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Sphingoid Bases as Possible Diagnostic Parameters

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Aim. To determine the concentrations and ratios of sphingoid bases, sphinganine and sphingosine, in the serum and urine of healthy individuals, as a basis for the normal value range, which may be useful in the diagnosis of diseases characterized by sphingolipid metabolism impairment. Possible sex differences were also investigated, as well as effects of hormonal changes on sphingoid base concentrations during pregnancy or menopause.

Method. Sphingolipids were extracted from the serum and urine and hydrolyzed. Sphinganine and sphingosine were determined by high performance liquid chromatography. The analysis included serum and urine samples of 20 men and 20 women, and urine samples of 5 healthy postmenopausal and 5 healthy pregnant women.

Results. Serum concentrations of free and total sphingoid bases showed no major variations in healthy individuals of both sexes: total sphingosine $28.28 \pm 8.96 \times 10^3$ pmol/mL in men and $22.52 \pm 10.19 \times 10^3$ pmol/mL in women ($p = 0.080$); total sphinganine $0.61 \pm 0.15 \times 10^3$ pmol/mL in men and $0.58 \pm 0.25 \times 10^3$ pmol/mL in women ($p = 0.574$). Urine concentrations showed greater variability. Hormonal changes associated with menopause or pregnancy significantly decreased the urinary concentrations of total sphinganine in postmenopausal women, and increased free sphinganine/sphingozine ratio.

Conclusion. Serum but not urine concentrations of sphingoid bases could be used as a sensitive indicator in the diagnosis of the diseases associated with sphingolipid metabolism impairment.

Key words: *blood chemical analysis; chromatography, liquid; reference values; sphingolipids; sphingosine; urine*

Sphingolipids are a group of lipids present in all eukaryotic cells (1). They are defined as compounds characterized by long-chain sphingoid bases, usually substituted with a long-chain fatty acid linked via an amide bond to produce ceramides. The most abundant long-chain bases in mammals are DL-erythro-sphinganine (d18:0) and D-sphingosine (d18:1). (Figs. 1 and 2). Free sphingoid bases are usually present in cells in low concentrations.

Sphingolipids have many biological functions (2-5). A number of genetic diseases cause impairments in the catabolism of complex sphingolipids, which is why sphingolipid degradation has been thoroughly studied. Several natural inhibitors of the sphingolipid metabolism have been identified, such as fumonisins, *Alternaria* toxins, and sphingofungins. Serine palmitoyltransferase and ceramide synthase, enzymes involved in the sphingolipid biosynthesis pathway (Fig. 2), are target substances for the action of fungal toxins. The inhibition of ceramide synthase results in the accumulation of sphinganine (6). As long-chain bases can easily diffuse across the cell membrane, the sphinganine accumulated in the cells may partially leave them and reach the blood and urine. Because these changes occur before any other signs of cellular lesion, increased concentrations of free sphinganine and sphinganine/sphingosine ratio (SA/SO) might be used as specific biomarkers in the early detection of ceramide synthase inhibition due to the intake of fumonisins and fumonisin-like substances (7-9). Some diseases of humans and animals have been related to mycotoxins that cause impairments of the sphingolipid metabolism (10).

We determined the concentrations and ratios of sphingoid bases (sphinganine and sphingosine) in the serum and urine of healthy subjects to establish normal range values. Such values may be useful in the diagnosis of diseases characterized by sphingolipid metabolism impairment. Also, possible sex differences in the concentrations of sphinganine and sphingosine were investigated, as well as possible modifications in the sphingoid base concentrations due to hormonal changes associated with menopause and pregnancy.

Material and Methods

Subjects

We determined free and total sphinganine and sphingosine concentrations in sera of 40 healthy blood donors (20 men and 20 women, mean($\pm$SD) age 29 ± 5 and 23 ± 4 years, respectively). The analysis also included urine samples of 40 healthy

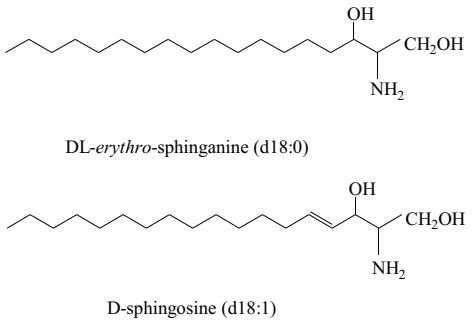

Figure 1. Structures of most common sphingoid bases in mammals.

