methods

Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS)

G. Liebisch,* W. Drobnik,* M. Reil,* B. Trümbach,* R. Arnecke,† B. Olgemöller,† A. Roscher,§ and G. Schmitz[1,*]

Institut für Klinische Chemie und Laboratoriumsmedizin,* Klinikum der Universität Regensburg, Franz-Josef-Strauss-Allee 11, 93042 Regensburg, Germany; Labor Becker,† Olgemöller & Partner, München, Germany; and Kinderklinik, Klinische Chemie/Biochemie,§ Ludwig Maximilian Universität München, München, Germany

Abstract Ceramide (CER) is an important signaling molecule involved in a variety of cellular processes, including differentiation, cell growth, and apoptosis. Currently, different techniques are applied for CER quantitation, some of which are relatively insensitive and/or time consuming. Tandem mass spectrometry with its high selectivity and sensitivity is a very useful technique for detection of low abundant metabolites without prior purification or derivatization. In contrast to existing mass spectrometry methods, the developed electrospray tandem mass spectrometry (ESI-MS/MS) technique is capable of quantifying different CER species from crude cellular lipid extracts. The ESI-MS/MS is performed with a continuous flow injection and the use of an autosampler, resulting in a high throughput capability. The collision-induced fragmentation of CER produced, in addition to others, a characteristic fragment of *m/z* 264, making a precursor ion scan of 264 well suited for CER quantitation. Quantitation is achieved by use of a constant concentration of a non-naturally occurring internal standard C8-CER, together with a calibration curve established by spiking different concentrations of naturally occurring CER. The calibration curves showed linearity over a wide concentration range and sample volumes equivalent to 10 μg of cell protein corresponding to about 20,000 fibroblasts were sufficient for CER analysis. Moreover this assay showed a detection limit at the subpicomole level. In summary, this methodology enables accurate and rapid analysis of CER from small samples without prior separation steps, thus providing a useful tool for signal transduction research.—Liebisch, G., W. Drobnik, M. Reil, B. Trümbach, R. Arnecke, B. Olgemöller, A. Roscher, and G. Schmitz. **Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS).** ***J. Lipid Res.*** **1999.** 40: **1539–1546.**

Supplementary key words ceramide quantitation • electrospray ionization • tandem mass spectrometry

Ceramide (CER) is an important intracellular second messenger (1) involved in diverse biological processes such as regulation of cell growth (2), apoptosis (3), and cellular senescence (4). A variety of different techniques are used for CER measurement.

Up to now the diacylglycerol (DAG) kinase assay is the most commonly used procedure for CER quantitation in the range of 25 to 2000 pmoles (5). This method utilizes the ability of DAG kinase to phosphorylate CER, resulting in the generation of radioactive ceramide-1-phosphate that can be detected by thin-layer chromatography (TLC). However, the specificity of this assay has recently been questioned by a report comparing the DAG kinase assay with a mass spectrometry method (6). Alternative methods include a variety of procedures, which use TLC for the detection of unlabeled (7) and radioactive labeled CER (8–10) as well as several methods using HPLC with prior derivatization steps. These latter methodologies are based either on the esterification of CER with benzoylchloride (11), 6-methoxy-α-methyl-2-naphthaleneacetic acid (12), or alternatively the derivatization of deacylated CER with *o*-phthaldialdehyde (OPA) and the subsequent separation of the fluorescent products with HPLC and UV/fluorescence detection (13). Recently, Yano et al. (14) published a method that allows detection of different CER species after TLC purification, conversion to anthroyl derivatives, and HPLC separation.

In the last years, mass spectrometry methodologies have

Abbreviations: APCI, atmospheric pressure chemical ionization; CER, ceramide; CID, collision-induced dissociation; cps, counts per second; CV, coefficient of variation; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ESI-MS/MS, electrospray tandem mass spectrometry; FCS, fetal calf serum; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; OPA, *o*-phthaldialdehyde; PBS, phosphate-buffered saline; PIS, precursor ion scan; SD, standard deviation; SDS, sodium dodecyl sulfate; TIC, total ion chromatogram; TLC, thin-layer chromatography.

[1] To whom correspondence should be addressed.

been developed for the detection of CER, which are independent of prior fluorescence derivatization of the analyte and thus avoid a potential source of error. However, these techniques still require an initial separation step of the crude lipid extract by HPLC. The detection is achieved either by atmospheric pressure chemical ionization (APCI) mass spectrometry (15) or by electrospray tandem mass spectrometry (ESI-MS/MS) (16). A qualitative determination of distinct CER species from relatively crude lipid extract was described by Gu et al. (17), who performed a precursor ion scan (PIS) for a specific CER fragment resulting from collision-induced dissociation (CID). However, this procedure still needed an initial purification of the lipid extract by silica gel column and, moreover, the sole addition of an unnatural CER species as internal standard did not allow quantitation.

Based on the method described by Gu et al. (17), we aimed to establish a methodological protocol that enables not only qualitative but quantitative determination of various CER species and works without any prior separation steps of the lipid extract.

