Quantitative analysis of ceramide molecular species by high performance liquid chromatography

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Abstract **A method was developed for quantitative analysis of molecular species of ceramide (N-acyl-sphingosine) and dihydroceramide (N-acyl sphinganine) by high performance liquid chromatography (HPLC). Various N-acyl chain-containing ceramides or dihydroceramides were semi-synthesized as standard materials and allowed to react with anthroyl cyanide, a fluorescent reagent. Anthroyl derivatives of ceramide and dihydroceramide containing C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} saturated N-acyl chain could be completely separated to each molecular species by reversed-phase HPLC equipped with fluorescence detector, although some ceramide molecular species containing monoenoic acyl chain were eluted together with saturated dihydroceramide species. Ceramide molecular species could be quantified using N-heptadecanoyl or N-tricosanoyl sphingosine as an internal standard, and the lower detection limit was below 1 pmol. This method was applied to the analysis of sphingomyelin and free ceramide in U937 cells. The analysis of the ceramide obtained by hydrolysis of sphingomyelin of U937 cells revealed that the ceramide moiety was mainly composed of N-palmitoyl sphingosine, N-nervonoyl sphingosine, and N-lignoceroyl sphingosine, representing 50.0, 27.4, and 6.7% of sphingomyelin, respectively. The total free ceramide and dihydroceramide of U937 cells was determined to be 254 ± 5 pmol/10^6 cells. Major molecular species of the free ceramide fraction were N-lignoceroyl, N-palmitoyl, and N-nerovonoyl sphingosine, representing 27.6%, 26.6%, and 13.6% of this fraction, respectively. Different distribution of free ceramide molecular species from sphingomyelin species may suggest that selective metabolism of molecular species occurs in the synthesis or degradation of sphingomyelin. These results indicate that the picomole level of molecular species of ceramide and dihydroceramide is successfully determined by fluorescence HPLC and that this newly developed method may be useful to reveal the metabolism and function of ceramide and related compounds in cultured cells.—**Yano, M., E. Kishida, Y. Muneyuki, and Y. Masuzawa. **Quantitative analysis of ceramide molecular species by high performance liquid chromatography. *J. Lipid Res.* 1998.** 39: **2091–2098.**

Supplementary key words HPLC • dihydroceramide • sphingomyelin • U937 cells

Ceramide (N-acyl sphingosine) is known as an important precursor common to the biosynthesis of glycosphingolipids and sphingomyelin (1, 2). Naturally occurring ceramide is composed of various molecular species resulting from a combination of C16–26 fatty acids and sphingosine (sphingenine). The N-acyl chain is thought to be mainly introduced by the acylation of sphinganine, and the resultant dihydroceramide (N-acyl sphinganine) is desaturated to form ceramide (3–5). The hydrolysis of sphingolipids also yields ceramide and may further lead to the generation of sphingosine (6, 7) and sphingosine phosphate (8, 9). Although the regulation of such metabolism of ceramide has not been fully understood, recent studies have established the existence of a sphingomyelin cycle (10, 11) or a sphingomyelin pathway (12) that operates in response to various agonists and stresses. This pathway is initiated by activation of sphingomyelinase, and a resultant hydrolysate, ceramide, is thought to act as a second messenger in the intracellular signaling pathway to apoptosis, cell cycle arrest, cell differentiation, cell propagation, or induction of cytokine synthesis (10–12). In addition, catabolites of ceramide, such as sphingosine (13–19) and sphingosine phosphate (20–24), are also suggested to play an important role in cell regulation.

Quantitative analysis of intracellular free ceramide has provided evidence for the close relationship of the ceramide level with the fate of various cells. Enzymatic determination of ceramide using *E. coli* diacylglycerol kinase and [γ-^{32}P]ATP has been used to determine the increased mass of free ceramide in activated cells because of its many advantages, such as broad linearity and applicability to crude lipid extract (25). Normal phase HPLC analysis of ceramide after attaching a fluorescent tag was also developed for low nanogram range quantitation of the ceramide (26). These assay systems can supply an answer to the present question about the level of intracellular free

Abbreviations: LCB, long chain base; sphingosine (sphingenine), LCB18:1; sphinganine, LCB18:0; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography. Fatty acids are expressed as the number of carbon atoms: number of double bonds in tables or figures.