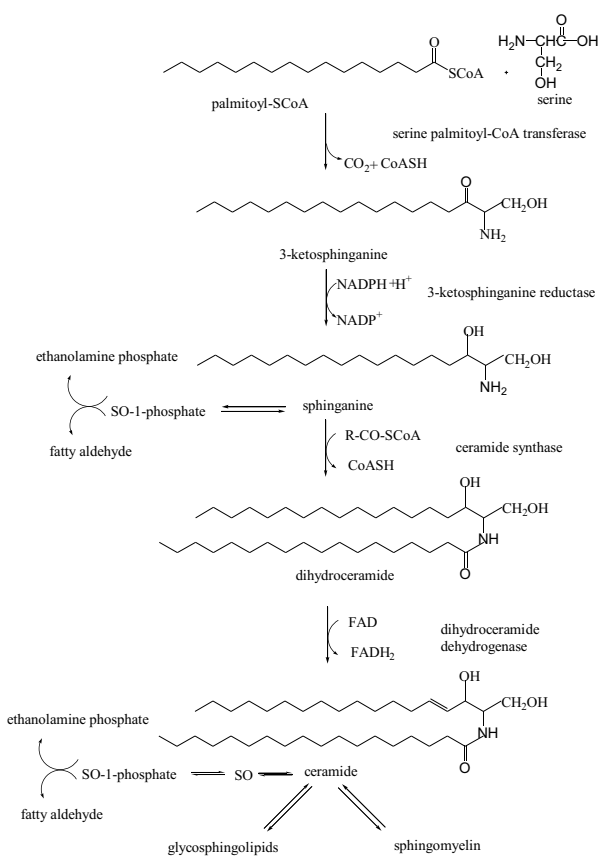

Figure 2. Biosynthetic pathway of sphingolipids.

blood donors (20 men and 20 women, mean age 28±7 and 23±4 years, respectively) and urine samples of 5 healthy postmenopausal women (mean age, 53±2 years) and 5 healthy pregnant women (mean age, 27±4 years). Serum and urine samples were collected at the Department of Transfusion Medicine, Institute of Immunology (Zagreb, Croatia), and urine samples of postmenopausal women and pregnant women were collected at the Department of Gynecology and Obstetrics, Zagreb University Hospital Center (Zagreb, Croatia).

Instruments

We analyzed the samples by high performance liquid chromatography using the following Perkin Elmer (Norwalk, CT, USA) equipment: isocratic pump with a piston (Series 10, Liquid Chromatograph), fluorescence detector (Model LC 240), interface (Series 900), column oven (Series 200), and software for chromatography (Turbochrom 4.1.2). Analytical column (Radial-Pak cartridge, Nova-Pak C_{18}, 10 cm x 0.8 cm, 4 m), column module (RCM 8x10), and the holder with a precolumn filter (Guard-Pak assembly, Nova-Pak C_{18}, 4 m) we used were manufactured by Waters Corporation (Milford, MA, USA). We used manual injector (model 7125) and 50- L injector loop manufactured by Rheodyne (Cotati, CA, USA), and 500- l syringe for loop filling with the sample by Hamilton Supelco (Bellefonte, PA, USA). A device for high performance liquid chromatography solvent filtration was manufactured by Millipore Corporation (Bedford, MA, USA).

The centrifuge, microcentrifuge (Mikro-242), vortex (EV-202), and water bath used in the study were manufactured by Tehtnica (Železniki, Slovenia); Univapo 100 H evaporator with Unicryo MC 21 cooling unit by Uniequip (Martinsried, Germany); ultrasonic bath by Iskra (Ljubljana, Slovenia); and TMA thermostat by Bodalec & Havoić, BTE-S (Dugo Selo, Croatia).

Chemicals

We used $CHCl_3$ (*pro analysis*) and CH_3OH (high performance liquid chromatography gradient grade) by J.T. Baker, Mallinckrodt Baker B.V. (Deventer, the Netherlands); CH_3OH (*pro analysis*) for extraction by Riedel de Hähn AG (Seelze, Germany); sphingoid base standards D-sphingosine (d18:1) and DL-erythro-sphinganine (d18:0) , orthophthaldialdehyde (OPA) and 2-mercaptoethanol by Sigma Aldrich Chemie GmbH (Steinheim, Germany); and NH_4OH, KOH and HCl by Kemika (Zagreb, Croatia). Sphinganine (d20:0) was a gift from Professor A.H. Merrill from Emory University, Atlanta, USA.

