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Liquid chromatography–tandem mass spectrometric determination of ceramides and related lipid species in cellular extracts

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Abstract

A liquid chromatography–electrospray ionization tandem mass spectrometric (LC–MS/MS) method was developed for the simultaneous qualitative and quantitative determination of sphingolipid metabolites such as ceramides, sphingisine, sphinganine, sphingomyelins, and ceramide 1-phosphates in the extracts of human promyelocytic leukemia cells (HL-60). The assay uses C_4 ceramide as an internal standard; simple liquid extraction; a short XTerra MS C_{18} (3 μm, 50 mm × 2.0 mm) column; a gradient mobile phase of 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (5/2/3 → 1/2/7); mass spectrometric detection using electrospray ionization. This LC–MS/MS method allowed the identification of 22 sphingolipid derivatives at pmol levels. In addition, this technique was successfully applied to analyze the changes of the sphingolipids profiles in cancer cells treated with apoptosis inducing agents, C_2 ceramide and H_2O_2.

Keywords: Sphingolipid; Ceramides; LC–MS/MS; HL-60 cells

1. Introduction

Ceramides (Cers) have recently been identified as key signal molecules which mediate many biological functions such as cell growth, differentiation, senescence and apoptosis [1–6]. It is now well established that ceramides can be generated and removed as a result of a number of available metabolic pathways [7]. Examples of ceramides formation include sphingomyelin hydrolysis and the *de novo* pathway of ceramide synthesis, both of which have demonstrated importance in mediating apoptotic effects [8–10]. Sphingolipid metabolites including ceramides exist at very low concentrations in cells but may have important biological effects as second messengers. Nevertheless, their mechanisms of action remain largely enigmatic. Most of these compounds are metabolically inter-convertible and structurally similar, and consequently the overall relationships of these lipids with biological responses are difficult to assign conclusively. Therefore, a sensitive and specific analytical method for determination of sphingolipid metabolites is required to understand an overall picture of their roles and biological consequences.

Various analytical tools for the identification and quantification of sphingolipid metabolites have been reported. The established methods include gas chromatography (GC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) [11–17] and enzyme- or antibody-based assays [18–20]. These procedures are insensitive, nonspecific, or time consuming. Also they cannot measure multiple sphingolipids simultaneously. Recently, mass spectrometric methods, especially LC/MS or LC/MS/MS methods have facilitated the identification of unique changes in specific ceramides from biological samples [21–24]. They allow the convenient and sensitive identification and quantitation of sphingolipid species. However, most analytic methods were available for several limited sphingolipid species.

In the present investigation, an LC–MS/MS method with electrospray ionization was developed to simultaneously detect 22 different sphingolipid metabolites including ceramides, sphingisine, sphinganine, sphingomyelins, and ceramide 1-phosphates from cellular extracts. In addition, the utility of this method was demonstrated by examining changes of these sphingolipids in human promyelocytic leukemia cells (HL-60) after the treatment with apoptosis-inducing agents.

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2. Experimental

2.1. Materials

N-Acetyl-D-sphingosine (C_2 ceramide (*d*18:1/2:0); C_2 Cer), *N*-hexanoyl-D-sphingosine (C_6 ceramide (*d*18:1/6:0); C_6 Cer), *N*-octanoyl-D-sphingosine (C_8 ceramide (*d*18:1/8:0); C_8 Cer), *N*-palmitoyl-D-sphingosine (C_{16} ceramide (*d*18:1/16:0); C_{16} Cer), *N*-stearoyl-D-sphingosine (C_{18} ceramide (*d*18:1/18:0); C_{18} Cer), ceramide 1-phosphate (*d*18:1/*n*, *n* = 16:0, 18:0, 20:0, 22:0 and 24:1; Cer1P C_n), sphingomyelin (*d*18:1/*n*, *n* = 16:0, 18:0 and 24:1; SM C_n), and D-*erythro*-dihydrosphingosine (sphinganine (*d*18:1); Sa) were purchased from Sigma Chemicals (St. Louis, MO, USA). *N*-Butanoyl-D-sphingosine (C_4 ceramide (*d*18:1/4:0); C_4 Cer), *N*-decanoyl-D-sphingosine (C_{10} ceramide (*d*18:1/10:0); C_{10} Cer), *N*-lauroyl-D-sphingosine (C_{12} ceramide (*d*18:1/12:0); C_{12} Cer), *N*-myristoyl-D-sphingosine (C_{14} ceramide (*d*18:1/14:0); C_{14} Cer) and *N*-arachidoyl-D-sphingosine (C_{20} ceramide (*d*18:1/20:0); C_{20} Cer) were obtained from Acros Organics (NJ, USA). D-*erythro*-1,3-Dihydroxy-2-amino-4-*trans*-octadecene (sphingosine (*d*18:0); So), sphingosine 1-phosphate (*d*18:1; So1P), sphingosylphosphorylcholine (SPC) and lysophosphatidic acid (LPA) were purchased from Biomol (PA, USA). Chloroform, methanol and tetrahydrofuran of spectroanalytical grade were purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grade. HPLC-grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA). High-purity nitrogen (99.999%) was obtained from Shin Yang Gas (Seoul, Korea). RPMI medium 1640, fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco BRL (Invitrogen Co., Grand Island, NY, USA).

