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C₁₈ Ceramide Analysis in Mammalian Cells Employing Reversed-Phase High Performance Liquid Chromatography Tandem Mass Spectrometry^a

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Abstract

Ceramides play an important role in diverse cellular functions such as differentiation, cell cycle progression, cell-cell adhesion, senescence and apoptosis. Here we report a method of extracting lipids from mammalian cells and quantifying ceramide, where the assay conditions were optimized for reproducibility, linearity, recovery, and sensitivity. Simultaneous chromatographic separations were carried out by reversed-phase high performance liquid chromatography coupled to electrospray ionization using a Pursuit 3 Diphenyl (50 × 2.0 mm) column and supported by a mobile phase consisting of acetonitrile plus 0.1% formic acid and 25 mM ammonium acetate. Ceramides were detected in the multiple reaction mode by tandem mass spectrometry in the positive ion mode and all extracted ion peaks were integrated for quantitative analysis. The limits of detection and quantification achieved were 0.2 picogram and 1.0 picogram on column, respectively. Using this method, we successfully quantified and compared differences in C₁₈ ceramide levels induced by two DNA damaging agents, mitomycin C and daunorubicin, and two apoptosis-inducing ligands, TNF- α and TRAIL. This work therefore describes a method that will be helpful for investigating how ceramide is regulated by different chemotherapeutic agents and will help us to better understand the mechanisms of signal transduction involving ceramide.

Keywords

Ceramide; Reversed-Phase HPLC electrospray ionization MS/MS; Quantification; Detection; TRAIL; Daunorubicin; TNF- α ; Mitomycin C; DNA Damage; Apoptosis

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Introduction

Sphingolipids are structural components of the biological membranes of all eukaryotic organisms. The central backbone of all sphingolipids is a sphingoid base which is covalently attached to an acyl chain by an amide bond, called N-acyl sphingosine or ceramide. The species of ceramide are defined by the number of carbon atoms attached to the amide-linked fatty acid moiety, which is linked to the sphingosine backbone shared by all species, though it should be noted that only even numbered carbon ceramides (C_2 to C_{28}) exist in nature [2]. Ceramide has been shown to play an important role in cellular processes such as signal transduction [3; 4], cell proliferation [5], cell-cell adhesion [6; 7], apoptosis [8] and senescence [9].

The generation and quantification of ceramide have received much attention in recent years because of its involvement in the cellular stress response [10; 11], however only a few studies have looked into the specificity of the various species of ceramide. Long chain ceramides, such as C_{16} to C_{24} , are believed to be the species generated following cellular stress, induced by agents such as apoptosis-inducing ligands, DNA damage, or heat shock [12]. We are particularly interested in C_{18} (Figure 1), since it is the most abundant molecular species of ceramide (long chain or short chain) present in mammalian cell membranes [13] and may have the greatest signal transduction effect. Interestingly, C_{18} is less researched than are other species of ceramide such as C_{16} .

Currently, there are a few methods in use for analysis of ceramide. Some of these are thin-layer chromatography (TLC) [14], high-performance liquid chromatography (HPLC), the diacylglycerol kinase assay (DAG) [15; 16], and mass spectrometry (MS) [17]. HPLC assays are more reliable than TLC-based assays for the separation of ceramide, while mass spectrometry is a more powerful tool of analysis than the DAG assay for ceramide detection and quantification, because of its capacity to selectively target specific analytes from a vast array of compounds in biological samples. Developed within recent years for use as an ion source, Electrospray ionization (ESI) possesses the ability to generate intact molecular ions of polar biomolecules and hence the capacity to qualitatively detect and quantitatively measure small molecules in biological fluids or tissues [18]. A recent advancement in mass spectrometry is the triple quadrupole technology, which enables robust ion sources and employs a fast-scanning mass analyzer to yield highly sensitive and reproducible results [18]. The multiple reaction monitoring mode (MRM) is one feature of the triple quadrupole technology which scans both precursor and product ions. This aids in achieving a lower limit of detection and greater sensitivity as opposed to scanning only the precursor ion. To date, separation, detection and quantification of ceramide by chromatography and mass spectrometry has proven to be the most effective technology, and hence is widely used [17; 18; 19; 20; 21; 22]. Optimizing these technologies for the detection and quantification of ceramide has been an area of interest in recent times and currently, there are a few methods with parameters to achieve this. However, these methods are not without limitations, which may include an extensive sample preparation or calibration time, the inability to perform synchronized measurements of different analytes, the requirement for large amounts of sample material, and most importantly, an inability to deliver the desired sensitivity.

