Arch Pharm Res Vol 22, No 3, 294-299, 1999

Improved Fluorescent Determination Method of Cellular Sphingoid Bases in High-performance Liquid Chromatography

Hong-Tak Yoon[1], Hwan-Soo Yoo[1], Bum-Kyu Shin[1], Woo-Jin Lee[1], Hwan-Mook Kim[2], Seon-Pyo Hong[3], Dong-Cheul Moon[1] and Yong-Moon Lee[1]

[1]College of Pharmacy, Chungbuk National University, Chongju 361-763, [2]Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, KIST, Taejon 305-600 and [3]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

(Received November 10, 1998)

Precolumn orthophthaldehyde (OPA) labeling method of sphingoid bases, sphingosine and sphinganine, was investigated to obtain high fluorescent detectability. In order to improve the fluorescent yield, we investigated the optimal solubility of sphingoid bases for five pre-incubation solvents by incorporating the heating procedure before OPA derivatization. The pre-incubation in ethanol prominently increased the fluorescent peak height of OPA derivative for each sphingoid bases in high performance liquid chromatography. About ten-fold increase of detectability was archived by pre-incubating lipid extracts pellets in ethanol at 60 °C for 30 min. Optimal derivatization was performed in 30 min at ambient temperature and the fluorescent intensity of OPA derivative was stable for two weeks at 4 °C. The detection limit of sphingosine was 0.1 pmol as injected amount. This method was applied to the determination of cellular sphingosine and sphinganine in various human lung cancer cells. This OPA procedure was prospective to be useful for quantitating the amount of sphingoid bases in other cancer cells.

Key words : Sphingosine, Sphinganine, Pre-incubation, OPA, Fluorescent derivatization, HPLC, Lung cancer cells

INTRODUCTION

Recent cumulative studies of sphingolipid signaling pathway stepwisely revealed that sphingomyelin (SM) degradation in inner membrane produces bio-active lipid second messengers which successively can activate the target enzymes by phosphorylation (Ghosh *et al.*, 1997). The triggered enzyme induce apoptosis, cell proliferation and differentiation by directly activating the target genes or mRNA level. This fact suggests that the regulation of SM metabolic pathway is very important to understand the roles of sphingolipids in cells. Among them, ceramides are primarily released from SM degradation by sphingomyelinase (SMase). And then, they are glycosylated to form cerebrosides or divided into long fatty acid chain and sphingosine (Hannun, 1994). The sphingoid bases, sphingosine and sphinganine (dehydrosphingosine), are known to strongly inhibit the protein kinase C (PKC) isoforms (Igarashi *et al.*, 1989). Although there have been some reports that sphingosine and its analogues induce apoptosis in some cell lines, sphingosine concentration below 10 μM might activate cell growth in other cell lines (Igarashi, 1997). Additional studies revealed that sphingosine-l-phosphate, a phosphorylated form of sphingosine, recruits intracellular calcium and activates cell proliferation (Olivera *et al.*, 1993; Spiegel *et al.*, 1995). These data suggested that cellular changes of sphingoid bases in low concentration exert the regulation of cellular proliferation.

In the most cell lines, the average amount of free sphingoid bases is below 10 pmol/10^6 cells (Riley *et al.*, 1993; Riley *et al.*, 1996). Therefore, low concentration of sphingoid bases limit for determining the relationship between them and their cellular effects (Van Veldhoven *et al.*, 1989).

o-Phthalaldehyde (OPA) reacts with primary amine group of sphingoid bases and forms resultant isoindole fluorophore in the presence of 2-mercaptoethanol. However, isoindole fluorophore is gradually disintegrated in ambient temperature because surrounding oxygen easily replaces the sulfur atom of 2-mercaptoethanol. These reaction make the isoindole structure unstable. Therefore, it is that the sensitive method which enables to measure

Correspondence to: Yong-Moon Lee, College of Pharmacy, Chungbuk National University, Chongju 361-763, Korea
E-mail: ymleefn@cbucc.chungbuk.ac.kr

the minute changes of sphingoid bases to clarify their physiological roles and cellular metabolic fates is continuously demanded. To analyze sphingoid bases by HPLC, various OPA derivatization methods have been proposed (Merrill *et al.*, 1988; Riley *et al.*, 1994a; Castegnaro *et al.*, 1996). But, they failed to establish proper solvent condition to dissolve extracted sphingoid bases and to apply the fluorogenic reaction with OPA reagent.