MATERIALS AND METHODS

Materials

C6-, C16-, and C18-CER were obtained from Alexis Deutschland GmbH (Grünberg, Germany), C8-CER were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany) and C2-, C24:1-CER were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Solvents for ESI-MS/MS were of HPLC grade purchased from J. T. Baker (Gross-Gerau, Germany). Lipid extraction solvents were of analytical grade from Merck (Darmstadt, Germany).

Cell culture

Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with l-glutamine, nonessential amino acids, and 10% fetal calf serum (FCS) in a humidified 5% CO_2 atmosphere at 37°C. The used cells were cultured skin fibroblasts from patients with Tangier disease or controls without genetic defect.

Sample preparation

Cells were rinsed two times with ice-cold phosphate-buffered saline (PBS) and either scraped in PBS, centrifuged at 800 *g*, and the resulting pellet was homogenized in distilled water by sonication or lysed in 0.2% sodium dodecyl sulfate (SDS). An aliquot of the cell homogenate was taken for protein determination. An amount of 0.5 μg of C6-CER (extraction standard) was added to the cell lysate. Lipids were extracted according to the procedure of Bligh and Dyer (18). C8-CER (internal standard) was added according to cell protein 0.5 μg/mg to the chloroform phase. To avoid loss of lipids the whole procedure was performed in glassware.

ESI-MS/MS

For tandem mass spectrometric analysis, a PE Sciex API 365 equipped with an IonSpray device was used. All standards and cell extracts were dissolved in an appropriate volume of 5 mm ammonium formate in methanol–chloroform 3:1 (v/v) just prior to analysis. For optimization of ESI and tandem mass spectrometer parameter, a CER standard mixture containing 1 μg/mL of each species was infused into the electrospray source via a 100 μm i.d. fused silica transfer line using a Harvard Apparatus pump at 5 μL/min. Thirty-five μL aliquots of calibrator and analytical samples were injected by an autosampler in a constant flow of 20 μL/min provided by PE series 200 micro lc pump. For positive precursor ion scan of 264 (PIS 264), the following analysis parameters were used, unless otherwise indicated: ion spray voltage 5900 V, orifice voltage 52 V, collision energy 35 V, collision gas nitrogen 5.0 (pressure $2.1 \cdot 10^{-5}$ torr), step size 1 amu, dwell time 15 ms, data collection window 5 min. Measurement and data analysis were done by the PE Sciex software LC Tune 1.3 and Sample control 1.3. The total ion chromatograms (TIC) of the precursor ion scan 264 were used for identification of the sample peak. An automatic Apple script detected the sample peak in the TIC and, based on the scans within this peak, the average intensities and the ratios to the internal standard of the different CER species peak were calculated.

Calibration lines

For calibration line measurement, a chloroform solution of cellular lipid extract was divided into equal amounts and 0.5 μg/mg C8-CER as internal standard was added. Different concentrations of distinct CER species were spiked, the chloroform was removed, and the samples were dissolved in 5 mm ammonium formate in methanol–chloroform 3:1 (v/v) to get the indicated concentration.

CER TLC analysis

Cellular lipids were separated by high performance thin-layer chromatography (HPTLC) with a modified procedure of Motta et al. (7). In brief, samples were dissolved in chloroform–methanol 1:1 (v/v) and applied to silica gel HPTLC. Plates were run twice with chloroform–methanol–acetic acid 190:9:1 (v/v), air dried, and stained by dipping in 10% cupric sulfate (in 8% phosphoric acid) and heated to 170°C. Quantitation was achieved by scanning with a Camag TLC scanner II.

DAG kinase assay

Cellular CER was determined as described by Van Veldhoven et al. (5) except that 1.25 μg of a high purity DAG kinase (Calbiochem-Novabiochem Corporation, La Jolla, CA) was used. Reaction product analysis was performed by high performance thin-layer silica gel 60 plates (Merck, Darmstadt, Germany), which were developed in the solvent chloroform–methanol–acetic acid 65:15:5 (v/v) and analyzed with a Berthold Tracemaster 20 TLC analyzer.

Protein determination

Protein concentrations were measured according to Smith et al. (19).

RESULTS

Fragmentation of CER

It has been shown previously that collision-induced dissociation (CID) of CER in positive ion mode generates mainly the characteristic product ions of *m/z* 264 and 282, whereas other sphingosine backbone metabolites, such as glucosylceramides, showed different fragmentation characteristics (17). The CER fragments resulted from the loss of N-linked fatty acid, one and two molecules water, respectively (**Fig. 1**). This dissociation pattern was confirmed for distinct CER species, varying in N-linked fatty acid moiety (2–24 carbons length), and a solvent and ionization additive was selected to generate a

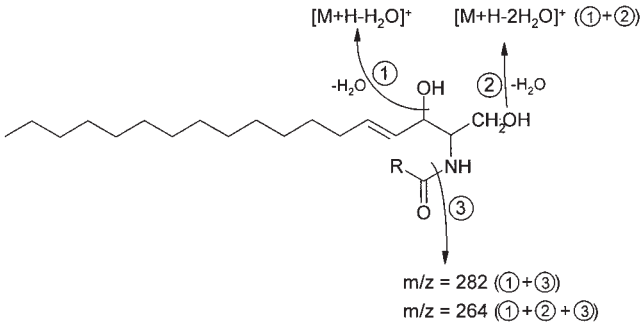

Fig. 1. CER fragmentation pattern.

maximum of positive ions. Analysis of a combination of different CER standards in various solvents revealed a mixture of 5 mm ammonium formate in methanol–chloroform 3:1 (v/v), which showed best ionization efficiency (data not shown). Electrospray parameter and collision offset were tuned to favor generation of the *m/z* 264 product ion, where optimal collision activation was found at 35 V with nitrogen 5.0 as collision gas (pressure $2.1 \cdot 10^{-5}$ torr).

