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Comparative study of naphthalene-2,3-dicarboxaldehyde and *o*-phthalaldehyde fluorogenic reagents for chromatographic detection of sphingoid bases

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Abstract

Naphthalene-2,3-dialdehyde (NDA) was developed as a precolumn labeling reagent for the fluorescent determination in a HPLC system of bioactive sphingoid bases, including sphingosine, sphinganine, and C_{20}-sphinganine. Cellular sphingoid bases generally exist in the range of 10~100 pmol/10^6 cells in a wide variety of cell types and tissues. This study aimed to obtain stable fluorescent derivatives of sphingoid bases and to increase their detectability at low concentrations. Sphingoid bases were reacted with NDA in the presence of cyanide ion to readily make an intensely fluorescent structure, 1-cyano-2-alkyl-benz[*f*]isoindole (CBI) and were then eluted separately on a reversed-phase C_{18} column with a simple mobile phase of 90% acetonitrile. For evaluating the NDA method, we compared the fluorescent intensity, elution profile, stability, and detectability of NDA derivatives with those of corresponding *o*-phthalaldehyde (OPA) derivatives. By monitoring the fluorescent intensity at the excitation wavelength of 252 nm and emission wavelength of 483 nm, NDA derivatives were sensitively determined at concentrations below 1.0 pmol of sphingoid bases in $1 \cdot 10^5$ U937 cells and were more stable than OPA derivatives. Linear calibration plots were obtained in the range studied (0.5~500 n*M*). The limit of detection for NDA derivatives of sphingoid bases was approximately 0.1 pmol ($S/N=3$). The method successfully measured the accumulation of sphingosine in U937 cells following *N,N*-dimethylsphingosine treatment, and of sphinganine following fumonisin B_1 treatment.

Keywords: Derivatization, LC; Naphthalene-2,3-dicarboxaldehyde; Sphingoid bases; Sphingosine; Sphinganine; Amines

1. Introduction

The biological activity of sphingosine in mammalian cells in vitro has been primarily reported to be inhibition of PKC (protein kinase C) with an IC_{50} value of 100 μ*M* [1,2]. Recent studies aimed at finding the direct target of sphingosine action have revealed the effects of sphingosine on various protein kinases: sphingosine inhibits calmodulin-dependent kinase [3] and the insulin receptor tyrosine kinase [4], but enhances the activities of diacylglycerol kinase [5] and casein kinase II [6]. Although there

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have been some reports that sphingosine and its analogues induce apoptosis in some cell lines [7–9], low concentrations of sphingosine might activate cell growth in several systems, including Swiss 3T3 cells [10], mesangial cells [11], Chinese hamster ovary (CHO) cells [12], and rat fibroblasts [13]. These conflicting data may be due to the rapid inter-conversion of the lipid molecules among ceramide, sphingosine, and sphingosine 1-phosphate, which depends on a cell's expression pattern and the activity of the converting enzymes.

In most cell lines, the amount of free sphingoid bases is in the range of 10~100 pmol/10^6 cells [14]. However, the low concentration of sphingoid bases in HL60 cells (25.0±2.8 pmol/10^6 cells) and U937 cells (13.8±1.2 pmol/10^6 cells) limits the ability to determine the relationship between the amount of the sphingoid bases and their cellular effects [15].

Several methods have been reported for the determination of sphingoid bases, including the fluorescent derivatization method using *o*-phthalaldehyde (OPA) [16], the chemical method by acylation with radioactive acetic anhydride [15] and the enzymatic method using sphingosine kinase [17] or diacylglycerol kinase [18]. The OPA method has routinely been used to measure the levels of sphingoid bases in cells because its good resolution in the reversed-phase mode enables reproducible quantification of sphingoid bases. To analyze sphingoid bases in biological samples or in organ tissues, various OPA methods have been proposed depending on the sample preparations [16,19,20].

OPA reacts with the primary amine group of sphingoid bases and forms an isoindole fluorophore in the presence of 2-mercaptoethanol [16]. However, OPA derivatives are sometimes unstable at room temperature. This is derived from the instability of OPA adducts and can be partially explained by the disintegration of isoindole structures [21]. Hence, a highly sensitive and more stable method is required when analyzing small samples or samples with low sphingoid base content.

