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# Observation of different ceramide species from crude cellular extracts by normal-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry

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**Normal-phase high-performance liquid chromatography (NP-HPLC) coupled to atmospheric pressure chemical ionization mass spectrometry (APCI-MS) allows qualitative analysis of endogenous ceramide and dihydroceramide species from crude lipid extracts utilizing chromatographic methods readily adaptable from commonly used thin layer chromatography (TLC) conditions. Qualitative information for the species comes from observation of differences in chromatographic and mass spectrometric behavior between species. Application to the analysis of ceramide and dihydroceramide from various cell lines is demonstrated. The results show the species profile in each cell line to be unique despite growth under identical conditions. The results from APCI-MS analysis corroborate and enhance information acquired from use of the diacylglycerol kinase assay for total ceramide measurement. This technique readily allows the previously difficult distinction between ceramide and dihydroceramide species. Copyright © 2003 John Wiley & Sons, Ltd.**

Multiple lines of research suggest an important role for ceramide in such diverse responses as the ability of cells to grow and divide,[1] cell differentiation,[2] cell cycle arrest,[3] cell senescence,[4] and apoptosis.[5] The observation that ceramide production increases in response to a broad spectrum of stress inducers and the nature of ceramide-mediated responses suggest a key role for ceramide as a mediator of intracellular stress signals.[6,7] The realization that upon agonist stimulation sphingomyelin (SM) serves as a reservoir for the release of ceramide led to the view that the SM cycle was a major pathway to ceramide formation.[8] According to this paradigm, agonist stimulation induced sphingomyelinase activation resulting in the cleavage of SM to ceramide and phosphorylcholine. The resulting increase in ceramide and decrease in SM production is usually transient, however, owing to the actions of sphingomyelin synthase in SM resynthesis and ceramide consumption.[8,9] It is now well established, however, that this is not the only route of ceramide generation and removal. The true complexity of ceramide metabolism is based on the possibility that modulation of ceramide levels can be the result of any number of available metabolic pathways (Fig. 1). Examples of alternate routes of ceramide formation include the *de novo* pathway of ceramide synthesis, which has shown importance in mediating apoptotic effects[10] and glucocerebrosidase-mediated cleavage of glucosyl ceramides.[11,12]

At this point the relevance of the specific route of ceramide generation to specific functions is not known. Neither do we know if there are differences in structure or subcellular localization of the ceramide generated by these different pathways. What is known, however, is that several variations of ceramide structure exist. In general, ceramide (Fig. 1) is comprised of a sphingosine backbone and fatty acid joined by an amide bond. The biologically important features of the molecule appear to be located at carbons 1–5 of the sphingosine backbone and consist of a primary and secondary hydroxyl group at the C1 and C3 positions, respectively, an amino group which serves the fatty acid linkage at C2, and a *trans* double bond located across C4 and C5. Significant variations in the sphingosine backbone include the absence of the double bond (resulting in dihydroceramide) and the presence of the additional C4 hydroxyl group for dihydroceramide (resulting in phytoceramides). Significant variation can also occur in the amide-linked fatty acid moiety including variations in chain length, unsaturation, or hydroxylation.[13] Studies have suggested that dihydroceramide does not have the biological activity of ceramide,[14] but the significance and activity of other variations is as yet undefined. These

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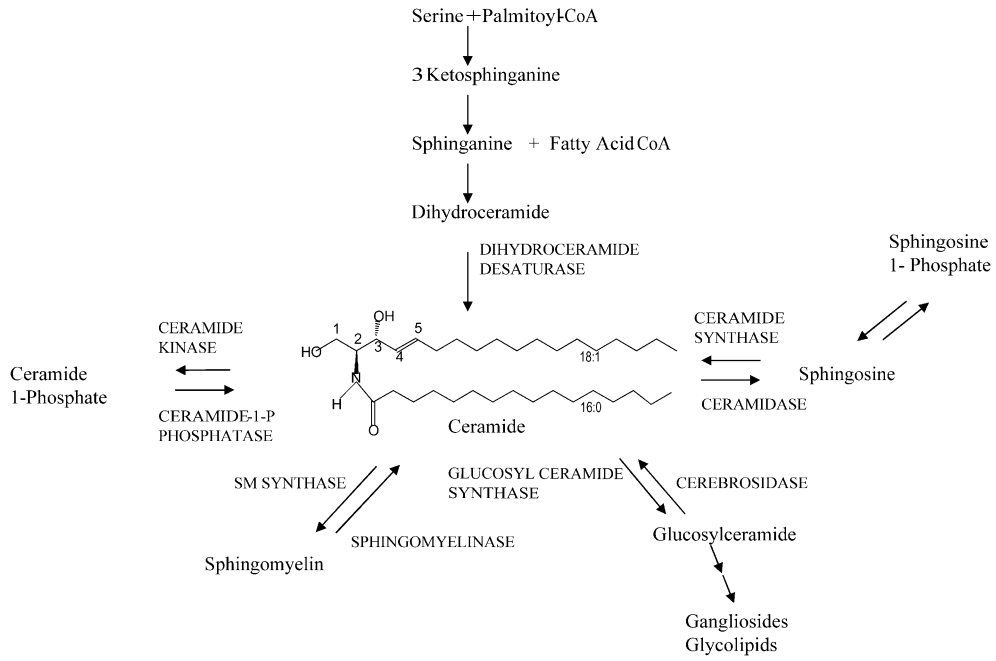


**Figure 1.** Structure and metabolism of ceramide. The complexity of ceramide metabolism is indicated by the number of possible routes of ceramide generation and removal. Enzymes directly acting to generate or remove ceramide are also labeled. *De novo* ceramide is generated by the top metabolic pathway ending in dihydroceramide desaturase which makes the final conversion from dihydroceramide to ceramide. Other possible routes to generate ceramide include ceramide synthase, cerebrosidase, sphingomyelinase, and ceramide phosphate phosphatase. Alternatively, ceramide levels can be cleared by ceramidase, glucosyl ceramide synthase, sphingomyelin synthase or ceramide kinase.

possibilities may, however, hold answers as to how one molecule can be implicated in so many regulatory responses.