Identification of CER species in crude lipid extract

Now the described specific fragmentation pattern was used to detect CER species from crude lipid mixtures. Under optimized conditions, precursor ion scan at *m/z* 264 (PIS 264) of different CER standards showed two distinct peaks for each CER species $[M + H]^+$ and $[M + H - H_2O]^+$. All precursor ion peaks obtained by the analysis of an unprocessed fibroblast lipid extract could be attributed to the mass of certain CER species. The main peaks were assigned to CER with fatty acid moiety lengths of C16, C22, C24, C24:1, each of them occurring unfragmentated $[M + H]^+$ and with a loss of one molecule water $[M + H - H_2O]^+$, respectively (**Fig. 2**). Additionally, fibroblast lipid extracts showed varying small quantities of C18-, C20-CER and the N-linked hydroxy fatty acids C18:1-OH and C22:1-OH CER species.

Principle of quantitation

For quantitative assessment, a constant amount of non-naturally occurring internal standard (C8-CER) was added to the lipid extracts. Although this is sufficient for comparison of different CER profiles measured under constant instrument settings, it allows neither quantitation nor relation of profiles measured under different conditions. Therefore, in order to achieve quantitative values, calibration curves were established by spiking different amounts of natural CER to lipid extracts. The obtained calibration lines showed high linearity for both unfragmented and water dissociated CER over a broad range (**Fig. 3**). In order to avoid matrix effects or compensate for machine instabilities, which affect ionization efficiency

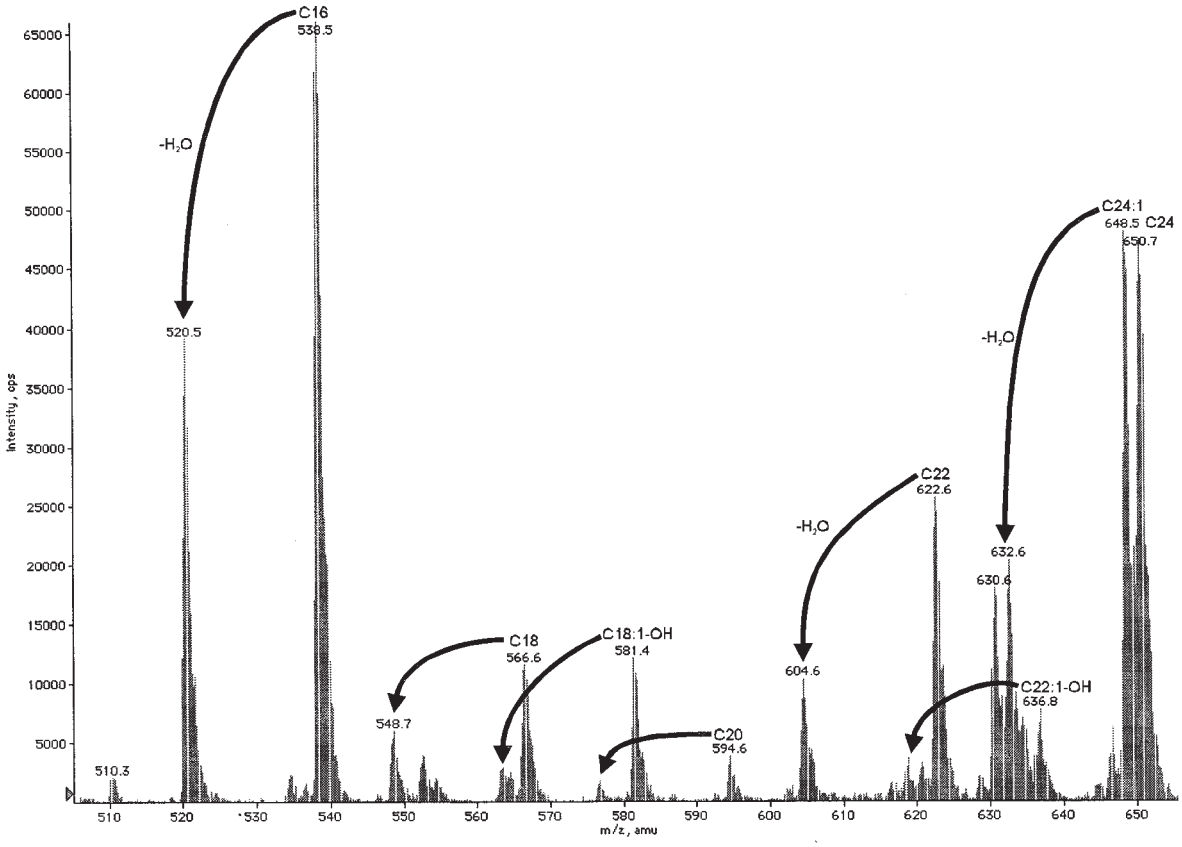

Fig. 2. Precursor ion scan 264 of fibroblast lipid extract. Precursor ion scan 264 of a crude fibroblast lipid extract with a concentration of 1 mg/mL and step size of 0.2 amu, 4 scans. The main CER species were C16, C22, C24:1, C24-CER each occurring as $[M + H]^+$ and without one molecule water $[M - H_2O + H]^+$, respectively.