In this report, we describe a newly developed, intensely fluorescent naphthalene-2,3-dialdehyde (NDA) derivatization for sphingoid bases that has high sensitivity [22,23]. The reaction principle is that NDA reacts with the primary amine group of sphingoid bases and forms a highly fluorescent structure, 1-cyano-2-alkyl-benz[*f*]isoindole (CBI) in the presence of cyanide ions (Fig. 1). The usefulness of this method was evaluated by comparing it with the typical OPA method. We were also able to determine with high sensitivity the changes in sphingoid base concentration following the treatment of U937 human myelocytic leukemia cells with fumonisin B_1 (FB_1) or *N*,*N*-dimethylsphingosine (DMS).

2. Experimental

2.1. Chemicals

HPLC-grade solvents were purchased from Tedia (Fairfield, OH, USA) and were used without further purification. NDA was obtained from Molecular Probes (Eugene, OR, USA). The NDA reagent was dissolved in methanol, protected from light by using amber glassware, and stored at 0 °C. Sphingosine, sphinganine, and *N*,*N*-dimethylsphingosine were supplied by Sigma (St. Louis, MO, USA). FB_1 was kindly supplied by Dr. H.S. Yoo, College of Pharmacy, Chungbuk National University and C_{20}-sphinganine by Dr. Merrill Jr., Department of Biochemistry, School of Medicine, Emory University.

2.2. High-performance liquid chromatography (HPLC)

The HPLC system consisted of Jasco (Tokyo, Japan) PU-980 high-pressure pumps, a 7125 injector with a 50 μl loop (Rheodyne, Cotati, CA, USA), a separation column (150 mm×4.6 mm I.D.) packed with Mightysil RP-18GP (Kanto Chemical, Tokyo, Japan), a Jasco FP-720 fluorescence detector, and a Hitachi D-2500 Chromato-Integrator. All chromatography was carried out using a mobile phase of 90% acetonitrile for the separation of NDA derivatives and 87% acetonitrile for OPA derivatives at a flow-rate of 1.0 ml/min. The column temperature was kept at 40 °C in a column oven (Hitachi 655A-52). The fluorescent derivatives were monitored at the excitation wavelength of 252 nm and the emission wavelength of 483 nm for NDA derivatives, and at the excitation wavelength of 340 nm and the emission wavelength of 455 nm for OPA derivatives.