Extraction

Sphingolipids were extracted from the serum and urine samples by the modified method of extraction described by Riley et al (7). The recovery rate in extraction procedure was 41 11%, which was in the range obtained by Riley et al (7). Sphinganine (d20:0) was used as an internal standard for quantitation and determination of sphinganine and sphingosine recovery. To 0.5 mL of the serum sample, 2.25 mL of a mixture containing $CHCl_3$:CH_3OH = 1:2 (v/v) and 0.150 mL of 2N NH_4OH solution were added, and the content was thoroughly mixed on a vortex. Then the mixture was incubated at 37 C for 1 h in a closed screw tube and cooled down to room temperature. Then 1.5 mL $CHCl_3$ and 3 mL alkaline water (0.1 mL 2 N NH_4OH + 250 mL distilled deionized water, pH = 8.0-10.0, prepared daily) were added and centrifuged for 20 min at 3000 rpm for phase separation. Upon centrifugation, the upper aqueous phase was discarded, and 3 mL alkaline water were added to the lower chloroform phase; the content was mixed and centrifuged. Rinsing of the chloroform phase with alkaline water was repeated, the aqueous phase removed, and the chloroform phase evaporated to dryness. Thus prepared, the sample could be stored overnight in nitrogen at +4 C or subjected to base or acid hydrolysis.

Base Hydrolysis

The objective of base hydrolysis is the splitting of acylglycerolipids (to obtain free sphinganine and sphingosine) and hydrolysis of lisosphingolipids (free sphingoid bases modified on the sphinganine or sphingosine C1 atom hydroxyl group). Base treatment is successful in the removal of phospholipids (phosphatidylethanol amine and phosphatidyl serine) that can react with orthophthaldialdehyde (OPA) reagent for sphingosine base derivation (11). This procedure does not release sphingoid bases from complex sphingolipids.

Base hydrolysis was performed by the modified method described by Riley et al (7). A dry extract was dissolved in 1 mL of 0.1 mol/L methanol KOH:$CHCl_3$ = 4:1 (v/v) mixture and placed for 1 min in ultrasonic bath, whereafter it was incubated at 37 C for 1 h. After the mixture was cooled down, 1 mL $CHCl_3$ and 1 mL alkaline water were added, gently mixed, and centrifuged for 20 min at 3000 rpm. Then the upper aqueous phase was discarded and lower chloroform phase washed with 2 mL alkaline water and centrifuged again for 10 min at 3000 rpm. The upper aqueous phase was removed, and the chloroform phase evaporated to dryness and stored at -20 C until analysis.

Acid Hydrolysis

The objective of acid hydrolysis is to obtain total sphinganine and sphingosine released from complex sphingolipids. Acid treatment leads to hydrolysis of amide-bound fatty acid and any group esterified on the sphingoid base C1 atom. Acid hydrolysis was carried out according to the method described by Yoo et al

(12). To a dry extract, 0.5 mL aqueous methanol HCl (1 N HCl, prepared immediately before use) were added, the tube was stoppered by a Teflon cap, and left to stay for approximately 15 h at 68 C. Upon cooling down to room temperature, 0.5 mL of saturated methanol KOH (30 g KOH were dissolved in 100 mL CH_3OH and filtered through wrinkled filter paper), 0.5 mL of alkaline water, 0.1 mL of 2N NH_4OH solution, and 0.6 mL of $CHCl_3$ were added; the mixture was stirred and centrifuged for 20 min at 3000 rpm. The aqueous phase was discarded, and the chloroform phase was washed 3 times with 0.9 mL alkaline water, evaporated to dryness, and stored at -20 C until analysis.

The initial serum volumes for base and acid hydrolysis were 0.5 mL and 50 L, respectively. For sphingolipid extraction from urine, greater sample volumes (10 mL for base hydrolysis and 2 mL for acid hydrolysis) were used because of lower sphinganine concentration in male urine. In this case, the sample was frozen and lyophilized in an evaporator with a cooling unit. Before extraction, 0.5 mL of distilled water were added.