In this report, we newly developed modified OPA procedure by inserting the pre-incubation and optimized OPA derivatization for sphingoid bases. By this method, we could demonstrate its usefulness for the determination of sphingoid bases with high sensitivity in various human lung cancer cells.

MATERIALS AND METHODS

Reagents and apparatus

D-sphingosine and sphinganine (DL-erythro-dihydrosphingosine) and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). OPA was purchased from Nakalai Tesque (Tokyo, Japan). The internal standard, C_{20}-sphinganine, was a generous gift from Dr. Merrill in Emory University (Atlanta, GA, U.S.A.). Other solvents for extracting sphingolipids and HPLC eluents were used as a HPLC analytical grade.

The analytical HPLC system was equipped with an L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne 7125 injector, a 655A-52 column oven (Hitachi) and a FP-720 fluorescent detector (Jasco, Tokyo, Japan).

Reagent preparation for OPA derivatization

OPA reagent solution was prepared by following steps. Twenty five milligrams of OPA and 25 μl of 2-mercaptoethanol were dissolved in 0.5 ml of ethanol. The total volume was adjusted to 50 ml with 3% borate buffer (adjusted pH to 10.5 with KOH) and stored at 4°C under nitrogen in the dark. This reagent solution was prepared weekly or whenever the reactivity with sphingoid base standards decreases.

For the preparation of stock solution of sphingosine, ten milligrams of sphingosine were dissolved in 1 ml of ethanol with brief ultrasonication.

The organic phase of cellular lipid extracts was evaporated in Speed Vac concentrator(Hanil, Seoul, Korea). The precipitates of lipid mixture were redissolved in 40 μl of ethanol. This ethanol solution was preincubated in 1.5 ml PTFE tube at 60°C for 30 min. The fluorescent OPA derivatization was successfully archived by adding 5 μl of OPA reagent solution to 40 μl of ethanol solution of lipid mixture and then incubated 30 min, at ambient temperature.

The alkaline solution for lipid extraction was prepared daily. The alkaline solution is prepared that 0.1 ml of 2N NH_4OH stock solution was added to 250 ml of deionized water and adjusted the pH to 9.0.

Selection of pre-incubation solvent

Ten microliters of ethanol stock solution of 1.0 μM sphingosine in test tube was evaporated *in vacuo*. Then, the test tubes with 40 μl of each organic solvent were pre-incubated at 60°C for 30 min. After finishing OPA derivatization in room temperature, the aliquot of 10 μl reaction mixture was directly injected into HPLC system.

Cell culture

Human lung cancer cell lines and human promyelocytic leukemia cell line, HL-60 cells, were kindly obtained from Korea Research Institute of Bioscience and Biotechnology, KIST. Cells were grown in RPMI 1640 supplimented with 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. Cells were subcultured at 37 °C in 5% CO_2 atmosphere.

Extraction of cellular sphingoid bases

The total population of 1×10^7 cells were harvested and rapidly precipitated by brief centrifugation of 1,000 rpm for 10 sec. After supernatant was removed, pellets were washed twice with 0.5 ml of phosphate-buffered saline (PBS, pH 7.2) and then precipitated again by above procedure. In order to extract sphingolipids, 1.5 ml of chloroform-methanol (1 : 2, v/v) mixture was directly added to the pellets. At the same time, 40 μl (40 pmol) of C_{20}-sphinganine (internal standard) stock solution (1.0 μM) was also added and mixed well. To transfer sphingoid bases to organic layer, 1 ml each of chloroform and alkaline solution (pH 9~10) were added and vortexed vigoriously before centrifugal separation. After lower organic layer was washed twice with 1 ml of alkaline solution, lower organic phase was dried over anhydrous Na_2SO_4 and then evaporated *in vacuo*.

To reduce byproducts in OPA reaction, alkaline hydrolysis with 1 ml of 0.1 M KOH in chloroform-methanol (1 : 2,v/v), was prepared and was incubated for 60 min at 37°C to break down cellular acylglycerolipids and phospholipids. After hydrolysis, the same extraction procedure above was applied again to the hydrolysates to extract free sphingoid bases.

Chromatography for sphingoid bases analysis

The OPA derivatives of sphingoid bases were separated by using 82% acetonitrile at a flow rate of 1.0 ml/min on reversed-phase column (Cosmosil 5C18-AR, 4.6 mm i.d.×150 mm) at 40°C. OPA derivative of sphingoid bases were monitored fluoromatrically at 340 nm for excitation wavelength and 455 nm for emission wavelength.