The importance of this class of lipid molecules has already led to the development and/or use of several techniques for ceramide analysis including the diacylglycerol kinase (DGK) assay,[15,16] metabolic labeling followed by quantitation on thin layer chromatography (TLC),[17] and derivatization for HPLC analysis.[18] The DGK assay has come to be the leading method for the rapid and convenient determination of total ceramide mass levels.[15,19,21] This assay is unable to distinguish either the biologically active ceramide from its inactive metabolic precursor dihydroceramide or changes in individual ceramide species. A combination of the above approaches is often used to attempt to circumvent these limitations. However, the emerging complexity of ceramide formation involving multiple regulatable metabolic enzymes and their possible topological restriction to specific compartments have necessitated use of more specific tools examining individual molecular species of ceramide and dihydroceramide.

MS methods have been shown as viable tools for lipid analysis[20] without need for chemical derivatization.[21,22] Recent development of these methods has been based on the introduction of atmospheric pressure ionization techniques for interfacing between liquid flow and MS. In the atmospheric pressure ionization techniques, analyte molecules are ionized at atmospheric pressures and then transferred to the high-vacuum conditions ordinarily required for mass spectrometric analysis. A leading method for achieving this interface has utilized electrospray ionization (ESI). Another interface between HPLC and MS that has shown promise is atmospheric pressure chemical ionization (APCI). Both the ESI and APCI ionization techniques have specific advantages and disadvantages that have been extensively reviewed.[23,24]

Despite the wealth of literature available, the choice of an appropriate LC/MS interface depends on the application and is not always obvious. Among previous reports, some have focused on multiple sphingolipid-based compounds with little interest in the specific ceramide composition. Others have been designed for more qualitative determination of ceramide composition, and still others have directed efforts for quantitation to those species found to be changing in their system. Even among those interested in the ceramide species, however, none simultaneously analyze both ceramide and dihydroceramide species. Investigators have utilized both ESI and APCI, each with either direct infusion or an HPLC interface.[20,25–39] In general, the methods share use of precursor ion scanning functions, most frequently conducted with use of a triple quadrupole mass spectrometer. Sullards has recently applied a variety of MS scan functions to the analysis of sphingolipids by ESI-LC/MS and LC/MS/MS on a triple quadrupole mass spectrometer. In this work they showed that quantitation methods based on LC/MS/MS with multiple reaction monitoring could have distinctive advantages over methods based on other types of LC/MS or LC/MS/MS scans.[31] Recently, ion trap mass spectrometry detectors with capability for tandem mass spectrometric detection have been used for quantitative LC/MS/MS analysis.[19]

In light of reports of highly effective LC/MS methods, we set out to adapt commonly used separation methods in our

laboratory, such that MS measurements could add molecular detail to support and confirm our preliminary work. We further sought to use MS methodologies that would minimize matrix effects, such as ion suppression, that could modify analyte response, facilitating qualitative comparisons of both ceramide and dihydroceramide species levels.

These goals led us to the use of normal-phase high-performance liquid chromatography (NP-HPLC) on silica stationary phases coupled to APCI-MS detection in an ion trap mass spectrometer. NP-HPLC seemed to be a natural fit, as this would allow utilization of existing expertise in NP separations on silica utilizing TLC. The coupling to APCI was made to take advantage of the greater applicability of APCI for hydrophobic analytes.[21] Suitability of the method for high-throughput analysis along with required quality control measures is described. Comparison of the method to well-accepted current methodologies such as the DGK assay for total mass analysis is noted.

## MATERIALS AND METHODS

### Materials

Ceramide type III (obtained from bovine brain sphingomyelin) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ceramide standards were synthesized from D-erythro-sphingosine and D-erythro-dihydrosphingosine backbones purchased from Avanti (Alabaster, AL, USA) as previously described.[40] Ramos cells (EBV-negative Burkitt lymphoma), HL-60 cells (promyelocytic leukemia), Molt-4 cells (acute lymphoblastic leukemia), and MCF-7 cells (breast adenocarcinoma, pleural effusion) were from ATCC (Rockville, MD, USA). RPMI 1640 (GIBCO, Gaithersburg, MD, USA), 10% FCS (Summit Biotechnology, CO, USA), 25 mM Hepes, 2 mM L-glutamine (GIBCO), 100 units/mL penicillin and 100 units/mL streptomycin (GIBCO) were purchased for cell culture. HPLC-grade ethyl acetate and iso-octane were purchased from Burdick and Jackson (Muskegon, MI, USA) for HPLC separation on Iatrobead (Iatron Laboratories, Tokyo, Japan) beaded silica columns ($4.6 \times 150$ mm, 5 micron). The helium bath/damping gas for the operation of the LCQ analyzer was of research grade (National Welders Supply, Charlotte, NC, USA).