2.2. Preparation of stock solutions and calibration standards

Each lipid was dissolved in ethanol to prepare the standard stock solution to a final concentration of 0.5 mM and stored at −20 °C. This solution was further diluted in ethanol to obtain working standard solutions at several concentrations. The stock solution was stable at −20 °C over a period of 6 month with the loss less than 2%.

Calibration standard samples were prepared by addition of the working standards to 3 ml of chloroform/methanol (1:2, v/v) giving a known amount of each compound. Thirty microliter of internal standard solution (C_4 ceramide (*d*18:1/4:0, 1.0 μg/ml in ethanol) was added to each sample to a final amount of 81.2 pmol. Calibration standard samples were analyzed according to the procedure described in Section 2.4. The calibration curves were constructed using 7 or 8 concentration points.

2.3. Cell culture

Human promyelocytic leukemia cells (HL-60) obtained from KCLB (Seoul, Korea) were grown in RPMI 1640 supplemented with 1.5 g/l sodium bicarbonate, 15% (v/v) FBS, and 100 units/ml of penicillin–streptomycin. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

2.4. Lipid extraction and preparation

The cells (8.8×10^6 cells) grown in 100 mM culture dishes were harvested, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in 5 ml of PBS. The internal standard (81.2 pmol of C_4 ceramide: 30 μl of 1 μg/ml ethanolic solution) and 3 ml of chloroform/methanol (1:2, v/v) were added to 4 ml aliquot of cell suspension. The samples were sonicated for 0.5 min and 0.8 ml of water was added. The mixtures were vortexed for 5 min and stood at room temperature for 30 min. After centrifugation at $2000 \times g$ for 5 min, the supernatant was taken and 1 ml of chloroform and 1 ml of water were added. The samples were vortexed and stood for 30 min for phase separation. The organic layer (3.5 ml) was taken and evaporated to dryness under nitrogen gas in a TurboVap evaporator (Zymark, Hopkinton, MA, USA). The dried residue was dissolved in 100 μl of 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7, v/v/v) and 10 μl was injected into the column.

2.5. Determination of protein concentrations

The protein concentrations of the samples (1 ml aliquot of cell suspension) were determined using the Bio-Rad protein Assay (Bio-Rad, USA). The measured sphingolipids were normalized to the protein concentrations.