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ceramide increased during the activation of cells, but do not provide any information concerning the metabolism of molecular species of the ceramide. Ceramide molecular species can be analyzed by mass spectrometry (MS). The identification and structure determination of ceramide molecular species have been carried out by gas chromatography/mass spectrometry (GC/MS) (27), and the molecular species of free ceramide in human serum has been determined (28). The recent development of electrospray MS and tandem mass spectrometry (MS/MS) provided a rapid, sensitive, and quantitative method for molecular species analysis (29, 30). The combination of MS/MS with liquid chromatography (LC/MS/MS) has made it possible to determine the intracellular level of sphingolipid metabolites including ceramide molecular species (31). However, this excellent method is not necessarily suitable for routine laboratory work, mainly because only a limited number of investigators have the opportunity to use LC/MS/MS . Furthermore, quantitative analysis using MS is inapplicable to most of the tracer experiments in which the incorporation of radioactive precursors should be measured simultaneously with mass quantitation. In this study, we developed a method for the analysis of ceramide molecular species using HPLC separation and fluorometric detection and applied this method to the quantitative analysis of molecular species of free ceramide in U937 cells.

MATERIALS AND METHODS

Chemicals

Sphingosine was purchased from Dorman Serdary Research Lab. (Ontario, Canada) or Sigma (St. Louis, MO). Sphinganine, various fatty acids, and *Bacillus cereus* sphingomyelinase (EC 3.1.4.12) were from Sigma. N-methyl-N-trimethylsilyl-trifluoroacetamide was obtained from Pierce (Rockford, IL). Oxalylchloride was from Aldrich (Milwaukee, Wis). Quinuclidine was purchased from Nacalai Tesque (Kyoto, Japan). 1-Anthroyl cyanide was from Wako (Osaka, Japan). RPMI 1640 medium, fetal bovine serum, l-glutamine, and kanamycin were obtained from GIBCO BRL (Grand Island, NY). Solvents used for HPLC analysis were commercially available as HPLC grade, and other chemicals were of reagent grade. The solvents used for chemical synthesis were dried with molecular sieves before use.

Semisynthesis of molecular species of ceramide and dihydroceramide

Molecular species of ceramide were semisynthetically prepared from sphingosine and free fatty acid. Fifteen milligrams of sphingosine was suspended in 4 ml dichloromethane, and 0.5 ml N-methyl-N-trimethylsilyl-trifluoroacetamide was added dropwise to the suspension at 4°C under an argon atmosphere. The trimethylsilyl ether (TMS) derivatization was approximately assessed by observing the solubility of the substrate. From 10 to 60 min after recognizing complete dissolution of sphingosine, a dichloromethane solution of fatty acid chloride, prepared from fatty acid and oxalylchloride, was added dropwise to the reaction mixture at 4°C. The reaction mixture was allowed to stand at room temperature for more than 30 min. After evaporating the solvent, the TMS derivative of ceramide was dissolved in ethyl acetate and washed with saturated $NaHCO_3$ solution and 0.3 m HCl three times, respectively. After recognizing the removal of the TMS group by thin-layer chromatography (TLC), the ceramide was purified by column chromatography (0.5 g BONDESIL SI 40 UM, Varian, Harbor City, CA) using hexan–ethylacetate as eluate. Molecular species of dihydroceramide were prepared in a similar manner using sphinganine as a substrate instead of sphingosine.

HPLC analysis of anthroyl derivatives of ceramide and dihydroceramide

Ceramide and dihydroceramide were converted to 1-anthroyl derivatives by the modified method of Goto et al. (32). Briefly, ceramide or dihydroceramide (less than 100 mg) was treated with 1-anthroyl cyanide (2 mg/ml) in 250 ml acetonitrile–dichloromethane 1:2 (by vol) containing 0.04% quinuclidine at 4°C overnight. The reaction was stopped by adding 100 ml methanol. It was confirmed by TLC with hexane–diethyl ether 50:50 (by vol) that all of ceramide reacted with anthroyl cyanide to make one newly observed fluorescent spot on TLC. The anthroyl derivative of ceramide or dihydroceramide was purified in a manner similar to the separation of ceramide or dihydroceramide. Anthroyl derivative of ceramide and dihydroceramide was eluted with 4–8% ethylacetate in hexane. The purified 1-anthroyl derivatives of ceramide and dihydroceramide were analyzed with an HPLC system equipped with a reversed-phase column (Merck Lichrosorb RP-18, 5 μm, 4 mm × 250 mm) and a fluorescence detector (Waters 474 Scanning Fluorescence Detector, excitation at 365 nm; emission at 412 nm). Acetonitrile–methanol–ethylacetate 12:1:7 (by vol) was used as the mobile phase, and the flow rate was 1.2 ml/min. ^{1}H-NMR spectra of eluted peaks revealed that the ratio of anthroyl moiety to ceramide backbone is 2 (data not shown), indicating that not only the primary hydroxyl group but also the secondary hydroxyl group reacted with anthroyl cyanide.

