ELSEVIER

Journal of Chromatography A, 876 (2000) 229–233

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Ceramide analysis utilizing gas chromatography–mass spectrometry

Klaus Raith[a,*], Jörg Darius[b], Reinhard H.H. Neubert[a]

[a]*Department of Pharmacy, Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University, Wolfgang-Langenbeck-Strasse 4, 06120 Halle (Saale), Germany*

[b]*Institute of Clinical Pharmacology, Otto-von-Guericke-University, Leipziger Strasse 44, 39120 Magdeburg, Germany*

Received 27 August 1999; received in revised form 24 January 2000; accepted 31 January 2000

Abstract

Suitable analytical methods are a prerequisite of a detailed investigation of ceramides. Therefore, a new gas chromatograph–mass spectrometry method with electron impact ionization was developed. Samples have been prepared for gas chromatography by the formation of volatile trimethylsilyl derivatives. The method provides high separation efficiency, sensitivity and specificity. Mass spectra facilitate the structural characterization of each species, because certain fragments indicate the fatty acid as well as the sphingoid base moiety. In a 30-mm run even very similar ceramides are baseline separated. The method is compared to a recently published assay for liquid chromatography–mass spectrometry.

Keywords: Ceramides; Fatty acids

1. Introduction

Ceramides are amides of fatty acids with long-chain sphingoid bases. In the course of an increasing research interest in ceramides, particularly as signal transducers and skin lipids, the need for powerful, specific analytical methods became obvious. The combination of chromatographic separation and mass spectrometry is best suited to solve this challenging problem.

The classical approach in ceramide analysis is based on thin-layer chromatography. If ceramides are to be analyzed in a general lipid extract, mostly a preseparation into polar and nonpolar lipids is followed by a separation into four to seven ceramide bands [1–3]. Since the silica plates select the ceramides predominantly according to number and position of hydroxy groups, each of these bands represents a certain structure of long-chain sphingoid base and fatty acid, irrespective of chain length.

If a more specific analysis is required, liquid or gas chromatography are the methods of choice. Ceramides may be hydrolyzed according to the method of Gaver and Sweeley [4]. Fatty acids as well as sphingoid bases can separately be analyzed by gas chromatography utilizing a variety of established methods. However, these data do not reveal information about the combination of both.

Since ceramides do not show a sufficient volatility, the polar hydroxy groups have to be subjected to derivatization. Different approaches have been reported, particularly yielding trimethylsilyl derivatives

*Corresponding author. Tel.: +49-345-552-5215; fax: +49-345-552-7292.

E-mail address: raith@pharmazie.uni-halle.de (K. Raith)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00144-8

[5] and permethylated ceramides [6]. GC analysis methods of these derivatives using flame ionization detection (FID) and mass spectrometry (MS), have been described [7–9], mostly relying on classical packed columns. Very few assays with the more powerful capillary columns have been presented for plasma [10,11] or hair ceramides [12] and ceramides from *Urtica dioica* [13], respectively.

HPLC is a possible alternative. UV and fluorescence detection require derivatization. In contrast, LC–MS does not and is more specific as well. However, the most commonly used electrospray (ESI) and chemical ionization interfaces generate only molecular ions but no fragment ions. If more structural information than molecular mass is needed, tandem mass spectrometry is required [14,15]. Compared to LC–MS–MS, GC–MS is a less expensive and widely used technique providing structural information already on the single MS stage. The electron impact ionization (EI) spectra are obtained under standardized conditions and therefore library searchable. Although first approaches to ESI-MS–MS libraries have been presented, the comparability of these spectra is much more difficult.

In the present paper, a general GC–MS method is presented and applied to commercially available ceramide mixtures obtained from bovine brain sphingomyelin. It is discussed in comparison with a state-of-the-art method of LC–MS–MS.