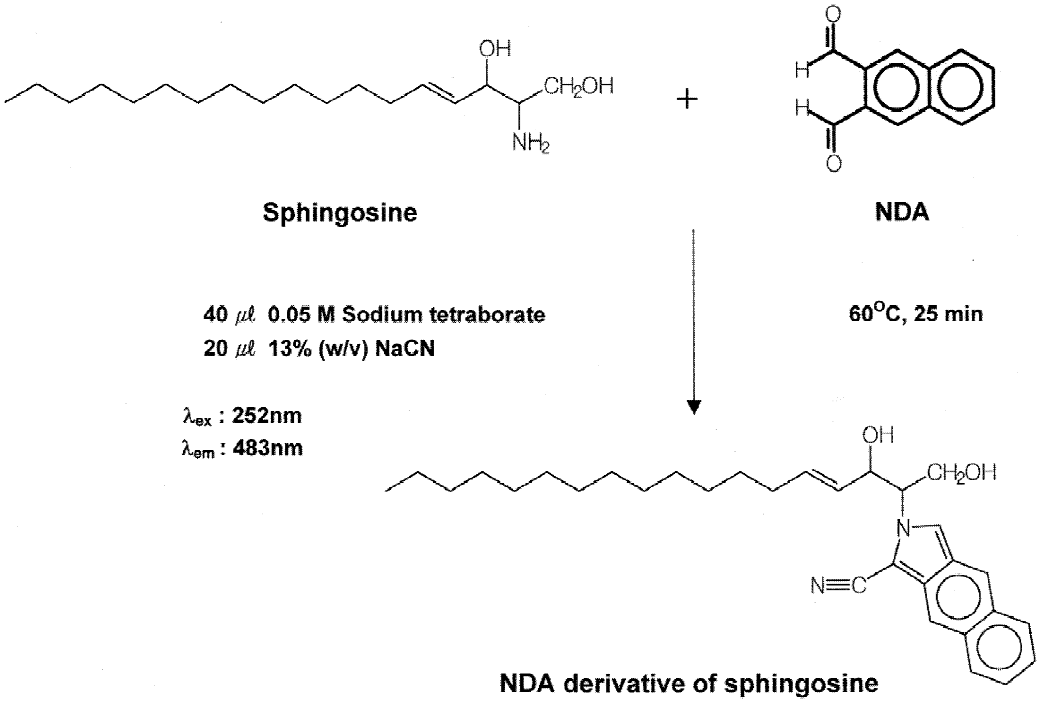

Fig. 1. Derivatization reaction of NDA with sphingosine.

2.3. Extraction of sphingoid bases in cultured cells

Human leukemia U937 cells were kindly provided by the Korea Research Institute of Bioscience and Biotechnology, KIST, South Korea. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/ml penicillin–streptomycin. Cells were sub-cultured every other day at 37 °C in a 5% CO_2 atmosphere.

Cells were harvested at scheduled intervals and washed twice with phosphate-buffered saline (PBS, pH 7.2). After brief centrifugation at 10 000 rpm for 10 s, 1.5 ml of $CHCl_3$–MeOH (1:2, v/v) and 20 pmol of C_{20}-sphinganine in an ethanol stock solution were added and mixed well with the cell pellets. Then, 1.0 ml each of $CHCl_3$ and alkaline solution were added to the tube. The alkaline water was freshly prepared by mixing 500 μl of 1 *M* KOH with 250 ml of distilled water; the pH of this solution was adjusted with drops of concentrated HCl solution.

The organic lower phase was washed twice with 1.0 ml of alkaline solution and was passed through an anhydrous Na_2SO_4 column to remove the water content. To digest the glycerolipids in the mixture, 1.0 ml of 0.1 *M* KOH in $CHCl_3$–MeOH (1:2, v/v) was added and the solution was incubated for 60 min in 37 °C. A 1.0 ml volume each of $CHCl_3$ and alkaline water were added to the tube after it had cooled. The organic lower layer was separated by brief centrifugation and then passed through an anhydrous Na_2SO_4 column. The organic phase was collected in a fresh tube and dried with a nitrogen stream [16].

2.4. Derivatization of sphingoid bases with NDA

The dried lipid extracts or sphingoid base standards for the calibration curve were dissolved in 40 μl of ethanol. NDA derivatization was accomplished by adding the following stock solutions: 40 μl 0.05 *M* sodium tetraborate ($Na_2B_4O_7$) buffer (pH 9.5), 20 μl 13% (w/v) NaCN, and 20 μl 50% (w/v) NDA. The tube was tightly sealed with PTFE film and heated at 60 °C in a water bath for 25 min. The reaction mixture was injected into the HPLC

system with an injection volume in the range of 10~50 μl.

2.5. Calibration curve

For calculating the calibration curve, two separate replicates of each of five different concentrations of sphingoid bases (10, 20, 30, 40, and 50 pmol) in 0.05 *M* borate buffer (pH 9.5) were performed in the presence of 20 pmol of C_{20}-sphinganine as an internal standard. Peak area ratio was expressed as the peak area ratio of each NDA derivative or OPA derivative of sphingoid bases versus the peak area of the OPA derivative of the internal standard.

2.6. Analysis of sphingoid bases changes by FB_1 or DMS treatment in U937 cells

U937 cells were plated onto 50 mm diameter dishes at 5.0×10^5 cells/ml and preincubated in RPMI 1640 with 10% (v/v) FBS at 37 °C for 12 h. A 2 ml volume of media was replaced with freshly prepared media containing 1.0, 2.0, or 4.0 μ*M* of FB_1 or 2.0 or 10.0 μ*M* of DMS and the cells were further incubated at 37 °C for 24 h. The extraction procedure and HPLC analysis described above were used to measure the levels of sphingoid bases. The concentration of sphingoid bases in cells was calculated from the equation for the linear regression ($y = ax + b$, where x is the concentration in pmol/1.0×10^6 cells in cultured cells and y is the peak area ratio of sphingosine or sphinganine versus C_{20}-sphinganine).