Separation of ceramide and dihydroceramide of U937 cells

U937 cells were obtained from the Riken Gene Bank (Wako, Japan) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and kanamycin at 37°C in a humidified atmosphere of 5% CO_2. Lipids of U937 cells were extracted by the method of Bligh and Dyer (33). In the extraction, heptadecanoyl or tricosanoyl sphingosine was added (50 ng/10^6 cells) as an internal standard for the determination of intracellular free ceramide. The total lipid extract was separated by TLC with hexane–diethyl ether–acetic acid 10:90:1 (by vol). The bands corresponding to free ceramide and phospholipids were scraped from the plates, and each lipid fraction was extracted by the method of Bligh and Dyer (33). The TLC separation excluded hydroxy fatty acid-containing species from free ceramide fraction. Also, phytosphingosine-containing species, another trihydroxy ceramide species, were probably separated from nonhydroxy fatty acid-containing ceramide. The free ceramide fraction was further treated with 0.03 m NaOH in 90% ethanol at 37°C for 30 min to degrade contaminated glycerolipids such as monoacylglycerol. Sphingomyelin was purified from the phospholipid fraction by TLC with chloroform–methanol–NH_4OH 65:25:5. Purified sphingomyelin was dissolved in 2 ml diethyl ether and combined with 2 ml sphingomyelinase solution (11 units/ml in 0.1 m Tris-HCl buffer, pH 7.2, containing 0.1% Triton X100 and 25 mm $MgCl_2$). The sphingomyelinase treatment was carried out by vigorous stirring of the mixture overnight, and the liberated ceramide was purified by TLC. Ceramide and dihydroceramide were converted to 1-anthroyl derivatives as previously mentioned.

Fatty acid analysis of sphingolipids

The N-acyl chain of sphingomyelin or the anthroyl derivative of ceramide was analyzed by gas–liquid chromatography (GLC).

In the case of sphingomyelin, mild alkaline treatment was carried out after the separation by TLC to remove contaminated glycerolipids as previously mentioned in the separation of free ceramide. The constituent fatty acids were methylated with 5% HCl in methanol at 80°C for 18 h. The methyl ester was extracted with hexane, and the fatty acid composition was determined using a Shimadzu GC 14A gas chromatograph analyzer with a capillary column (Omegawax TM25, 30 m × 0.25 mm, Supelco, Bellafonte, PA).

RESULTS AND DISCUSSION

We first semi-synthesized standard molecular species of ceramide and dihydroceramide. Although ceramide can be synthesized by direct reaction of sphingosine with fatty acid chloride, the use of TMS derivative of sphingoid base allowed its amino group to completely and selectively react with acyl chloride in an equimolar reaction mixture. In addition, because TMS derivatization significantly increased the solubility of sphingoid bases in organic solvents, the reaction with acyl chloride was more rapid. The high selectivity and rapid reaction were beneficial for the synthesis of many kinds of molecular species. Also, this synthetic method is useful to conveniently obtain heptadecanoyl or tricosanoyl sphingosine, which is needed as an internal standard for the quantitative analysis by HPLC but is not commercially available. The following fluorescence labeling was performed below 4°C because the by-products increased at a higher temperature. Fluorescence-labeled ceramide or dihydroceramide was separated from reagents by column chromatography. This step was somewhat time-consuming, but many kinds of standard materials could be simultaneously separated by use of small columns. This was also the case with handling many samples of free ceramide from living cells cultured separately. Thin-layer chromatography was an alternative procedure for the separation of fluorescence-labeled ceramide, but the hydrolysis of a portion of the fluorescent label was observed after extraction from silicic acid using a chloroform:methanol solution.

Figure 1 shows HPLC separation of anthroyl derivatives of N-acyl sphingosine and N-acyl sphinganine. Species containing C16–C24 (even carbon number) saturated fatty acid were found to be completely separated within 40 min by isocratic elution. Among the species composed of the same sphingoid base, the retention time of N-oleoyl derivative was close to that of the N-palmitoyl derivative, but separation was sufficient even when the ratio of the amounts of the two species was 1/20 (data not shown). On the other hand, some monoenoic acid-containing ceramide species could not be separated from saturated acid-containing dihydroceramide species. For example, N-docosenoyl sphingosine (LCB18:1–22:1) was eluted together with N-stearoyl sphinganine (LCB18:0–18:0). Ceramide molecular species containing odd carbon number fatty acids were also semi-synthesized and applied to this HPLC system. N-heptadecanoyl sphingosine was eluted at a retention time similar to that of N-palmitoyl sphinganine or N-oleoyl sphinganine (data not shown). Also, the

