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Separation, identification and quantitation of ceramides in human cancer cells by liquid chromatography–electrospray ionization tandem mass spectrometry

M. Fillet[a,*], J.-C. Van Heugen[b], A.-C. Servais[a], J. De Graeve[b], J. Crommen[a]

[a]*Department of Analytical Pharmaceutical Chemistry, Institute of Pharmacy, University of Liège, CHU, B36, B-4000 Liège 1, Belgium*
[b]*Department of Environmental Toxicology, University of Liège, CHU, B35, B-4000 Liège 1, Belgium*

Abstract

Ceramides are important intracellular second messengers that play a role in the regulation of cell growth, differentiation and programmed cell death. Qualitative and quantitative analysis of these second messengers requires sensitive and specific analytical methods to detect endogenous levels of individual ceramide species and to differentiate between them. Nine synthetic ceramides were separated by liquid chromatography coupled to tandem mass spectrometry on a C_{18} bonded silica column. The lipids were eluted in gradient elution mode using a mixture of water, acetonitrile and 2-propanol as mobile phase. They were detected by reaction monitoring performed on positive ion electrospray generated ions. Collision-induced fragmentations conducted on ceramides produced a well characteristic product ion at m/z 264, making multiple reaction monitoring (MRM) well suited for various ceramides quantitative measurements. After optimization of the extraction step, the proposed methodology was able to identify and quantify different ceramide species issued from human cancer cells. The method could be validated for C_{16}, C_{18} and C_{20} ceramides, quantified at the nanogram level. The validation exhibits good results with respect to linearity, accuracy and precision.

Keywords: Ceramides; Sphingolipids; Lipids

1. Introduction

In addition to their major structural role as a barrier for cell permeability and as a matrix for the association of membrane proteins, sphingolipids play a key role in the signal transduction and cell regulation.

Sphingomyelin hydrolysis to ceramides occurs through the action of sphingomyelin-specific forms of phospholipase C called sphingomyelinases. In addition to the breakdown of sphingomyelin to ceramide, a mechanism for the generation of ceramide exists through the action of ceramide synthase. Accurate and precise analytical methods, able to quantify selectively the various ceramide molecular species at low concentrations, are crucial for elucidating their function and metabolism.

Various experimental approaches have demonstrated that ceramides are key signaling molecules generated in response to a variety of stresses that mediate growth arrest, differentiation, senescence, apoptosis or an immune response [1–6]. Nonetheless, their mechanisms of action remain largely unknown. In view of the increasing interest in sphingolipid metabolites, sensitive and specific methods are required to measure their endogenous levels.

*Corresponding author. Tel.: +32-4-366-4354; fax: +32-4-366-4347.

E-mail address: marianne.fillet@ulg.ac.be (M. Fillet).

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Structurally, the ceramides exhibit a long amino alcoholic chain covalently bound via an amide linkage to a fatty acyl moiety (cf. Fig. 1). These molecules can vary in length, degree of unsaturation and hydroxylation, giving rise to a complex and diverse group of compounds. These lipids have proven to be very difficult to analyse due to their apolar nature, diversity and relatively low levels in biological samples.

Until now, the diacylglycerol (DAG) kinase assay is the most commonly used procedure for ceramide quantitation [7,8]. The DAG kinase phosphorylates ceramide into a radioactive product which is detected by thin-layer chromatography (TLC). The principle limitations of the DAG kinase assay as well as the TLC measurement are their requirement of high sample volumes and their lack of discrimination capability between different ceramide species. Sever-