In this report, we describe an analytical methodology for extracting, separating, detecting, and quantifying ceramide using advanced reversed-phased HPLC ESI-MS/MS systems. We utilized C₁₇ ceramide (Figure 1) as an Internal Standard (IS) since it has properties that are similar to C₁₈ and other ceramides of mammalian cells, yet it is not a naturally occurring species [18]. To demonstrate the application of this method we examined the generation of C₁₈ ceramide during intrinsic and extrinsic apoptosis using mitomycin C, daunorubicin, TNF- α , and TRAIL.

Mitomycin C is a clinically active antineoplastic agent commonly used in combination chemotherapy regimens to treat a variety of tumors [1; 23] and has been reported to act as an alkylating agent that induces DNA damage by crosslinking the DNA [24; 25]. Because it is the prototype member of a class of drugs referred to as bioreductive agents, elucidation of its mechanism of action continues to generate considerable interest and controversy.

Daunorubicin is an anthracycline which has been reported to mediate cytotoxicity by drug-induced damage to DNA via free radicals, intercalation-induced distortion of the double helix, or by stabilization of the cleavable complex formed between DNA and topoisomerase II [26]. There is, however, controversy regarding its mechanism of action [8; 27; 28]. The extrinsic apoptotic pathway is initiated by the binding of specific signaling molecules to their receptors. Two of these molecules are tumor necrosis factor- α (TNF- α) [29; 30] and TNF-related apoptosis inducing ligand (TRAIL) [29; 31], both members of the tumor necrosis factor family of proteins. Since TRAIL has been shown to induce apoptosis in a wide array of epithelial-derived tumor cells, it has been proposed as a new molecular target in anti-cancer research [32].

Our method provides increased sensitivity, while requiring less sample preparation and calibration times than current options. Using this rapid, exceedingly sensitive, and efficient method, pertinent questions regarding ceramide's role in signal transduction cascades can now be addressed. This general method can also be applied to a variety of other biological systems.

Materials and Methods

Materials

Ceramide (Cer) was purchased from Avanti Polar Lipids (Alabaster, AL). All organic solvents were HPLC gradient, ACS certified, and were of the highest purity and quality commercially available. Mitomycin C was purchased from Roche Applied Science (Indianapolis, IN), dissolved into dimethylsulfoxide (DMSO) as a stock at 4 mg/ml, and then added to cell cultures for a final concentration of 8 μ g/ml. Daunorubicin was purchased from Sigma Aldrich (St Louis, MO) and was dissolved into methanol as a stock at a concentration of 440 μ M, with a final concentration of 10 μ M in cell cultures. TRAIL was purchased from Peprotech Inc (Rock Hill, NJ) and was dissolved in phosphate-buffered saline (PBS) as a stock at a concentration of 100 μ g/ml, and a final concentration of 50 ng/ml was used to treat cells. TNF- α was purchased from R&D Systems (Minneapolis, MN) and was diluted into PBS as a stock at 1 μ g/ml and used at a concentration of 5 ng/ml to treat cells. Cycloheximide was purchased from Sigma (St Louis, MO) and was prepared as a stock at a

concentration of 5 mg/ml and stored at -20°C until use. A final concentration of 5 $\mu\text{g/ml}$ was used in cell cultures along with TRAIL and TNF- α to inhibit *de novo* protein synthesis.