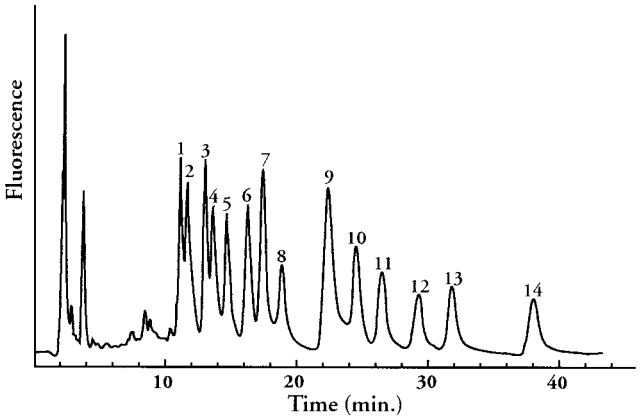

Fig. 1. Separation of anthroyl derivatives of molecular species of ceramide and dihydroceramide by HPLC. Molecular species of ceramide and dihydroceramide were semisynthetically prepared as described in Materials and Methods. Each species was reacted with 1-anthroyl cyanide, and the resultant anthroyl derivative was purified by silicic acid column chromatography. Seventeen species of purified anthroyl derivatives were mixed and separated by HPLC on a reversed-phase column (Merck LiChrosorb RP-18, 4 mm × 250 mm) with the mobile phase of acetonitrile–methanol–ethyl acetate 12:1:7 (by vol) at a flow rate 1.2 ml/min. Each peak was detected using a spectrofluorometer with excitation at 365 nm and emission at 412 nm. Molecular species corresponding to each peak number were: peak 1, LCB18:1–18:1; peak 2, LCB18:1–16:0; peak 3, LCB18:0–18:1; peak 4, LCB18:1–20:1 and LCB18:0–16:0; peak 5, LCB18:1–18:0; peak 6, LCB18:0–20:1; peak 7, LCB18:1–22:1 and LCB18:0–18:0; peak 8, LCB18:1–20:0; peak 9, LCB18:1–24:1 and LCB18:0–20:0; peak 10, LCB18:1–22:0; peak 11, LCB18:0–24:1; peak 12, LCB18:0–22:0; peak 13, LCB18:1–24:0; and peak 14, LCB18:0–24:0.

retention time of N-tricosanoyl sphingosine was close to that of N-docosanoyl sphinganine (data not shown). However, these two odd carbon number acid-containing species were completely separated from other ceramide species (data not shown). The plot of retention time versus the carbon number of saturated acid-containing species possessing either sphingosine or sphinganine base did not show a linear relationship (**Fig. 2**). The relationship could be approximated by the curve of involution ($R2 > 0.99$), as shown in Fig. 2, or a curve of secondary degree ($R2 > 0.99$). This was also the case with species containing monounsaturated fatty acids. These approximate equations may be useful to identify unknown peaks in the HPLC profile. In fact, we could determine the retention times of eicosenoyl-sphingosine (LCB18:1–20:1) and docosenoyl-sphinganine (LCB18:0–22:1), which were not prepared as standard materials, in the analysis of natural ceramide from U937 cells.

As previously mentioned, N-heptadecanoyl or N-tricosanoyl sphingosine was not separated from some dihydroceramide molecular species. However, the dihydroceramide level is considered to be much lower than that of the ceramide in human serum (28) and some murine tissues (34). In this case, N-heptadecanoyl or N-tricosanoyl sphingosine can be used as an internal standard to approximately quantify the molecular species of ceramide,

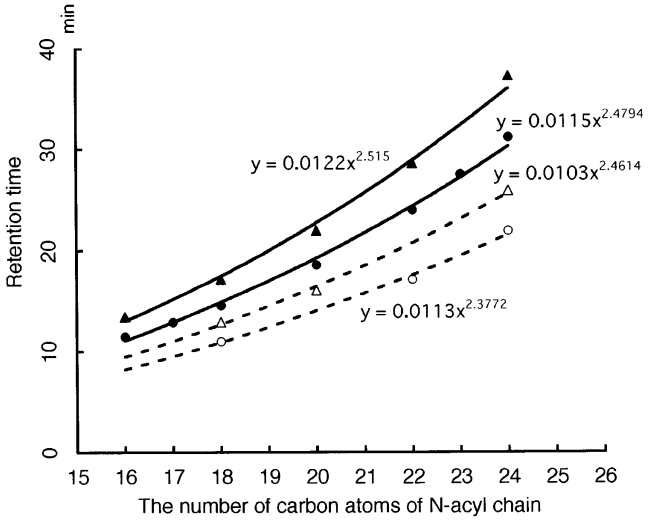

Fig. 2. The relationship between retention time and carbon number of N-acyl chain in the separation of molecular species of ceramide and dihydroceramide by HPLC. Each molecular species was plotted with its carbon atom number of the N-acyl chain versus the retention time observed in Fig. 1. N-saturated fatty acid sphingosine(—●—), N-monounsaturated fatty acid sphingosine (—○—), N-saturated fatty acid sphinganine(—▲—), N-monounsaturated fatty acid sphinganine(—△—). Assuming that each relationship is approximated by involution, the curves and equations were filled in the figure.