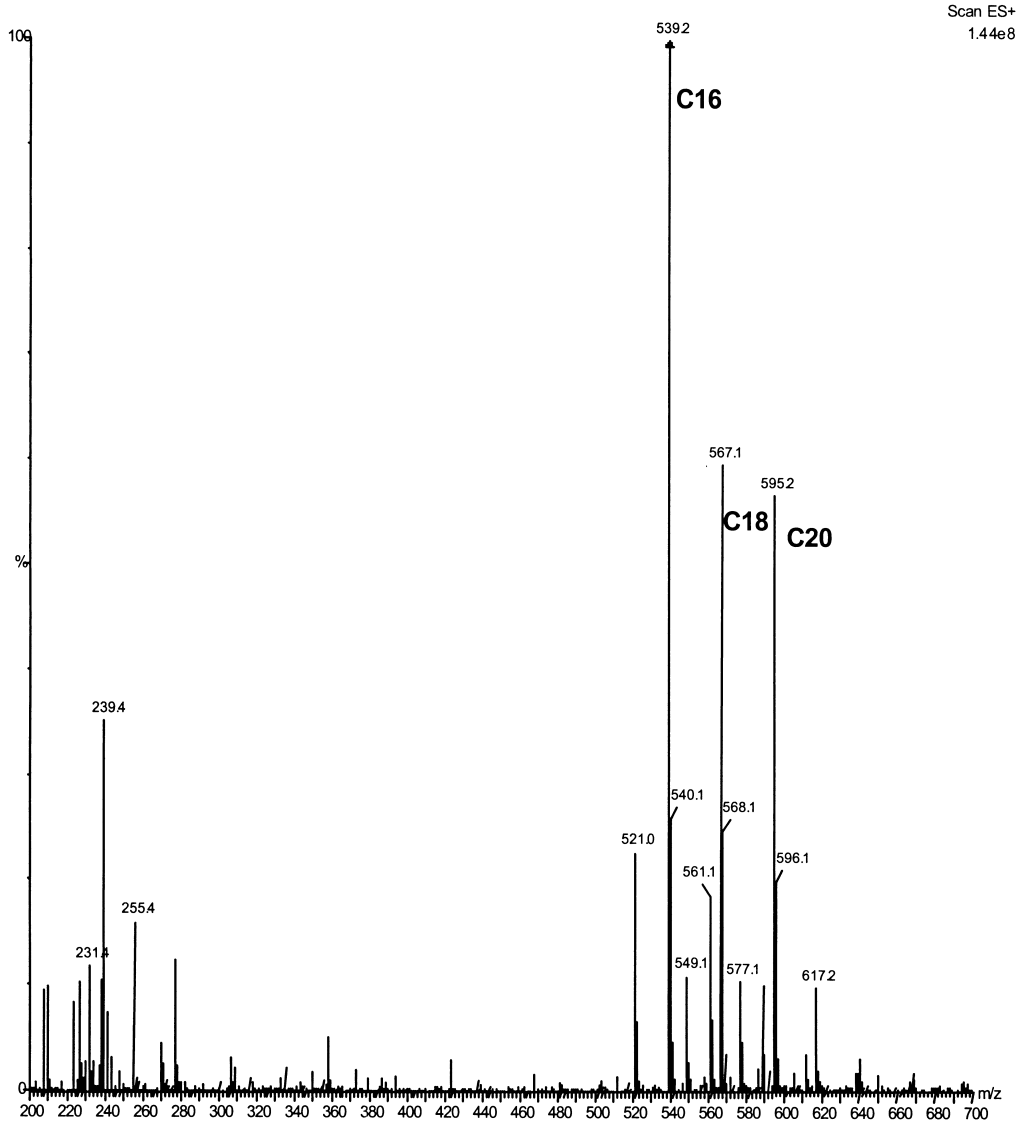

Fig. 1. MS spectra of C_{16}, C_{18} and C_{20} ceramides.

al other methods are also available for quantitation of sphingolipid metabolites by liquid chromatography (LC). Since the ceramides lack a chromophore group to allow a sensitive UV measurement, fluorescent or radioisotope detection after derivatization were perferred [9–11] All the above described procedures are time-consuming and tricky derivatization steps might often be source of error hampering the accurate determination of the ceramides levels. The use of an evaporative light-scattering detector for the ceramide visualization and identification provides direct analysis without any prior derivatization [12].

In the last few years, mass spectrometry methodologies have been developed for the detection of ceramides [13–17]. This technique is also a powerful tool for determining endogenous, physiologically active compounds because of its high selectivity and sensitivity. Electrospray ionization mass spectrometry (ESI-MS) has been successfully used for several years for the analysis of peptides and proteins and it has been more recently extended to the analysis of other species like carbohydrates and lipids [15,17].

This work is focused on the separation and the identification of standard ceramides by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS–MS). The extraction procedure of lipids from human cells was optimized in order to obtain sufficient sensitivity. In human cancer cells, the endogenous ceramides were identified and quantitatively measured. Finally, the procedure was validated for C_{16}, C_{18} and C_{20} ceramides and some analytical data are presented.