2.6. Liquid chromatography and mass spectrometry

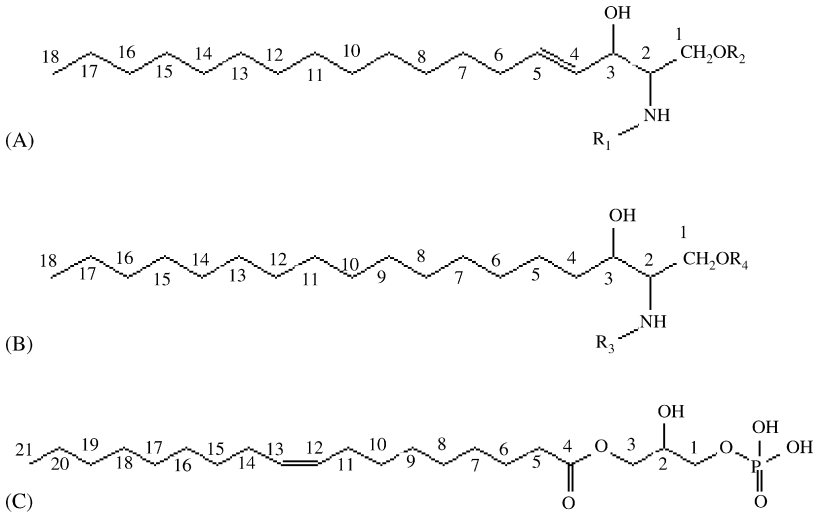
The high-performance liquid chromatography (HPLC) system was performed using LC-10ADvp binary pump system, SIL-10ADvp autosampler and CTO-10ASvp oven (Shimadzu, Kyoto, Japan). The analytical column was an XTerra MS C_{18} (50 × 2.0 mM i.d., 3 μm, Waters, MA, USA) maintained at 60 ± 1 °C. The HPLC mobile phases consisted of 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (5/2/3, v/v/v) (A) and 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7, v/v/v) (B). A gradient program was used for the HPLC separation at a flow rate of 0.2 ml/min. The initial buffer composition was 100% of A, then linearly changed to 100% of B in 17 min and maintained for 1.0 min, followed by re-equilibration to initial condition for 2.0 min. Each run time was 20.0 min.

The HPLC system was coupled on-line to an SCIEX API2000 triple–quadrupole mass spectrometry (Applied Biosystems, Concord, Canada) equipped with a Turbo Ion Spray source. Electrospray ionization (ESI) was performed in the positive mode with an ionspray voltage of 5400 V and in the negative mode with an ionspray voltage of −4100 V. Nitrogen gas was used as the nebulizing, turbo spray and curtain gas with the optimum values set at 30, 80 and 30, respectively (arbitrary units). The heated nebulizer temperature was set at 400 °C. The mass spectrometer operated with low and unit resolution for Q1 and Q3, respectively. Multiple reaction monitoring (MRM)

Table 1
Selected ions and calibration results for sphingolipids ($n = 3$)

Sphingolipids	Selected ion[a] (m/z)	Detection limit (pmol)	Calibration range (pmol)	Regression line				
				Slope		Intercept		R^2[b]
				Mean	S.D.[c]	Mean	S.D.	
C_4 Cer	$370/264^+$	–	–	–	–	–	–	–
C_6 Cer	$398/264^+$	0.4	3.8–754.5	0.01736	0.000071	−0.02	0.020	0.999
C_8 Cer	$426/264^+$	0.1	7.0–704.7	0.0204	0.00011	−0.08	0.030	0.999
C_{10} Cer	$454/264^+$	0.7	3.3–661.2	0.00363	0.000025	−0.003	0.0064	0.999
C_{12} Cer	$482/264^+$	6.2	6.2–622.7	0.001046	0.0000100	−0.006	0.0024	0.999
C_{14} Cer	$510/264^+$	2.9	2.9–588.4	0.001485	0.0000071	−0.001	0.0016	0.999
C_{16} Cer	$538/264^+$	0.3	2.8–557.7	0.0243	0.00014	−0.06	0.029	0.999
C_{18} Cer	$566/264^+$	0.3	2.7–530.1	0.0261	0.00019	−0.04	0.039	0.999
C_{20} Cer	$594/264^+$	0.3	2.5–505.0	0.00932	0.000059	−0.02	0.011	0.999
So	$300/282^+$	1.0	5.0–1001.7	0.01334	0.000070	−0.07	0.027	0.999
So1P	$380/264^+$	–	–	–		–		–
Sa	$302/284^+$	0.5	9.9–995.0	0.01309	0.000065	−0.14	0.024	0.999
SPC	$465/184^+$	1.3	3.2–645.7	0.01169	0.000062	−0.02	0.015	0.999
SM C_{16}	$704/184^+$	–	–	–	–	–	–	–
SM C_{18}	$732/184^+$	–	–	–	–	–	–	–
SM C_{24}	$814/184^+$	–	–	–	–	–	–	–
Cer1P C_{16}	$616/79^-$	–	–	–	–	–	–	–
Cer1P C_{18}	$644/79^-$	–	–	–	–	–	–	–
Cer1P C_{20}	$672/79^-$	–	–	–	–	–	–	–
Cer1P C_{22}	$700/79^-$	–	–	–	–	–	–	–
Cer1P $C_{24:1}$	$726/79^-$	–	–	–	–	–	–	–
LPA	$435/153^-$	–	–	–	–	–	–	–

[a] Selected ion for MRM, parent ion/fragment ionpolarity.
[b] R is the linear correlation coefficients for the calibration.
[c] S.D., standard deviation.