RESULTS AND DISCUSSION

Improved OPA procedure for sphingoid bases

The most important point of OPA derivatization is to adjust the pH and thiol concentrations for reactions and the stability of fluorescent isoindole products.

Although Merrill *et al.* (1988) originally reported the OPA method for sphingisine and sphinganine in cultured cells, reaction media might be too hydrophylic to dissolve the sphingoid bases completely. But, if the composition of reaction media were changed to hydrophobic condition to dissolve well the cellular sphingoid bases from the lipid extractant, the reaction yield of OPA derivatives was abruptly decreased.

Based on this finding, we investigated the optimal procedure to increase reaction yield of OPA to sphingosine by inserting pre-incubation procedure. The suitable incubation conditions were adjusted to 60°C for 30 min in consideration of solvent volatility. The pre-incubation with ethanol resulted in relatively higher yield than other tested organic solvents (Fig. 1). This data indicates that the pre-incubation procedure is necessary to dissolve sphingoid bases completely to obtain proper sensitivity in HPLC. The modified OPA method enabled to increase the peak height of sphingosine ten times more than reported Merrill's method (1988) (Fig. 2).

The fluorescent response for OPA derivatives of sphingosine was stable for two weeks in HPLC system (data not shown), even though that of OPA derivatives of amino acids was reported to be unstable and gradually disintegrated after OPA reaction.

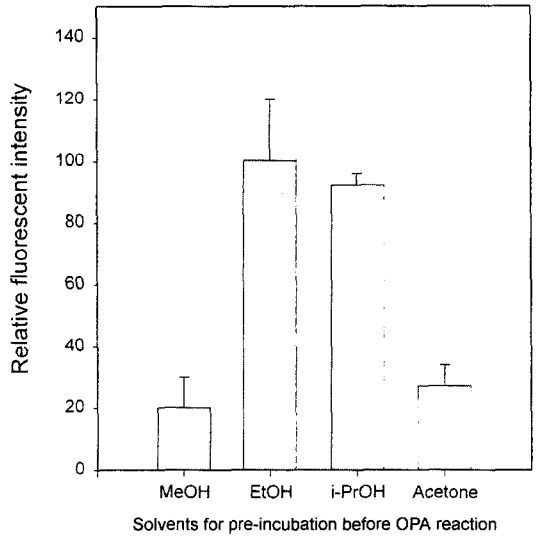

Fig. 1. Effect of pre-incubation solvents on the fluorescence intensity of OPA derivatives. Ten pmols of sphingosine were pre-incubated at 60°C for 30 min and monitored by HPLC-fluorescent detection system after OPA derivatization. Each column and bar represented mean±S.D. (n=3).

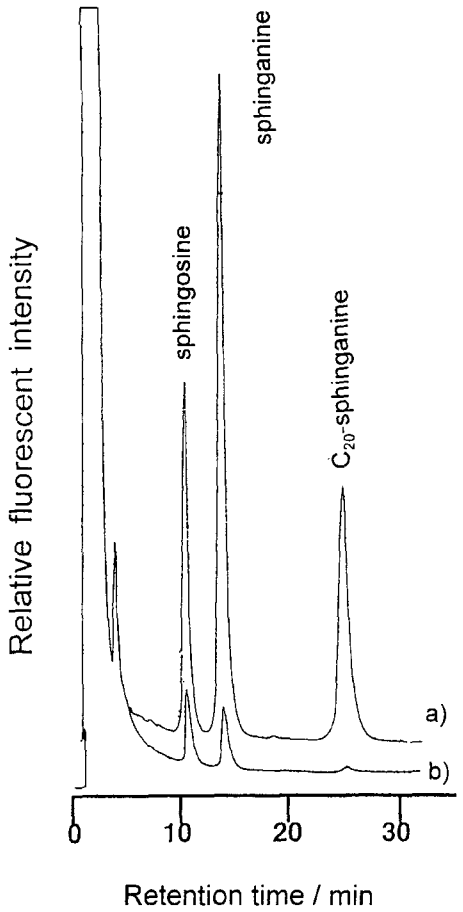

Fig. 2. Improved fluorescent intensity of standard sphingoid bases (25 pmol/10 μl injection) by (a) improved OPA method and (b) the original OPA method was depicted as reference at the same chromatographic conditions. HPLC conditions were described in Meterials and Methods.

