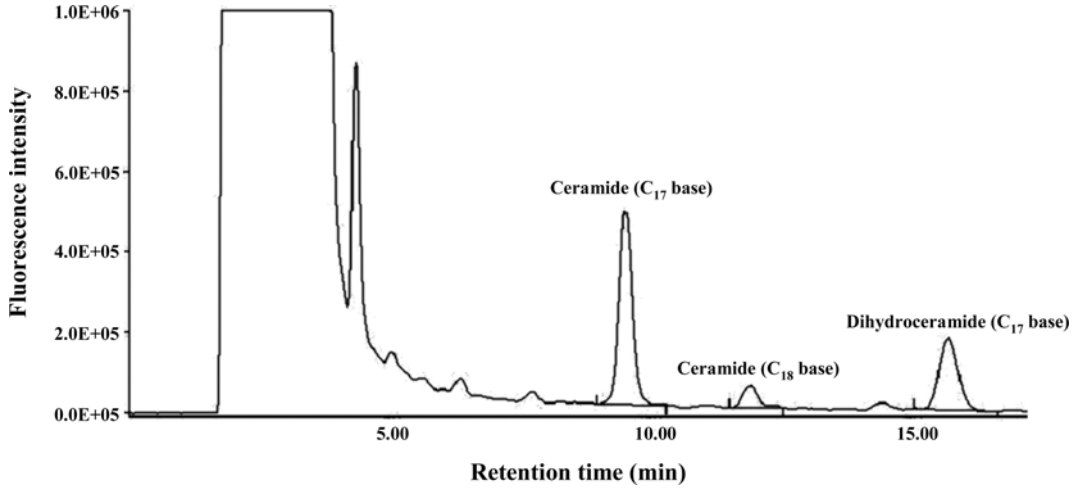

Fig. 3. HPLC chromatogram of hair sphingolipids under optimal conditions for the determination of ceramide (C_{17} base), ceramide (C_{18} base) and dihydroceramide (C_{18} base). Human hairs were obtained from Korean female volunteers. Each hair sample was cut every 5 cm from the proximal root end and crude lipids were extracted from approximately 20 mg of hair fibers with 1 mL ethanol at 37°C for 1 h following the addition of ceramide (C_{17} base) as an internal standard. Ceramide and dihydroceramide were separated from other lipids by TLC, deacylated to release sphingosine and dihydrosphingosine, respectively, and analyzed with HPLC.

Table I. Intra-day and inter-day validations for determination of ceramide and dihydroceramide

Added amount (μM)	Ceramide			Dihydroceramide		
	Measured (mean ± S.D. [a])	CV [b] (%)	Recovery (%)	Measured (mean ± S.D.)	CV (%)	Recovery (%)
Intra-day (n =5)						
1.00	1.04 ± 0.01	0.96	103.52	0.94 ± 0.04	4.26	94.55
10.00	9.41 ± 0.55	5.84	94.10	10.15 ± 0.15	1.48	101.50
20.00	21.05 ± 0.72	3.42	103.75	19.21 ± 1.38	7.18	96.35
Inter-day (n =5)						
1.00	0.95 ± 0.02	2.11	96.50	1.01 ± 0.06	5.94	101.15
10.00	10.60 ± 0.44	4.15	105.20	9.65 ± 0.55	5.70	96.50
20.00	18.95 ± 0.27	1.42	94.75	20.22 ± 1.00	4.95	101.11

[a] Standard deviation. [b] Coefficient of variation.

endogenously occurring ceramide and dihydroceramide containing a C_{18} sphingoid base backbone for separation by TLC. Otherwise, the contents of sphingoid bases may be included in the amounts of ceramide and dihydroceramide. The Rf value of the ceramide-dihydroceramide band on the TLC plate was 0.54, while Rf values of sphingosine and dihydrosphingosine bands were 0.09 and 0.06, respectively (Lee et al., 2007).