3. Results and discussion

3.1. Increased sensitivity of NDA derivatives of sphingoid bases

NDA derivatized products of amino acids [24,25], bioactive peptides [26–28] are generally monitored at the excitation wavelength of 442 nm. Alternatively, the monitoring of NDA products at the excitation wavelength of 420 nm was available on column-switching technique [29–31]. NDA derivative of fumonisin B_1, a fungal toxin which selectively inhibits ceramide synthase in sphingolipid metabolic pathway, monitored at the excitation wavelength of 410 nm and at the emission wavelength of 483 nm in HPLC–fluorescent detection systems [32]. Based on the fact that sphingoid base-like structure of fumonisin B_1 and its fluorescent spectrum of NDA derivative [32], the detection wavelength of NDA derivative of sphingoid bases was initially established at 410 nm for excitation. For the purpose of obtaining highly sensitive detection, the fluorescence of NDA derivatives of sphingosine were measured at the shorter excitation wavelengths of 252 or 283 nm. These results were compared to the results obtained at 410 nm, the excitation wavelength used. The fluorescence of OPA-sphingosine derivatives at 340/455 nm (excitation [ex.]/emission [em.]) was simultaneously measured as a reference.

The optimized elution profile (Fig. 2) depicts a

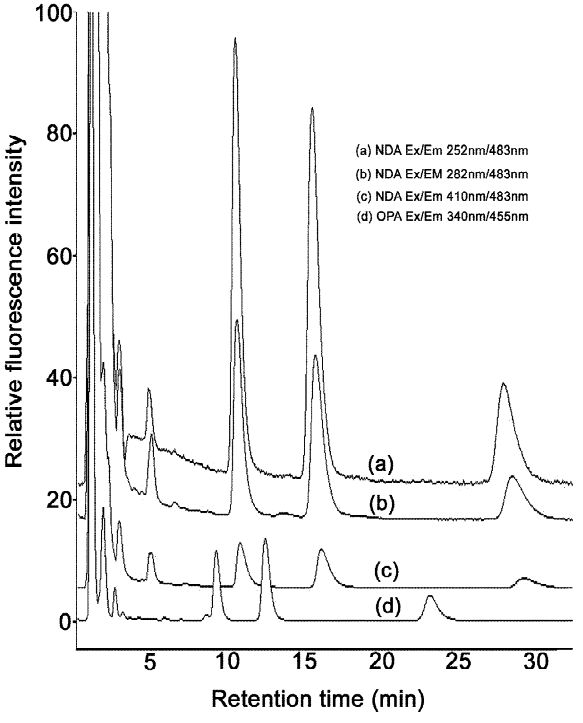

Fig. 2. Comparative chromatogram of sphingoid base derivatives. NDA derivatives were monitored fluorometrically at (a) 252 nm/483 nm (ex./em.), (b) 282 nm/483 nm (ex./em.), and (c) 410 nm/483 nm (ex./em.). (d) A reference chromatogram of OPA derivatives monitored at 340 nm/455 nm (ex./em.). The standard ethanol solution containing 100 pmol of each sphingoid bases per 40 μl was derivatized and then was injected 10 μl of total reaction mixture (injected amount: ca. 8.33 pmol of each sphingoid base).