Extraction of cellular sphingoid bases

During analysis, we followed up the extraction procedure of sphingoid bases established by Merrill *et al.* (1988). When secondary extractant pass through Na_2SO_4 mini-column, it would be desirable to allow to stand for flowing down slowly of all organic solution by gravity in order to remove aqueous content completely. This step is very important to establish the recovery and to obtain reliable reproducibility, even though it takes about 10 min per extraction of sample.

The synthetic C_{20}-sphinganine used as an internal standard simplifies the sphingoid bases quantitation. It was reported the mean extraction efficiencies±S.D. of sphinganine were 35±13% (n=23) and 44±17% (n=9) for rat liver and kidney tissue, respectively (Riley *et al.*, 1994b).

The alkaline digestion of cellular glycerophospholipids enabled to remove possible detection interference in

chromatographic elution and shortened the analysis time.

The most important procedure in this study is pretreatment with ethanol to dissolve sphingoid bases before OPA derivatization. For complete dissolution of sphingoid bases, the heating at 60°C for 30 min was essential.

Isocratic separation of sphingoid bases

In chromatographic separation, It was desirable to simplify the composition of mobile phase for general use. For the separation of the OPA derivatives of sphingoid bases with simple mobile phase, 82% acetonitrile, was satisfied enough to separate three sphingoid bases, sphingosine (12 min), sphinganine (15 min) and C_{20}-sphinganine (28 min) within 30 min. In this chromatographic conditions, stereoisomers of sphinganine, *threo*-(faster) and *erythro*-sphinganine (later), could be partially separable in this chromatographic condition (data not shown), but *erythro*-sphinganine, the natural major form, is only found from the extracts of cultured cell lines.

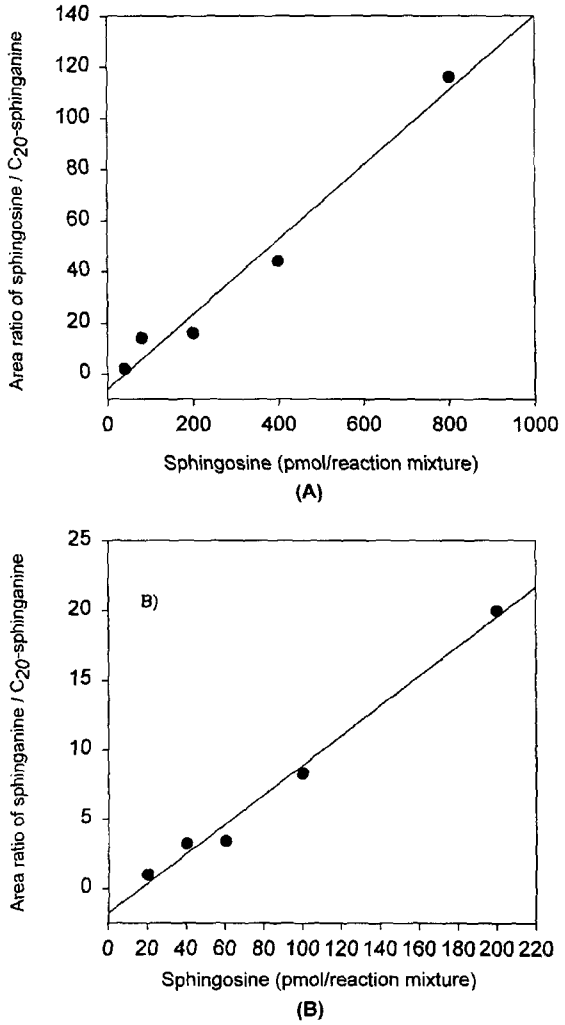

Fig. 3. Calibration curves of (A) sphingosine and (B) sphinganine

Calibration curve

Using internal standard, C_{20}-sphinganine (100 pmol), each amount of sphingoid bases was estimated by the peak area ratio. Bacause chromatographic analysis by isocratic elution makes the peak small and broad in retention time duration, the most of peak height ratio of each peak is not reasonable even though the fluorescent response of each sphingoid bases is same. The quantitative linear range of sphingoid bases was tested from 50 pmol to 800 pmol for sphingosine and from 20 pmol to 200 pmol for sphinganine which reflected the concentration range of endogenous sphingoid bases in cultured cells (Fig. 3). The concentration of endogenous sphingoid bases was calculated by the formula, $Y=0.1066X-1.7756$ ($R^2=0.987$) for sphingosine and $Y=0.1462X-6.0531$ ($R^2=0.974$) for sphinganine, respectively. It was suggested the use of proper internal standard, C_{20}-sphinganine reduces the variation of estimated amount of the sphingoid bases in biological samples.