2. Experimental

2.1. Chemicals

C_2, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and C_{20} ceramides were obtained from Acros (Gell, Belgium).

Water, acetonitrile, 2-propanol, ethanol, formic acid were of HPLC grade from Merck (Darmstadt, Germany).

Nitrogen (alphagaz 1) and argon (alphagaz 2) were purchased from Air Liquide (Milmort, Belgium).

2.2. Instruments and methods

The high-performance liquid chromatograph (HPLC) is a HP 1100 series; it is equipped with a binary pump, a vacuum degasser, a thermostated column compartment and an autosampler, all from Agilent Technologies (Waldbronn, Germany). The HPLC separations were performed at 70°C on a RP C_{18} Nucleosil AB column (5 μm, 70×2 mm I.D.) from Macherey–Nagel (Düren, Germany). The mobile phase was a gradient mixture formed as follows: A, water–acetonitrile–2-propanol (8:1:1, v/v/v); B, acetonitrile–2-propanol (9:1, v/v). The steps of the gradient program were: 0 min 35:65 (A–B), 2 min 35:65 (A–B), 7 min 10:90 (A–B), 13 min 10:90 (A–B), 15 min 0:100 (A–B), 16 min 35:65 (A–B), 18 min 35:65 (A–B). The mobile phase was degassed for 10 min in an ultrasonic bath before use. The flow-rate was 0.450 ml/min. The sample volume injected was 6 μl.

MS detection was carried out using a Ultima triple quadrupole instrument (Micromass, Manchester, UK) operating under MassLynx 3.5 and configured with a Z-spray electrospray ionization source.

Source conditions were as follows: positive ion electrospray, capillary voltage 3.0 kV, cone voltage 14 V, source temperature 145°C, desolvation temperature 450°C, cone gas flow (nitrogen) 94 l/h and desolvation gas flow (nitrogen) 552 l/h. The MS–MS fragmentation of the molecular ion was achieved with a collision energy of 27 eV (collision gas:argon).

2.3. Preparation of standards

Standard solutions for ESI-MS–MS optimization were prepared by dissolving the ceramides in a mixture of ethanol–formic acid (99.8:0.2, v/v) to reach a concentration of 1 μg/ml.

2.4. Cellular lipid extraction

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), scraped in PBS, centrifuged at 800 *g* and the resulting pellet was homogenized in distilled water by sonication. An aliquot of the cell homogenate was taken for protein determination.

Then, 10 ng of C_{12} ceramide (as internal standard) was added to the cell lysate (the latter corresponding

to 500 μg of proteins). Lipids were extracted using the Folch's partition with a mixture of chloroform–methanol (2:1, v/v), centrifuged at 1500 *g*, and finally washed with chloroform–methanol–water (3:48:47, v/v/v). The organic phase was evaporated near to dryness under a gentle stream of dry nitrogen. The samples were reconstituted by vortexing with 200 μl of a mixture ethanol–formic acid (99.8:0.2) until complete dissolution.

To avoid any loss of lipids, the whole procedure was performed in siliconized glassware.

2.5. Cell preparation

HCT116 human colon carcinoma cells were cultured in McCoy medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 1% 200 m*M* L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Ovcar ovarian carcinoma cells, U937 lymphoid cells and MCF7 A/Z breast cancer cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum, 1% 200 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.6. Preparation of spiked samples for the validation

Before the extraction process, five cell lysates (corresponding to 500 μg of proteins from HCT116 cells) were spiked with a constant amount of internal standard (10 ng of C_{12}) and with increasing amounts of C_{18} and C_{20} (0, 2, 4, 8 and 16 ng) and of C_{16} (0, 5, 10, 20 and 50 ng). The lipids extraction were conducted according to the procedure described in Section 2.4.

3. Results and discussion

3.1. Optimization of the MS detection

The MS analysis of ceramides was first investigated by direct introduction of the reference compounds dissolved in an ethanol–formic acid (99.8:0.2) mixture using the electrospray interface in the positive mode of ionization.

For each of the tested ceramide, two main peaks were observed corresponding respectively to the $[M+H]^+$ and $[M+H-H_2O]^+$ ionic species (Fig. 1). Parameters such as the capillary and cone voltages, as well as the cone and the desolvation gas flows were optimized in order to generate the highest protonated molecular ion under a stable spray (cf. Section 2.2).