Recovery, precision, and accuracy

The results of intra-day analysis for the recoveries of ceramide and dihydroceramide standards were obtained by spiking each compound at 1, 10, and 20 μM into human hair lipid extracts (Table I). For intra-day analysis, the recovery from 10 μM dihydroceramide sample ranged from 93.55% - 102.50%, with a mean recovery of 101.50%, while the recovery from 10 μM ceramide ranged from 91.10% - 101.15%, with a mean recovery of 94.10%. The SD and CV for intra-day precision of 10 μM dihydroceramide sample were 0.15% and 1.48%, respectively.

For inter-day analysis, recovery from the 10 μM dihydroceramide sample ranged from 92.50% - 102.15% with a mean recovery of 96.50%, while the recovery from 10 μM ceramide ranged from 98.55% - 109.70% with a mean recovery of 105.20%. The SD and CV for inter-day precision of 10 μM dihydroceramide were 0.55% and 5.70%, respectively.

Linearity

The calculation of ceramide and dihydroceramide contents based on HPLC peak area was based on 100 pmol of added ceramide (C_{17} base). A calibration curve was generated using human hair fibers containing 1, 2, 5, 10, 20, 50 and 100 mg dry hair weight, and 100 pmol of ceramide (C_{17} base) standard. The ratios of endogenously occurring ceramide (C_{18} base) and dihydroceramide (C_{18} base) to synthetic ceramide (C_{17} base) were lineally proportional to the dry weight of human hair samples at the range of 1-50 mg, with linearity of 0.9695 and 0.9898, respectively, between hair samples and HPLC peak area ratio (Fig. 4). The analytical method using HPLC equipped with fluorescence detector lowers the detection limit to the fmol level. OPA-fluorescence derivatization of sphingosine and dihydrosphingosine released from ceramide and dihydroceramide, respectively, by SCDase deacylation is very simple and the derivative is stable under 4°C for 24 h. The limits of detection and quantification for ceramide extracted from hair fibers were 0.1 (S/N = 3) and 1 pmol (S/N = 10), respectively.

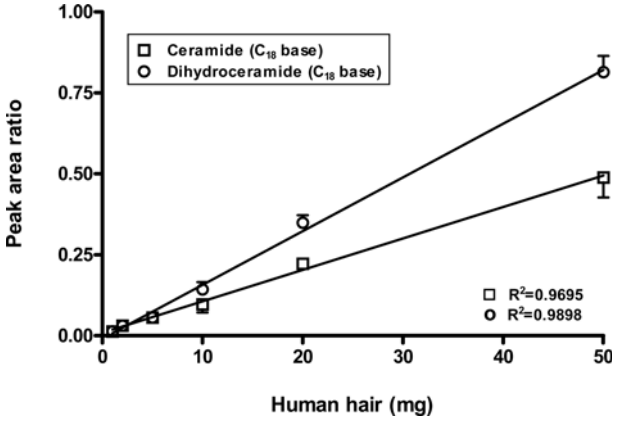

Fig. 4. Linearity between hair weight and fluorescence peak area ratio of either ceramide (C_{18} base) or dihydroceramide (C_{18} base) to ceramide (C_{17} base). The crude lipid was extracted, and ceramide and dihydroceramide were separated by TLC. The SCDase deacylation of ceramide and dihydroceramide continued for 12 h. The released sphingosine and dihydrosphingosine were analyzed by HPLC. Ceramide (C_{18} base) and dihydroceramide (C_{18} base) were divided by ceramide (C_{17} base), an internal standard. Values are expressed as the mean ± S.D. (n = 3-5).