### Cell culture

Ramos cells (EBV-negative Burkitt lymphoma), HL-60 cells (promyelocytic leukemia), Molt-4 cells (acute lymphoblastic leukemia), and MCF-7 cells (breast adenocarcinoma, pleural effusion) were grown in RPMI 1640 supplemented with 10% FCS, 25 mM Hepes, 2 mM L-glutamine, 100 units/mL penicillin and 100 units/mL streptomycin under standard incubator conditions (humidified atmosphere, 95% air, 5% $CO_2$, 37°C).

Cells were split from $1 \times 10^6$ cells/mL in 10% FCS, washed to remove media containing secreted IgM and plated at $0.4 \times 10^6$/mL in 2% FCS and allowed to equilibrate for 2 h prior to treatment. The goat anti-human IgM Fc5u fragment specific antibody (Jackson Immunoresearch, PA, USA) was incubated at 10 μg/mL with Ramos cells for 6 and 24 h, extracted by the method of Bligh and Dyer,[41] and used for determination of lipid phosphate, and analyzed by DGK and MS ($\sim 2 \times 10^6$ cells for each).

### Sample preparation

For analysis, 1–2 million cells were pelleted, harvested in methanol, and extracted by the method of Bligh and Dyer.[41] Aliquots of the lower organic phase were used for phosphate determination and for MS analysis.

### NP-HPLC

All separations were conducted using Iatrobead beaded silica columns ($4.6 \times 150$ mm, 5 micron) in the normal-phase mode of operation. This specially formulated silica column was chosen because of its normal-phase properties coupled with much higher stability than ordinary silica columns. Elutions were completed on an Agilent (Palo Alto, CA, USA) model 1100 HPLC system equipped with a binary pumping system, with a flow rate of 0.5 mL/min and gradient elution as follows. A: 0–1 min, 100% iso-octane; B: 1–16 min, ramp from 100% iso-octane to 100% ethyl acetate; C: 16–30 min, 100% ethyl acetate; D: 30–35 min, ramp from 100% ethyl acetate to 100% iso-octane.

### APCI-MS

MS analyses were conducted using a ThermoFinnigan (Foster City, CA, USA) LCQ ion trap mass spectrometer. The nebulization and auxiliary gas flows for the ion sources were obtained from the boil-off from the house liquid nitrogen supply. For APCI-MS, the entire flow from the HPLC column was directed to the APCI source. The nebulization probe heater was operated at 450°C. For all experiments, source ion optics were adjusted to accomplish desolvation of ions while minimizing fragmentation of analyte ions in the inlet region of the mass spectrometer. (**WARNING**: Owing to the combustible nature of the normal-phase solvents coupled with the high voltage of APCI, it is essential to use a nitrogen rather than a compressed air supply and to divert the MS drainage tube to hood ventilation.) The triple quadrupole instrument utilized a 90:10 mixture of argon/nitrogen for the collision gas in the second quadrupole region during MS/MS experiments requiring collision-induced dissociation (CID).

### Diacylglycerol kinase assay

Ceramide level evaluation was performed using the *E. coli* diacylglycerol kinase assay.[15,18,21] Briefly, the lipids were incubated at room temperature for 30 min in the presence of β-octylglucoside/dioleoylphosphatidylglycerol micelles, 2 mM dithiothreitol, 5 μg of proteins from the diacylglycerol kinase membranes, and 2 mM ATP (mixed with [γ-32P]-ATP in a final volume of 100 μL). After the lipids had been extracted by the Bligh and Dyer method,[41] the reaction products were separated by TLC in chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5), and the radioactivity associated with the phosphatidic acid and the ceramide phosphate was measured. Ceramide and diacylglycerol levels were quantitated by using external standards and were normalized to phosphate.

## RESULTS

Initially, $C_{16}$ ceramide and $C_{16}$ dihydroceramide standard solutions were infused directly to determine the feasibility of ionization using an APCI source. MS conditions, including

potentials on ion optic components and temperatures on the inlet capillary, were adjusted to optimize ionization and fragmentation patterns for later analysis. It was observed that ceramides produced singly protonated ions that would lead to facile single loss of $H_2O$. Collisional activation prompted further fragmentation leading to loss of up to two $H_2O$ molecules and/or a fatty acid moiety. Commonly, mass product ions with an *m/z* of 264 for ceramides and 266 for dihydroceramides were observed. Standards could be shown to undergo two successive dehydration reactions from the sphingosine/sphinganine bases upon collisional activation. These observations correlate well with previous reports for other related compounds.[20,31] Figure 2 shows MS/MS spectra for standard $C_{16}$ ceramide and $C_{16}$ dihydroceramide. Figure 2 also shows the structures of $C_{16}$ ceramide (Fig. 2(A)) and $C_{16}$ dihydroceramide (Fig. 2(B)) and lists the *m/z* values for the fragments obtained under collisional activation. The relative ease of the initial loss of $H_2O$ for $C_{16}$ ceramide is shown by near complete removal of the *m/z* 538 precursor ion, and the absence of the *m/z* 300 and 282 fragments (suggesting rapid dehydration prior to cleavage of the fatty acid chain). In contrast, the presence of residual unfragmented *m/z* 540 precursor ions under similar activation conditions indicates a higher stability for the $C_{16}$ dihydroceramide to dehydration, although an initial