3.2. MS fragmentation of the ceramides

It has been shown previously that collision-induced dissociations (CID) of ceramides in electrospray, positive ion mode, mainly generate the characteristic product ions of *m/z* 264 and *m/z* 282, respectively, whereas other sphingosine metabolites provided different fragmentation pathways [14]. These fragments resulted from the loss of the *N*-linked fatty acid moiety and one or two molecules of water (cf. Fig. 2). This dissociation pattern could be confirmed for ceramides species varying in this *N*-linked fatty acid moiety (2–20 carbons into the chain).

The collision energy was optimized in order to reach a maximum intensity for the fragment ion of *m/z* 264 resulting in a minimum response for the

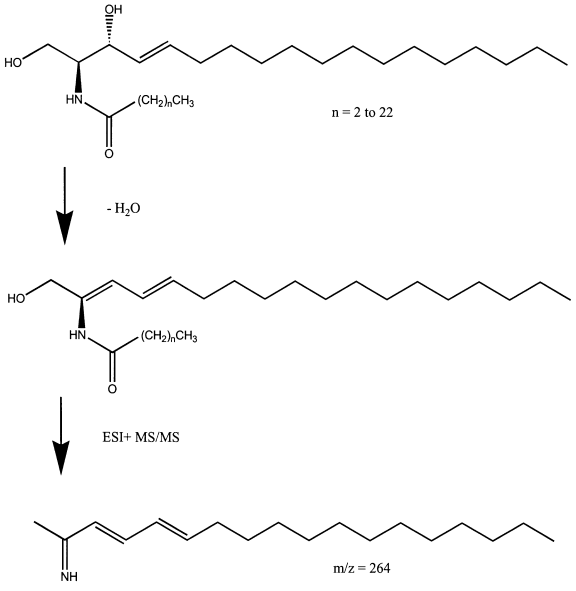

Fig. 2. Structure of ceramide and fragmentation process.

molecular ionic species (Fig. 3). The fragment ion with the highest m/z ratio (m/z 264) was selected for quantitative MS detection in the multiple reaction monitoring (MRM) mode. As MRM is based on the transition involving the specific molecular ion and a characteristic fragment ion, high detection selectivity as well as high signal-to-noise ratios could be obtained providing a clean reversed-phase (RP) LC–MS–MS-MRM chromatogram.

To increase the sensitivity, two MRM signals were traced and added for the evaluation of each ceramide. The first MRM signal is $[M+H]^+ \rightarrow m/z$ 264 while the second corresponds to the $[M+H-H_2O]^+ \rightarrow m/z$ 264 transition.