Preparation of Standards

Ceramides were precisely weighed and dissolved in ethanol to produce stock solutions of 0.01 mg/ml for both C₁₇ and C₁₈. Further dilutions were made using acetonitrile to create a series of C₁₇ and C₁₈ working stock solutions (9 levels) of 10, 7.5, 5.0, 2.5, 1.0, 0.5, 0.1, 0.05, and 0 ng/ml. A 500 ng/ml C₁₇ work stock solution was also made for use as an internal standard, also in ethanol. Stock solutions were stored at -80°C and all working stock solutions were stored at -20°C . When ready for use, all working stock solutions were thawed and sonicated at room temperature before use. Standard curves were constructed using unweighted linear regression of the peak areas of the analyte (y) versus the corresponding concentrations of the analyte (x, ng/ml).

Instrumentation

High-performance liquid chromatography (HPLC) was performed using Agilent Technologies Model 1200 (Agilent Technologies, Santa Clara, CA) series equipped with a quaternary pump and linked with an HTC PAL autosampler (CTC Analytics) housing 6 $\times$ 96-well sample plates. A Pursuit 3 Diphenyl reversed-phase column, 50 $\times$ 2.0 mm i.d. 3 μm (Varian, Walnut Creek, CA) was chosen for the separation of ceramide. The injection volume was 25 μl . The temperature of column and the autosampler was room temperature. The needle wash was performed for 5 seconds in MeOH. The mobile phases were - A: 0.1% formic acid, and 25 mM ammonium acetate in water, B: 100% acetonitrile. The flow rate was 0.8 ml/min with the gradient represented by following times and percent of mobile phase B: 0.10, 30; 1.10, 95; 3.00, 95; 3.20, 95; and 5.00, 30. Each sample ran for a total of 5 minutes, including re-equilibration. The ion source employed was the electrospray ionization interface (ESI) of an Agilent Technologies 6410A Triple Quadrupole MS/MS system (Agilent Technologies, Santa Clara, CA) and ionization was performed in the positive ion mode. The ESI was maintained with gas temperature of 300°C and drying gas (nitrogen) flow of 10 liters/minute resulting in nebulization pressure of 45 psi. The capillary (ion spray) voltage was 4500 V and the Electron-Multiplier-Voltage (EMV) was 600 V. The multiple reaction monitoring (MRM) mode was operated at a dwell time of 150 ms, collision energy was 26 V and the fragmentor was 130 V for both compounds. Monitoring ions were m/z 566.5 (precursor ion) - m/z 264.2 (product ion) for C₁₈ and m/z 552.5 (precursor ion) - m/z 264.2 (product ion) for C₁₇ which served as the internal standard (IS). The instrumental MassHunter software with qualitative analysis and quantitative features were used to process the data.

Cell Culture

U2OS cells, derived from a human osteosarcoma, were obtained from the ATCC (Manassas, VA) and were cultured in McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented to contain 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g/ml}$) (Sigma). 1×10^6 cells were cultured in 6 cm plates for 24 hours in medium for adherence before being used for experiments.