Chromatographic Conditions

Sphingoid bases were analyzed by high performance liquid chromatography. OPA reagent, reacting with sphingoid base via its amino group, was used for sphingoid base derivation before their column application. OPA reagent was prepared by dissolving 5 mg of reagent and 5 L of 2-mercaptoethanol in 0.1 mL of ethanol, making up to 10 mL with boric buffer (pH adjusted to 10.5 by 1 mol/L KOH). Prepared reagent can remain stable for 7 days if stored in the dark at +4 C.

Standards

Standard mixture was prepared by adding 5 L of each 10 mol/L D-sphingosine (d18:1), DL-erythro-sphinganine (d18:0) and sphinganine (d20:0). Then 485 L of mobile phase and 100 L of OPA reagent were added. The mixture was stirred and left for 20-30 min at room temperature, and then filtered and injected on the column.

Samples

The sample was prepared for high performance liquid chromatography by addition of 250 L of mobile phase to dry extract (obtained by base or acid hydrolysis). After that, the sample was mixed on a vortex for 1 min, and 50 L of OPA reagent were added. Then the mixture was mixed on a vortex for 30 s, filtered by centrifugation through a 0.45- m-pore size filter (for 1 min) and left at room temperature for 1 h before injection. Particular sphingoid bases in the sample were identified by comparing the sample chromatogram with the standard mixture chromatogram.

High Performance Liquid Chromatography Conditions

A mixture of $CH_3OH:H_2O$ = 9:1 (v/v) was used as a mobile phase after being filtered through nitrocellulose filter paper of 0.45- m-pore size and degassed by the passage of helium for 3-5 min. The flow rate was 2 mL/min, and column oven temperature was 30 C. The analyses were performed at excitation wavelength of 334 nm and emission wavelength of 440 nm. The injected sample volume was 50 L.

Statistical Analysis

The distribution of all variables within subgroups was tested with Kolmogorov-Smirnov test and no deviation from normal was found. Analysis was performed by use of ANOVA with Scheffé post-hoc test or t-test for independent samples with Levene's test for equality of variance (13). The possible statistically significant sex differences in the concentration and ratio of sphingoid bases were assessed by t-test at 5% level of significance. We used STATISTICA for Windows 2000 (Version 6.0, StatSoft, Inc., Tulsa, OK, USA) statisical package for all statistical analyses.

Results

Sphingosine Bases in Serum and Urine of Healthy Subjects

Sphingolipid extraction from the serum and urine samples was followed by base and acid hydrolysis, and high performance liquid chromatography determination of free and total sphinganine and sphingosine concentrations. Sphingoid bases were identified by comparing their retention times with the standard retention times in the same conditions (Fig. 3). The analysis included serum samples of 20 men and 20 women (Table 1), and urine samples of 20 men and 20 women (Table 2). Urinary free and total sphinganine, free sphingosine, and total sphinganine/sphingosine ratio were significantly higher in women than in men (Table 2). There were no statistically significant sex differences in the concentration and ratio of sphingoid bases in the serum.

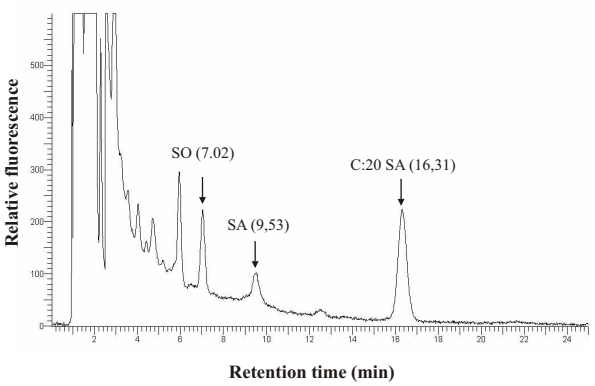

Figure 3. High performance liquid chromatography chromatogram of free serum sphinganine (SA) and sphingosine (SO) in a healthy man.

Sphingosine Bases in Urine of Healthy Postmenopausal and Pregnant Women

The sex differences observed in urine concentrations of sphingosine and sphinganine were presumed to be due to hormonal differences between men and women. Therefore, sphingoid bases were analyzed in the urine of 5 healthy postmenopausal women. The measured concentrations of free sphinganine and sphingosine and total sphinganine and sphingosine were lower than the mean values obtained by the analysis of urine samples from healthy premenopausal women serving as a control group (Table 3). These values, as well as SA/SO ratios, were significantly

Table 1. Serum concentrations of free and total sphingosine (SO), sphinganine (SA), and SA/SO ratio in healthy subjects