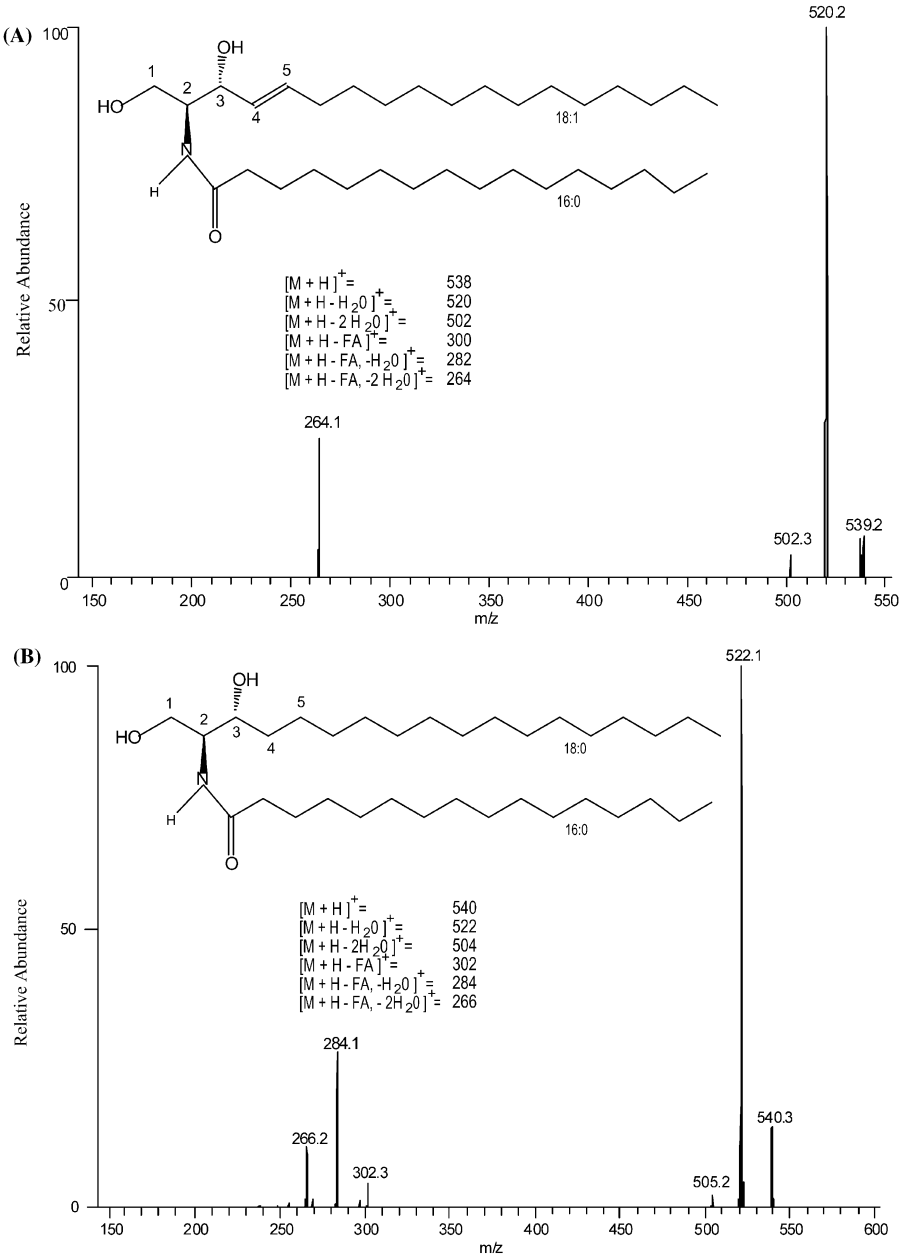


**Figure 2.** Direct Infusion MS/MS of (A) $C_{16}$ ceramide (*m/z* 538) and (B) dihydroceramide (*m/z* 540). In order to provide qualitative analysis of ceramide structure, standards were ionized and fragmented as described in Methods. Efficient fragmentation of the respective precursor ions required an relatively high collision energy of 33% CID (on an arbitrary scale of collision energy provided in the data system) showing the stability of these molecules. Superimposed on the spectra are the structures for the ceramide and dihydroceramide under study, as well as a list of relevant fragments found.

loss of the fatty acid moiety followed by dehydration is shown by the presence of *m/z* fragments 284 and 302. These results demonstrate an ability of APCI-MS to detect and structurally characterize both ceramide and dihydroceramide in a manner consistent with previous reports for similar compounds.

In order to achieve efficient HPLC separation of ceramide species, several columns, solvents and flow parameters were evaluated. Separations with a flow of ethyl acetate and isooctane on a silica column were used, as similar systems are often used for TLC separations of the ceramide species. For these initial studies, type III ceramide was used for the optimization of separation conditions utilizing APCI-MS detection. Examination of data from the analysis of this mixture of ceramides yielded chromatographic peaks containing pairs of mass spectrometric peaks indicating fragmentation patterns (1:2 ratio of precursor to loss of water) that allowed preliminary compound identifications to be made without further analysis with MS/MS detection (Fig. 3). Use of a normal-phase Iatrobead column with a gradient of isooctane/ethyl acetate provided separation of ceramide species present in this standard mixture. Figure 3(B) shows that the first ceramide peak was composed largely of ceramides with fatty acid chains from 22 to 26 carbons in length. Both the precursor and single dehydration ions are labeled for each of these compounds. Figure 3(C) shows a similar pattern for the second ceramide peak whose composition was largely due to ceramides with fatty acid lengths of 16 to 20. A relative rough comparison of areas of $[M+H-H_2O]^+$ chromatographic peaks gave a composition of 6% $C_{26:1/0}$, 57% $C_{24:1/0}$, 5% $C_{22:0}$, 2% $C_{20:0}$, 27% $C_{18:0}$, and 4% $C_{16:0}$. (The designations 24:1/0 and 26:1/0 refer to the presence of a mixture of ceramides of the same fatty acid length differing only in the presence or absence of a single is double bond on the fatty acid chain.) These results closely matched standard product information ratios and therefore helped suggest the potential for the future development of this approach for the quantitative analysis of ceramide species.

