

Detection and Analysis of Ceramide in Skin and Blood in a Healthy Chinese Population

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Synopsis

To explore the significance of ceramide in the skin barrier and its potential utility within the cosmetics industry, an accurate and high-speed method was used to detect the types of ceramides in the skin and blood of a healthy Chinese population. Forearm cortical skin stratum corneum samples were obtained from four healthy subjects using a noninvasive method. In addition, these subjects were collected intravenously to obtain blood samples. Ceramides were detected in skin and blood samples using high-performance liquid chromatography coupled with specialized high-resolution Fourier Transform mass spectrometry machine. Data were analyzed using full-flow lipid analysis software. Peaks representing ceramides were detected in all skin samples and some blood samples. The results show that ceramides in skin are predominantly long-chain ceramides, but mainly short-chain in the blood. Simple and fast qualitative and quantitative analysis of ceramide in the skin and blood provides a basis for the precise addition of ceramide in future skin care products and the metabolic regulation and prevention of various diseases.

INTRODUCTION

The stratum corneum (SC) of the epidermis is the main barrier to prevent the entry of external substances into the human body and reduce trans-epidermal water loss (1). Intercellular lipids mainly include ceramide (about 50%), cholesterol (about 25%), free fatty acids (about 15%), and a small amount of phospholipids. Ceramides combined with cornified envelope have a physiological function in corneocyte flexibility (2).

Ceramide is closely related to many diseases. Decreased ceramide content in the SC will destroy the skin barrier, including atopic dermatitis (AD), psoriasis, and other diseases (3).

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Ceramides can be used as a moisturizer to treat dry skin. Many kinds of ceramides can be used synergistically to improve skin barrier function (4). Ceramides can also reduce the severity of skin diseases, reduce pain, improve the efficacy of other drugs, reduce adverse reactions to drugs, accelerate the recovery of diseases, and improve quality of life (5). Ceramides have been used in topical formulations of AD (6). Ceramides can also reduce the reactivity to environmental pollution and skin sensitivity (7). Ceramide in the blood is used as a biomarker for predicting cardiovascular diseases (8) and pulmonary cystic fibrosis (9) and other diseases. Increase of ceramide contents in the blood may also be related to the risk of Alzheimer's disease and can predict cognitive impairment (10). Therefore, determination of the ceramide content qualitatively and quantitatively is very important for assessing skin condition and occurrence and development of various diseases.

Busman (11) measured intracellular ceramide species using high-performance liquid chromatography (HPLC) coupled to atmospheric pressure-ionization mass spectrometry (MS). Zhixin (12) extracted human epidermis SC using tape stripping, and then quantified ceramides using normal-phase liquid chromatography combined with dynamic multi-reaction monitoring MS. Twelve ceramide subclasses were found, including CER[NDS], CER[NS], CER[NP], CER[NH], CER[ADS], CER[AS], CER[AP], CER[AH], CER[EODS], CER[EOS], CER[EOP], and CER[EOH].

Here, we identified the ceramide kinds and measured its content in skin and blood samples using HPLC and high-resolution MS and then analyzed them. The difference in ceramide in different tissues has not been detected by using precise methods. The high-throughput mass spectrometer has detected ceramide, and the detection result has high accuracy and the detection speed is very fast.

MATERIALS AND METHODS

SUBJECTS

Subject inclusion criteria: healthy Chinese volunteers (18–65 y old, no gender requirements), no history of skin disease, no skin care moisturizers or other topical preparations used within 1 mo before the study, no allergic reaction to cyanoacrylate, no history of phototherapy in a year, no history of drug use in the past month, and no history of chronic wasting disease.

Subject exclusion criteria: The trial does not include juvenile children, pregnant women, lactating women, and patients who use hormone replacement therapy or immunotherapy.

The study was approved by the Research Ethics Committee of the Anhui Medical University and conformed to the Declaration of Helsinki. After being completely notified of the procedures, written informed consent was obtained from all participants. Four volunteers participated in our study, and we collected and processed their blood samples and skin SC samples.

MATERIALS AND LIPID STANDARDS

Chemicals. Methanol and chloroform were obtained from Sinopharm Group Chemical Reagent Company Limited, ultrapure water was obtained by Thermo ultrapure water machine, and cyanoacrylate glue was purchased from Deli Group Company Limited (Ningbo, China).