Extraction Procedure

Since the organic solvents used for extraction affect the recovery of lipids, we investigated the effect of using methanol with chloroform and acetonitrile with chloroform. We noted that when acetonitrile/chloroform (1:2) was used for extraction, there was approximately 60% less yield than when methanol/chloroform (1:2) was used for the extraction of C₁₈, while no change was noted for C₁₇. Hence we conclude that extraction using methanol/chloroform (1:2) is efficient for both C₁₈ and C₁₇. Cells were harvested at the indicated times by removing medium and washing adherent cells with 1 ml PBS. 1 ml of methanol was added to plates and cells were scraped and transferred into a 5.5 ml glass vial with screw top. Cells were sonicated for 5 minutes and 100 µl of the solution was removed for protein concentration measurements. 20 µl of a 500 ng/ml C₁₇ solution were added to the remaining cells in the glass tube to obtain a final concentration of 10 ng of C₁₇ internal standard (IS) by the end of the extraction procedure. 2 ml of chloroform were added to the cells and the suspension was vortexed for 5 seconds followed by 30 minutes of sonication. Lysates were spun for 5 minutes at 3000 rpm. The lower chloroformic layer was transferred to a new tube leaving the upper methanolic and the middle proteinous layers. When we extracted lipids and performed HPLC-MS/MS after a single extraction with methanol/chloroform (1:2), we found that the recovery of C₁₇ was below 50%, while the recovery of C₁₈ was over 75%. However, double extraction with methanol/chloroform (1:2) yielded over 92% recovery for both C₁₇ and C₁₈. Hence, the upper methanolic and the middle proteinous layers from the first extraction were subjected to a second extraction. 1 ml of methanol and 2 ml of chloroform were added to the upper layers for re-extraction and the tube containing the chloroformic layer was closed and placed on ice. Re-extraction was performed by vortexing, sonicating, and spinning as indicated above. Again, the lower layer was removed and added to the tube on ice, which was then dried under low nitrogen gas at room temperature. The samples were reconstituted with 1 ml acetonitrile, vortexed and stored at -80°C until analysis. All ceramide measurement experiments were normalized for protein content using BioRad's D_c Protein Assay (BIORAD Laboratories, Hercules, CA). We dissolved our cell suspension in 1% Sodium Dodecyl Sulfate (SDS).

Accuracy and Precision of the Assay

Analytical errors were investigated by using theoretical (spiked) calibration samples. Working solutions of C₁₇ or C₁₈ were added to either methanol alone or to a suspension of U2OS cells in methanol to achieve the indicated concentrations by the end of the extraction procedure. Lipids were extracted on the same day and three replicates of each sample were analyzed. All tabulated results were standardized based on the endogenous and/or background concentrations detected for each zero point in a sample run and calculated as the difference between the detected concentration for each concentration point and the zero point concentration. Endogenous concentration refers only to the zero point for C₁₈ since C₁₇ is a non-naturally occurring ceramide. The recovery was determined as a percentage [calculated detected concentration (with cells)/detected concentration (no cells)] × 100.

Results

Validation of the Method

It has been reported that to increase the sensitivity for detecting ceramides, ESI should be performed in the positive ion mode [21]. We injected 2.5% of the extracted lipids on the Pursuit 3 Diphenyl reversed-phased column (50×2.0 mm i.d. $3 \mu\text{m}$) and applied a flow rate of 0.8 ml/minutes for a period of 5 minutes. Ceramides were stable on each sample run during the ESI giving the protonated molecular ion as the major ion, also aided by the addition of 25 mM NH_4Ac to the mobile phase A in order to minimize the formation of salt-adducts. Using tandem mass spectrometry (MS/MS) and MRM to increase our selectivity and to minimize the time required to scan and detect our ions of interest, we identified the precursor (566.5 for C_{18} and 552.5 for C_{17}) and product (252.1 and 264.2 for C_{18} and 264.2 for C_{17}) ions of C_{18} and C_{17} (Table 1). The common product ion at m/z 264.2 was due to the loss of fatty acid and two hydroxyl groups from the compounds [33].

C_{17} and C_{18} solutions were injected individually to find the retention time (RT). The individual retention time for both C_{17} and C_{18} was 2.27 minutes. A solution containing both C_{17} and C_{18} was injected under the same conditions to assess whether the combination of both had any effect on the RT. As is shown in Figure 2, there was no effect on the RT. One highlight of our analytical method was that the total run time for each sample including calibration time was only 5.0 minutes. With this short run time, we could analyze up to 288 samples per day.