Ceramide: R1 = fatty acid; R_2 = H = H

Ceramide 1-phosphate: R_1 = fatty acid; $R_2 = PO_2(OH)_2$

Sphingosine : R_1 = H; R_2 = H

Sphingosine : R_3 = H; R_4 = H

Sphingosine 1-phosphate: R_1 = H; $R_2 = PO_2(OH)_2$

Sphingomyelin : R_1 = fatty acid; $R_2 = PO_4N^+(CH_3)$

Sphingosylphosphorylcholine : R_1 = H; $R_2 = PO_4N^+(CH_3)$

Fig. 1. Chemical structures of sphingolipids and their metabolites; (A) $d18{:}1^{\Delta 4}$; (B) $d18{:}0$; and (C) lysophosphatidic acid.

detection was employed using nitrogen as the collision gas (4 arbitrary value) with a dwell time of 20 ms for each transition, collision energies of 29, 19, 25, 21, 31, 40, −130, and −30 eV for Cers, So, So1P, Sa, SPC, SMs, Cer1P, and LPA, respectively. The optimal collision energy values were obtained by automatic tuning process with each standard injected by syringe pump. Monitoring ions of each sphingolipid are shown in Table 1. The data acquisition was ascertained by Analyst 1.3.1 software.

2.7. Assay validation

The concentration ranges for calibration curves cover the cellular concentrations expected in our experimental studies. The calibration curves for sphingolipids standards in cellular extracts were generated by plotting the peak area ratio (analyte/IS) versus the concentrations in the calibration standards samples by least-square linear regression.

The accuracy and precision (presented as the coefficient of variation; CV) of the assay were determined using validation samples at three concentrations. Accuracy (%) was determined from percentage ratio of measured nominal concentration (mean of measured/nominal × 100). The CV and accuracy for intra-day were evaluated by the analysis of five samples at each concentration. The CV and accuracy for inter-day assay were assessed for 5 days.

2.8. Application of the assay

For induction of apoptosis, HL-60 cells were treated with 5 μM of C_2 ceramide (*d*18:1/2:0) or 10 μM of hydroperoxide incubated for 30 min, and washed with PBS. Then, the cell suspension was prepared according to Section 2.4, and analyzed by LC–MS/MS. After analysis, percent changes to control cells were calculated by peak area ratio.