Apparatuses. Q Exactive quadrupole-electrostatic field track well high-resolution FT MS machine and Ultimate 3000 HPLC instrument were obtained from Thermo Fisher Scientific Company (Waltham, MA), Heraeus Fresco 17 Centrifuge and Vacuum freeze dryer were provided by Shanghai Thermo Fisher Biotechnology Company Limited, JK-100B Ultrasonic Cleaner was purchased from Hefei Jin Nick Machinery Manufacturing Company Limited (Hefei, China), Millex-HV needle type filter was obtained from Shanghai Hehe Technology Company Limited (Shanghai, China), and EYELA rotary evaporator was from the N-1300 series Tokyo Physical and Chemical Equipment Company Limited (Tokyo, Japan).

PRETREATMENT OF SKIN SC SAMPLES

SC sample acquisition. A thin layer of cyanoacrylate-containing glue was coated onto one end of a slide. A 2.5×2.5 -cm area close to the volar side of the forearm was prepared. A force of 1 newton was used to press the slide to the forearm for 1 min followed by carefully stripping. This was repeated at the same site for three times.

Dissolution. Epidermis on the slides was placed in a 100-ml beaker followed by immersion in 5 ml of chloroform and 25 μ l of methanol (chloroform:methanol, 99.5:0.5, v:v).

After mixing well, the stripped corneocytes in chloroform methanol were placed in JK-100B ultrasonic cleaner and subjected to ultrasonic (100 W) shock for 20 min until fully dissolved.

Filtration. The above suspension was then transferred to a round-bottom flask using a syringe and a 0.22- μ m filter.

Drying. The flask was connected to a rotary evaporator and evaporated at 40°C until the liquid evaporated.

Redissolution. One milliliter of methanol was added to the flask, mixed well, and then the liquid was transferred to a 2-ml centrifuge tube.

Centrifuge. The sample was subjected to centrifugation (2,000 r/min) for 10 min at 4°C and the supernatant transferred to a new 2-ml centrifuge tube, followed by testing.

PRETREATMENT OF BLOOD SAMPLES

- (i) Two hundred microliters of the blood sample was transferred to a 15-ml centrifuge tube.
- (ii) Then, 1.5 ml of methanol was added into the centrifuge tube and vortexed until fully mixed.
- (iii) Five milliliters of isopropanol was added into the centrifuge tube and vortexed.
- (iv) Then, 1.25 ml of ultrapure water was added to the centrifuge tube and vortexed.
- (v) The mixture was then allowed to stand for 15 min at about 23°C to separate into layers. The supernatant (isopropanol layer) was taken for analysis.
- (vi) The supernatant was freeze-dried with a vacuum freeze dryer. Then, 200 μ l of a 1:1 mixture of isopropanol and acetonitrile was added and centrifuged. The supernatant

was collected and placed in a sample bottle for testing. The steps were repeated three times.

CHROMATOGRAPHIC CONDITIONS

Columns were BEH C18 Shield columns (100 × 2.1 mm, 1.7- μ m particles). The column temperature was maintained at 40°C. Mobile phase A = 60:40 acetonitrile (ACN)/H₂O + 10 mmol NH₄HCO₂, 0.1% HCOOH; Mobile phase B = 90:10 isopropanol (IPA)/ACN + 10 mmol NH₄HCO₂, 0.1% HCOOH, and reverse column gradient wash with a flow rate of 0.3 ml/min.

MASS SPECTROMETRIC CONDITIONS

The instrument was operated in both positive and negative ion electrospray ionization full-scan mass ddMS² mode. First-order MS detects the mass-to-charge ratio and intensity of all charged ions, and second-order MS is the further dissociation of the parent ion peptide. First-order resolution is 70 × 10³ and secondary resolution is 17.5 × 10³. Lipid-Search software was used for data processing.