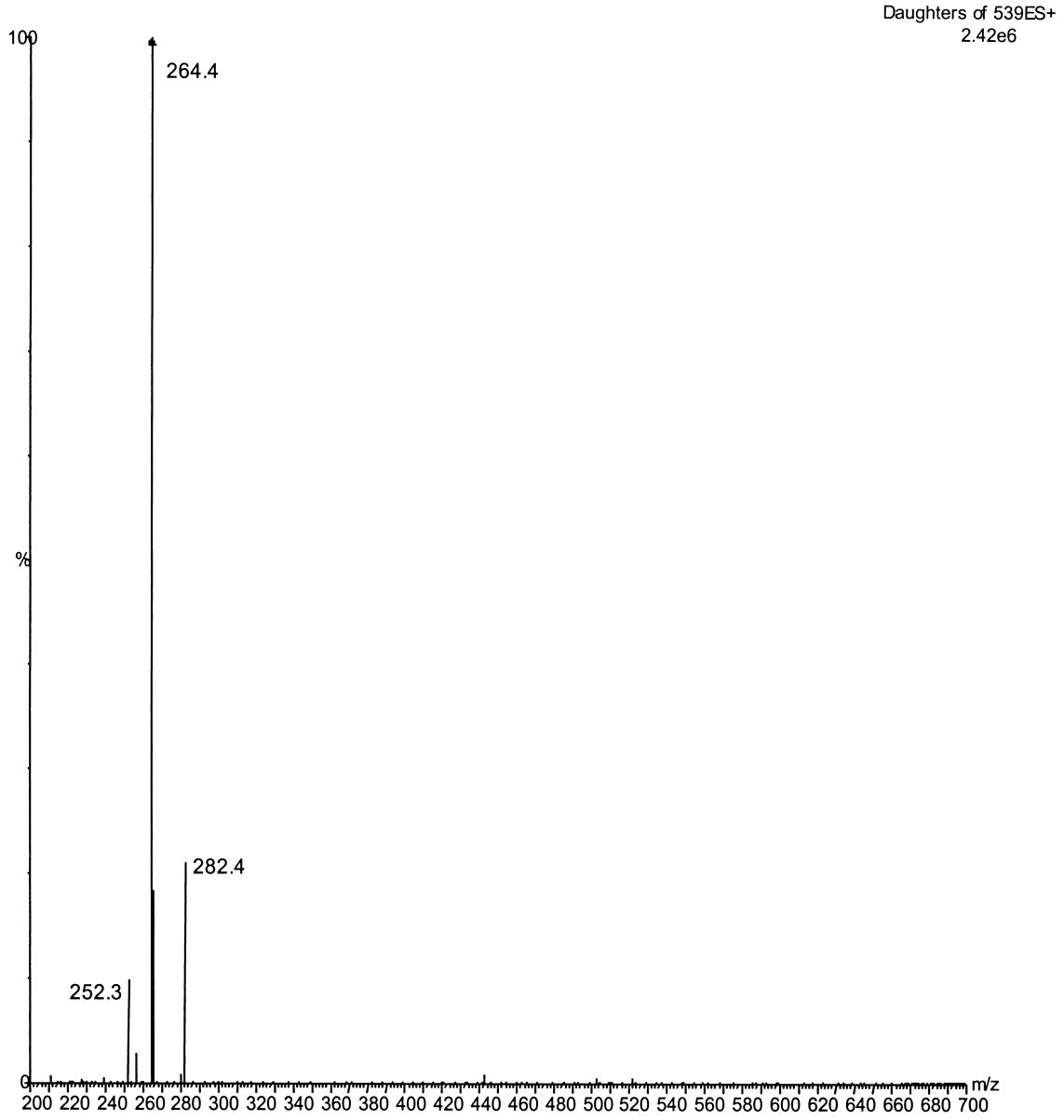

Fig. 3. MS–MS spectrum of C_{16} ceramide.

3.3. Selection of the LC conditions

The next step of this study concerned the optimization of the LC separations of the nine commercialy available ceramides (C_2, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and C_{20} ceramides, respectively).

The best separation of ceramide species was obtained on a reversed-phase column (C_{18} Nucleosil AB column). Among a large number of potential buffers and organic solvents compatible with the MS–MS detection, the mixture water–acetonitrile–2-propanol provided the best results in terms of selectivity. A multiple step gradient (cf. Section 2.2) has been developed in order to reach high selectivity for the compounds with short migration times. The MRM chromatogram recorded for the ceramide synthetic mixture is depicted in Fig. 4.

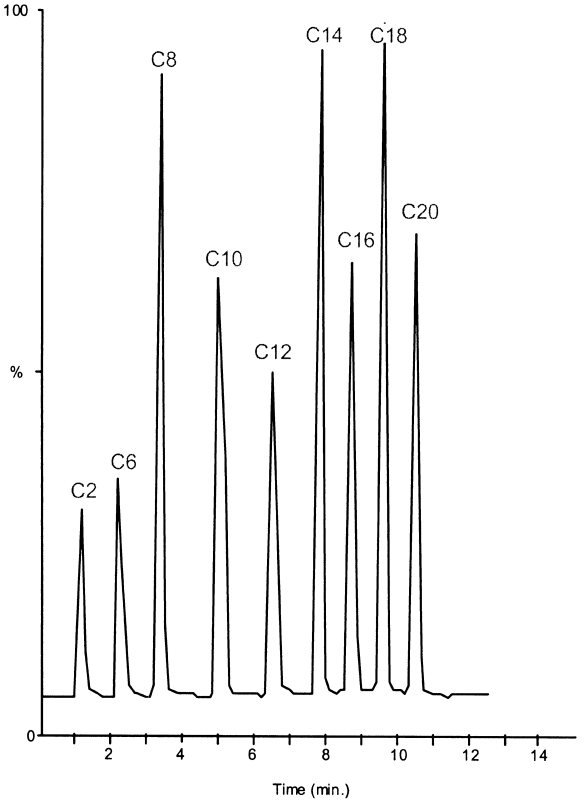

Fig. 4. MRM chromatogram of nine standard ceramides (1 μg/ml) by LC–ESI-MS–MS. All conditions described in Section 2.2.