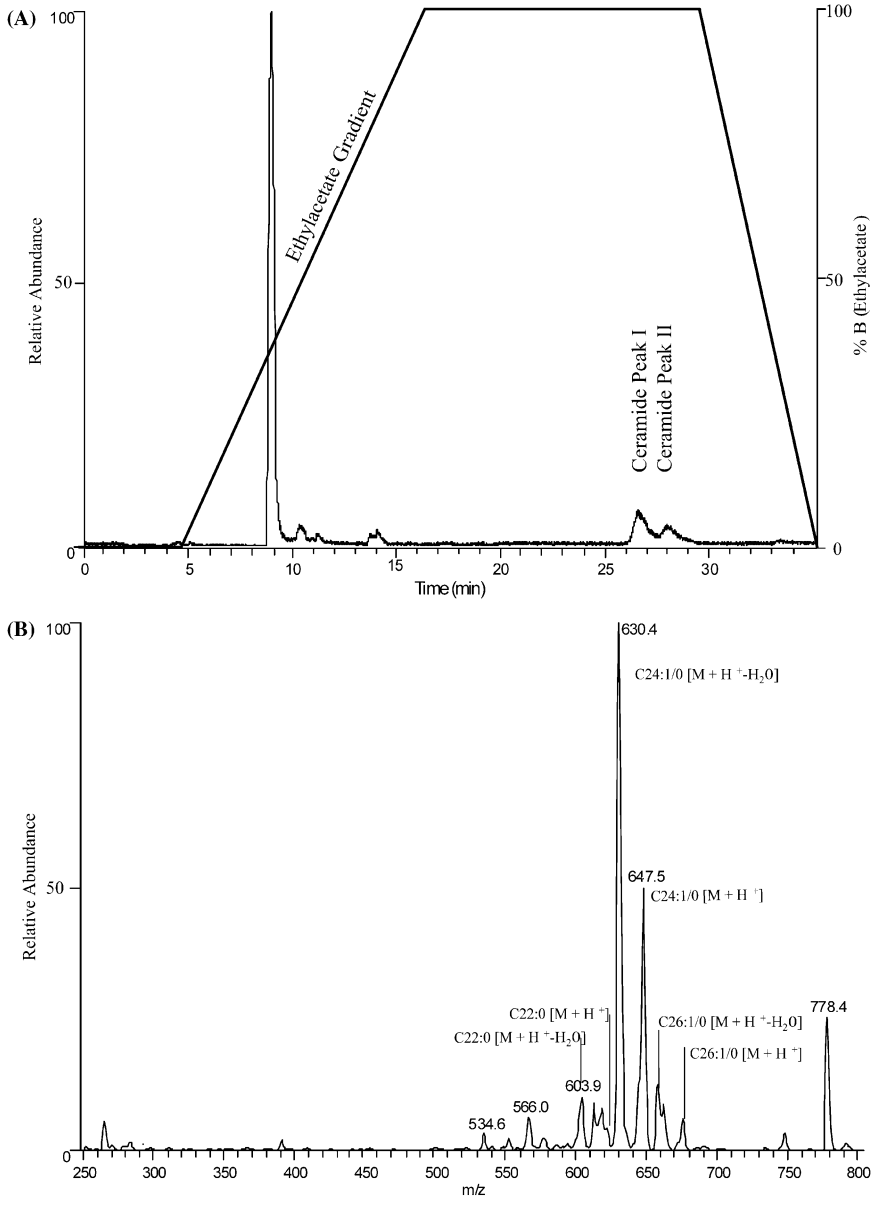


**Figure 3.** APCI LC/MS of type III ceramide. (A) shows the TIC chromatogram whereas (B) and (C) show MS spectra from the first and second ceramide peaks, respectively.

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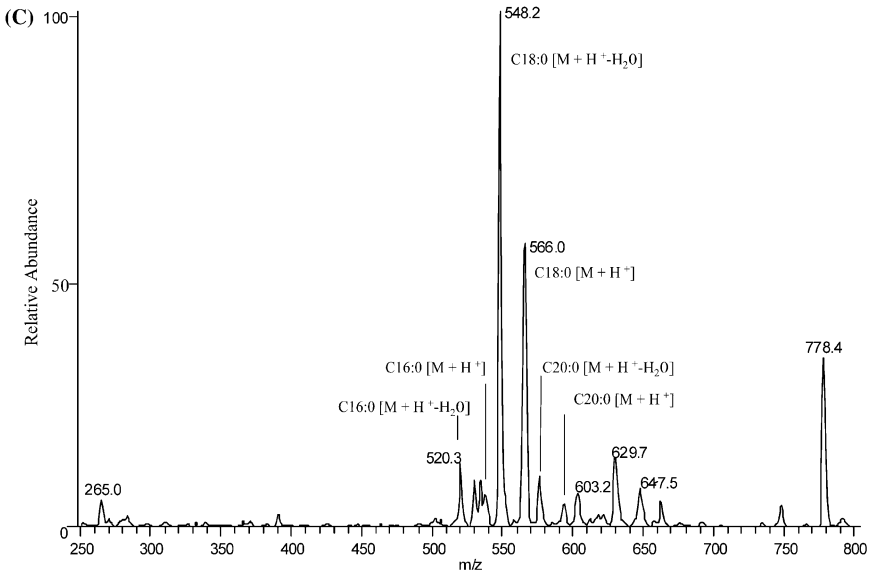