RESULTS

DETECTION OF SKIN SAMPLES

Q Exactive peaks were observed in all skin samples with reaction intensities between 10⁵ and 10⁷. Figure 1 list the peak extraction areas of the major long-chain ceramide in the

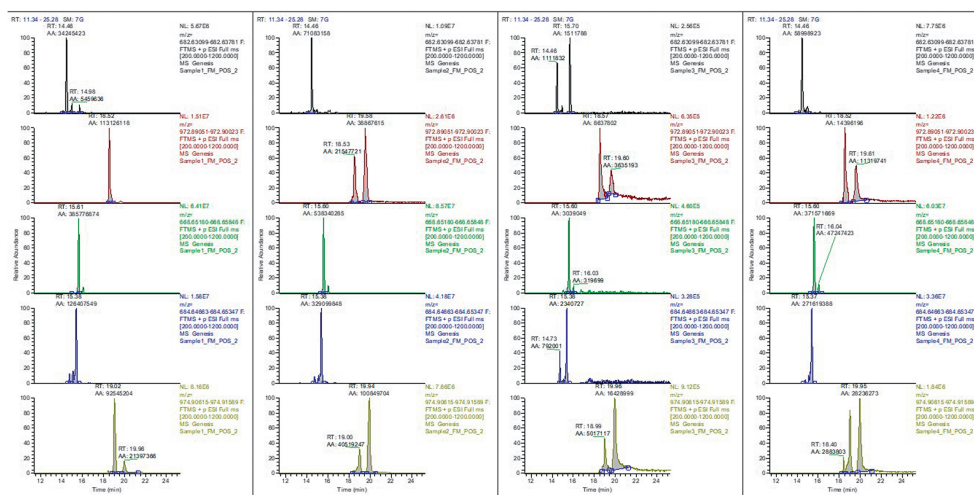


Figure 1. After dissolution, filtration, drying, redissolution, and centrifuging, the first-order extraction peaks of ceramide subclasses in the four skin SC samples were obtained by chromatography.

skin SC of the four volunteers. LipidSearch database is then matched with lipids that may be present in the lipid profile.

CERAMIDE MATCHING ANALYSIS

Taking skin samples as an example, we detected multiple ceramides from the human SC. According to the first-order peak of the sample, the possible lipid type obtained was Cer(d18:0/24:0). The lipid information of the LipidSearch database was matched and analyzed according to the secondary peak map, and the red results are the matching fragments in the database (Figure 2). Finally, the chemical formula of the lipid was $C_{42}H_{85}NO_3$. The structural formula and specific information are shown in Figure 3. In the same way, we also determined that the chemical formula of Cer(d18:0/24:0+O) is $C_{42}H_{85}NO_4$, the chemical formula of Cer(d18:1/24:0) is $C_{42}H_{83}NO_3$, and the chemical formula of Cer(d18:0/pO/24:0) is $C_{42}H_{85}NO_5$.

Relative quantitative analysis was performed based on the ceramide reaction peak area. Four samples of skin detected the concentration of $C_{42}H_{85}NO_3$ (Table I). Because of the quality error of skin samples and individual differences, the concentration of the same ceramide is different. It is believed that $C_{42}H_{85}NO_3$ is the main type of ceramide in the skin.

DETECTION OF BLOOD SAMPLES

Some peaks can be observed in samples of blood from four volunteers, mainly corresponding to short-chain ceramides, as shown in Figure 4. The lipid information of the LipidSearch database was matched and analyzed according to the secondary peak map. Finally, the chemical formula of the lipid was $C_{20}H_{39}NO_3$ and $C_{42}H_{85}NO_3$. Table II lists the peak

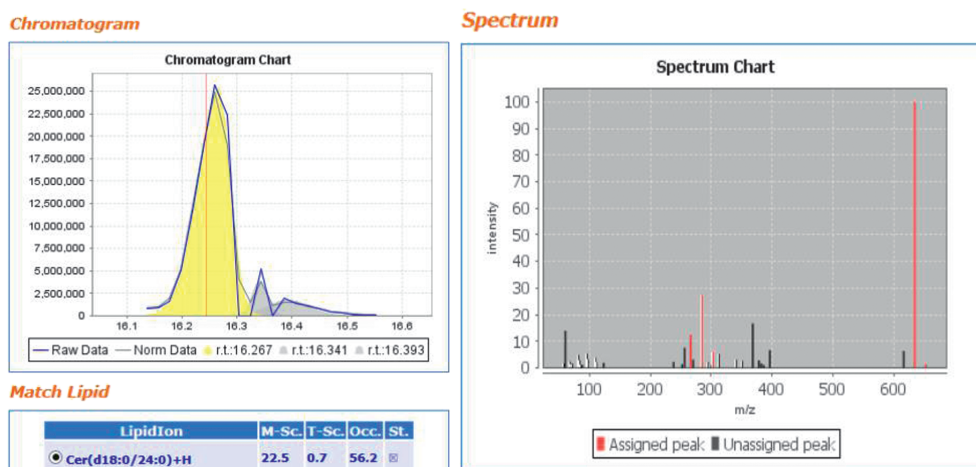
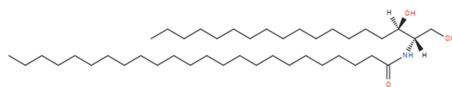


Figure 2. According to the first-order peak of the chromatographic chart, the possible lipid types obtained were matched in the LipidSearch database, and the lipid information was analyzed. The red results are the matched fragments in the database.

