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Liquid chromatography/electrospray ionisation-mass spectrometry method for the quantification of sphingosine and sphinganine in cell cultures exposed to fumonisins

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Abstract

Fumonisins, mycotoxins produced by *Fusarium verticillioides*, are potent inhibitors of the de novo sphingolipid biosynthesis via inhibition of the key enzyme ceramide synthase. The cellular response to a fumonisin exposure is obvious as an alteration of the ratio of the sphingoid bases sphingosine (SO) and sphinganine (SA). We developed a new column liquid chromatography/electrospray ionisation-mass spectrometry (LC–ESI-MS) method for the rapid, simultaneous and quantitative determination of these bases in cell cultures of immortalised human kidney epithelial cells (IHKE cells). For sample preparation, cell lysates were only diluted, centrifuged and directly used for LC–MS measurements. Quantification was carried out using phytosphingosine (PSO) as an internal standard. Detecting the protonated molecule $[M+H]^+$ signals of SO (m/z 300) and SA (m/z 302) in the selected ion monitoring (SIM) mode, detection limits of 10 pg for SO (signal-to-noise ratio $S/N=3:1$) and 25 pg for SA ($S/N=3:1$) were established. The average recovery for SO and SA was higher than 90% for control IHKE-cells, respectively. The developed LC–ESI-MS method allows the sensitive, selective and rapid monitoring of sphingosine and sphinganine in cell matrices with a drastically reduced time for sample preparation.

Keywords: Mycotoxins; Sphingosine; Sphinganine; Fumonisins

1. Introduction

The fumonisin mycotoxins are structurally related compounds, mainly produced by *Fusarium verticillioides* (formerly *F. moniliforme*) and occur as one of the most common contaminants in corn and corn-based foods and feeds, world-wide [1]. The most prevalent of the fumonisins, fumonisin B_1 (FB_1), shows a species-specific toxicity as its ingestion causes a range of syndromes in animals, including leukoencephalomalacia (ELEM) in horses [2], pulmonary oedema in pigs [3] and hepatotoxic, nephrotoxic, and carcinogenic effects in rats [4,5]. The question if these toxins show a positive correlation to human esophageal cancer rates in South Africa [6] and China [7] remains unanswered. However, most recently a National Toxicology Program (NTP) long-term feeding study provided clear evidence for the carcinogenic activities of FB_1 in female mice and

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male rats [8]. The mode of action of fumonisins is believed to be the disruption of the de novo biosynthesis of sphingolipids, a class of lipids playing key roles in cell growth and differentiation [9]. Fumonisins bear a remarkable structural similarity to sphingosine (SO) (Fig. 1) and have been found to inhibit ceramide synthase [10], a key enzyme in the sphingolipid metabolism which is responsible for the acylation of sphinganine (SA) and SO. This disruption of the biosynthetic pathway of sphingolipid biosynthesis leads to increased levels of SA and decreased levels of complex sphingolipids such as ceramide, sphingomyelin, cerebrosides, gangliosides, and sulfatides [10–12]. Elevations in SA and changes in the ratio sphinganine/sphingosine (SA/SO) have been observed in various animal species consuming fumonisin-contaminated feed, particularly in the sera and urine of vervet monkeys [13,14], ponies [15], chickens [16], rabbits [17], pigs [18], rats [19], mice [20] as well as in humans [21,22]. Further these changes in free sphingoid bases have been described for a number of cell cultures including rat primary hepatocytes [23], cultured renal cells [24] and cultured cerebellar neurons [25]. Not least since the SA/SO ratio has been proposed as a possible biomarker of consumption of fumonisin-contaminated feed [18,26], there has been increasing interest in quantifying SA and SO from tissues, blood and urine. Several methods are available for the determination of these compounds by high-performance liquid chromatography (HPLC), most of which are modifications of the method by Merrill et al. [27]: after extraction with organic solvents, the two sphingoid bases are determined as their *o*-phthaldialdehyde (OPA) derivatives using fluorescence detection [26,28–30]. The major disadvantages of this method are fluorescent interferences caused by matrix components making a definite and accurate quantitative determination difficult. Other methods are based on the formation of more stable compounds such as aminoquinolylcarbonyl [31], biphenylcarbonyl [32] or *p*-nitrophenylacetyl [33] derivatives. However, these methods also cannot eliminate the analytical problems with coelution of interfering compounds. In addition, there is a method for the quantification of sphingoid bases by enzymatic treatment [34]. Additionally, all these procedures for the determination of sphingolipid bases are time-consuming and labor-intensive as sample preparation requires many steps. Thus, the objective of this study was to develop a rapid method allowing a reliable determination of SA and SO in cell cultures using the high selectivity and sensitivity of liquid chromatography/electrospray ionisation-tandem-mass spectrometry (LC–ESI-MS). To demonstrate the utility of this method, we exposed human proximal tubule-derived cells (immortalised human kidney epithelial cells, IHKE) to fumonisins which had been described