**Figure 3.** Continued.

Separation of dihydroceramides was optimized using a combination of ceramide type III and individual dihydroceramide standards. Figure 4(A) shows a distinctive elution pattern of compounds based on binding to the Iatrobead silica gel. Higher MW ceramides eluted first, and the presence of the *trans*-4–5 double bond in ceramide also substantially affected the binding properties, such that ceramides elute 2.5 min earlier than dihydroceramides of identical fatty acid chain length. Error bars are included to indicate standard deviations for measured retention times. Lines are included only to aid in visualization of general trends in elution behavior. Observation of a mathematical relationship between retention time and chain length is beyond the scope these experiments. Figure 4(A) illustrates that reproducible separations were obtained of approximately 2.5 min between ceramide and dihydroceramide species of identical chain length, and 0.5 min between ceramides or dihydroceramides differing in two carbons in length[3] and shows the retention times for ceramides and dihydroceramides of length 14 to 26. Whereas the proximity of the allylic *trans*-4–5 double bond of the sphingoid base to the 3-OH of ceramide apparently affected the retention of ceramide by the stationary phase such that ceramide and dihydroceramide species could be separated, introduction of a double bond on the fatty acid moiety (as in $C_{24:1}$ and $C_{26:1}$) had no such effect on retention. For example, although $C_{24:0}$ ceramide ($C_{18:1}$ backbone) and $C_{24:1}$dihydroceramide ($C_{18:0}$ backbone) each contain a single double bond and have identical *m/z* values of 649.5, $C_{24:0}$ ceramide eluted at 25.77 min while $C_{24:1}$ dihydroceramide eluted at 28.18 min. However, both $C_{24:1}$ and $C_{24:0}$ ceramide eluted together as did both $C_{24:1}$ and $C_{24:0}$ dihydroceramide. Figure 4(B) shows the overlay of synthetic standards on data obtained from 46 separate ceramide mixtures from cell extracts showing that the behavior of both standards and samples on the Iatrobead silica column are reproducible. Table 1 is a tabular representation of these and other MS data. This table lists the average retention times including standard error, and the average *m/z* values of $[M+H]^+$ and $[M+H-H_2O]^+$ ions followed by the mass ranges used for their analysis in the LCQUAN program.

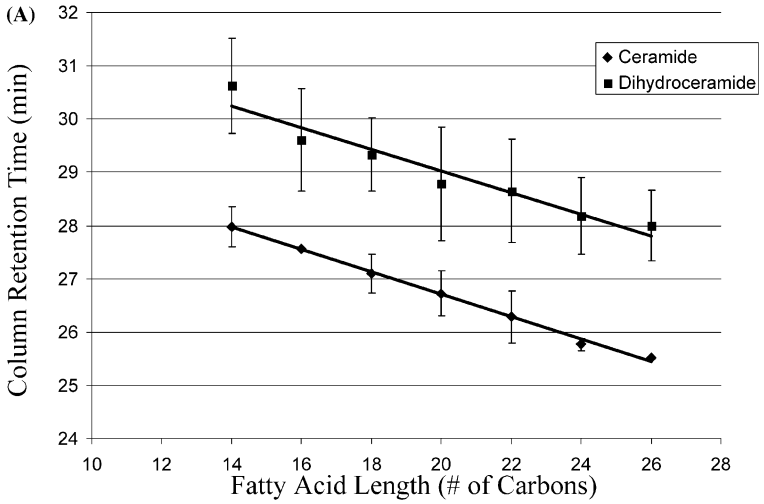


**Figure 4.** Retention of ceramide and dihydroceramide compounds on an Iatrobead column as a function of chain length.

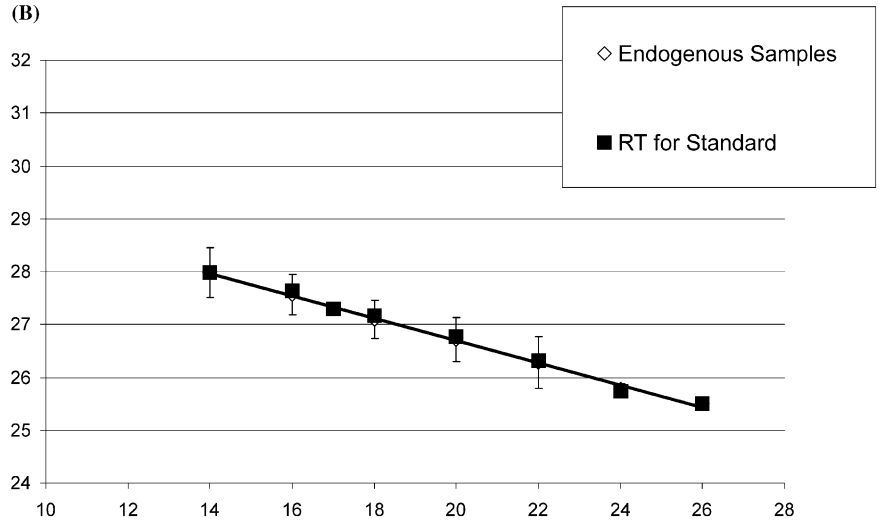


**Figure 4.** Continued.

Compounds are distinguished by chromatographic retention time, mass (*m/z* value), and intensity ratios between the two MS peak areas associated with the each compound (i.e. the precursor and the singly dehydrated ion).