baseline resolution of the standard sphingoid base mixture. As expected, the elution order is to a large extent related to the hydrophobicity of the derivatized sphingoid bases: the more hydrophobic NDA derivatives have longer retention time in the chromatographic run. By measuring NDA derivatives at the excitation wavelength of 252 nm, the peak area of the NDA derivatives was significantly increased. For instance, a 10-fold increase in the fluorescence emission of NDA derivatives at 483 nm was obtained with excitation at 252 nm, compared to the fluorescence of OPA derivatives at 340/455 nm. Therefore, it was determined that to obtain high signal for NDA derivatives, monitoring at 252/483 nm is optimal in this HPLC system (Fig. 3). The Jasco FP-720 fluorescence detector enabled us to detect the small fluorescent signals of NDA derivatives above 0.1 pmol ($S/N=3$) in a conditioned eluent system. Moreover, NDA reactivity for the amine group in each of the sphingoid bases was almost identical. The NDA fluorescent response ratio of sphinganine versus sphingosine was calculated to be 1.1, whereas the OPA ratio was above 1.5 in our experimental system. These results suggest that it is possible for NDA to reduce the error due to differential fluorescence intensities of derivatized sphingoid bases. The established NDA derivatization allowed the determination of sphingoid bases with the same reactivity and with sensitivity at least 10-fold higher than the OPA method.

3.2. Increased stability of NDA-sphingoid bases derivatives

The OPA method for sphingoid base quantification by HPLC has been applied reproducibly to various biological samples with good precision [16,19,20]. However, a potential disadvantage of the OPA method for the analysis of amino acids and peptides is that the amino acid derivatives are sometimes unstable [21]. In this case, the instability of OPA derivatives mainly originates from the liable breakdown of the isoindole structure. Furthermore, 2-mercaptoethanol, which is an essential reagent for

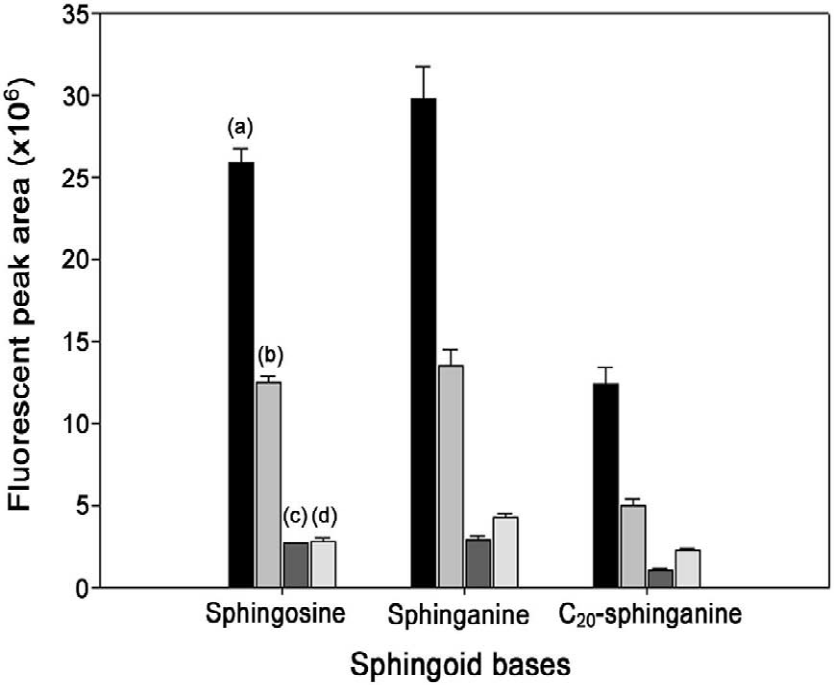

Fig. 3. Enhanced fluorescent intensity of NDA derivatives at various excitation wavelengths. NDA derivatives were monitored fluorometrically at (a) 252 nm/483 nm (ex./em.), (b) 282 nm/483 nm (ex./em.), and (c) 410 nm/483 nm (ex./em.). (d) OPA derivatives were monitored at 340 nm/455 nm (ex./em.). Values represent the mean±S.E. of triplicate experiments.

producing OPA adducts, undergoes oxidization from the reaction solution over time [33]. Therefore, it is recommended to freshly prepare the OPA reaction buffer before use. Because of this instability, it is sometimes difficult to detect sphingoid bases that exist in small amounts in HL-60 or U937 cells using the OPA method. In this study, we compared the stability of each fluorescent signal by derivatizing sphingoid bases with the OPA or NDA method and then allowing them to stand at room temperature. The replicates of a single sample of derivatized sphingosine were analyzed over 5 days. The fluorescent signal of the OPA derivatives decreased rapidly to 63.2±4.8% at 24 h, 51.3±6.3% at 48 h, and 26.2±6.4% at 120 h compared to initial signal in three separate analyses, while the fluorescent signal of NDA derivatives decreased less drastically to 86.4±5.9% at 24 h, 82.8±7.1% at 48 h, and 57.2±8.3% at 120 h in three separate analyses. The excellent stability of the NDA derivatives will facilitate the determination of a small amount of sphingoid bases in cells.