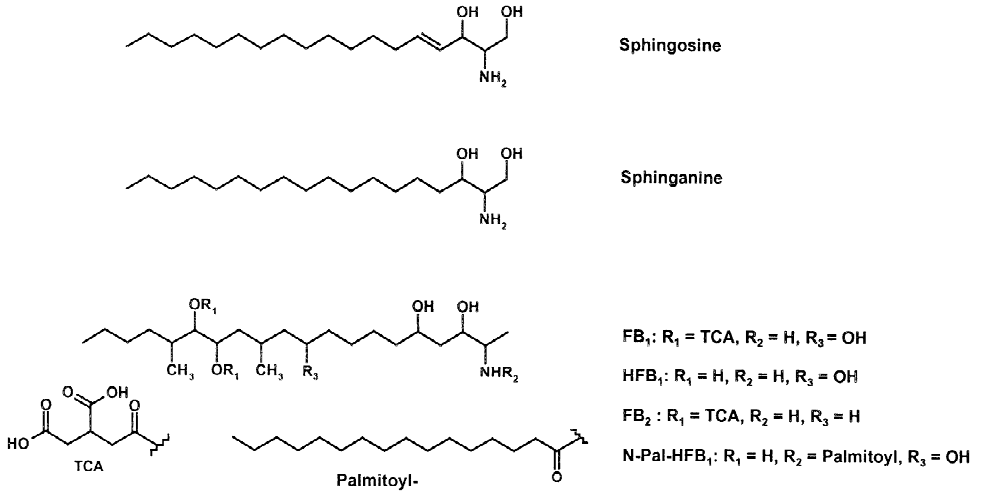

Fig. 1. Chemical structures of sphingosine, sphinganine, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), hydrolysed fumonisin B_1 (HFB_1) and *N*-palmitoyl-hydrolysed fumonisin B_1 (N-Pal-HFB_1).

to inhibit ceramide-synthase, especially FB_1, fumonisin B_2 (FB_2), hydrolysed fumonisin B_1 (HFB_1) and *N*-palmitoyl-hydrolysed fumonisin B_1 (N-Pal-HFB_1) (Fig. 1) [12,35,36]. Thereby HFB_1 is known as the hydrolysed product of FB_1 formed during alkaline treatment by removing the two tricarballylic acid groups from the 20-carbon backbone. Its enzymatic conversion by ceramide synthase with palmitoyl-coenzyme A results in the formation of the metabolite N-Pal-HFB_1 [36]. After incubating IHKE-cells with these substrates, we determined changes in the SO and SA levels using phytosphingosine (PSO) as an internal standard.

2. Experimental

2.1. Chemicals

DMEM/Ham's-F12 media and fetal calf serum were obtained from Biochrom KG (Berlin, FRG). Bicinchoninic acid was from Pierce; FB_1 was obtained from Alexis Biochemicals (Grünberg, Germany). Hydrolysed fumonisin B_1 was produced from FB_1 according to the method of Hopmans et al. [37]. FB_2 was obtained from M. Trucksess, FDA (WA, USA), N-Pal-HFB_1 was synthesised according to Humpf et al. [36]. Fumonisins are potential carcinogens and should be handled with care. Sphingosine, sphinganine and phytosphingosine hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Water, methanol, both of HPLC grade, and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany).