The method was utilized for the analysis of endogenous lipid samples. MS/MS analysis was conducted on APCI $[M+H]^+$ ions and corresponding fragment ions of ceramides extracted from both Ramos and Molt-4 cells, and the results were compared with standards. $C_{14:0}$-$C_{26:0}+C_{24:1}$ and $C_{26:1}$ were detected along with the corresponding dihydroceramide species under full scan analysis. MS and MS/MS chromatograms provided confirmation for the structural assignments associated with endogenous ceramides. These results further confirm previous observations that the predominant endogenous sphingoid backbone was $C_{18:1}$.[42]

Figure 5 compares basal ceramide and dihydroceramide species information by DGK (Fig. 5(A)) and APCI-MS (Fig. 5(B)) for four cell lines, Molt-4, MCF-7, HL-60, and Ramos cells, all grown under identical culture conditions. MS analyses leading to quantitative estimates were performed in triplicate. Error bars are included to indicate standard deviations for the measured intensities. Notably, the DGK assay reveals a classical ceramide doublet, and the relative ratios of the upper to lower ceramide bands as seen in the DGK assay correlates well with the relative abundances of very long chain and long chain ceramide and dihydroceramides as seen by APCI-MS. In all cell lines, the predominant ceramide species were $C_{16}$ and $C_{24:1/0}$. In addition, the relative abundance of each ceramide was roughly 10-fold greater than its corresponding dihydroceramide counterpart.

**Table 1.** Average retention times for ceramide and dihydroceramide species detected including standard error in column retention,[3] and the average *m/z* values of $[M+H]^+$ and $[M+H^+-H_2O]^+$ ions followed by the ranges used for their quantitative analysis in the LCQuan program. In addition to differences in the masses of precursor and loss of $H_2O$ values listed in the table, substantial differences occurred in relative abundances of these ions on the order of a 1:2 ratio for ceramides and a 5:1 ratio for dihydroceramides, respectively. This is reflected in the detection windows as these take into account the mass range encompassing the largest peak, i.e., the loss of $H_2O$ for ceramides and the unfragmented precursor for dihydroceramides

| | *m/z* of precursor | *m/z* of precursor−$H_2O$ | Detection window | Retention times (min) | RT Std Dev (min) |
|---|---|---|---|---|---|
| Ceramide | | | | | |
| $C_{26:1/0}$ ~(2:1) | 675.5/677.5 | 657.5/659.5 | 656.2–660.8 | 25.51 | Normalized |
| $C_{24:1/0}$ ~(2:1) | 647.5/649.5 | 629.5/631.5 | 628.2–632.8 | 25.77 | 0.11 |
| $C_{22:0}$ | 621.5 | 603.5 | 602.2–604.8 | 26.28 | 0.49 |
| $C_{20:0}$ | 593.5 | 575.5 | 574.2–576.8 | 26.72 | 0.42 |
| $C_{17:0}$ (intstd) | 551.5 | 533.5 | 532.2–534.8 | 27.29 | STD |
| $C_{18:0}$ | 565.5 | 547.5 | 546.2–548.8 | 27.10 | 0.36 |
| $C_{16:0}$ | 537.5 | 519.5 | 518.2–520.8 | 27.56 | 0.38 |
| $C_{14:0}$ | 509.5 | 491.5 | 490.2–492.8 | 27.98 | 0.47 |
| $C_{6:0}$ | 397.5 | 379.5 | 378.2–380.8 | ~33.00 | STD |
| Dihydroceramide | | | | | |
| $C_{26:1/0dh}$ ~(2:1) | 677.5/679.5 | 659.5/661.5 | 676.2–680.8 | 28.00 | 0.67 |
| $C_{24:1/0dh}$ ~(2:1) | 649.5/651.5 | 631.5/633.5 | 648.2–652.8 | 28.18 | 0.73 |
| $C_{22:0dh}$ | 623.5 | 605.5 | 622.2–623.8 | 28.65 | 0.97 |
| $C_{20:0dh}$ | 595.5 | 577.5 | 594.2–596.8 | 28.78 | 1.07 |
| $C_{18:0dh}$ | 567.5 | 549.5 | 566.2–568.8 | 29.34 | 0.69 |
| $C_{16:0dh}$ | 539.5 | 521.5 | 538.2–540.8 | 29.61 | 0.97 |
| $C_{14:0dh}$ | 511.5 | 493.5 | 510.2–512.8 | 30.62 | 0.89 |