Linearity, Limit of Detection, and Limit of Quantification

Three sets of standard curve samples were prepared using the nine (9) calibration standards as described in the experimental procedures section. Calibration standards were consecutively injected from the lowest concentration to the highest concentration and all data points were collected on the same day to create the calibration curve. Two blank samples (acetonitrile) were also run between each calibration standard. Unweighted linear regression was used to fit all data points into a single curve. Analysis was repeated on the following 2 days and Figure 3 shows a typical standard curve. The standard curve can be extended up to 50 ng/ml and maintain a R^2 value of 0.9972. The limit of detection (LOD) achieved was 0.2 picogram (or 0.3 fmol), defined as a peak signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) was 1.0 picogram on column (or 1.7 fmol) represented by the lowest point on the calibration curve. Analyses were reproducible and accurate. The Relative Standard Deviation (RSD) ($n=6$) for quantification (peak area) as demonstrated by three quality control (QC) samples for C_{18} were as follows: 1 pg on column (0.05 ng/ml) was 6.2%, 20 pg on column (1 ng/ml) was 2.9% and 200 pg on column (10 ng/ml) was 5.8% for C_{18} .

Recovery

For any analytical method to be useful, recovery is important. To optimize recovery, all extraction and analysis procedures were performed using glassware [34]. We assessed recovery using five levels of spiked (theoretical) samples, either with or without 1×10^6 U2OS cells. Both C_{17} and C_{18} were used to spike samples at the same concentration, and

extractions were carried out together for all samples. Samples were analyzed on consecutive days. Our results show that our method yielded very high recovery for both C₁₇ and C₁₈ from cells (Table 2). C₁₇ recovery ranged from 92.2% to 98.9% while C₁₈ recovery ranged from 92.2% to 105.2%. Our calculations show our ability to recover C₁₇ and C₁₈ from samples that included cells, which is the most relevant calculation for our purpose.

Application of Method

The development of our method for analyzing C₁₈ ceramide was applied to determine ceramide's involvement in the mechanism of DNA damaging and apoptosis-inducing agents. We utilized two DNA damaging agents, mitomycin C and daunorubicin, and two apoptosis-inducing agents, TNF- α and TRAIL, to treat U2OS cells for up to 16 hours. Here we show that daunorubicin induced a nearly 250% increase in ceramide levels by 8 hours, and maintained this level for up to 16 hours. In contrast, treatment with mitomycin C induced only a 50% increase in ceramide by 8 hours, increasing by another 10% within 16 hours (Figure 4a). When comparing TNF- α and TRAIL similar, but greater, effects were seen. TNF- α caused a 400% increase by 6 hours, which decreased with time, while TRAIL did not significantly alter the ceramide levels when compared to TNF- α (Figure 4b). This data demonstrates that ceramide is strongly induced following exposure of U2OS cells to daunorubicin and TNF- α , much less induced following exposure to mitomycin C, and not induced following exposure to TRAIL.

Discussion and Conclusion

We have quantitatively optimized the detection for the C₁₇ and C₁₈ molecular species of ceramide by their fragmentor and collision energies (ionization and dissociation), enhancing the sensitivity while ions are being monitored concurrently irrespective of their differences in dissociation kinetics. By optimizing these two parameters we enhanced sensitivity by preventing molecular decomposition before entry into the first quadrupole and this compensates for difference in the rates of dissociation of the various molecular species [18].

Our IS was added prior to extraction of lipids to enhance its use from being only an IS for HPLC-MS/MS to being used concurrently as an extraction standard. This is a more efficient process when compared to adding the IS immediately before HPLC-MS/MS. Additionally, we have controlled for extraction losses by protein quantifications using aliquots from the solution for extraction immediately before the extraction process is started. Experiments for assessing the efficiency of the HPLC-MS/MS section of our method as it relates to losses were carried out using samples that were spiked with known concentrations of either C₁₇, C₁₈, or both immediately before HPLC-MS/MS. Separate runs were compared for reproducibility.