3.3. Comparison of the linearity of fluorescent responses of OPA and NDA derivatives

To compare the fluorescent intensity of OPA and NDA derivatives on a chromatogram, various low amounts of sphingoid bases (10~50 pmol) were carried through the extraction and alkaline hydrolysis, and were finally derivatized either with OPA or NDA. The estimates in peak area ratio of NDA derivatives and OPA derivatives of sphingoid bases versus the peak area of the OPA derivative of C_{20}-sphinganine demonstrated that the calibration curve of the NDA derivatives appears as a straight line with a steep slope, which is indicative of the sensitive responsiveness to changes in concentration ($y=0.78x+0.93$, $r=0.991$ for sphingosine; $y=1.08x+0.27$, $r=0.988$ for sphinganine), whereas the plot of the OPA derivatives has relatively a gentle slope as the concentration increases ($y=0.05x+0.06$, $r=0.988$ for sphingosine; $y=0.06x+0.16$, $r=0.972$ for sphinganine). The calibration curves made by the NDA method remained as straight lines up to 200 pmol of sphingoid bases.

Additionally, we found high fluorescence when the concentrations of sphingoid bases derivatized with NDA were estimated in various populations of U937 cells (Fig. 4). In this practical analysis, the calculated amounts of free sphingosine and free sphinganine were 6.08±0.81 and 0.79±0.10 pmol/10^6 cells, respectively. Based on analytical data obtained from OPA derivatization in the same sample, the amount of free sphingosine and free sphinganine were calculated to be 6.82±0.35 and 1.33±0.10 pmol/10^6 cells, respectively. When the cell number was below 6.0×10^5 cells, the fluorescent signal of OPA was not observed and therefore it was not possible to quantify the levels of both sphingosine and sphinganine with the OPA method. The fluorescent intensity of NDA derivative of

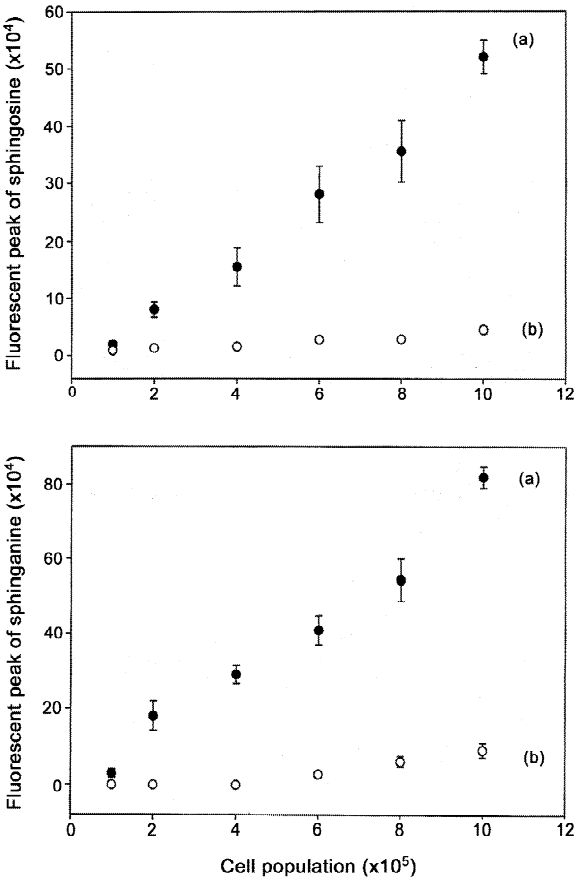

Fig. 4. Comparison of the detectability of NDA (a) and OPA (b) derivatives in various cell populations. The calculated population of U937 cells was harvested and the sphingoid base content was measured. Values represent the mean±S.E. of triplicate experiments.

sphingoid bases was consistent with cell number above 1.0×10^5 cells, although many steps including partitioning, washing, alkaline hydrolysis and passing through anhydrous Na_2SO_4 column were applied for the pre-treatment of a practical sample. The major advantage of the NDA method is that it is sufficiently sensitive to quantify the concentration of sphingoid bases in a small volume of U937 cells.