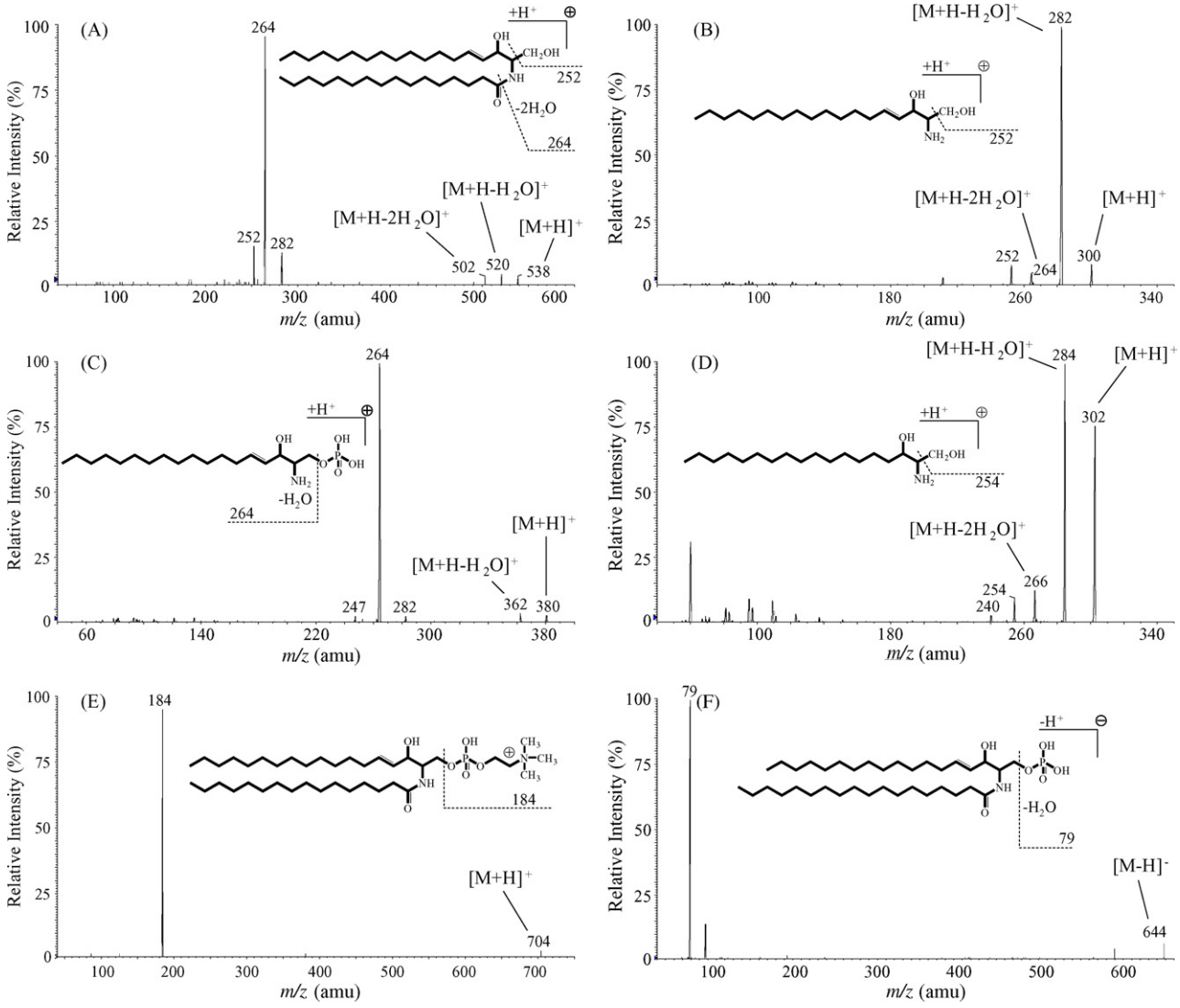


Fig. 2. Product ion mass spectra of (A) C_{16} ceramide (*d*18:1/16:0), (B) sphingosine (*d*18:1), (C) sphingosine 1-phosphate (*d*18:1), (D) sphinganine (*d*18:0), (E) sphingomyelin (*d*18:1/16:0), and (F) ceramide 1-phosphate (*d*18:1/18:0). Major fragment ion with the highest abundance was used for quantitation of each sphingolipid.

3. Results and discussion

3.1. Ionization and mass spectrometric characteristics

Sphingolipids have similar molecular structures with differences in their length of alkyl chains and head groups as shown in Fig. 1. These alkyl chain length and head groups provide distinct chromatographic and mass spectral characteristics of the compounds. All sphingolipids used in this study excluding for ceramide 1-phosphates were analyzed by MS in the positive ion mode. All of the sphingolipids showed a protonated or deprotonated molecular ion in electrospray ionization, and collision-induced dissociation gave simple MS/MS spectra with one or two predominant fragment peaks. Representative product ion mass spectra of protonated ceramide (*d*18:1/16:0), sphingosine (*d*18:1), sphingosine 1-phosphate (*d*18:1), sphinganine (*d*18:0), sphingomyelin (*d*18:1/16:0) and ceramide 1-phosphate (*d*18:1/18:0) are shown in Fig. 2.

Ceramides (Fig. 2A) provided a common product ion at *m/z* 264 by elimination of fatty acid and two hydroxyl groups. Sphingosine (*d*18:1) (Fig. 2B) and sphinganine (*d*18:0) (Fig. 2D) mainly showed the dehydrated ion ($[M+H–H_2O]^+$) in MS/MS. Sphinosine 1-phosphate gave the fragment peak by loss of water and phosphate (Fig. 2C). Sphingomyelins (SM) provided the distinctive product ion at *m/z* 184 based on the phosphocholine group (Fig. 2E). Ceramide-1-phosphates were analyzed in the negative ion mode and the common fragment ion at *m/z* 79 based on the phosphoryl group was observed in MS/MS spectra (Fig. 2F). Detection mode and the fragment ions selected for MRM analysis for the lipid derivatives are summerized in Table 1.