Analysis of sphingoid bases in lung cancer cells

The elution profiles for extracts from lung cancer cells did not show any intrinsic interference after 10 min and also depicted baseline separation for each

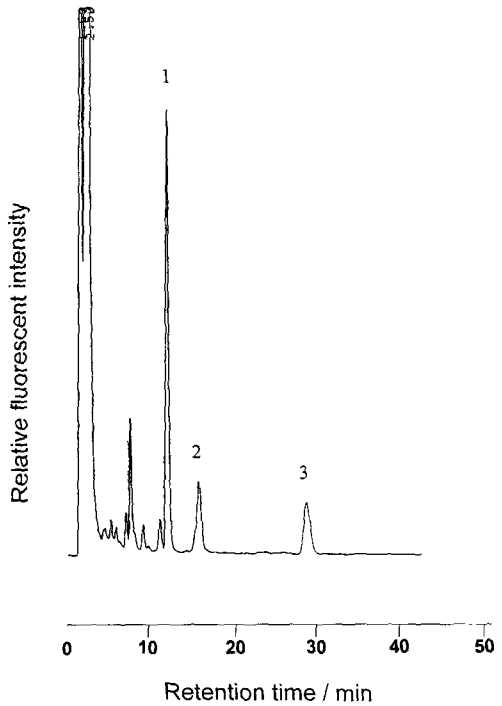

Fig. 4. Chromatogram of OPA derivative of sphingoid bases in LCSC#1 cells (human lung cancer cells). Peaks: 1, sphingosine; 2, sphinganine; 3, internal standard

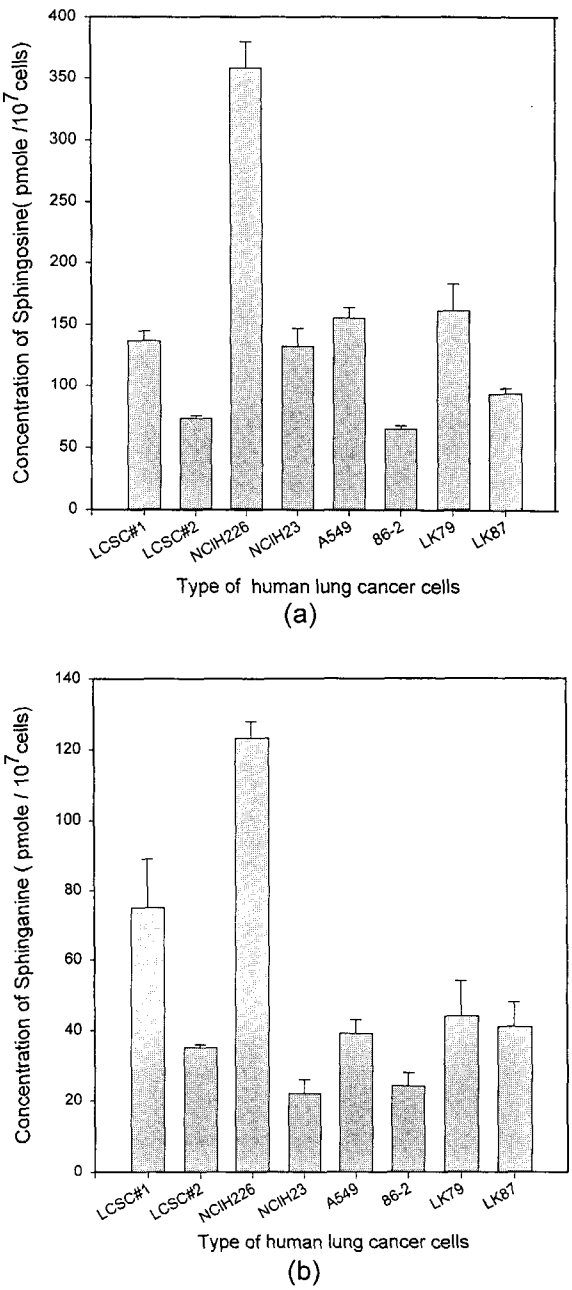

Fig. 5. Quantitation of sphingoid bases in lung cancer cells by this method. The values are expressed as mean±S.E. (n=3). Each Figure represents the concentration of sphingosine(a) and sphinganine(b), respectively.

sphingoid bases and C_{20}-sphinganine (Fig 4). From three separated experiments, the amounts of sphingosine and sphinganine in lung cancer cells, were estimated in the range from 50 pmol to 150 pmol for sphingosine and from 20 pmol to 80 pmol for sphinganine, respectively (Figs. 5a, b). In all cases, sphingosine contents is ca. 2-3 times higher than those of sphinganine in accordance of those of former reports (Wang *et al.*, 1991; Riley *et al.*, 1994b). Extraordinarily, NCIH226 cells showed higher levels of sphingoid bases.