2.2. Cell culture

Human immortalised proximal tubule cells (IHKE cells, passage 170–180) were kindly provided by S. Mollerup, National Institute of Occupational Health, Norway. They were cultured as described by Tveito [38] in DMEM/Ham's-F12 medium (100 μl/cm^2 culture area) enriched with 13 mmol/l $NaHCO_3$, 15 mmol/l Hepes, 36 μg/l hydrocortisone, 5 mg/l human apotransferrin, 5 mg/l bovine insulin, 10 μg/l mouse epidermal growth factor, 5 μg/l Na-selenit, and 10% fetal calf serum at pH 7.3 at 37 °C in 5% CO_2. Protein content was determined with the bicinchoninic acid (BCA) assay [39] from Pierce (via KMF Laborchemie, Sankt Augustin, FRG).

2.3. Apparatus

Chromatographic separation was carried out by an Applied Biosystems 140b pump (Bai, Bensheim, Germany). For sample injection, a SunChrom Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used. LC–ESI-MS analyses were conducted on a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and mass spectrometric evaluation were carried out on a personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT).

2.4. Sample preparation

Cells were incubated with 200 μl cell lysis buffer (Apo-Alert caspase-3 fluorescence kit, Clontech) for 10 min on ice, harvested and centrifuged at 16 000 *g* for 10 min at 4 °C. To 40 μl supernatant (90 μl for control sample), 50 μl (no methanol for control sample) methanol and 10 μl of a phytosphingosine solution (1 μg/ml methanol) were added and the mixture centrifuged at 8000 *g* for 10 min.

2.5. Mass spectrometric analysis of sphinganine and sphingosine

For LC–ESI-MS, chromatographic separations were carried out on a Waters Symmetry C_{18} column (150×2.1 mm I.D., 5 μm; Waters, Milford, MA, USA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was 0.05% TFA in methanol (v/v). HPLC was programmed as follows: isocratic step at 60% B for 1 min, linear gradient to 99% B at 6 min followed by an isocratic step at 99% B for 3 min. The column was equilibrated for 5 min at the starting conditions. The flow-rate was set to 200 μl/min, and the injection volume was 10 μl (20 μl for control sample). For pneumatically assisted electrospray ionisation, the spray capillary voltage was set to 3.5 kV and the temperature of the heated capillary acting simultaneously as repeller electrode (20 V) was 200 °C.

Nitrogen served both as sheath (70 p.s.i.; 1 p.s.i. = 6894.76 Pa) and as auxiliary gas (10 U). The mass spectrometer was operated in the selected ion monitoring (SIM) mode, detecting positive ions $[M+H]^+$ of sphingosine (mass/charge ratio m/z 300), sphinganine (m/z 302), and phytosphingosine (m/z 318) at a total scan duration of 1 s. Quantitative evaluations were based on the peak area ratios of sphingosine and sphinganine in comparison to that of phytosphingosine. The data are presented as mean values ± SD of two Petri dishes. All analyses were performed in duplicate.