3.4. Reproducibility and recovery

The reproducibility of the NDA method was measured at the excitation wavelength of 252 nm by 10 repeated injections of a mixture containing NDA derivatives of sphingosine, sphinganine, and C_{20}-sphinganine at a concentration of 20 pmol. The RSDs for sphingosine, sphinganine, and C_{20}-sphinganine were 3.2, 6.7, and 8.0%, respectively. By following the OPA method under the same extraction procedures, the RSDs for sphingosine, sphinganine, and C_{20}-sphinganine were 4.9, 3.7, and 6.1%, respectively. The somewhat higher variation obtained with the NDA method might be due to the chromatographic baseline drift that accompanies the increased sensitivity.

The recovery with the NDA method was determined by adding a mixture of standard sphingoid bases into a cell lysate of 1.0×10^6 cells at concentrations of 5.0, 10.0, and 20.0 pmol. The recovery obtained for various concentrations of sphingosine and sphinganine was between 96.7 and 103.8%.

3.5. Quantitation of the changed amount of sphingoid bases in U937 cells following treatment with FB_1 or DMS

Fumonisin B_1 is a potent inhibitor of ceramide synthase, the enzyme that catalyzes the acylation of sphinganine in the de novo biosynthesis of sphingolipids [34]. Therefore, the sphinganine concentration in cells should be abruptly increased by treatment with FB_1. To evaluate the practical usefulness of the NDA method for detecting small changes, cells were incubated for 24 h in low concentrations of FB_1 with small differences between the concentrations (1.0, 2.0, and 4.0 μM FB_1) and then the change in the amount of sphinganine in the cells was determined.

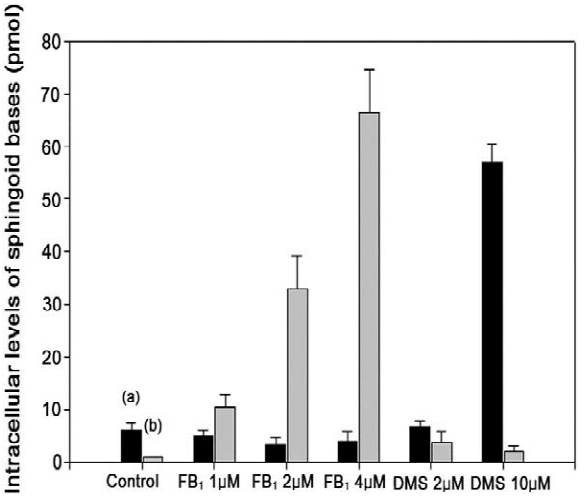

Fig. 5. The determination of the changed concentration of sphingoid bases derived from the inhibitory effects of FB_1 or DMS treatment on U937 cells. The amounts of sphingosine (a) and sphinganine (b) in cells after FB_1 or DMS treatment for 24 h were measured by the NDA method. Values represent the mean±S.E. of triplicate experiments.

DMS is a competitive inhibitor of sphingosine kinase that produces sphingosine 1-phosphate, a bioactive second messenger [35]. We treated cells with DMS for 24 h. As shown in Fig. 5, a selective increase of sphinganine following FB_1 treatment and of sphingosine following DMS treatment was determined simultaneously at the pmol level by the NDA method.

In conclusion, our results indicate that the NDA method reported here is useful for the quantitative determination of sphingoid bases. The high stability and intense fluorescence of NDA products extend the quantitative range, and therefore will allow the detection of small amounts of sphingoid bases. These outstanding advantages of the NDA method can be attributed to both the stable fluorescent structure of CBI and the high molar absorptivity of its extended aromatic structure.

Acknowledgements

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