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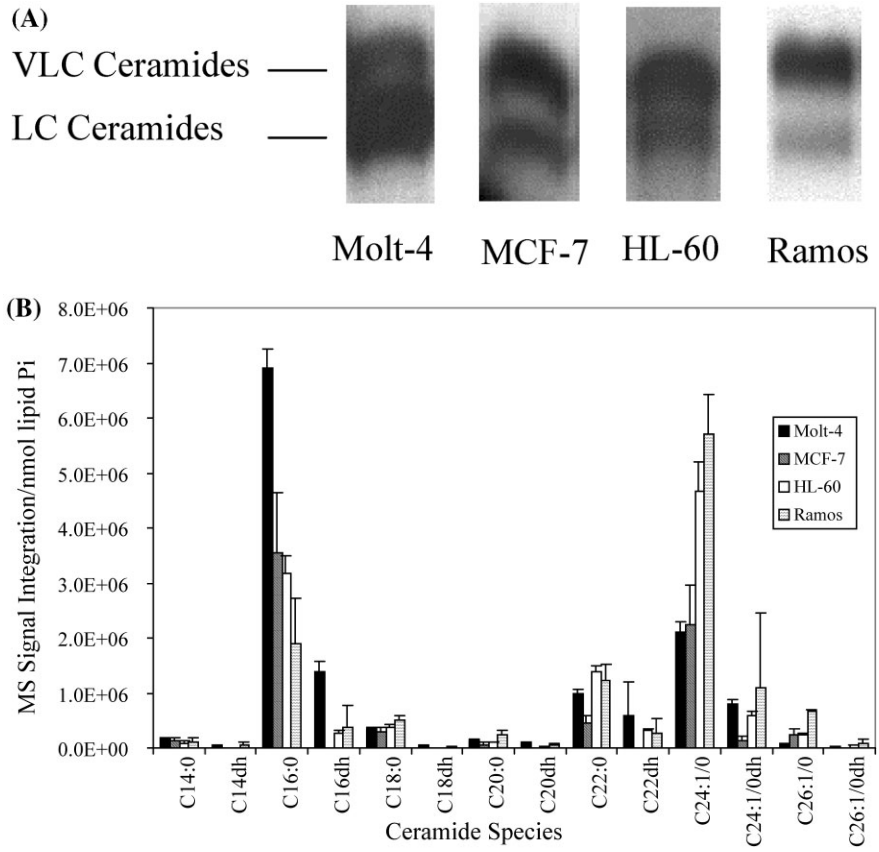


**Figure 5.** Ceramide profiling by DGK and APCI-MS. (A) Representative DGK assay autoradiographs from four cell lines grown under identical culture conditions showing the classical ceramide doublet composed of long chain (LC) and very long chain (VLC) ceramide and dihydroceramide species. (B) Ceramide and dihydroceramide species profiles by APCI-MS from the same four cell lines.

Notably, although each cell line was grown under identical culture conditions, the ceramide profile differs substantially suggesting perhaps differences in fatty acid uptake and/or metabolism and/or differences in ceramide metabolism between these cell lines. We conclude that APCI-MS adds significant qualitative information that may lead to insights into ceramide metabolism previously unattainable by the current DGK methodology. Under full-scan analysis, with reliance on APCI source-induced fragmentation, access to structural information is sacrificed in favor of increased sensitivity. In practical terms, this only impacts qualitative analysis of the specific ratio of saturated vs. singly unsaturated fatty acid for a ceramide of equal fatty acid length. It should be noted that the described LC/MS experiment does not distinguish species such as $C_{24:0}$ from $C_{24:1}$, although this is possible with methods based on MS/MS detection.

## DISCUSSION

Previously published methods for ceramide determination have involved both ESI and APCI-MS techniques, which have utilized both direct infusion[20,23–29,37] and HPLC-interfaced introduction of samples.[26,31–36] The HPLC separations prior to MS analysis have utilized either normal-phase[26,33,35] or reversed-phase chromatography.[31,32,34,36] Whereas ESI has been the dominant ionization technique,[20,23–29,32,33,35] there have also been reports on the use of APCI for ceramide analysis.[31,33,34] One of these reports utilizing ESI[20] shows direct quantitation, and others show relative comparisons using standards.[24–29,31,34,37] No previous studies of the ceramide species have simultaneously analyzed for both the ceramide and dihydroceramide species.

Here we have reported the use of APCI as the interface for LC/MS analysis of ceramides and dihydroceramides on an ion trap mass spectrometer. Using this approach, we were able to identify endogenous ceramides and dihydroceramides from 14 to 26 carbons in length, each possessing an 18-carbon sphingoid base, with certain very long chain species also possessing a single double bond on the fatty acid moiety. It was observed that APCI source fragmentation ratios could facilitate lipid identification. APCI-MS should allow for development of quantitative comparisons between species in the same sample as well as between the same species in different samples. APCI allows a degree of in-source decomposition that is reproducible and can be used to determine compound identity. There is ordinarily no sample-related ion suppression effect or salt adduction in APCI, simplifying data interpretation.

The need for prior HPLC separation has been questioned recently,[20] suggesting that the mass difference between ceramides is sufficient for independent analysis, particularly in the precursor scanning mode. However, here it has been

shown that use of chromatographic separation can allow a determination to be made for ceramide and dihydroceramide species. Furthermore, the chromatographic step adds confidence to mass analysis by reducing the possibility that other structurally similar compounds may interfere with the analysis.

The normal-phase Iatrobead column was chosen for this work because of its use of specially formulated silica material with normal-phase properties but with higher stability. Designed to take up to 15% $H_2O$, the Iatrobead silica material allows one to reverse the column and flush polar compounds with MeOH/$H_2O$. Despite repeated use for several hundred crude lipid sample injections, we have observed no change in column retention or other evidence of degradation. The quality of the separations and the stability of the column obviated the need for either base hydrolysis or column prepurification, steps that could contribute to the introduction of error, necessitate larger sample sizes, and require higher labor input. Optimization of normal-phase HPLC separation allowed for a more ready comparison of results from other well-established rapid analytical techniques utilizing normal-phase TLC separations. The ability of LC/MS to compliment existing analytical methodologies, while simultaneously adding significant information, suggests that this may become a valuable tool in ceramide analysis.

This combination of normal-phase chromatography and APCI described here, both eminently well suited to non-polar analytes such as ceramide, provides the basis for effective qualitative and future quantitative studies of ceramides and dihydroceramides, and, further, provides the basis for the convenient development of new LC/MS lipid methods based on the adaptation of other well-developed TLC methodologies.

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