3.4. Comparison of the ceramides patterns observed in four cancer cell lines

Once the LC and MS conditions were fully optimized, identification of endogenous ceramides was achieved using four different cell lines: HCT116 human colon carcinoma cells, Ovcar ovarian carcinoma cells, U937 lymphoid cells and MCF7 A/Z breast cancer cells. In all of them, five ceramide species (C_{16}, C_{18}, C_{20}, C_{22}, C_{24}) could be retrieved but in different ratios. The MRM chromatograms of the endogenous ceramides retrieved in the different cell lines are reported in Fig. 5.

3.5. Method validation

In order to generate quantitative results for those C_{16}, C_{18} and C_{20} ceramides, calibration curves were established by spiking different amounts of the corresponding natural ceramides (C_{16}, C_{18} and C_{20}) to HCT116 cell lysate (amount corresponding to 500 μg of proteins). For quantitative assessment, a constant amount (10 ng) of non-naturally occurring C_{12}-ceramide internal standard was added to the mixture before starting the lipid extraction.

All values were expressed as the ratio of the peak areas of the natural ceramide to that of the internal standard. This was done in order to cope for possible variations in the extraction yield or/and to compensate for some instabilities in the MS parameters, which could affect ionization efficiency and ion counts.

3.5.1. Linearity

Calibration graphs were constructed with five levels in the range 0–16 ng for the C_{18} and C_{20} ceramides and in the range 0–50 ng for the C_{16} ceramide. Three determinations were performed at each concentration level ($n=3$). Linear regression lines were calculated by plotting normalised peak areas (ratios of peak areas of the natural ceramide to that of the internal standard) versus the spiked ceramide amount using the least-squares method with the hypothesis of homoscedasticity [18,19].

The linearity of the calibration curve was determined tracing each ceramide with two added MRM signals: $[M+H]^+$ and $[M+H-H_2O]^+$ to m/z 264. Straight lines and coefficient of determination