when a sufficient amount of such odd carbon number fatty acid-containing ceramide is added to the test samples. In addition, all of the molecular species, including dihydroceramide species eluted with an internal standard, can be precisely determined from two separate chromatograms, one of which is for the sample with an internal standard, the other is for the sample without an internal standard. The latter chromatogram gives the molar ratio of all of molecular species in the sample, and the area corresponding to dihydroceramide eluted with odd carbon number acid-containing species can be calculated from the molar ratio to one of the other species. For further confirmation of the effectiveness of odd carbon number fatty acid-containing species as an internal standard, the recovery of ceramide containing very long chain fatty acids, such as N-nervonoyl sphingosine and N-docosanoyl sphingosine, against N-heptadecanoyl sphingosine was assessed during the course of derivatization and purification. As shown in **Fig. 3**, the plot of the measured amount versus the added amount (1–18 pmol) of ceramide species was approximated by a straight line, which was extrapolated to the origin of the graph. This indicated that the sub-picomole level of ceramide molecular species could be measured by this quantitative method. In fact, when this assay system was applied to the quantitative analysis of the ceramide molecular species of living cells, the addition of 1 pmol N-nerbonoyl species to the lipid extract of U937 cells resulted in an increase in the corresponding amount of this species (data not shown). The upper detection limit was not precisely assessed, but it was at least 20 times as much as the amount of an internal standard which could be determined by this method (data not shown).

To assess whether this HPLC system is applicable to molecular species analysis of naturally occurring ceramide, the molecular species distribution of the sphingomyelin of U937 cells was determined after liberation of the ceramide moiety by sphingomyelinase treatment. **Figure 4** shows the HPLC separation of the ceramide moiety of sphingomyelin from U937 cells. Each molecular species was identified by comparing its retention time with that of synthetic ceramide molecular species (**Table 1**), and the identification of the predominant species (peaks 3, 11, and 16) was confirmed by the detection of corresponding fatty acid moiety in each fraction using gas chromatography. Three predominant molecular species eluted at peaks 3, 11, and 16 were identified as N-palmitoyl sphingosine (LCB18:1–16:0), N-nervonoyl sphingosine (LCB18: 1–24:1), and N-lignoceroyl sphingosine (LCB18: 1–24:0), representing 50.0, 27.4, and 6.7% of total ceramides derived from sphingomyelin, respectively. Gas chromatographic analysis of the N-acyl chain of the total sphingomyelin fraction revealed that the acyl moiety of U937 sphingomyelin is mainly composed of palmitate, nervonate, and lignocerate and that each percentage was 47.0 ± 4.4, 24.8 ± 4.0 and 8.6 ± 1.7, respectively. This N-acyl chain composition almost agrees with our HPLC data for the distribution of the predominant molecular species. As to minor molecular species, some difference was observed in oleic acid composition between fatty acid analysis and molecular species analysis. GC analysis indicated that sphin-

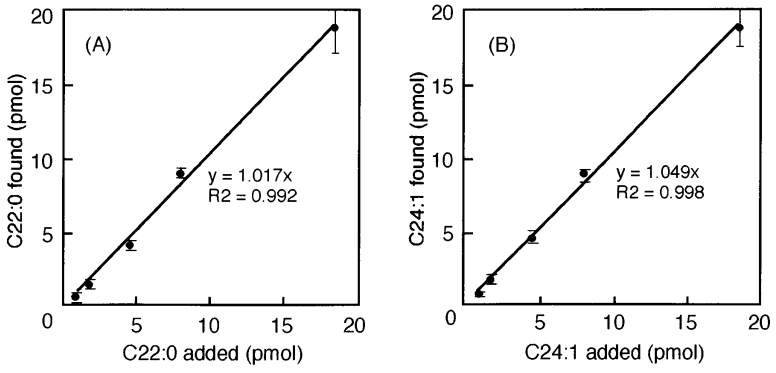

Fig. 3. Accuracy of determination of ceramide molecular species by HPLC analysis. From 1 pmol to 18 pmol of N-docosanoyl sphingosine or N-nervonoyl sphingosine was added into separate tubes with 5 pmol N-heptadecyl sphingosine. The ceramides were converted to anthroyl derivatives and quantified by HPLC analysis as described in the text. The amounts determined from the ratio to N-heptadecyl sphingosine were plotted versus the added amounts. (A) N-docosanoyl sphingosine; (B) N-nervonoyl sphingosine.