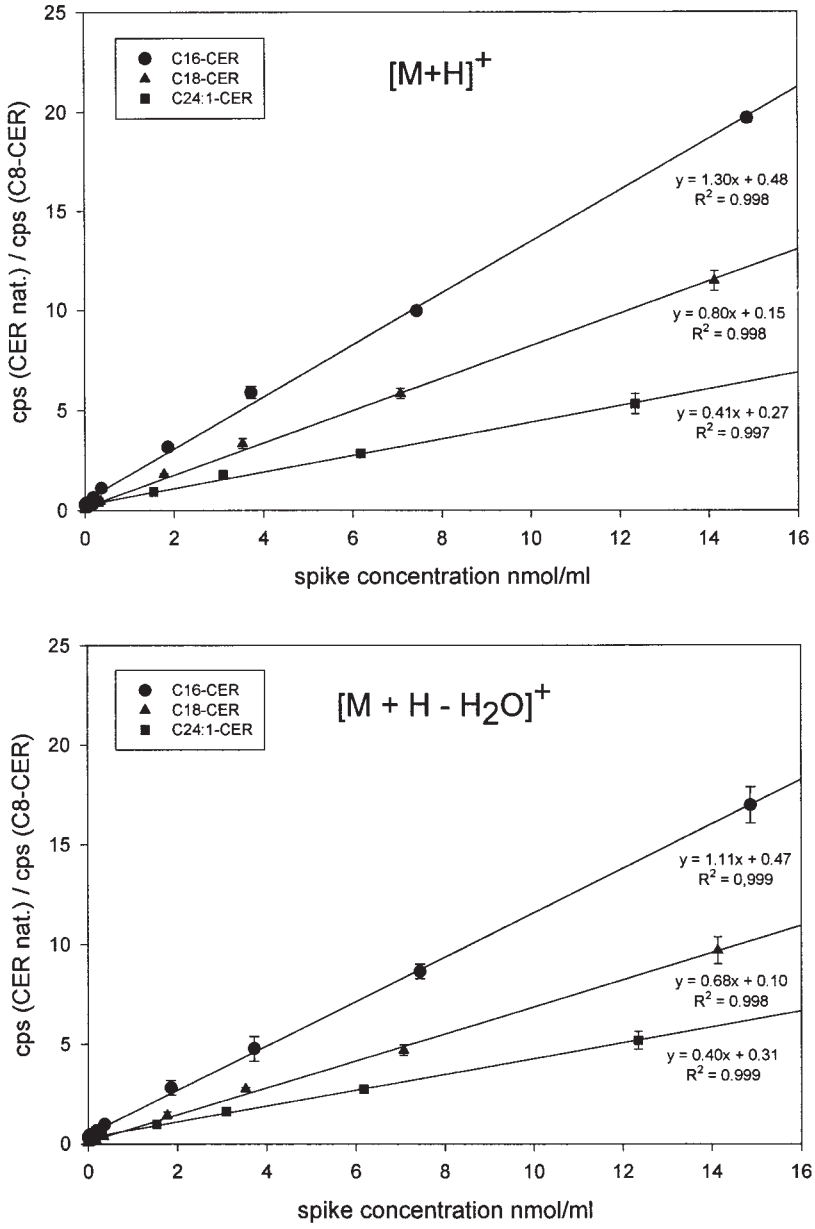

Fig. 3. Calibration lines. Different concentrations of naturally occurring CER species C16, C18, C24:1 were spiked to aliquots of a fibroblast lipid extract. Samples were measured in triplicate; the equation shown resulted from a linear least square fit of spiked concentration and the ratio of the counts per second (cps) of natural CER to cps C8-CER.

and consequently ion counts, all values were expressed as the ratio of counts per second (cps) from the natural CER and the internal standard (C8-CER). Analysis of lipid extracts from different primary fibroblast cultures revealed that the slope of the calibration curves was constant within a 10% error range (**Table 1**). Moreover, calibration line slopes of fibroblasts from Tangier disease patients, an inherited HDL-deficiency syndrome, did not differ from slopes of control fibroblasts. While there was no significant change of the calibration line slope observed up to 300 runs, altered slopes may occur after heavy pollution (Table 1, set 2) or after mass spectrometer maintenance (Table 1, set 3). Pollution of the ESI-MS/MS particularly affected the long-chain CER species by reducing the slope of the calibration curve and the sensitivity. Molar values were calculated from the CER/C8-CER ratio by division through the mean of the calibration curve slopes. For commercially unavailable CER species, the slope of the closest related CER species was used for calculation, e.g., slope of C24:1-CER for C24 and C22-CER. Both the $[M + H]^+$ and the $[M + H - H_2O]^+$ forms of CER were used for quantitation and delivered similar results (**Table 2**). However, in the case of C18:1-OH-CER, the values for $[M + H]^+$ and $[M + H - H_2O]^+$ were found to be markedly different. Except for C18:1-OH-CER, the most precise values were achieved when both peaks were combined for