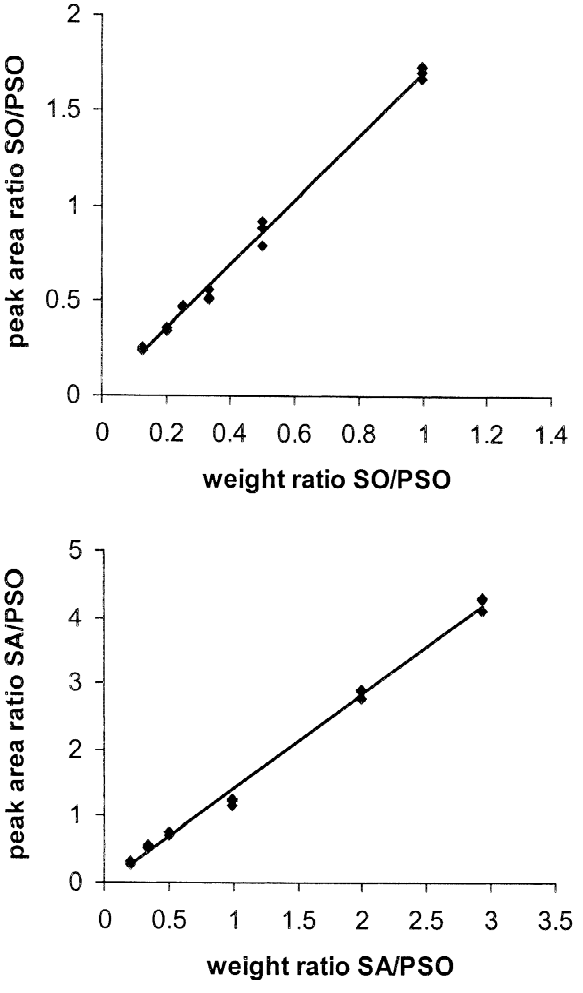

Fig. 2. Calibration curves for sphingosine (SO) and sphinganine (SA), showing the relationship between the weight ratios of SO or SA to phytosphingosine (PSO) and the resulting peak area ratios.

2.6. System calibration, detection limit, and recovery

The analytical system was calibrated with standard curves for sphingosine and sphinganine, which were prepared as follows: mixtures of phytosphingosine and either sphingosine or sphinganine in various weight ratios (SO/PSO: 1:8 up to 1:1, SA/PSO 1:5 up to 3:1; these weight ratios were chosen due to the expected SO and SA concentrations in the cell lysates) were analysed. The resulting peak area ratios of the ions with m/z 318 (phytosphingosine) to m/z 300 (sphingosine) or m/z 302 (sphinganine) were plotted against the weight ratios (Fig. 2). The limit of detection (LOD) was determined with standard solutions. Recoveries were determined by adding 5 and 25 ng SO and SA to lysates (100 μl) of control cells with known native contents of the analytes. All analyses were carried out in duplicate.

3. Results and discussion

As described, several methods are available for the determination of SO and SA using labor-intensive, nonspecific or time-consuming procedures. On the other hand, mass spectrometry is a powerful tool for determining endogenous physiologically active compounds with high selectivity and sensitivity. Several of our own studies as well as literature data have proven that the combination of liquid chromatography with electrospray mass spectrometry is useful for the analysis of fumonisins [40,41] and structurally related compounds such as sphingolipids [42–44]. Thus, we were encouraged to develop a method for the quantitative determination of SO and SA in cell cultures that is based upon LC–ESI-MS. A further reason for developing this method was to reduce the time required for the extraction procedure and thus to minimize the steps for sample preparation and clean-up.

Sphingosine and sphinganine are effectively ionised by the electrospray process, resulting mainly in the protonated molecule $[M+H]^+$. Fig. 3 shows a typical electrospray mass spectrum of SO and SA with the $[M+H]^+$ at m/z 300 (SO) and m/z 302 (SA) demonstrating that SO and SA could effectively be transformed into protonated ions ensuring the