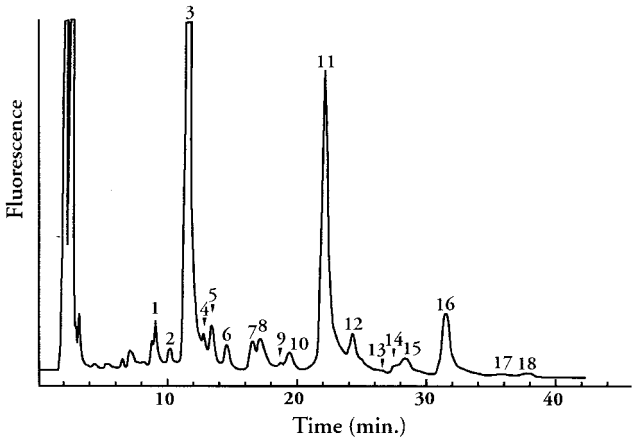

Fig. 4. HPLC separation of molecular species of ceramide and dihydroceramide obtained by the hydrolysis of sphingomyelin of U937 cells. Sphingomyelin of U937 cells was isolated as described in Materials and Methods and hydrolyzed by *Bacillus cereus* sphingomyelinase. The resultant ceramide and dihydroceramide were converted to anthroyl derivatives, and the molecular species were analyzed by HPLC as described in the text or the legend of Fig. 1. The identified molecular species corresponding to each peak number is shown in Table 1.

gomyelin contained 2–5% of oleic acid as its N-acyl chain, but only a negligible amount of oleate-containing species was detected by HPLC analysis. Incomplete removal of lysophospholipids from the sphingomyelin fraction by mild alkaline treatment might result in overestimation of oleate by GC analysis. The sum of sphinganine-containing peaks except for peak 11 was 12.5%, indicating that the sphingomyelin fraction of U937 cells contained only a small percentage of sphinganine-containing species.

TABLE 1. Distribution of molecular species of sphingomyelin from U937 cells

Peak Number[a]	Molecular Species		% Distribution[b]
1	not identified		
2	not identified		
3	LCB 18:1–16:0		50.5 ± 1.3
4		LCB 18:0–18:1	0.2 ± 0.1
5	LCB 18:1–20:1	LCB 18:0–16:0	1.1 ± 0.2
6	LCB 18:1–18:0		1.2 ± 0.1
7		LCB 18:0–20:1	2.0 ± 0.2
8	LCB 18:1–22:1	LCB 18:0–18:0	2.4 ± 0.2
9	LCB 18:1–20:0		0.3 ± 0.1
10		LCB 18:0–22:1	1.3 ± 0.2
11	LCB 18:1–24:1	LCB 18:0–20:0	27.7 ± 1.5
12	LCB 18:1–22:0		3.4 ± 2.0
13		LCB 18:0–24:1	0.7 ± 0.1
14	not identified		
15		LCB 18:0–22:0	2.1 ± 0.2
16	LCB 18:1–24:0		6.8 ± 0.2
17	not identified		
18		LCB 18:0–24:0	0.2 ± 0.1

Molecular species of ceramide moiety of sphingomyelin of U937 cells were analyzed as shown in Fig. 4. Each peak except for peaks 5 and 10 was identified by comparing the retention time with that of synthetic ceramide molecular species. Retention times LCB18:1–20:1 and LCB18:0–22:1 were estimated from the data shown in Fig. 2. Values represent the mean percent distribution ± SD calculated from three separate determinations.

[a]Each peak number is shown in Fig. 4.

[b]Unidentified peaks were excluded from the calculation of % distribition. Each unidentified peak did not exceed 1% of the total peak area.

The HPLC method was also applied to the quantitative analysis of the molecular species of the free ceramide fraction in U937 cells. N-tricosanoyl sphingosine was used as an internal standard, and an elution profile is shown in **Fig. 5**. The chromatogram for the sample without N-tricosanoyl sphingosine was also obtained (data not shown), and the amounts of all molecular species were determined as shown in **Table 2**. The total of free ceramide and dihydroceramide was calculated as 254 ± 5 pmol/10^6 cells (159 ± 3 ng/10^6 cells), assuming that unidentified peaks were not the ceramide and dihydroceramide species. The amount of free ceramide in cultured cells has been determined by the method using *E. coli* diacylglycerol kinase and [γ-^{32}P]ATP(25) or normal phase HPLC (26). For example, HL60 cells have been reported to contain 150 pmol (35) or 108 ng (26) free ceramide/10^6 cells. Our data concerning the free ceramide level in U937 cells were higher than those observed in HL60 cells, but the order of magnitude of the amount was the same. In the free ceramide fraction of U937 cells, N-palmitoyl sphingosine (LCB18:1–16:0), N-nervonoyl sphingosine (LCB18:1–24:1), and N-lignoceroyl sphingosine (LCB18:1–24:0) were found to be the predominant species (Fig. 5, Table 2). These species were also predominant in ceramide derived from sphingomyelin. However, the proportions of N-palmitoyl sphingosine and N-lignoceroyl sphingosine were different from those in sphingomyelin. The proportion of N-palmitoyl sphingosine in the free ceramide fraction was one-half as much as that in the sphingomyelin constituents. In contrast, the proportion of N-lignoceroyl species was approximately twice as much as that in sphingomye-