	Concentration (mean ± SD) (range)				
Parameter	men (n = 20)	women (n = 20)	t	df	p
free SA (pmol/mL)	2.80 ± 2.00 (1.00-8.00)	2.50 ± 1.00 (1.00-5.00)	0.783	34	0.439
free SO (pmol/mL)	9.80 ± 4.00 (4.00-19.00)	8.60 ± 3.00 (2.00-15.00)	1.035	34	0.308
free SA/SO	0.29 ± 0.12 (0.14-0.57)	0.31 ± 0.13 (0.13-0.60)	-0.439	34	0.664
total SA ($\times 10^3$ pmol/mL)	0.61 ± 0.15 (0.31-0.81)	0.58 ± 0.25 (0.29-1.09)	0.567	34	0.574
total SO ($\times 10^3$ pmol/mL)	28.28 ± 8.96 (13.68-44.84)	22.52 ± 10.19 (7.56-41.98)	1.804	34	0.080
total SA/SO	0.02 ± 0.01 (0.02-0.06)	0.03 ± 0.01 (0.02-0.05)	-1.054	34	0.299
free/total SA (%)	0.459	0.431			
free/total SO (%)	0.035	0.038			

Table 2. Urinary concentrations of free and total sphingosine (SO), sphinganine (SA), and SA/SO ratio in healthy subjects

Parameter	Concentration (mean ± SD) (range) men (n = 20)	Concentration (mean ± SD) (range) women (n = 20)	t	df	p
free SA (pmol/mL)	0.06 ± 0.05 (0.02-0.20)	3.60 ± 3.08 (0.20-11.58)	-3.117	16.003	0.007
free SO (pmol/mL)	0.46 ± 0.29 (0.10-0.94)	17.18 ± 13.84 (0.87-41.77)	-2.806	16.004	0.013
free SA/SO	0.18 ± 0.12 (0.04-0.49)	0.25 ± 0.08 (0.16-0.46)	-2.015	33	0.052
total SA (pmol/mL)	2.85 ± 1.64 (0.60-6.20)	23.00 ± 21.00 (1.86-41.76)	-3.103	16.114	0.007
total SO (pmol/mL)	103.66 ± 74.64 (7.48-264.44)	177.79 ± 167.19 (16.69-668.61)	-1.677	21.862	0.108
total SA/SO	0.04 ± 0.02 (0.02-0.08)	0.11 ± 0.05 (0.02-0.23)	-6.753	33	0.0001
free/total SA (%)	2.105	15.652			
free/total SO (%)	0.444	9.663			

Table 3. Urinary concentrations of free and total sphingosine (SO), sphinganine (SA), and SA/SO ratio in healthy, postmenopausal, or pregnant women

Parameter	Concentration (mean ± SD) healthy (n = 20)	postmenopausal (n = 5)	pregnant (n = 5)	t	df	p
free SA (pmol/mL)	3.60 ± 3.08	0.21 ± 0.09	1.72 ± 2.41			
free SO (pmol/mL)	17.18 ± 13.84	1.31 ± 0.41	3.80 ± 4.71			
free SA/SO	0.25 ± 0.08	0.15 ± 0.03	0.40 ± 0.13*			
total SA (pmol/mL)	23.00 ± 21.00	6.66 ± 0.66		2.519	16.064	0.023
total SO (pmol/mL)	177.79 ± 167.19	96.09 ± 47.94		1.063	20	0.300
total SA/SO	0.11 ± 0.05	0.08 ± 0.04		1.506	20	0.148

*Statistically significant difference ($p = 0.005$), ANOVA ($F = 11.213$; $df = 2.24$; $p < 0.001$) with Scheffé post hoc test.

lower and almost equal to the values measured in the control group of men.

The concentration and ratio of sphingoid bases were also analyzed in urine samples from 5 healthy pregnant women. The mean values of free sphinganine and sphingosine were lower than the values recorded in the control group of women, whereas SA/SO ratio was significantly greater (Table 3).

Discussion

We found that urine concentrations of sphingoid bases were higher in women than in men and differed depending on the hormonal status of women (premenopause vs postmenopause). On the other hand, there were no significant differences between men and women in the serum concentrations of sphingoid bases.