CONCLUSION

To determine sphingoid bases in cultured cells, the pretreatment of ethanol at 60°C for 30 min before the precolumn derivatization of sphingoid bases by OPA, profoundly improved the fluorescent signal in HPLC system. This was explained that the trace amount of sphingoid bases in cellular lipid extracts was not completely dissolved in OPA reaction media. We successfully modified the original Merrill's method apply to other cell lines which have trace amount of sphingoid bases.

ACKNOWLEDGEMENTS

This study was supported by the Academic Research Fund (GE96-101) of the Ministry of Education, Republic of Korea. Also, we acknowledged to Dr. A. Merrill Jr for his kind gift, C_{20}-sphinganine.

REFERENCES CITED

Castegnaro, M., Garren, L., Gaucher, I. and Wild, C. P., Development of a new method for the analysis of sphinganine and sphingosine in urine and tissues. *Nat. Toxins*, 4, 284-290 (1996).

Ghosh M., Strum, J. C. and Bell, R. M., Lipid biochemistry: functions of glycerolipids and sphingolipids in cellular signaling. *FASEB J.*, 11, 45-50 (1997).

Hannun, Y. A., The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.*, 269(5), 3125-3128 (1994).

Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa T., Ghendy, K. and Racker, E., Effect of chemically well-defined sphingosine and its N-methyl derivatives on protein kinase C and sre kinase activities. *Biochemistry*, 28, 6796-6800 (1989).

Igarashi, Y., Functional roles of sphingosine, sphingosine- l-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. *J. Biochem.*, 122, 1080-1087 (1997).

Merrill, A. H., Wang, E., Mullins, R. E., Jamison, W. C. L., Nimkar, S. and Liotta, D. C., Quantitation of free sphingosine in liver by high-performance liquid chromatography. *Anal. Biochem.*, 171, 373-381 (1988).

Olivera, A. and Spiegel, S., Sphingosine-l-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature*, 365, 557-559 (1993).

Riley, R. T., An, N. H., Showker, J. L., Yoo, H. S., Norred, W. P., Chamberlain, W. J., Wang, E., Merrill, Jr., A. H., Motelin, G., Beasley, V. R. and Haschek, W. M., Alteration of tissue and serum sphinganine to sphingosine ratio: an early biomarker of exposure to

fumonisins-containing feeds in pigs. *Toxicol. Appl. Pharmacol.*, 108, 105-112 (1993).

Riley, R. T., Wang, E. and Merrill, Jr., A. H., Liquid chromatographic determination of sphinganine and sphingosine: use of the free sphinganine-to-sphingosine ratio as a biomarker for consumption of fumonisins. *JAOAC*, 77 (2), 533-540 (1994a).

Riley, R. T., Hinton, D. M., Chamberlain, W. J., Bacon, C. W., Wang, E., Merrill, A. H. and Voss, K. A., Dietary fumonisin B1 induces disruption of sphingolipid metabolism in Sprague Dawley rats: a new mechanism of nephrotoxicity. *J. Nutr.*, 124, 594-603 (1994b).

Riley, R. T., Wang, E., Schroeder, J. J., Smith, E. R., Plattner, R. D., Abbas, H., Yoo, H. S. and Merrill, Jr., A. H., Evidence for sphingolipid metabolism as a contributing factor in the toxicity and carcinogenicity of fumonisins. *Nat. Toxins*, 4, 3-15 (1996).

Spiegel, S. and Milstein, S., Sphingilipid metabolites; members of a new class of lipid second messengers. *J. Membrane Biol.*, 146, 225-237 (1995).

Van Veldhoven, P. P., Bishop, W. R. and Bell, R. M., Enzymatic quantification of Sphingosine in the picomole range in cultured cells. *Anal. Biochem.*, 183, 177 189 (1989).

Wang, E., Norred, W. P., Bacon, C. W., Riley, R T. and Merril, A. H., Inhibition of sphingolipids biosynthesis by fumonisins. Implication for diseases associated with Fusarium moniliforme. *J. Biol. Chem.*, 266, 14486-14490 (1991).