2. Materials and methods

2.1. Chemicals

Ceramide III (also coded as Cer [NS] [3]), a non-hydroxy fatty acid ceramide mixture, and ceramide IV (also coded as Cer [AS] [3]), a 2-hydroxy fatty acid ceramide mixture, both prepared from bovine brain sphingomyelin, were obtained from Sigma (Deisenhofen, Germany) (see Fig. 1). Following the nomenclature in Refs. [1,3], ceramide III (Sigma) should be classified as ceramide II, since it has a sphingosine-type instead of a phytosphingosine-type base. The derivatization reagents *N*,*O*-bis-(trimethylsilyl)-acetamide (BSA), trimethylchlorosilane (TMCS), *N*-trimethylsilylimidazol (TSIM), hexamethyldisilazane (HMDS) and pyridine (99+%) were purchased from the same supplier.

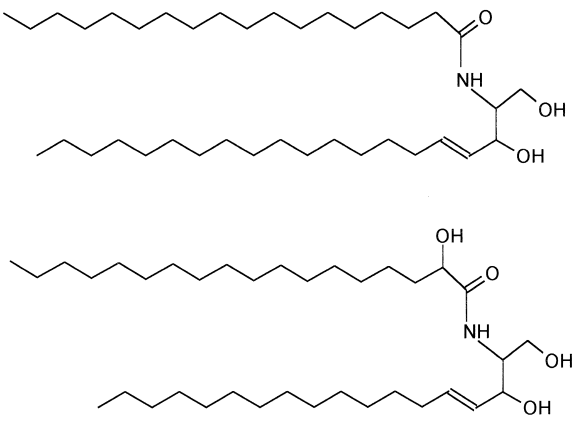

Fig. 1. C_{18} fatty acid species of ceramide III (top) and ceramide IV (bottom).

2.2. Sample preparation

Several protocols have been tested (see Section 3). The finally approved derivatization procedure was as follows: to 1 mg ceramide 0.5 ml of a mixture BSA–TMCS–TSIM (3:2:3, v/v/v) were added. Subsequently, the tubes were subjected to 3 h of heating to 75°C in a thermoblock.

2.3. GC–MS

GC–MS analyses were carried out using a gas chromatograph HIP 5890 Series II coupled to a mass spectrometer HP 5989 A MS-Engine (Hewlett-Packard, Waldbronn, Germany). This apparatus was equipped with a capillary column Optima (30 m× 0.32 mm I.D., film thickness 0.1 μm; Macherey–Nagel, Duren, Germany). The carrier gas was helium (0.6 bar, at 260°C; flow approximately 1.2 ml/min). A total of 0.5 μl of each sample was injected splitless. The injector and the transfer line were kept at 320 and 330°C, respectively. Initially, the oven temperature was held at 260°C and then increased at 3°C/min up to 360°C and kept at this temperature for 20 min.

3. Results and discussion

Since ceramides are relatively large and polar lipids, they have to be subjected to derivatization before GC. Three protocols have been tested. Success was surveyed by checking the ESI mass spectra, which are easily accessible by introducing a diluted solution via syringe pump to an ESI-MS. Under single MS conditions (quadrupole or ion trap), the molecular mass can be obtained [14,15].

Firstly, 200 μg of ceramide were treated with 100 μl pyridine, 20 μl HMDS and 10 ml TMCS and kept for 20 min at room temperature [7]. This was principally successful but not complete. Furthermore, in the case of ceramide IV precipitation has been observed. Secondly, 6.8 mg of ceramide were treated with 0.5 ml TSIM (1 h at 60°C) and subsequently with 0.3 ml BSA and 0.15 ml TMCS (overnight at 60°C). This approach was successful but time consuming. Thirdly, 0.5 ml of a mixture BSA–TMCS–TSIM (3:2:3) were added to 1 mg of ceramides and subjected to thermostatting for 6–24 h at 60°C. This method was further modified in the way described in Section 2. Thus, the two to three hydroxy groups of each ceramide molecule could completely be transferred into the trimethylsilylated forms.To achieve structural information, ionization was implemented throughout by EI.