TABLE 1. Slope of calibration lines of different days

	C6	C16	C18	C24:1
		$[M + H]^+$		
Set 1		1.49 ± 0.14	0.93 ± 0.09	0.42 ± 0.01
Set 2	1.35 ± 0.07	1.23 ± 0.05	0.68 ± 0.04	0.08 ± 0.01
Set 3	1.20 ± 0.08	0.36 ± 0.04	0.37 ± 0.05	0.51 ± 0.05
		$[M + H - H_2O]^+$		
Set 1		1.23 ± 0.13	0.73 ± 0.07	0.41 ± 0.03
Set 2	0.50 ± 0.11	0.50 ± 0.23	0.25 ± 0.12	0.02 ± 0.01
Set 3	0.52 ± 0.05	0.14 ± 0.03	0.16 ± 0.03	0.04 ± 0.01

Different fibroblasts were spiked with natural CER species. Calibration lines were generated by linear least square fit of the ratio CER/C8-CER against the spiked concentration. Regression coefficients R^2 were above 0.98 and each line consisted of at least 5 different spike concentrations. The displayed values are mean ± SD of the regression line slope from 4 different fibroblast cultures. Three control and one with monogenetic defect.

TABLE 3. Serial dilution of a calibration line

Dilution Factor	C6 Slope	C6 Ord.	C16 Slope	C16 Ord.	C18 Slope	C18 Ord.	C24:1 Slope	C24:1 Ord.
				$[M + H]^+$				
1	1.19	0.03	1.89	1.65	1.17	0.22	0.18	0.54
2	1.18	0.01	1.88	1.58	1.14	0.23	0.15	0.56
4	1.07	0.02	1.68	1.51	1.01	0.19	0.16	0.52
8	1.10	0.02	1.69	1.58	1.07	0.19	0.14	0.52
16	0.88	0.02	1.40	1.40	0.83	0.17	0.11	0.45
				$[M + H - H_2O]^+$				
1	0.66	0.01	1.21	1.31	0.80	0.15	0.12	0.45
2	0.61	0.01	1.19	1.26	0.70	0.15	0.11	0.47
4	0.63	0.01	1.16	1.21	0.67	0.13	0.10	0.45
8	0.56	0.01	1.07	1.18	0.62	0.13	0.08	0.43
16	0.55	0.01	0.95	1.12	0.58	0.12	0.07	0.39

CER species were spiked to fibroblast lipid extract corresponding to 1 mg/ml cellular protein concentration. The samples were serial diluted 2-fold resulting in a protein concentration of 62.5 μg/ml. Calibration lines were generated by linear least square fit of the ratio CER/C8-CER against the spiked concentration. All regression coefficients R^2 were above 0.98 and each line consisted of 8 different spike concentrations. The displayed values are the slope and the ordinate of the regression equation.

calculation $[M + H]^+ + [M + H - H_2O]^+$, as the majority of the CER specific signal was included (Table 2).

Influence of the analyte concentration

In order to investigate whether the concentration of cellular lipid extract is critical for an exact quantitation, a serial twofold dilution of spiked cellular lipid extracts was performed (**Table 3**). This experiment showed a characteristic feature of ESI analysis, because in contrast to reduced CER concentrations, the ion counts were not affected in a similar way. Thus, at a 16-fold dilution, the observed counts were only 30% reduced compared to the undiluted samples. This is most likely due to the suppression of ESI by matrix components, e.g., salts, and therefore a dilution of these matrix components may compensate for the reduced signal due to lower CER concentrations. Dilution reduced the slope of the calibration line but did not alter the molar values, because this was accompanied with a reduced CER/C8-CER ratio for the unspiked samples. Consequently the calculated amounts for the naturally occurring CER species were constant over the whole tested dilution range. Because of a significant slope reduction and loss of sensitivity from the 8-fold (equivalent to 125 μg cell protein/mL) to 16-fold (equivalent to 62.5 μg cell protein/mL) dilution, we preferred 250 μg cell protein/mL as standard concentration and 35 μL as injection volume, making an aliquot of less than 10 μg cell protein sufficient for quantitation of different CER species.

Extraction standard

We also introduced an extraction standard (C6-CER) to exclude incomplete CER extraction. A certain amount of C6-CER was added to the cell homogenate and the found amount of C6-CER was compared with the expected mass. In all measured samples an almost complete C6-CER recovery was found, varying from 90% to 105%.