We found two reports on serum SA/SO ratio derived from the analysis of serum of healthy subjects: one that included subjects from France (9 men and 10 women) and South Africa (13 women of Asian origin), whose SA/SO ratio ranged from 0.09 to 0.78 (14), and the other that included a greater number of subjects from South Africa and Kenya and produced similar results (15). A cross-sectional study of 265 residents of Linxian, China, which examined potential demographic, physiologic, and dietary correlates of serum sphinganine and sphingosine (16) found significant differences in the SO measurements (in the serum) depending on age, menstruation status, serum cholesterol, retinol, carotenoids, tocopherols, and fresh vegetable and fruit consumption in the summer season. In our study, the concentrations of sphingoid bases obtained by acid hydrolysis were considerably higher than those obtained by base hydrolysis, because the former procedure leads to total base release, whereas the results obtained by base hydrolysis represent both free sphingoid bases and those incorporated in lysosphingolipids. The concentrations of sphingosine (obtained by base and acid hydrolysis) exceeded the concentrations of sphinganine (3.5-fold increase in base hydrolysate in both men and women, and 46- and 39-fold increase in acid hydrolysate in men and women, respectively). This could be explained by the fact that sphinganine is an intermediary product on the biosynthesis pathway, whereas sphingosine is formed by degradation of complex or dietary sphingolipids.

Our results showed that there was no statistically significant sex difference either in the concentrations of free and total sphinganine and sphingosine, or in the SA/SO ratio. This was consistent with other studies (14-16). The fact that the sphingoid base concentrations measured in our study were lower than those reported elsewhere (14,15) could be explained by genetic or environmental factors or different methodologies.

Several studies on SA/SO ratio obtained by urine analysis of healthy women and men showed similar results (15,17-19). Likewise, serum and urine concentrations of free and total sphingosine in our study exceeded those of sphinganine. Results of the high performance liquid chromatography analysis of sphingoid base concentrations pointed to statistically significant sex differences in the concentrations of free sphinganine and sphingosine (60- and 37-fold in women, respectively). Statistically significant sex differences were also recorded in the concentration of total sphinganine (8-fold greater concentration in women) and total SA/SO ratio (greater in women). The concentrations of sphinganine obtained by base hydrolysis in some male urine samples were below detectable levels. The reason for these sex differences is unknown, but they may be related to hormonal differences. Also, there were great interindividual variations in the sphingoid base concentrations, which were more pronounced in women. This is consistent with results of other studies reporting on both inter- and intra-individual differences in urinary sphingoid base concentrations recorded during a several-month follow-up (18). The variability in the levels of SO and SA was greater than in the SA/SO ratio. The study car-

ried out by van der Westhuizen et al (15) yielded no statistically significant sex differences in the sphingoid base concentrations in the urine. Solfrizzo et al (17) observed free sphinganine and sphingosine concentrations in women during various periods with and without genital tract inflammation, and found significant differences for both sphinganine and sphingosine concentrations. During the period of genital tract inflammation, these concentrations were considerably higher, whereas SA/SO ratio showed no major changes. Our female subjects were healthy, thus the interindividual variation in the sphingoid base concentrations may have been due to the sampling performed at different phases of the menstrual cycle.

The differences observed in the free and total sphinganine and sphingosine concentrations between the control groups of healthy men and women prompted us to analyze urinary sphingoid base concentrations in healthy postmenopausal women and pregnant women. Our results suggest that sex hormones may be involved in the regulation of sphingolipid metabolism because the urinary concentrations of sphinganine and sphingosine in postmenopausal women approached those found in the urine of healthy men rather than those recorded in premenopausal healthy women. We think that these differences could be a consequence of hormonal alterations associated with menopause in women. The results of the analysis of urinary sphingoid base concentrations and ratio in healthy pregnant women also suggested hormonal alterations associated with gestation. There is little data on the effect of sex hormones on the regulation of sphingolipid metabolism, and most refer to the metabolism of gangliosides (20,21). To reach more definite conclusion on the hormone effect on the sphingoid base metabolism, we have started a series of studies on the topic.

In conclusion, our study showed significant variation in the urinary concentration of free and total sphingosine and sphinganine in healthy individuals. The variation is more pronounced in women, thus limiting the diagnostic value of sphingoid base determination. In contrast, serum concentrations of free and total sphingoid bases showed no major variation in healthy individuals of both sexes, indicating their potential diagnostic value as early indicators of the intake of fumonisin and other structurally similar mycotoxins, as well as in the diagnosis of the diseases associated with sphingolipid metabolism impairment.

Acknowledgment

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