Under the described conditions, very efficient separations have been observed. Fig. 2 shows the separation of two commercially available ceramide mixtures. The ceramides have retention times between 17 and 29 min. The retention order follows the fatty acid chain length. The unsaturated species have slightly shorter retention times than the saturated species with the same chain length. The 3-fold silylated ceramide IV species reach the detector later than the twice silylated ceramide III species. The gas chromatograms show an astonishing similarity to the electrospray mass spectra of these mixtures [14,15]. The ceramide species have been identified by interpreting the related EI mass spectra. Figs. 3 and 4 show spectra of ceramide IV and III species, respectively.

In Refs. [7,16] some EI spectra of ceramides were presented and discussed in detail. Although ceramide IV was not discussed there, the fragment ions of ceramide IV species could be assigned to equivalent structures. Typical fragments are $[M-15]$ (abstraction of methyl) and $[M-90]$ (abstraction of trimethylsilanol). The molecular mass of the entire trimethylsilylated ceramide can be calculated by addition of two fragments resulting from a split between C_2 and C_3 of the sphingosine chain (example in Fig. 3: 311+570=881; molecular mass of ceramide itself: 665 (subtraction of three trimethylsilyl groups)). The fragments m/z 311 as well as $m/z=243$ (abstraction of fatty acid amide and tetradecyl), $m/z=336$ (abstraction of fatty acid amide and trimethylsilanol) and $m/z=426$ (abstraction of fatty acid amide) are characteristic for C_{18} sphingosine. The ions $m/z=103$, $m/z=132$ and $m/z=157$, which were described in Ref. [7], could also be confirmed. Furthermore, a large trimethylsilyl peak was detected at $m/z=73$.

Fig. 4 shows an EI spectrum of a ceramide III species for comparison. The molecular mass is 709 (after subtraction of two trimethylsilyl groups $M=566$). The fragment $m/z=398$ indicates a stearic acid moiety in the ceramide. We initially expected, that the mass analyzer should be able to handle masses up to 1000 u since the molecular mass of the trimethylsilylated ceramides is relatively high ($M+216$ in the case of three hydroxy groups). But all intense and significant ions were in a range that most commercially available single quadrupole mass spectrometers are able to cope with.

The EI spectra enabled an unequivocal identification of each ceramide following the rules pointed out in Ref. [7].

Quantitative determinations have been performed to a limited extent up to now. Using compound specific ions with higher masses, the limit of detection in full-scan mode (S/N 3:1) is expected to be in the low ng range for the single ceramides contained in the mixture (in 0.5 μ1 derivatization mixture, splitless injection). The selected ion monitoring (SIM) mode enables a factor 10 improvement in sensitivity. The knowledge of the fragmentation principles of ceramides (see above) facilitates the selection of appropriate ions for SPA, even to search for substances in concentrations below the full-scan detection limit.