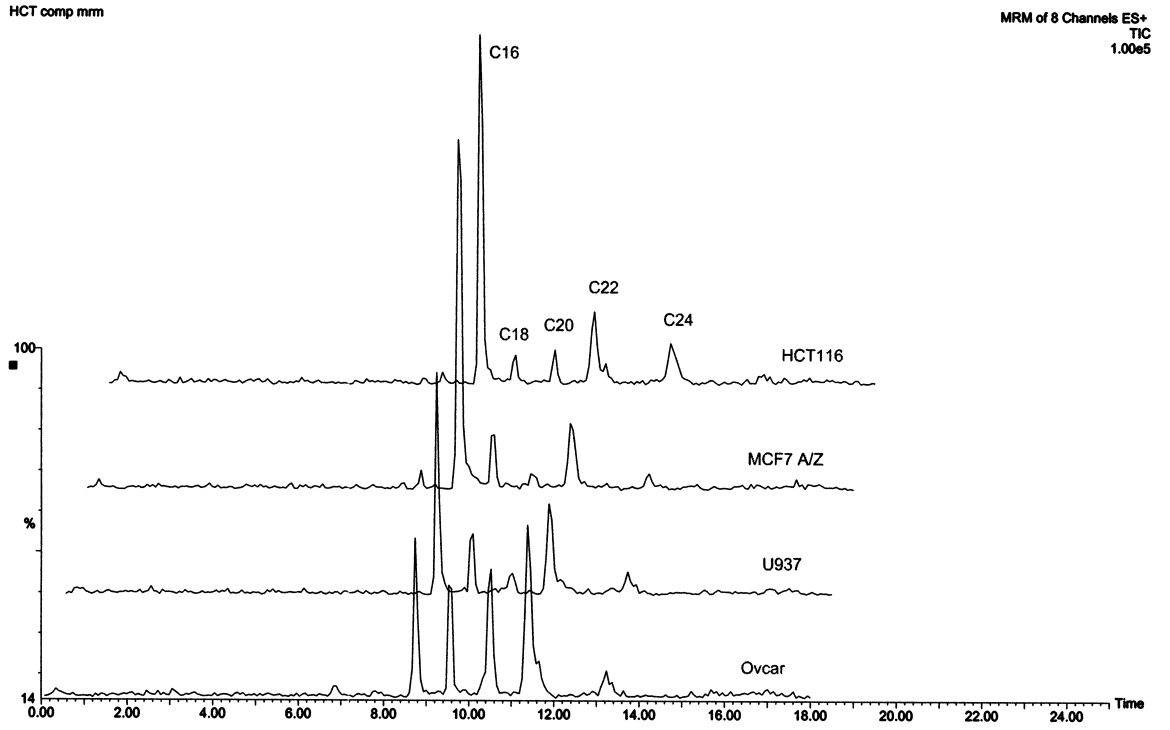

Fig. 5. MRM chromatogram of endogenous ceramides by LC–ESI-MS–MS in four cancer cell lines, HCT116 (colon), MCF7 A/Z (breast), U937 (lymphoid), Ovcar (ovarian). Time scale in minutes.

(r^2) of 0.9976 for the C_{16} ceramide, 0.9973 for the C_{18} ceramide and 0.9977 for the C_{20} ceramide were obtained (cf. Fig. 6). The linearity was also confirmed by an analysis of the variance (ANOVA) [18,19]. The regression parameters are given in Table 1.

3.5.2. Limits of detection and of quantitation

Limits of detection (LOD) and quantitation (LOQ) correspond to signal-to-noise ratios of 3 and 10, respectively.

The LOD, the lowest amount of C_{18} ceramide that could be detected, was 0.073 ng for 500 μg of HCT116 proteins, which corresponds to 124 fmol. The LOQ, the lowest amount of C_{18} ceramide that could be quantified was 0.245 ng, which corresponds to 413 fmol.

For the C_{16} ceramide, the LOD and the LOQ were 0.253 and 0.844 ng, respectively, for 500 μg of HCT116 proteins. For the C_{20} ceramide, the LOD and the LOQ were 0.318 and 1.060 ng, respectively.

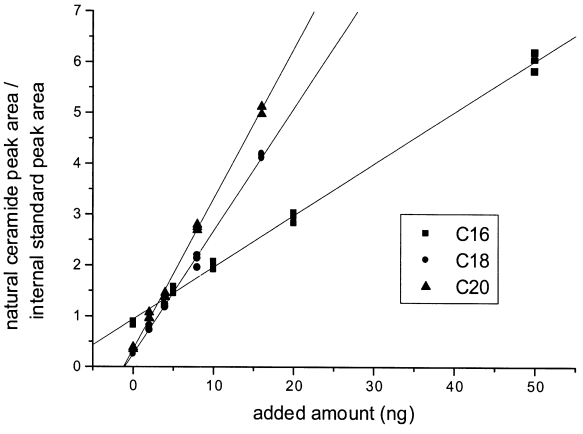

Fig. 6. Calibration curves for C_{16}, C_{18} and C_{20} ceramides.

Table 1
Validation results

Validation criteria	C_{16}	C_{18}	C_{20}
Calibration range (ng/500 μg proteins)	0–50	0–16	0–16
Calibration points	5	5	5
Coefficient of determination (r^2)	0.9976	0.9973	0.9977
Accuracy (k=3, n=15)			
Mean recovery±CI (%) at 4 ng added	ND	102.8±5.5	98.2±4.2
Mean recovery±CI (%) at 10 ng added	97.5±4.1	ND	ND
Mean recovery±CI (%) at 16 ng added	ND	104.6±4.6	104.6±5.1
Mean recovery±CI (%) at 50 ng added	100.2±2.3	ND	ND
Repeatability (k=3, n=6, RSD%)			
at endogenous level	5.97	13.13	13.34
at 4 ng added	ND	4.26	3.46
at 10 ng added	4.43	ND	ND
at 16 ng added	ND	4.00	4.23
at 50 ng added	2.23	ND	ND
Intermediate precision (k=3, n=18, RSD%)			
at endogenous level	10.26	13.43	18.54
at 4 ng added	ND	4.98	4.74
at 10 ng added	4.61	ND	ND
at 16 ng added	ND	4.68	4.32
at 50 ng added	3.87	ND	ND
LOD (ng/500 μg proteins)	0.253	0.073	0.318
LOQ (ng/500 μg proteins)	0.844	0.245	1.060
Estimation of endogenous level (ng/500 μg proteins)	9.226	1.043	1.165

ND: not determined.