3.2. Selection of the LC conditions

The next step of this study was focused on the optimization of chromatographic separations of 22 different lipid species. The best separation of sphingolipids was obtained using a reversed-phase C_{18} column (XTerra MS C_{18} column). In addition, the best results for peak shape and selectivity could be achieved using 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran system (data not shown). The selective ion chromatograms for standard mixtures of 22 lipid species are shown in Fig. 3A. All classes of sphingolipids including ceramides were well separated and eluted within 15 min. HL-60 cell extract was analyzed according to the above-optimized HPLC condition, and subsequently 12 endogenous sphingolipids including ceramides, sphingosine, sphinganine, sphingomyelin, and ceramide 1-phosphates were successfully identified (Fig. 3B). Most existing analytic methods using LC–MS could be useful only for several limited sphingolipid species whereas our method allows simultaneous analysis of various sphingolipids with good separation and high sensitivity.

3.3. Method validation

The calibration curve for each sphingolipid was constructed at the concentrations ranging from about 2 to 1000 pmol and

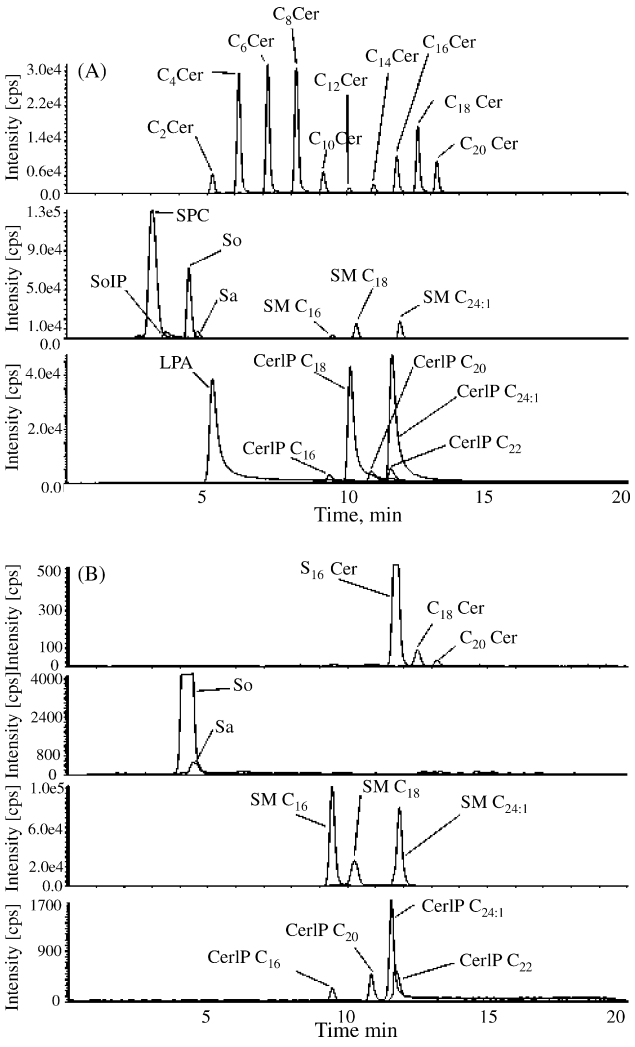


Fig. 3. LC–MS/MS selected ion chromatograms of ceramides and their metabolites: (A) standard mixtures of ceramides and their metabolites and (B) extracts of HL-60 cells. Cer, ceramide; SPC, sphingosylphosphorylcholine; So1P, sphingosine 1-phosphate; So, sphingosine; Sa, sphinganine; SM, sphingomyelin; LPA, lysophosphatidic acid; Cer1P, ceramide 1-phosphate.

processed by weighted least-square linear regression analysis. All sphingolipid metabolites showed good linearity over the ranges of concentration employed with correlation coefficients greater than 0.999. The calibration curves of 11 sphingolipids are described in Table 1. The intra and inter-day variations of 11 sphingolipids are summarized in Table 2. For all sphingolipids tested, the intra-day CVs were less than 7.4%, and the intra-day accuracies were between 96.7 and 114.0% at the low, middle, and high concentrations. The inter-day experience also showed good accuracy and reproducibility with CVs less than 13.8% and accuracies between 95.2 and 115.0%. The detection limits for 11 sphingolipids were ranging from 0.1 to 6.2 pmol with CV and accuracy less than 20%. Collectively, this LC–MS/MS method is considered to be useful for studies on the biological significance of ceramides and the related metabolites. Only mixtures were available in case of sphingosine 1-phosphate, lysophosphatidic acid, sphingomyelins and ceramide 1-phosphates. Therefore, method validation for them was performed by using peak

area ratio of the compounds versus internal standard (data not shown).