C42H85NO3



LM ID	LMSF02020012	
Common Name	Cer(d18.0/24.0)	
Systematic Name	N-(tetracosanoyl)-sphinganine	
Synonyms	C24DH Cer, N-(tetracosanoyl)-dihydroceramide; N-(tetracosanoyl)-dihydroceramide, Cer[NDs]	
Exact Mass	651.6529 (neutral)	Calculate m/z: <input type="text" value="((Select m/z)"/>
Formula	C ₄₂ H ₈₅ NO ₃	
Category	Sphingolipids [SP]	
Main Class	Ceramides [SP02]	
Sub Class	N-acylsphingamines (dihydroceramides) [SP0202]	
PubChem Compound ID (CID)	5283577	
METABOLOMICS ID	-	
KEGG ID	-	
HMDB ID	HMDB11768	

Figure 3. According to the matching identification information in the LipidSearch database, the structure and information of the corresponding compound can be found in the LIPID MAPS.

areas of the first-order extraction peaks of the major ceramides in the volunteer blood sample. In the four blood samples, C₂₀H₃₉NO₃ and C₄₂H₈₅NO₃ showed good and stable concentrations (Table II). Therefore, we believe that C₂₀H₃₉NO₃ and C₄₂H₈₅NO₃ are the main types of ceramide present in the blood.

DISCUSSION

The skin plays an important role in protection and defense as the first physiological barrier of the human body (13). Lipid components play an important role in the skin barrier and cooperate to maintain the health of the epidermis. Triglycerides (TG) mainly store and provide energy, phospholipids are the main components of biofilms to prevent water

Table I
Major Long-Chain Ceramides Measured in the Epidermis

Sample	C42H85NO3		
	Area	Concentration	Average (ng/ml)
Sample 1	13,718,882	34.25	33.63
	13,254,807	32.91	
	13,538,412	33.73	
Sample 2	11,638,420	28.22	27.59
	11,503,732	27.83	
	11,123,588	26.72	
Sample 3	17,879,947	43.09	43.06
	17,690,822	42.70	
	18,031,713	43.40	
Sample 4	8,222,066	23.34	22.78
	7,765,799	22.40	
	7,856,601	22.59	

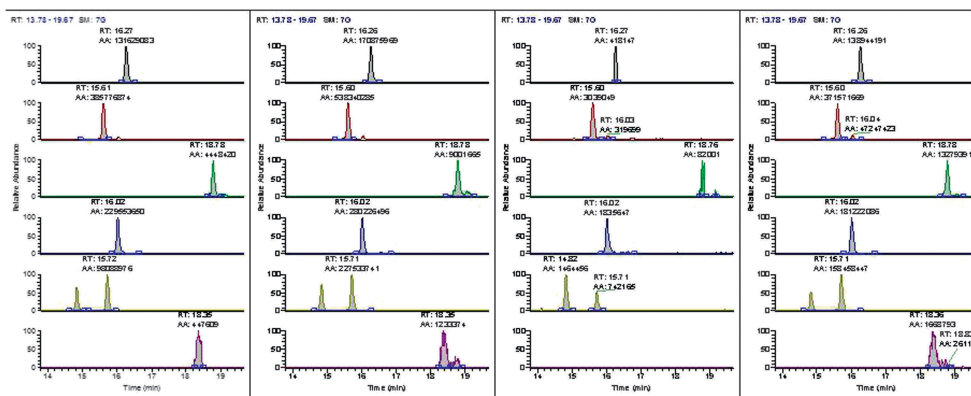


Figure 4. After centrifuging, drying, dissolution, and recentrifuging, the first-order extraction peaks of ceramide subclasses in the four blood samples were obtained by chromatography.

loss and foreign body invasion, and ceramides are the main effectors in the late stage of barrier repair and participate in the proliferation and differentiation of keratinocytes together with sphingosine to maintain the functional metabolism of the skin. The process of cell proliferation and differentiation maintains the functional metabolism of the skin and participates in the immune response (14). It also plays an important role in apoptosis, growth, differentiation, aging, immune-related signal transduction, and apoptosis and also has cosmetic effects such as adhesion, moisturizing, and antiaging (15).