The LC/MS/MS-MRM method developed in this paper demonstrated the lowest detection (LOD is 0.2 picogram or 0.3 fmol) and quantification limit (LOQ is 1.0 picogram or 1.7 fmol) for the quantification of ceramide in biological entities compared with previous methods whose limits included: 4 nmol [35-36], 13 pmol [22], 100 pmol [37], and 25 pmol [38], 50 fmol [39], and 10 fmol [40]. This method was also validated by good recoveries of 92.2% to 98.9% for C₁₇ and 92.2% to 105.2% for C₁₈, good reproducibility

(Table 2) and linearity ranged from 0.05 ng/ml (equivalent to 1.0 picogram on column with 20 μ l injection) to 10 ng/ml (equivalent to 200 picogram on column with 20 μ l injection) (Figure 3) when compared to other methods [41-43]. This linearity was extended to 50 ng/ml (equivalent to 1000 picogram on column with 20 μ l injection, and data not shown).

Time is of the essence with all procedures, and so we aimed to minimize the sample preparation and calibration times. We achieved this and found that it took one person approximately 4 hours and 30 minutes to prepare 50 samples and 4 hours and 10 minutes to calibrate and analyze these 50 samples. This means that using our method, we can both prepare and analyze 50 or more samples in any given work day. Compared with previous method, our LC/MS/MS-MRM method significantly reduced the time for sample analysis (5 minute per sample for running LC/MS/MS analysis employed in our method versus 15 minutes or longer needed in other methods).

Next, we have applied this LC/MS/MS quantification method for the measurement of C18 ceramide in cell lines treated with different kinds of reagents. As expected, we found that treatment of U2OS cells with TNF- α induced an easily measured, five-fold increase in the level of cellular ceramide. TNF- α is a well-known inducer of ceramide, and has been reported to induce apoptosis via a sphingomyelinase-mediated breakdown of sphingomyelin to ceramide [8; 44; 45; 46]. Our positive results in this system, which correlate well with previous findings using other analytical approaches, serve to validate our method.

Unlike the case with TNF- α , where there is general agreement that engagement of the pathway leads to the induction of ceramide, the ability of TRAIL to induce ceramide production is much less defined, and it may be that this ability is cell-type specific. Our laboratory used an indirect approach, the use of an inhibitor, to show that TRAIL-induced apoptosis is probably not mediated by ceramide pathways in U2OS cells [47]. Similar conclusions were also reached by another group [48]. However, it should be noted that Dumitru and Gulbins, by using four different cell types: T-splenocytes, BJAB, L929 and A549 cells, and utilizing DAG to measure ceramide, found that TRAIL was indeed able to induce a ceramide response [49]. Furthermore, they found that this increase was mediated by the activation of acid sphingomyelinases. In our study, we found no significant increase in ceramide levels following treatment of U2OS cells with TRAIL, providing further evidence that the TRAIL response is cell-type specific. It could be argued that the lack of a ceramide response by TRAIL could be due to the resistance of U2OS cells to this cytokine [50]; however, we have previously shown that U2OS cells are, in fact, sensitive to TRAIL and die *via* apoptosis [47]. In any case, the sensitivity of our method, which is significantly greater than the methods previously used, allows us to conclusively demonstrate that TRAIL does not induce the generation of ceramide, at least in U2OS cells.

Daunorubicin has been reported to induce ceramide-mediated apoptosis [27; 51; 52], and the mechanism was reported to be through *de novo* synthesis [27]; [28]. Consistent with this, our study showed a greater than three-fold increase in ceramide following treatment with daunorubicin. Currently, little is known about the effect of mitomycin C on ceramide generation. One study utilized the DAG assay and reported a 4.0 fold of elevation of ceramide following mitomycin C treatment using non-aged cells (IRM90, human diploid

lung fibroblasts) [53]. In addition, it has been reported that ceramide is generated by the hydrolysis of sphingomyelin following treatment of cells with mitomycin C [34]; this is the same pathway of ceramide generation reported for TNF- α . In our analysis, we found that compared to TNF- α and daunorubicin, mitomycin C generated a relatively small ceramide response of about 50%. This indicates that the degree of ceramide generation after DNA damage with mitomycin C may be cell-specific.