3.4. Determination of sphingolipids in human cancer cells by LC–MS/MS after treatment with apoptosis inducing agents

The validated method was applied to analyze the changes of ceramides and the related lipids in HL-60 cells after treatment of apoptosis inducing agents. Endogenous sphingolipid metabolites are present in HL-60 cells at various concentrations (Fig. 3B), and the basal levels of them in HL-60 cells were determined. The concentrations of C_{16} ceramide (*d*18:1/16:0), C_{18} ceramide (*d*18:1/18:0), C_{20} ceramide (*d*18:1/20:0), sphingosine (*d*18:1), and sphinganine (*d*18:0) in HL-60 cells were 202.8, 43.7, 13.7, 32.5, and 15.7 pmol/mg protein, respectively. Sphingomyelins and phsophoceramides could not be quantitated due to the lack of single reference standards but the peaks corresponding to these compounds could be identified in HL-60 cells.

The levels of sphingolipids and its metabolites produced endogenously were measured after the treatment of HL-60 cells with apoptosis inducing agents, C_2 ceramide (*d*18:1/2:0) and H_2O_2. The results were presented as relative percentage of the control (Fig. 4). Significant changes in sphingolipids profile were made by each apoptosis inducing agent, but their patterns were a little different depending on the agent. In both cases, there were significant increases in C_{16} ceramide and ceramide 1-phosphates which are involved in initiation of apoptosis. However, some diffenences were shown in C_{20} ceramide,

Table 2
Intra- and inter-day validation for the determination of sphingolipids in HL-60 cells ($n = 5$)

Compound	Added (pmol)	Intra-day				Inter-day			
		Found (pmol)		CV[a] (%)	Accuracy (%)	Found (pmol)		CV (%)	Accuracy (%)
		Mean	S.D.[b]			Mean	S.D.		
C_6 Cer	3.8	4.0	0.13	3.3	106.9	4.1	0.13	3.2	109.5
	75.4	73	1.9	2.6	97.0	74	2.9	3.9	97.7
	754.5	766	5.3	0.7	101.5	759	9.6	1.3	100.6
C_8 Cer	7.0	7.6	0.15	2.0	107.5	7.4	0.51	6.9	105.3
	140.9	140	2.5	1.8	99.3	142	4.2	3.0	100.6
	704.7	705	9.5	1.3	100.1	710	23	3.2	100.1
C_{10} Cer	3.3	3.4	0.16	4.8	102.3	3.5	0.16	4.6	106.7
	66.1	64	4.1	6.4	96.7	64	3.3	5.2	97.3
	661.2	650	28	4.3	98.0	660	26	3.9	99.6
C_{12} Cer	6.2	7.1	0.53	7.4	114.0	7.4	0.72	9.8	114.3
	124.5	125	3.1	2.5	100.8	119	5.8	4.9	95.2
	622.7	630	14	2.3	100.4	630	13	2.0	100.6
C_{14} Cer	2.9	2.97	0.029	1.0	101.1	3.0	0.14	4.6	102.4
	58.8	59	2.7	4.6	100.7	58	1.5	2.6	99.2
	588.4	594	9.2	1.5	100.9	590	9.1	1.5	100.3
C_{16} Cer	2.8	2.97	0.075	2.5	106.5	3.2	0.44	13.8	115.0
	55.8	56	3.6	6.4	100.5	53	2.8	5.3	95.6
	557.7	550	15	2.6	99.2	560	12	2.2	99.9
C_{18} Cer	2.7	2.70	0.015	0.6	102.0	3.0	0.22	7.3	112.0
	53.0	54	2.7	5.1	101.4	51	3.4	6.6	96.4
	530.1	530	21	3.9	100.3	530	11	2.0	100.3
C_{20} Cer	2.5	2.56	0.039	1.6	106.8	2.8	0.30	10.5	112.1
	50.5	51	1.2	2.3	100.4	49	3.4	6.9	96.7
	505.0	492	9.3	1.9	97.4	500	17	3.4	99.4
So	5.0	4.94	0.028	0.6	98.6	4.8	0.16	3.3	95.2
	100.2	107	2.1	2.0	106.5	103	4.0	3.9	102.6
	1001.7	1050	71	6.7	105.0	1020	47	4.6	101.7
Sa	9.9	10.1	0.37	3.7	101.4	9.7	0.44	4.5	97.3
	199.0	210	13	6.1	103.5	206	7.0	3.4	103.4
	995.0	985	3.5	0.4	99.0	990	12	1.2	99.4
SPC	3.2	3.24	0.078	2.4	100.5	3.4	0.16	4.7	105.2
	64.6	65	4.2	6.4	101.4	65	3.1	4.8	100.5
	645.7	640	21	3.2	98.8	640	13	2.0	99.3