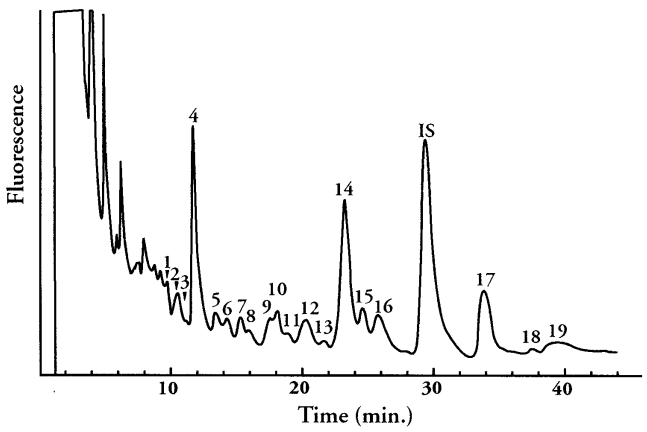

Fig. 5. HPLC separation of molecular species in free ceramide fraction of U937 cells. N-tricosanoyl sphingosine (50 ng/10^6 cells) was added in the extraction of the total lipid of U937 cells. The free ceramide fraction was separated by TLC and converted to anthroyl derivatives. The molecular species of ceramide and dihydroceramide were separated by HPLC as described in the text or the legend of Fig. 1. The identified molecular species corresponding to each peak number is shown in Table 2. The peak of N-tricosanoyl sphingosine is marked IS.

TABLE 2. Molecular species composition of free ceramide and dihydroceramide of U937 cells

Peak Number[a]	Molecular Species	pmol/10^6 Cells	ng/10^6 Cells	% Distribution
1	not identified			
2	not identified			
3	LCB 18:1–18:1	2.5 ± 1.5	1.5 ± 0.9	1.0 ± 0.6
4	LCB 18:1–16:0	53.8 ± 0.5	30.6 ± 1.3	21.9 ± 0.6
5	LCB 18:0–18:1	7.7 ± 1.1	4.9 ± 0.8	3.3 ± 0.6
6	LCB 18:1–20:1 LCB 18:0–16:0	11.8 ± 0.3	6.6 ± 0.2[d]	4.7 ± 0.1
7	LCB 18:1–18:0	8.9 ± 1.1	5.3 ± 0.5	3.5 ± 0.4
8	not identified			
9	LCB 18:0–20:1	9.7 ± 1.5	6.0 ± 0.7	3.8 ± 0.5
10	LCB 18:1–22:1 LCB 18:0–18:0	13.1 ± 1.0	8.6 ± 0.5[d]	5.2 ± 0.2
11	LCB 18:1–20:0	15.5 ± 1.5	9.5 ± 0.7	6.1 ± 0.5
12	LCB 18:0–22:1	7.4 ± 5.2	4.8 ± 3.4	1.9 ± 2.2
13	LCB 18:1–24:1 LCB 18:0–20:0	57.0 ± 2.4	38.8 ± 1.1[d]	22.6 ± 0.5
14	not identified			
15	LCB 18:1–22:0	19.7 ± 1.2	12.7 ± 0.6	7.7 ± 0.4
16	LCB 18:0–24:1	3.8 ± 0.7	2.5 ± 0.4	1.5 ± 0.2
	not identified[b]			
	LCB 18:0–22:0[b]	9.4 ± 0.4[c]	6.2 ± 0.2	3.7 ± 0.1
17	LCB 18:1–24:0	28.1 ± 0.1	19.1 ± 0.1	11.1 ± 0.2
18	not identified			
19	LCB 18:0–24:0	6.3 ± 0.0	4.3 ± 0.1	2.5 ± 0.0
Total		254.0 ± 5.0	159.4 ± 3.0	100.0

Molecular species of the free ceramide fraction from U937 cells were analyzed as shown in Fig. 5. Each peak was identified as described in the legend of Table 1. The amount of each species was calculated from the ratio of each peak area to LCB18:1–23:0 added as an internal standard (IS). In this determination, the areas corresponding to peaks hidden by the addition of the internal standard were calculated from the ratio to other species, which was obtained from a separate chromatogram for the sample without LCB18:1–23:0 and subtracted from the apparent peak area of LCB18:1–23:0 (IS in Fig. 5). Values represent the mean ± SD calculated from three separate determinations.