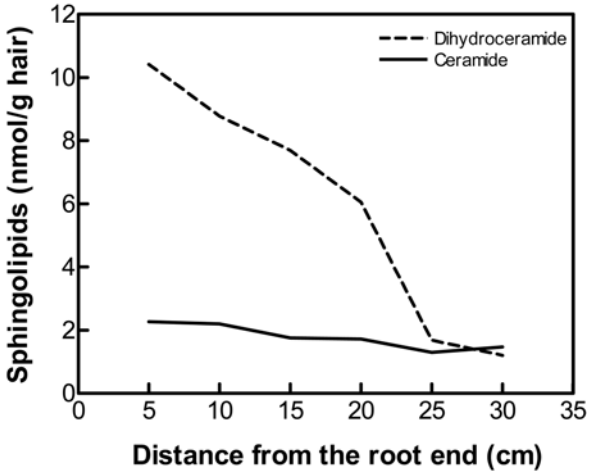

Fig. 5. Relationship between the distance from the proximal root end and levels of sphingolipids. Hair samples were obtained from healthy Korean female volunteers with 30 cm-long hairs and 6 groups of hair fibers were collected by cutting every 5 cm from the proximal root end to the distal tip end. Straight line and broken line represent ceramide and dihydroceramide, respectively.

Table II. Ceramide and dihydroceramide levels of hair fibers from the proximal root end to the distal tip end

Distance from the root end (cm, n = 5)	Ceramide (nmol/g)	Dihydroceramide (nmol/g)
0- 5	2.27 ± 0.22	10.42 ± 3.19
5-10	2.20 ± 0.94	8.77 ± 4.60
11-15	1.76 ± 0.54	7.69 ± 2.29
15-20	1.72 ± 0.67	6.05 ± 1.78
20-25	1.30 ± 0.42	1.68 ± 0.70
25-30	1.47 ± 0.15	1.20 ± 0.11

Dihydroceramide as a sensitive biomarker for hair growth and aging

Dihydroceramide content was highest in the hair sample obtained from the most proximal root end and gradually decreased along the fibers (Fig. 5). Dihydroceramide content was 10.42 ± 3.19 nmol/g hair fibers, while content of ceramide was 2.27 ± 0.22 nmol/g hair fibers (Table II), showing that dihydroceramide content of the first 5 cm hair from the root end was higher than ceramide by 4.5 folds. The ratios of dihydroceramide to ceramide contents in 0-10, 11-20 and 21-30 year-old groups were increased with values of 0.6 ± 0.22, 1.2 ± 0.22 and 1.6 ± 0.22, respectively, while the ratio in 31-40 year-old group was decreased to 1.1 ± 0.22 (Fig. 6A). Thus, *de novo* synthesis of dihydroceramide may be increased until the age of 30 and then decreased. However, we did not see any difference of dihydroceramide to ceramide ratio between male and female (Fig. 6B).