In conclusion, one can state that the described

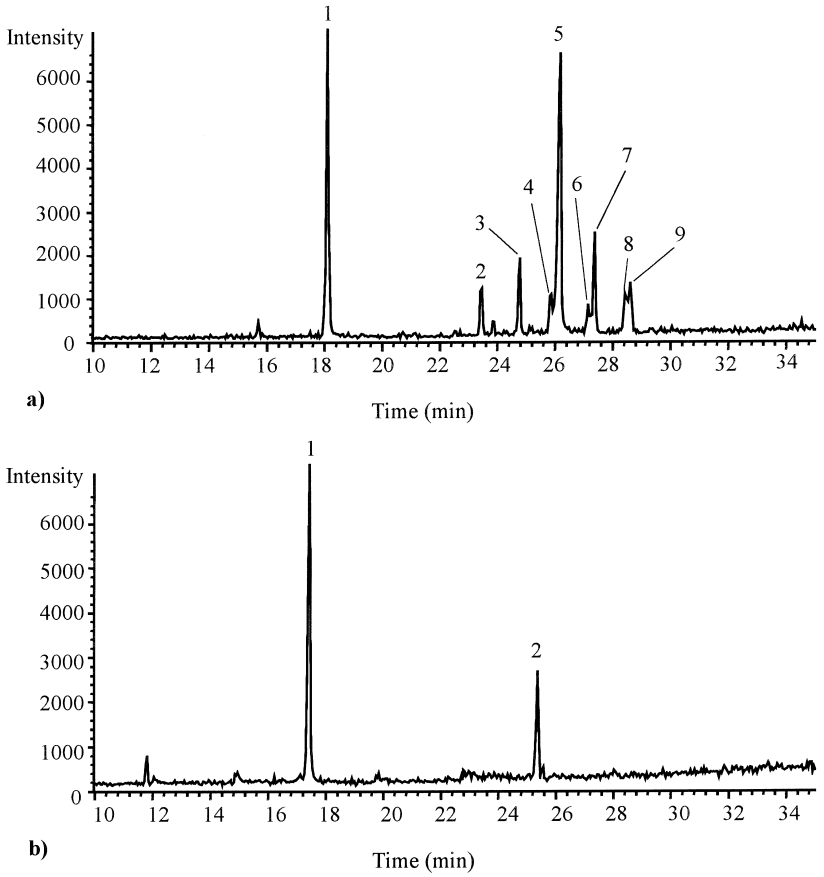

Fig. 2. GC–MS separation (ion chromatogram, $m/z=311$) of ceramide mixtures. The long-chain base is C_{18} sphingosine throughout. The acyl moiety varies in chain length and presence or absence of a double bond. Each peak represents a certain acyl sphingosine. The code for each fatty acid is: number of carbons:number of double bonds (e.g., 18:0 stands for 2-hydroxystearic acid and stearic acid, respectively, (a) Ceramide IV (Sigma), 2-hydroxy fatty acid ceramides; (1) 18:0, (2) 22:0, (3) 23:0, (4) 24:1, (5) 24:0, (6) 25:1, (7) 25:0, (8) 26:1, (9) 26:0 (b) Ceramide III (Sigma), non-hydroxy fatty acid ceramides; (1) 18:0, (2) 24:1. See text for further explanation.

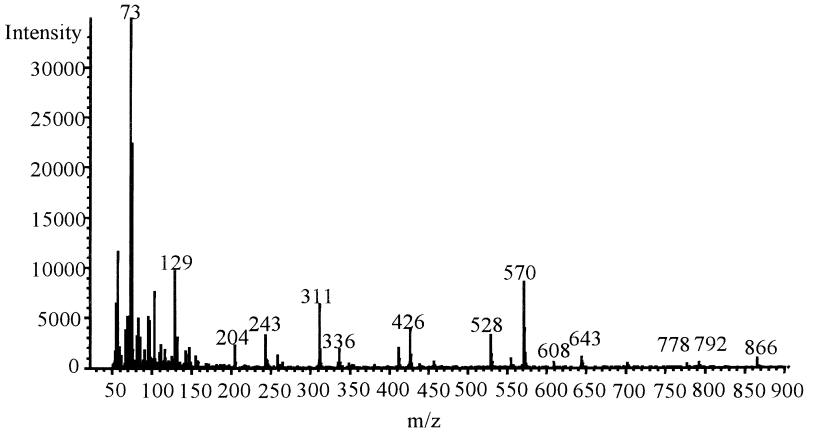

Fig. 3. EI mass spectrum of Tris-(trimethylsilyl)-*N*-(2-hydroxylignoceroyl)sphingosine. See text for explanation.