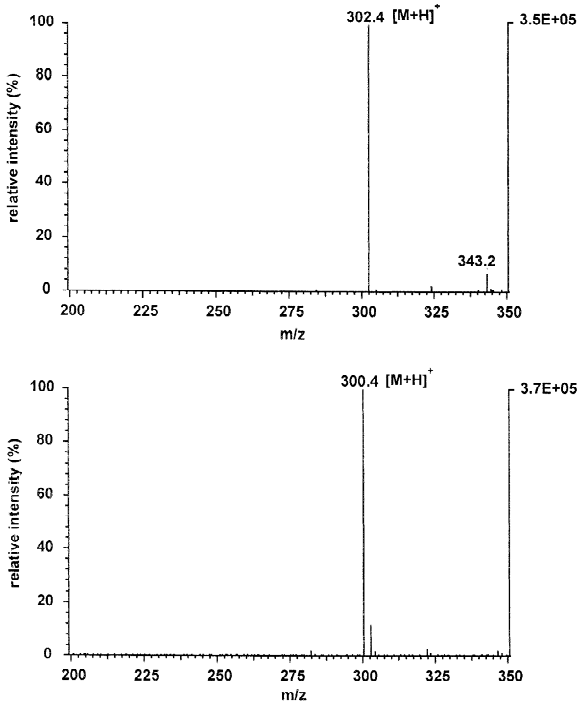

Fig. 3. Positive electrospray mass spectra of sphinganine (A) and sphingosine (B).

applicability of the ESI process for SO and SA analysis. Concerning the sample work-up, many methods for sphingoid base extraction have been developed most of which are modifications of the method described by Merrill et al. [27,45], using a mixture of chloroform and methanol for sample extraction followed by several steps for clean-up. For reasons of time and working effort, we analysed SO and SA after dilution with methanol, addition of definite amounts of the internal standard PSO and a brief centrifugation (10 min), directly from cell lysate aliquots. For chromatographic separations on a conventional reversed-phase column, we tested a methanol/water gradient, which was initially developed for the analysis of FB_1 [46]. Fig. 4 shows a typical LC–MS chromatogram of a sample of IHKE-cells which was incubated with FB_1 (10 μM) for 24 h. Sphinganine (6.90 min) was separated from SO (6.61 min) which was coeluting with the internal standard PSO (6.54 min). However, because the peak areas for quantification were taken from the single-ion chromatogram of each individual analyte, baseline separation is not necessary. Although the product ion spectra (data not shown) of SO, SA and PSO obtained by collision-induced dissociation

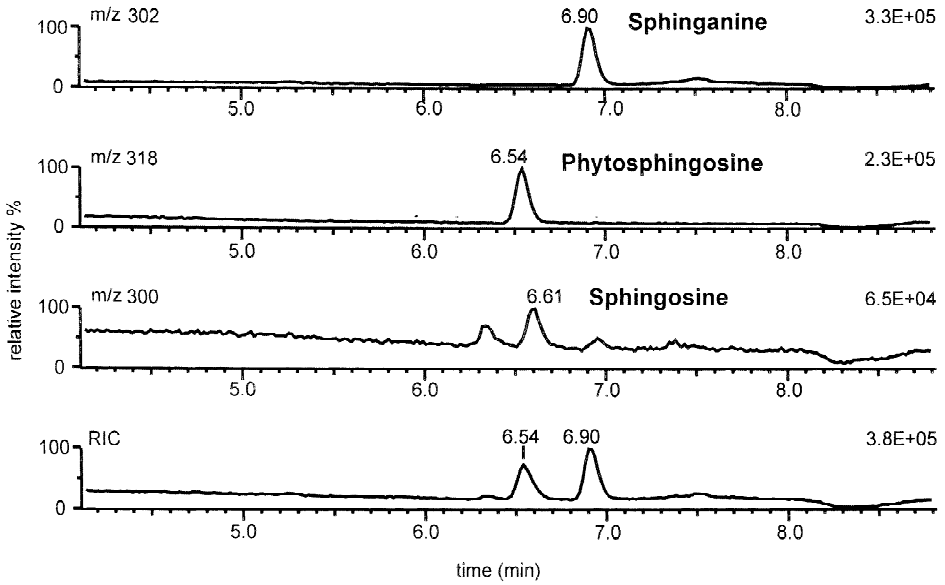

Fig. 4. LC–ESI-MS analysis of IHKE-cells incubated with FB_1 (10 μM, 24 h). Monitored m/z ratios were 300 (sphingosine), 302 (sphinganine) and 318 (phytosphingosine). RIC, reconstructed ion chromatogram.