[a] CV, coefficient of variation.
[b] S.D., standard deviation.

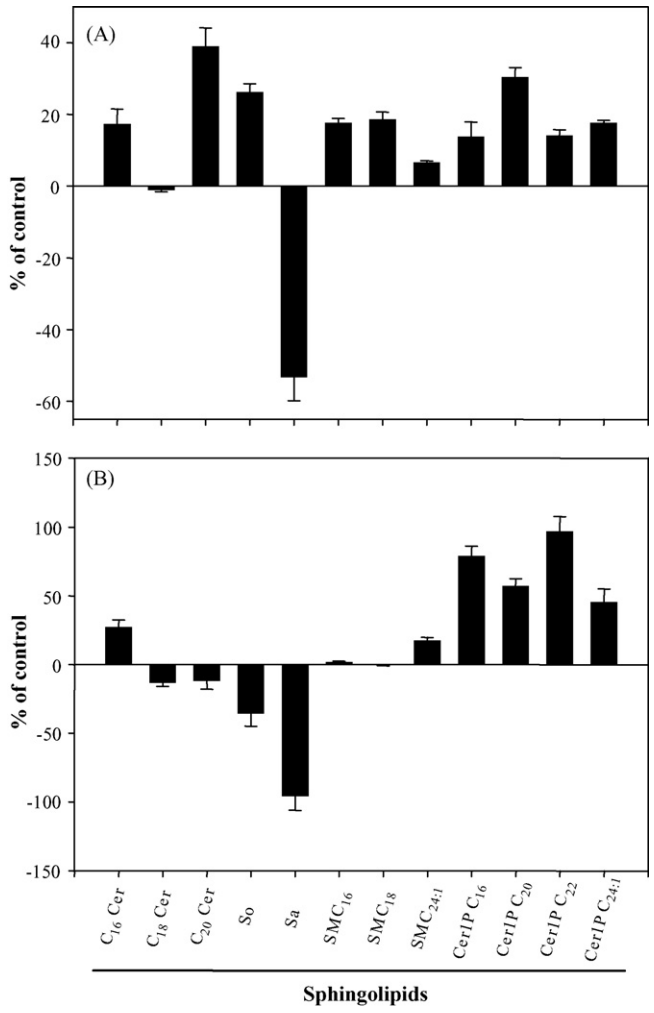


Fig. 4. Changes in the levels of ceramides and related lipid species in HL-60 cells after treatment of apoptosis inducing agents: (A) C_2 ceramide (*d*18:1/2:0) (5 μM); (B) hydrogen peroxide (10 μM); Cer, ceramide; So, sphingosine; Sa, sphinganine; SM, sphingomyelin; Cer1P, ceramide 1-phosphate.

sphingosine, and sphingomyelins levels. The developed analytical method enabled to monitor the overall changes of various sphingolipids in cellular extracts. Although these results are not enough to draw any evident conclusion on the role of each sphingolipid, but, at least suggest that each sphingolipid can be correlated with specific metabolic pathway in cell signal transduction.

4. Conclusion

An LC/ESI/MS/MS analysis method has been developed for the identification and determination of ceramides and related lipids in cellular extracts. The method was successfully applied to monitor the changes of these lipids in HL-60 cells triggered either by exogenous C_2 ceramide (*d*18:1/2:0) or by hydrogen peroxide. The described method can be a valuable tool in the cell signaling cascade studies of sphingolipids cycle.

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