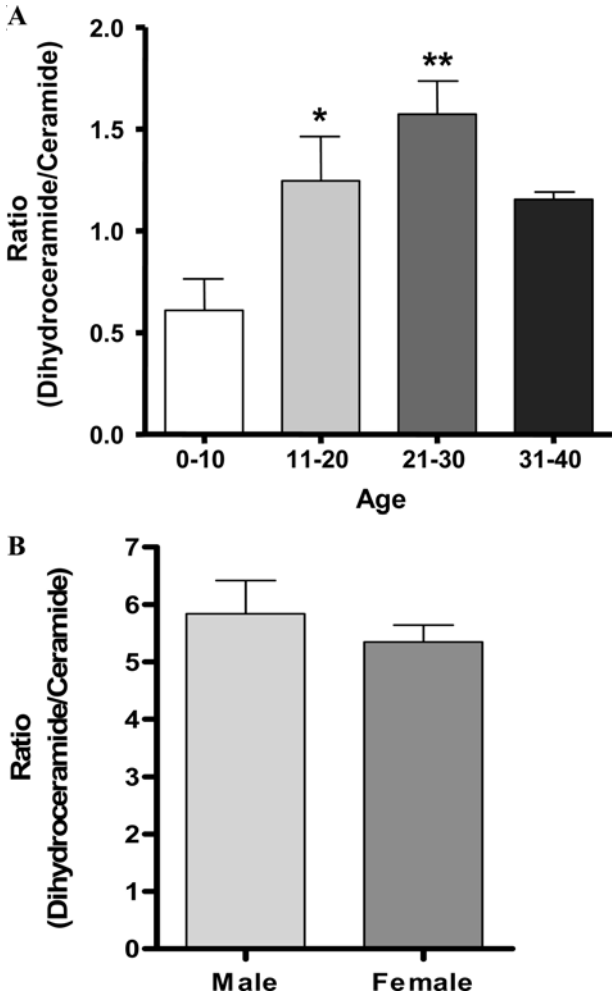

Fig. 6. The effects of age and gender on the ratio of dihydroceramide to ceramide in human hair fibers. (**A**) The samples were divided into 4 groups based on age, 0-10, 11-20, 21-30 and 31-40 year-old female volunteers. (**B**) Human hairs were obtained from adult volunteers. The hair samples were collected by cutting the first 0-5 cm from the proximal root end and analyzed by HPLC following ethanol extraction, TLC purification and SCDase deacylation. Values are expressed as the mean ± S.D. (n = 3 - 6). Differences among age groups with $*p < 0.05$ and $**p < 0.01$.

DISCUSSION

Ceramides are involved in the regulation of diverse cellular functions such as cell growth, differentiation, senescence, apoptosis and immune response. Ceramide and dihydroceramide are present in trace amounts along with cholesterol and cholesterol sulfates, while other lipids such as free fatty acids, triglycerides, squalene and wax esters are abundant (Wertz and Downing, 1988; Hussler et al., 1995b; Kon et al., 1998; Masukawa et al., 2005a; Masukawa et al., 2005b; Masukawa and Tsujimura, 2006). Ceramides in hair and skin perform water retention and barrier func-

tion (Masukawa and Tsujimura, 2006; Masukawa et al., 2006). Ceramide and dihydroceramide quantitation may reveal their functions in hair.

The objective of this study was to develop a quantitation method of endogenously occurring ceramide and dihydroceramide with good reproducibility and high sensitivity from human hair fibers. In this study, we decreased the variability by adding ceramide (C_{17} base) as an internal standard to samples prior to lipid extraction, eliminating variations due to extraction efficiency, TLC purification and HPLC analysis. The analytical conditions for the determination of ceramide and dihydroceramide with this method were validated from other biological samples, including human plasma, mouse tissues and plasma, and cultured cells (Lee et al., 2007). Addition of ceramide (C_{17} base) to hair samples minimized method variability from lipid extraction, TLC separation, enzymatic deacylation and HPLC analysis. The HPLC profiles of ceramide and dihydroceramide from human plasma without TLC purification with this analytical method showed many non-specific peaks, and the amounts of ceramide and dihydroceramide were not able to be determined. In this study, TLC separation of ceramide and dihydroceramide provides a clean HPLC chromatogram with an internal standard peak (Fig. 3). OPA derivatization following SCDase deacylation provides high sensitivity at the fmol level and excellent specificity for the detection of ceramide and dihydroceramide. Ceramide content was also determined using LC-MS spectrometry from human hair lipids with a fmol LOD (Masukawa and Tsujimura, 2006). However, the LC-MS method requires expensive equipment and sophisticated techniques not available to most investigators.

In this study, the HPLC peak of dihydroceramide was higher than that of ceramide (Fig. 3). Dihydroceramide levels were decreased with the distance along the hair from the proximal root end to the tip, but ceramide levels were not decreased (Masukawa et al., 2005b; Masukawa and Tsujimura, 2006). This decrease may be due to shampooing, conditioning, brushing, and exposure to light and heat. Dihydroceramide may be a potential biomarker for metabolism of hair root cells. The lowest amount of hair sample needed was 1 mg in dry weight, which is equivalent approximately 10 cm of hair fibers from the proximal root end (Fig. 5), and the analysis can be achieved with a couple of hair fibers. The ratio of dihydroceramide to ceramide contents was increased until 30 year-old ages (Fig. 6A) because of the increased levels of dihydroceramide, potentially acting as a biomarker for ageing. The ratios of ceramide and dihydroceramide contents in human hair were similar in male and female (Fig. 6B).

In conclusion, we developed a sensitive and reproducible analytical method for determining ceramide and dihydroceramide levels in human hair. Dihydroceramide appears to be a sensitive biomarker for age and hair length, and may play a role in the diagnosis of both stress and senescence-related diseases.

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