Influence of the extraction matrix

In signal transduction processes it is necessary to freeze the second messenger profile at distinct time points, therefore the influence of 0.2% sodium dodecyl sulfate (SDS), a fast cell lysing, anionic detergent, on ESI-MS/MS CER analysis was investigated (**Table 4**). Calibration lines of lipid extracts from cells either lysed in water by sonication or in 0.2% SDS were measured and both matrixes showed high linearity and low variance. SDS caused a

TABLE 2. Comparison of different CER peaks

	C6	C16	C18	C18:1-OH	C22	C24:1	C24
				$[M + H]^+$			
pmol/mg	4501	327	20	595	72	225	304
CV (%)	2.6	4.6	7.9	3.2	7.8	5.4	5.0
				$[M + H - H_2O]^+$			
pmol/mg	4593	250	15	329	64	253	250
CV (%)	4.2	5.8	13.8	5.2	4.2	5.1	4.0
				$[M + H]^+ + [M + H - H_2O]^+$			
pmol/mg	4536	262	16	459	81	254	305
CV (%)	2.5	4.1	3.8	3.8	3.5	3.7	3.6

The displayed values were the means of an aliquoted fibroblast lipid extract (in pmol/mg cell protein) and the coefficients of variation (CV) of the unfragmented CER $[M + H]^+$, CER without water $[M + H - H_2O]^+$, and the sum of both (n = 10).

TABLE 4. Comparison of water and 0.2% SDS as matrix for lipid extraction

Cell Homogen.	C6	C16	C18	C24:1
		$[M + H]^+$		
Water	1.28 ± 0.10	0.43 ± 0.04	0.46 ± 0.04	0.61 ± 0.06
0.2% SDS	1.20 ± 0.10	0.34 ± 0.03	0.35 ± 0.03	0.49 ± 0.04
		$[M + H - H_2O]^+$		
Water	0.60 ± 0.04	0.21 ± 0.02	0.16 ± 0.01	0.07 ± 0.01
0.2% SDS	0.51 ± 0.05	0.13 ± 0.01	0.10 ± 0.01	0.04 ± 0.01

Lipid extracts prepared from cells either homogenized in water by sonication or lysed in 0.2% SDS were spiked with natural CER species. Calibration lines were generated by linear least square fit of the ratio CER/C8-CER against the spiked concentration. Regression coefficients R^2 were above 0.99 and each line consisted of at least 5 different spike concentrations. The displayed values are mean ± SD of the regression line slope from 3 different fibroblast cultures.

slight decrease in calibration slope and sensitivity, due to a 20% reduction in ion counts.

Comparison with other methodologies

It was previously shown that Tangier fibroblasts are characterized by an increased cellular concentration of CER (20). In order to compare the ESI-MS/MS method with other methodologies conventionally used for ceramide quantitation, the CER content from control and Tangier disease fibroblasts was determined with thin-layer chromatography, DAG kinase assay, and ESI-MS/MS, respectively (**Table 5**). While similar results were obtained for ESI-MS/MS and the TLC determination, the DAG kinase assay showed much higher CER levels. All methods showed significantly higher total CER in Tangier disease fibroblasts. For ESI-MS/MS, the total CER concentration was calculated as the sum of the different CER species. The data obtained with ESI-MS/MS demonstrate that elevation of total CER concentration in Tangier fibroblasts is due to a similar increase in different CER species and is not caused by the elevation of a single CER species.

Limits of detection

In order to account for matrix effects, the limits of detection were investigated in spiked cellular lipid. For naturally occurring CER species, the detection limit was defined by the first spiked concentration showing a significant signal increase. The following limits were determined in a spike experiment with matrix concentration 0.25 mg/mL for the sum of both $[M + H]^+$ and $[M + H - H_2O]^+$ CER peaks: C16-CER 0.3 pmol, C18-CER 0.3 pmol, C24:1-CER 0.3 pmol.

TABLE 5. Comparison of TLC and ESI-MS/MS

Assay	Control	Tangier
TLC	2.1 ± 0.8	4.5 ± 2.2[a]
ESI-MS/MS	2.0 ± 0.4	3.6 ± 1.1[a]
DAG kinase	12.0 ± 1.0	18.9 ± 2.7[a]

CER content from control and Tangier disease fibroblasts was assessed with a thin-layer chromatography (TLC) assay and ESI-MS/MS and DAG kinase assay. The shown values are mean ± SD of four independent experiments for TLC, two for ESI-MS/MS and DAG kinase, respectively. Each experiment consisted of three control and two Tangier fibroblast cultures.

[a] Significantly different from control $P < 0.01$.