Our method demonstrates high accuracy, reproducibility, and linearity for C₁₇, C₁₈ and for C₁₇ and C₁₈ when analyzed together. Lipids were extracted from cells using a double extraction procedure using methanol:chloroform, 1:2 and were analyzed using reversed-phased HPLC-MS/MS on a Pursuit 3 Diphenyl column and in the positive ion mode. Our method is fast, accurate, and reliable for quantifying ceramide in mammalian cells. This analytical method can enhance investigations into how ceramide is regulated by different chemotherapeutic agents as well as the mechanism(s) of signal transduction where ceramide is involved. It can also be applied to other biological systems.

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Figure 1.
Molecular structures of C₁₈ and C₁₇ (Internal Standard)

Figure 2.

Ion chromatograms of MRM scans of C₁₈ and C₁₇ overlapped. Both C₁₈ and C₁₇ were analyzed together in the same sample and assessed for their retention times.

Figure 3.

Standard Curve for quantification of C₁₈ ceramide. A typical C₁₈ standard curve was constructed using unweighted linear regression of the peak areas. The x-axis is the concentration of the analyte in ng/ml and the y-axis shows the peak area of the analyte. The R² for this peak is 0.9976 and is an average R² obtained from three experiments. Nine (9) levels between 0 and 10 ng/ml were used.

a **b****Figure 4.**

a - The ceramide response to DNA damaging agents. Daunorubicin (DRB) (10 μ M) and Mitomycin C (MMC) (8 μ g/ml) were used to treat U2OS cells for 0, 8, and 16 hours before harvesting, extracting, and analysis for ceramide content by HPLC-MS/MS. All experiments were performed in triplicates and error bars represent the standard deviation from the mean. The level of ceramide without any DNA damage was the zero time point.

b - The ceramide response to apoptotic cytokines. TNF- α (5 ng/ml) and TRAIL (50 ng/ml) were used to treat U2OS cells at 0, 6, 12, and 16 hours before harvesting, extracting, and analysis for ceramide content by HPLC-MS/MS. All experiments were performed in triplicates and error bars represent the standard deviation from the mean. The level of ceramide without any treatment with apoptotic cytokines was the zero time point.

Table 1Parameters optimized for C₁₇ and C₁₈

	RP-HPLC Retention Time (mins)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Dwell (msec)	Collision Energy (V)
>C ₁₇ Ceramide (ISTD)	2.27	552.5	264.2	130	150	26
^a C ₁₈ Ceramide	2.27	566.5	252.1	130	150	26
^b C ₁₈ Ceramide	2.27	566.5	264.2	130	150	26

^aQualifier ion^bQuantifier ion

Table 2Recovery of C₁₇ and C₁₈

C ₁₇			
Theoretical Concentration ^a	Detected concentration (without cells) ^b	Detected concentration (with cells) ^c	% Recovery ^d
0	0	0.033	-
1	1.3452	1.2497	92.9 ± 2.7
5	5.0247	4.7534	94.6 ± 3.2
10	9.6811	9.5746	98.9 ± 4.0
50	50.0544	46.1501	92.2 ± 1.1
C ₁₈			
Theoretical Concentration ^a	Detected concentration (without cells) ^b	Detected concentration (with cells) ^c	% Recovery ^d
0	0	0.623 ^e	-
1	1.196	1.2582	105.2 ± 2.7
5	5.0854	5.0396	99.1 ± 2.9
10	10.1892	9.9345	97.5 ± 3.2
50	50.0742	46.1684	92.2 ± 2.4

^aSolutions of the indicated ceramide in acetonitrile (ng/ml)

^bThe mean of the triplicate measurement of spiked solutions into methanol (no cells present) (ng/ml)

^cThe mean of the triplicate measurement of spiked solutions into methanol (cells present) (ng/ml)

^dCalculated as the percentage of the detected concentration (with cells) (triplicate average) compared with the detected concentration (without cells) (triplicate average)

^eEndogenous level of C₁₈