Table 1
Recoveries of sphinganine and sphingosine in IHKE cells

Sample	Addition (ng/100 μl cell lysate)	Recovery (%)
1	Sphingosine (5)	99±2.0
2	Sphinganine (5)	101±2.0
3	Sphingosine (25)	92±1.0
4	Sphinganine (25)	102±5.0

(CID) of the protonated molecule using argon as collision gas, reveal specific signals for the application of selected reaction monitoring (SRM), we decided on the SIM mode. The technique of SRM guarantees high selectivity because coeluting matrix compounds are excluded from detection. But the SIM mode which is as sufficiently specific and sensitive as the SRM mode as our results demonstrate, allows analysis of SO and SA on benchtop single-quadrupole, which are available in many laboratories. For this reason, method development and validation were performed in our studies in the SIM mode. The analytical procedure was further characterised by means of calibration curves, the detection limits, and recoveries. A calibration curve was made with pure mixtures of standard solutions of PSO and either SO or SA in different weight ratios (see Experimental section) each injected at least three times. The peak area ratios were plotted against the corresponding mass ratio, and the resulting diagrams showed linear curves with correlation coefficients of $r = 0.994$ for SO and $r = 0.995$ for SA (Fig. 2). The limit of detection (LOD) was determined by the use of standard solutions: 10 pg for SO and 25 pg for SA were detected with a signal-to-noise ratio of 3:1. Recoveries of SO and SA with values higher than 90% (Table 1) were more than satisfying. The recoveries reported in the literature with the use of OPA-derivatives of SO and SA from urine and serum range from 55% [28] to 80–96% [29,30]. However, since the matrices, the analytical techniques and the internal standards used in this references are basically different from our approach, the methods can scarcely be compared to each other. To test the efficiency of the developed procedure, various samples of IHKE-cells exposed to different fumonisins were analysed for their SO- and Sa-levels. The determined concentrations are listed in Table 2. Whereas SO levels nearly remained unchanged, all samples showed an elevation of SA levels after incubation to fumonisins for 24 h, indicating that ceramide synthase in IHKE-cells was inhibited by all substrates. As can be seen from the results, the accumulation of SA by FB_1 and FB_2 (10 μ*M*, 25 μ*M*) is in same range whereas the effects are lower for HFB_1 and N-Pal-HFB_1. These findings confirm those from previous studies with HT29 cells supporting the fact that in equal concentrations, FB_1 is more effective than HFB_1 in disrupting the sphingolipid metabolism [47] and demonstrating the acceptance of the developed method.

4. Conclusion

In summary, LC–ESI-MS in combination with the selected ion monitoring (SIM) mode is a very sensitive and useful method for the determination of

Table 2
Accumulation of free sphinganine and sphingosine in IHKE cells after treatment with FB_1 (10 μ*M*), FB_2 (10, 25 μ*M*), HFB_1 (10, 25 μ*M*), and N-Pal-HFB_1 (10, 25 μ*M*) for 24 h

Fumonisin	Concentration (μ*M*)	Sphingosine SO (pg/μg protein[a])	Sphinganine SA (pg/μg protein[a])	SA/SO
No	Control	1.4±0.5	0.6±0.1	0.4
FB_1	10	1.8±0.2	35.8±1.7	19.8
FB_2	10	2.2±0.5	21.8±1.1	9.9
	25	1.5±0.2	39.7±1.6	26.5
HFB_1	10	2.7±0.8	3.5±0.5	1.3
	25	1.9±0.5	5.5±0.6	2.9
N-Pal-HFB_1	10	2.1±0.3	12.0±0.9	5.7
	25	2.4±0.4	18.2±2.5	7.5

[a] The amount of protein found in 100 μl cell lysate was in the same range (ca. 180 μg).

SO and SA in cell cultures and has several advantages compared to other methods. First, cell lysates were only diluted, centrifuged and directly used for LC–MS analysis and a further time consuming clean-up procedure is not necessary. Secondly, the problem of coelution of interfering compounds can be overcome by the selectivity of LC–MS using the SIM mode. The rapidity, sensitivity and specificity of the proposed method make it suitable for accurate determination of SO and SA in cell cultures exposed to fumonisins.

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