DISCUSSION

The aim of the current study was to establish a methodological protocol that enables not only qualitative but quantitative determination of various CER species from crude lipid extracts using tandem mass spectrometry. The method was based on the previous observation that CER in positive ion mode generate mainly the characteristic product ions of m/z 264 and 282, whereas other sphingosine backbone metabolites show different fragmentation characteristics (17). The high specificity of the PIS 264 almost exclude any incorrect measurement of non-CER molecules. Accordingly, all precursor ion peaks obtained by the analysis of an unprocessed fibroblast lipid extract could be attributed to the mass of certain CER species. The quantitation of different CER species was achieved by the use of a constant amount of a non-naturally occurring internal standard (C8-CER), together with a calibration curve established by spiking different concentrations of natural CER species. Molar values were calculated from the CER/C8-CER-ratio by division through the mean of the calibration curve slopes, whereas for commercially unavailable CER species, the slope of the closest related CER species was used for calculation. Although the application of closely related CER species for calculation permits no exact quantitation of these species, it should allow a good estimate and, at least within one run, allows the comparative analysis of the respective ceramide species. However, in the case of C18:1-OH-CER, the values for $[M + H]^+$ and $[M + H - H_2O]^+$ were found to be markedly different, suggesting that the nonhydroxy C18-CER slope used was not well suited for calculation of hydroxy fatty acid species. An extraction standard was included in order to account for loss of CER during lipid extraction. The calibration curve obtained with this method showed linearity over a wide concentration range. Additional evidence for the specificity of the described method is derived from the fact that both the CER specific molecule peak $[M + H]^+$ and the water-lost form $[M + H - H_2O]^+$ revealed similar results. These features, together with the minimal preanalytic requirements, the low sample amount equivalent to about 10 μg of cell protein, and the rapid analytical process, make the developed methodology well suited for the analysis of signal transduction processes. Moreover, as ESI-MS/MS was performed with a continuous flow injection and an autosampler, the described methodology is suitable for high throughput analysis.

Recently, the standard procedure for CER analysis, the DAG kinase assay, was the subject of controversy. Main criticism was raised from a study showing that FAS ligation resulted in an elevation of intracellular CER concentration, as measured by DAG kinase assay, although analysis by a mass spectrometry method showed no CER induction (6). In contrast, another report, which compared the DAG kinase assay with a HPLC derivatization method,

yielded similar results for both methods (13). In this study we observed similar quantitative values for total CER in human skin fibroblasts using either TLC or the sum of the different CER species as determined by ESI-MS/MS. In contrast, the DAG kinase assay yielded approximately 6-fold higher concentrations. This observation may point to the presence of substances in the lipid extract that interfere with the detection principle of the DAG kinase assay as suggested by Watts et al. (6). However, a principal limitation of the DAG kinase assay as well as of TLC is their inability to discriminate between different CER species and/or de novo formed dihydroceramide. The discrimination between CER and dihydroceramides is of particular importance, as only the unsaturated ceramide species and not the dihydro forms are currently regarded as active signaling molecules (1, 3). Because the presented ESI-MS/MS method is able to distinguish between ceramides and dihydroceramides, it should offer an advantage compared to the other methods. Moreover, for differentiating the pathways of ceramide generation either via sphingomyelinase activation or de novo synthesis (21), the determination of the precursor dihydroceramide may be of interest. Although the current protocol does not include the quantification of dihydroceramides, this can also be achieved with ESI-MS/MS and will be subject of future work. Although the quantitation by TLC allows a limited separation of different CER species, this technique requires high sample volumes. CER analysis using radiolabeled precursors is not the method of choice for CER quantitation and even the investigation of kinetics may be limited by non-homogenous labeling of distinct cellular pools.

Currently available procedures which are principally capable of separating different CER species, e.g., HPLC, need initial derivatization steps, as CER lacks a chromophore allowing a sensitive UV or fluorescence detection (11–14). All these techniques are time consuming and tricky derivatization steps are often a source of error. In the past years mass spectrometry was discovered for the detection of sphingolipids and especially CER analysis. The methodology offers the advantage that fluorescence derivatization is not necessary, as CERs are identified by their mass and their fragmentation characteristics. However, in contrast to the method described in this manuscript, currently existing methodological protocols still need prior separation of the lipid extract by HPLC (15, 16). Moreover, the method described by Gu et al. (17) was improved at several points. First, C8-CER as internal standard was more closely related to naturally occurring CER species than the C2-CER formerly used. Second, with utilization of calibration lines it is possible to quantify the spiked CER species exactly and estimate the closely related CER species, making results comparable to those achieved under different machine conditions. Third, this protocol does not need additional purification of the lipid extract. Fourth, using a continuous flow and an autosampler, the described methodology provides high throughput capability.

In summary, the established ESI-MS/MS method for the quantitation of different CER species may be a powerful tool in signal transduction research and offers several advantages compared to already existing methods. Moreover, a major advantage of using MS/MS as a platform for quantitation of CER is that this technology can be used in a similar way to quantify other metabolites of the sphingolipid and glycerophospholipid signaling pathways. Thus, the simultaneous assessment of multiple second messengers could be achieved within a reasonable time and from a limited amount of material. The need for such a multiplex signal analysis, especially in the sphingolipid pathway, is underlined by the fact that nearly all metabolites can readily be converted to each other and are known to have second messenger function with sometimes opposite cellular effects (22). Thus, future development using ESI-MS/MS as a technology platform should enable a rapid, simultaneous analysis of multiple lipid second messenger from crude lipid extract, allowing a more detailed interpretation of complex signaling processes.

This work was supported by Deutsche Forschungsgemeinschaft (DFG DR 348/2-1) (to Wolfgang Drobnik).

Manuscript received 3 February 1999 and in revised form 3 May 1999.