In addition to the results obtained using high-performance chromatography, more than 300 other lipids were detected under positive ion conditions, mainly ceramide and TG. More than 30 species were detected under negative ion conditions, all of which were ceramides. In addition to the ceramide compounds of interest, other lipids have been identified, such as phosphatidylcholine, lysophosphatidylcholine, TG, sphingomyelin, phosphatidylethanolamine, lysophosphatidylethanolamine, etc. Methods of collecting

Table II
Major Ceramides Measured in Blood Samples

Sample	C20H39NO3			C42H85NO3		Average (ng/ml)
	Area	Concentration	Average (ng/ml)	Area	Concentration	
Sample 1	481,000	5.76	6.05	75,182,885	212.59	220.99
	507,350	6.06		79,309,046	224.56	
	531,062	6.33		79,746,893	225.83	
Sample 2	1,293,308	15.07	15.52	86,415,078	245.18	246.73
	1,380,635	16.07		88,538,303	251.34	
	1,325,935	15.44		85,897,911	243.68	
Sample 3	498,855	5.96	7.17 (5.38 × 200/150 = 7.17)	80,564,935	228.20	220.13
	435,820	5.24		77,344,272	218.86	
	408,079	4.92		75,436,264	213.32	
Sample 4	540,450	6.44	6.13	73,712,246	208.32	203.46
	493,675	5.9		70,995,731	200.44	
	505,256	6.04		71,401,759	201.62	

skin SC include surgical resection, tape stripping, cyanoacrylate stripping, mechanical scraping, sanding, and organic solvent dissolution methods. Compared with other collection methods, the cyanoacrylate stripping method selected in this study is simple, reproducible, and can extract lipids from the skin to the greatest extent in a noninvasive manner. In this study, HPLC was used with high-resolution MS and LipidSearch software to determine ceramide constituents in the skin and blood. The method is simple, rapid, has ultrahigh resolution, good stability, high sensitivity, and can be used for qualitative and relative quantitative analysis of ceramide in samples.

Ceramide is less in the epidermis and difficult to extract from the skin. Because of the complexity of various ingredients, it is difficult for the cosmetics industry to mass produce them. A ceramide analog (pseudoceramide) was artificially synthesized (16). However, how to effectively produce pseudoceramides that are very similar to natural ceramides still needs further research. This study provides a methodological basis for the detection and extraction of ceramide content. The application of high-throughput liquid MS can thoroughly detect and explore the composition and proportion of each ceramide content and also explore the effect of different types of ceramide on the skin barrier. This research has great significance for the future development of skin care products.

According to the number of C atoms, ceramides are divided into long-chain (C chain > 40) and short-chain (C chain \leq 40) ceramides. Our research shows that long-chain ceramide is the main type in the skin, mainly $C_{42}H_{85}NO_3$. The long-chain and short-chain ceramides were measured in the blood at the same time, and the long-chain ceramides were significantly higher in the blood than in the skin, which may be related to factors such as vascular endothelial cell metabolism or tissue density. There are different distributions of ceramide in different tissues of the human body. The long-chain ceramides present in the skin SC help maintain the skin barrier, reduce water loss, and participate in adhesion. The blood is rich in short-chain ceramides, induces apoptosis, and participates in processes such as differentiation and aging (17,18). It can be used as a new type of exogenous agonist to regulate tissue metabolism together with cytokines (19). Long-chain ceramide is part of intracellular signal transduction and induces cell migration (20), which may have synergistic effects with short-chain ceramides, which needs to be studied further.

Of course, this experiment still had some limitations: the sample size is small, only the skin on the inside of the forearm is selected, the age range of volunteers is relatively small, and there may be some one-sidedness. In the future, ceramide contents of different individual ages and different body parts need to be further studied. It is also important to analyze differences in ceramide from different ethnic groups or any skin disease.

The study used liquid chromatography mass spectrometer to efficiently detect ceramides in the skin and blood and compared the types and contents of ceramide, which provided an effective method and basis for exploring the metabolic changes of ceramide in the skin barrier and various systemic diseases.

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