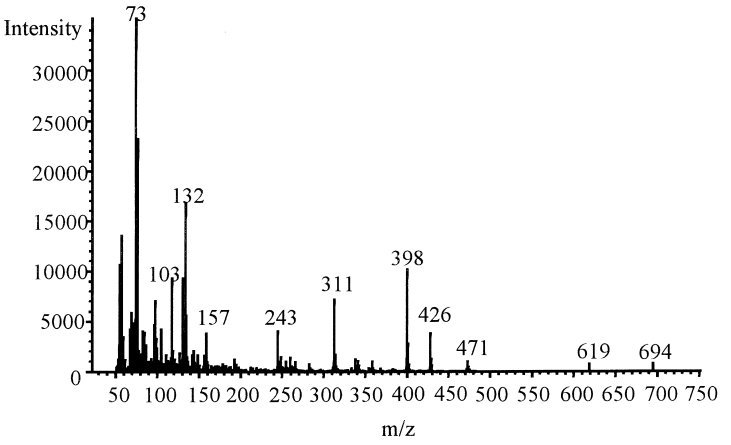

Fig. 4. EI mass spectrum of bis-(trimethylsilyl)-*N*-stearoylsphingosine. See text for explanation.

GC–MS method for ceramides provides efficient separation and an unequivocal structure identification by means of a library of searchable electron impact spectra. It even has the option of sensitive quantification The requirement of derivatization is considered to be a disadvantage of GC, especially in comparison to the recently presented LC–MS assay. The presence of the trimethylsilylated ceramides in a surplus of derivatization reagents may cause a contamination of the GC–MS system and thereby affect quantification stability. This can be detrimental in the case of high sample throughput, but at least in part compensated by the use of an appropriate internal standard.

The high performance of the described GC–MS method for ceramides with respect to separation and substance identification can only be reached in LC–MS by using a sophisticated LC–MS–MS procedure. Because of the higher price compared to a GC–MS quadrupole system, tandem MS is not yet standard equipment in most laboratories. Furthermore, the application of appropriate MS–MS parameters requires an additional analysis, and the number of simultaneously available spectra is limited. Therefore GC–MS is not only a viable alternative in ceramide analysis, but in many cases even superior.

References

[1] P.W. Wertz, D.T. Downing, J. Lipid Res. 24 (1983) 759.
[2] A.P.M. Lavrijsen, I.M. Higounenc, A. Weerheim, E. Oestmarin, E.E. Tuinenburg, H.E. Boddé, M. Ponec, Arch. Dermatol. Res. 286 (1994) 495.
[3] K.J. Robson, M.E. Stewart, S. Michelsen, N.D. Lazo, D.T. Downing, J. Lipid Res. 35 (1994) 2060.
[4] R.C. Gaver, C.C. Sweeley, J. Am. Oil Chem. Soc. 42 (1965) 294.
[5] B. Samuelsson, K. Samuelsson, Biochim. Biophys. Acta 164 (1968) 121.
[6] R.T.C. Huang, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 1306.
[7] K. Samuelsson, B. Samuelsson, Chem. Phys. Lipids 5 (1970) 44.
[8] M.G. Horning, S. Murakami, E.C. Horning, Am. J. Clin. Nutr. 24 (1971) 1086.
[9] W. Krivit, S. Hammarström, J. Lipid Res. 13 (1972) 525.
[10] A. Kuksis, J.J. Myher, Adv. Chromatogr. 28 (1989) 267.
[11] A. Kuksis, J.J. Myher, K. Geher, J. Lipid Res. 34 (1993) 1029.
[12] G. Hussler, G. Kaba, A.M. Francois, D. Saint-Leger, Int. J. Cosmet. Sci. 17 (1995) 197.
[13] R. Kraus, G. Spiteller, Liebigs Ann. Chem. (1991) 125.
[14] K. Raith, R. Neubert, Rapid Commun. Mass Spectrom. 12 (1998) 935.
[15] K. Raith, R. Neubert, Anal. Chim. Acta 403 (2000) 295.
[16] B. Samuelsson, L. Samuelsson, J. Lipid Res. 10 (1969) 47.