[a] Each peak number is shown in Fig. 5.

[b] These two peaks were hidden by the addition of LCB18:1–23:0.

[c] The ratio to other species was determined by separate HPLC analysis of the sample without an internal standard, and the composition of this species was calculated from the ratio.

[d] The weights were calculated on the assumption that these peaks were composed of sphingosine-containing species only.

lin. The free ceramide fraction also contained higher proportions of other long carbon chain acid-containing species, such as N-docosenoyl sphingosine (LCB18:1–22:1), N-arachidoyl sphingosine (LCB18:1–20:0) and N-docosanoyl sphingosine (LCB18:1–22:0), although the proportion of N-nervonoyl sphingosine (LCB18:1–24:1) was almost the same as that in sphingomyelin. These differences may indicate that selective metabolism of molecular species occurs in the synthesis or degradation of sphingomyelin. Presuming that peak 13 was composed of only ceramide species (LCB18:1–24:1), the sum of dihydroceramide species was calculated as 26.6% of the free ceramide fraction at its maximum. This proportion is higher than that estimated as the proportion of dihydroceramide-containing species (dihydrosphingomyelin) in sphingomyelin. Different from ceramide, dihydroceramide did not appear to retain specified fatty acids such as palmitate, nervonate, and lignocerate.

Our results indicate that the molecular species of ceramide and dihydroceramide are successfully determined by fluorescence HPLC. Sphingolipids from mammalian tissues have been reported to contain hydroxyl fatty acids and phytosphingosine especially in brain glycosphingolipids (36). Hydroxy acid-containing ceramide or phytosphingosine-containing ceramide derivatives can be separated from ceramide or dihydroceramide by silicic acid column chromatography (37) or TLC (38). HPLC analysis of hydroxy fatty acid-containing ceramide after benzoylation has been developed by several investigators (39–41). The ceramide fraction used in our analysis was separated from hydroxy fatty acid-containing ceramide and probably from phytosphingosine-containing ceramide by TLC. For the analysis of molecular species of these compounds using anthroyl cyanide as a labeling reagent, HPLC condition should further be examined, because 1-anthroyl cyanide may react with another hydroxyl group. Also, it has been reported that sphingolipids possess C_{20}-sphingosine and C_{20}-dihydrosphingosine (36). C_{20}-LCB-containing ceramide derivatives are probably fractionated together with ceramide and dihydroceramide by TLC. There is the possibility that some peaks in HPLC profiles (Fig. 4, Fig. 5) containe C_{20}-LCB-containing ceramide or dihydroceramide. However, such C_{20}-LCB have been mainly found in glycosphingolipids (36, 42) and are very minor components in free sphingosine fraction (43) or sphingomyelin (44). Therefore, it may be reasonable to speculate that the free ceramide fraction contains only negligible amounts of C_{20}-LCB-containing species in most tissues. Ceramide metabolism has recently attracted much attention because of its proposed role as an intracellular signal transducer (10–12). Hydrolysis of sphingolipids, especially sphingomyelin, and de novo synthesis of ceramide from dihydro-

ceramide are thought to mainly determine the intracellular level of free ceramide, which may be related to the fate of cells such as cell differentiation and apoptosis. Evidence has indicated that cell activation with various ligands and stresses or treatment with some toxic reagents increases the ceramide level. However, it has not been fully understood how ceramide metabolism is regulated or how the function of the ceramide is exerted. In addition, there is only little information concerning the roles of each molecular species of ceramide. The ceramide retains high amounts of very long chain fatty acids, such as lignoceric acid and nervonic acid, which are unusual in glycerolipids. Molecular species analysis will be a useful tool to elucidate the enzymatic mechanism and the physiological meaning of the enrichment of these acids in sphingolipids. Furthermore, there is some evidence indicating that ceramide molecular species may be selectively metabolized and exert a specified function. It has been suggested that turnover rates may differ among ceramide molecular species and that the molecular species preferentially used for biosynthesis may differ between sphingomyelin and glycosphingolipids (45).Our HPLC data indicated that there may be selective metabolism of ceramide molecular species in the synthesis or degradation of sphingomyelin. Ji et al. (46) reported that the biological activity of long chain fatty acid-containing ceramide may be stronger than that of C_2-ceramide. This implies that the biological activity of ceramide may be different according to the carbon number of the acyl chain. These lines of evidence indicate that the metabolism and function of sphingolipid molecular species should be more precisely elucidated. In addition to the methods previously described for quantitative analysis of ceramide (25–31), our HPLC method will serve as a new tool for clarifying the metabolism and function of each molecular species of sphingolipids.

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