3.5.3. Accuracy

The method accuracy was determined at two different levels ($n=6$) covering the same range as that used for linearity (4 and 16 ng for C_{18} ceramide and C_{20}; 10 and 50 ng for C_{16}). Mean recoveries with confidence intervals (CI at $P>0.05$) are presented in Table 1. As the theoretical value of 100% was included in the confidence interval, the test procedure could be considered as accurate over the range studied.

3.5.4. Precision

Method precision was determined by measuring repeatability and intermediate precision (between-day precision) ($n=6$). The study was carried out during 3 days ($k=3$), at three levels (0, 4 and 16 ng for C_{18} and C_{20} ceramides; 0, 10 and 50 ng for C_{16} ceramide). The RSD values were estimated from repeatability and intermediate precision variances, respectively [19,20].

As depicted from the data of Table 1, acceptable results with respect to precision could be obtained. The RSDs at the endogenous level, which is close to the LOQ for C_{20} ceramide, are relatively high but still acceptable for analysis conducted on such biological samples.

4. Conclusions

The method developed for the separation of ceramides present in human cancer cells using a

LC–ESI-MS–MS procedure was found to be linear, accurate, precise and sensitive to the nanogram level.

This method enables accurate and precise analysis for ceramides issued from relatively small samples providing a useful tool for signal transduction research. In the future, this procedure will be used for studying the changes of endogenous ceramides concentrations that could occur during cellular stress and apoptotic process.

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References

[1] C.J. Gamard, G.S. Dbaibo, B. Lui, L. Obeid, Y. Hannun, J. Biol. Chem. 272 (1997) 16474.
[2] T. Okazaki, T. Kondo, M. Tashima, Cell Signal 10 (1998) 685.
[3] Y.A. Hannun, Science 272 (1996) 1855.
[4] L.M. Obeid, Y.A. Hannun, J. Cell Biochem. 58 (1995) 191.
[5] J.D. Fishbein, R.T. Dobrowsky, A. Bielawska, S. Garrett, Y.A. Hannun, J. Biol. Chem. 268 (1993) 9255.
[6] M.E. Venable, Y.L. Lee, M.J. Smith, A. Bielawska, L.M. Obeid, J. Biol. Chem. 67 (1995) 27.
[7] P.P. Van Veldhoven, W.R. Bishop, D.A. Yurivich, R.M. Bell, Biochem. Mol. Biol. Int. 36 (1995) 21.
[8] A. Olivera, J. Rosenthal, S. Spiegel, Anal. Biochem. 223 (1994) 306.
[9] A.H. Merrill, E. Wang, R.E. Mullins, W.C.L. Jamison, S. Nimkar, D.C. Liotta, Anal. Biochem. 171 (1988) 373.
[10] M. Previati, L. Bertolaso, M. Tramarin, V. Bertagnolo, S. Capitani, Anal. Biochem. 233 (1996) 108.
[11] A. Olivera, S. Speigel, Nature 365 (1993) 557.
[12] T. McNabb, A. Cremesti, P. Brown, A. Fischl, Anal. Biochem. 276 (1999) 242.
[13] F.M. Rubino, L. Zecca, S. Sonnino, Biol. Mass Spectrom. 23 (1994) 82.
[14] M. Gu, J.L. Kerwin, J.D. Watts, R. Aebersold, Anal. Biochem. 244 (1997) 347.
[15] M. Mano, Y. Oda, K. Yamada, N. Asakawa, K. Katayana, Anal. Biochem. 244 (1997) 291.
[16] L.H. Couch, M.I. Churchwell, D.R. Doerge, W.H. Tolleson, P.C. Howard, Rapid Commun. Mass Spectrom. 11 (1997) 504.
[17] A. Karlsson, P. Michelsen, O. Goran, J. Mass Spectrom. 33 (1998) 1192.
[18] D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics: a Textbook, Elsevier, Amsterdam, 1988, p. 75.
[19] J. Caporal-Gautier, J.M. Nivet, P. Algranti, M. Cuilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu, R. Russoto, S.T.P. Pharma Prat. 2 (1992) 202.
[20] C. Hartmann, Analusis 22 (1994) M19.