REFERENCES

1. Gomez-Munoz, A. 1998. Modulation of cell signalling by ceramides. *Biochim. Biophys. Acta.* **1391:** 92–109.
2. Jayadev, S., B. Liu, A. E. Bielawska, J. Y. Lee, F. Nazaire, M. Y. Pushkareva, L. M. Obeid, and Y. A. Hannun. 1995. Role for ceramide in cell cycle arrest. *J. Biol. Chem.* **270:** 2047–2052.
3. Pena, L. A., Z. Fuks, and R. Kolesnick. 1997. Stress-induced apoptosis and the sphingomyelin pathway. *Biochem. Pharmacol.* **53:** 615–621.
4. Venable, M. E., J. Y. Lee, M. J. Smyth, A. Bielawska, and L. M. Obeid. 1995. Role of ceramide in cellular senescence. *J. Biol. Chem.* **270:** 30701–30708.
5. Van Veldhoven, P. P., W. R. Bishop, D. A. Yurivich, and R. M. Bell. 1995. Ceramide quantitation: evaluation of a mixed micellar assay using E. coli diacylglycerol kinase. *Biochem. Mol. Biol. Int.* **36:** 21–30.
6. Watts, J. D., M. Gu, A. J. Polverino, S. D. Patterson, and R. Aebersold. 1997. Fas-induced apoptosis of T cells occurs independently of ceramide generation. *Proc. Natl. Acad. Sci. USA.* **94:** 7292–7296.
7. Motta, S., M. Monti, S. Sesana, L. Mellesi, R. Ghidoni, and R. Caputo. 1994. Abnormality of water barrier function in psoriasis. Role of ceramide fractions. *Arch. Dermatol.* **130:** 452–456.
8. Andrieu, N., R. Salvayre, J. P. Jaffrezou, and T. Levade. 1995. Low temperatures and hypertonicity do not block cytokine-induced stimulation of the sphingomyelin pathway but inhibit nuclear factor-kappa B activation. *J. Biol. Chem.* **270:** 24518–24524.
9. Smith, E. R., and A. H. J. Merrill. 1995. Differential roles of de novo sphingolipid biosynthesis and turnover in the "burst" of free sphingosine and sphinganine, and their 1-phosphates and N-acyl-derivatives, that occurs upon changing the medium of cells in culture. *J. Biol. Chem.* **270:** 18749–18758.
10. Tepper, A. D., J. G. Cock, E. de Vries, J. Borst, and W. J. van Blitterswijk. 1997. CD95/Fas-induced ceramide formation proceeds with slow kinetics and is not blocked by caspase-3/CPP32 inhibition. *J. Biol. Chem.* **272:** 24308–24312.
11. Iwamori, M., C. Costello, and H. W. Moser. 1979. Analysis and quantitation of free ceramide containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine by high-performance liquid chromatography. *J. Lipid Res.* **20:** 86–96.
12. Previati, M., L. Bertolaso, M. Tramarin, V. Bertagnolo, and S. Capitani. 1996. Low nanogram range quantitation of diglycerides and ceramide by high-performance liquid chromatography. *Anal. Biochem.* **233:** 108–114.
13. Garzotto, M., J. M. White, Y. Jiang, D. Ehleiter, W. C. Liao, F. A.

Haimovitz, Z. Fuks, and R. Kolesnick. 1998. 12-*O*-tetradecanoylphorbol-13-acetate-induced apoptosis in LNCaP cells is mediated through ceramide synthase. *Cancer Res.* **58:** 2260–2264.
14. Yano, M., E. Kishida, Y. Muneyuki, and Y. Masuzawa. 1998. Quantitative analysis of ceramide molecular species by high performance liquid chromatography. *J. Lipid Res.* **39:** 2091–2098.
15. Couch, L. H., M. I. Churchwell, D. R. Doerge, W. H. Tolleson, and P. C. Howard. 1997. Identification of ceramides in human cells using liquid chromatography with detection by atmospheric pressure chemical ionization-mass spectrometry. *Rapid Commun. Mass. Spectrom.* **11:** 504–512.
16. Mano, N., Y. Oda, K. Yamada, N. Asakawa, and K. Katayama. 1997. Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. *Anal. Biochem.* **244:** 291–300.
17. Gu, M., J. L. Kerwin, J. D. Watts, and R. Aebersold. 1997. Ceramide profiling of complex lipid mixtures by electrospray ionization mass spectrometry. *Anal. Biochem.* **244:** 347–356.
18. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
19. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid [published erratum appears in *Anal. Biochem.* 1987. **163:** 279]. *Anal. Biochem.* **150:** 76–85.
20. Drobnik, W., G. Liebisch, C. Biederer, B. Trümbach, G. Rogler, P. Müller, and G. Schmitz. 1999. Growth and cell cycle abnormalities of fibroblasts from Tangier disease patients. *Arterioscler. Thromb. Vasc. Biol.* **19:** 28–38.
21. Bose, R., M. Verheij, F. A. Haimovitz, K. Scotto, Z. Fuks, and R. Kolesnick. 1995. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell.* **82:** 405–414.
22. Igarashi, Y. 1997. Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. *J. Biochem.(Tokyo)* **122:** 1080–1087.