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Rapid method to determine sphinganine/sphingosine in human and animal urine as a biomarker for fumonisin exposure

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Abstract

The widespread occurrence of fumonisins in maize and maize-based foods and feeds demands the development of rapid and reliable methods for the analysis of suitable biomarkers in biological fluids in order to assess human and animal exposure to these important mycotoxins. The increase in the ratio of free sphinganine/sphingosine (SA/SO) in urine has been recently proposed as a biomarker to evaluate exposure to fumonisins. The presently available method for the determination of SA and SO in biological samples is labor intensive, time consuming and insufficiently accurate. A new method has been proposed for the determination of SA and SO in human and animal urine which is more precise and accurate, and drastically reduces the number of steps during extraction and clean-up. The method is essentially based on the use of silica minicolumn clean-up of the chloroform extract from alkalinized urine. The final extract is derivatized with *o*-phthaldialdehyde reagent and SO and SA are determined by reversed-phase HPLC with fluorimetric detector. Urine samples spiked with SO, SA standards at concentrations ranging from 1.5 to 15 ng/ml have given mean recoveries higher than 80% and precision (coefficient of variation) lower than 10%. Detection limit for SO and SA was 0.1 ng/ml.

Keywords: Fumonisin; Sphinganine; Sphingosine

1. Introduction

Fumonisin mycotoxins are structurally related compounds, produced mainly by *Fusarium moniliforme* and *F. proliferatum*, which occur largely in maize and maize-based foods and feeds [1–3]. Fumonisins, mostly fumonisin B_1 and B_2, show a species-specific toxicity as they cause leukoencephalomalacia (LEM) in horses, pulmonary edema (PE) in pigs, hepatotoxicity, nephrotoxicity and hepatocellular carcinoma in laboratory rats, and the question if these toxins are involved in human oesophageal cancer (EC) remains unanswered [4]. Toxicological studies with animals show that horse is the most sensitive species, and it has been recommended that a maximum fumonisin level of 5 μg/g should be allowed in horse feed to avoid the danger of inducing LEM. Similarly for swine, a maximum level of 10 μg/g has been recommended to avoid PE [3]. Because of the great resistance of beef cattle and poultry to fumonisin toxicity, maximum feed levels of 50 μg/g have been suggested [3]. High levels of fumonisins (up to 20 μg/g) have been found in maize based food such as extruded maize, maize flour and polenta in Italy [1,5–7]. The high levels of fumonisins in Italian maize products are of concern, because previous studies showed a positive correlation between maize consumption and

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increased risk of EC in northeastern Italy [8]. A similar situation has been found in Transkei (South Africa) where the high incidence of EC has been statistically associated with the ingestion of fumonisin contaminated maize [9]. A recent report confirmed the general distribution of fumonisin worldwide, but was inadequate to estimate the global exposure of populations to fumonisins because of the limited number of samples and the big variation in maize consumption in different countries [3]. For example the estimated daily intake of maize in Transkei, South Africa is 460 g/person against 4 g/person in Switzerland and 7 g/person in Germany and United Kingdom [3]. Moreover, maize consumption within the same country can vary tremendously such as in Italy where polenta is widely consumed in the northeastern part of the country and only occasionally consumed in the south. Another problem which may lead to major errors in estimating the exposure of populations to fumonisins or other mycotoxins is the sampling, because often the analyzed samples are not representative of the whole bulk. The combination of these factors clearly shows that a reliable measure of mycotoxin exposure can hardly be performed by analyzing a number of food samples. The analysis of suitable biomarkers in biological fluids is a useful tool to assess human and animal exposure to mycotoxins. Direct measurement of fumonisins in biological fluids is not a good indicator of their consumption as these toxins are quickly eliminated after ingestion [10]. A useful biomarker to evaluate animal exposure to fumonisins has been recently proposed by Riley et al. [11] which involves the measurement of the free sphinganine-to-sphingosine ratio (SA/SO) in urine and serum. This ratio becomes elevated when animals are exposed to adequate amounts of fumonisins. The imbalance of SA/SO in biological fluids is a direct consequence of the disruption of sphingolipid metabolism caused by fumonisins. These compounds, due to their structural similarity with SA and SO (Fig. 1), provoke the inhibition of sphingolipid biosynthesis through inhibition of the sphinganine (sphingosine) *N*-acyl transferase (also known as ceramide synthase) [12]. This leads to an accumulation of free SA and an increase in the ratio of free SA to free SO in tissues and biological fluids after exposure to fumonisins [11]. The disruption of sphingolipid metabolism is a molecular event common to the above diseases caused by fumonisins and occurs at an early stage after the ingestion of the toxins.

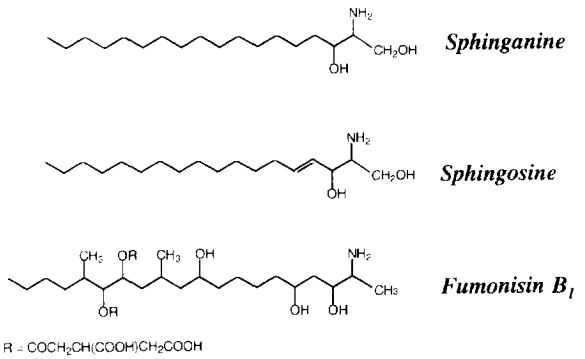

Fig. 1. Chemical structure of sphinganine, sphingosine and fumonisin B_1.

This paper reports a rapid method for the analysis of free SA and free SO in human and animal urine which improves the existing one considerably [11] and is suitable for the evaluation of human and animal exposure to fumonisins.

2. Experimental

Stock solutions (4.8 μg/ml) of sphinganine (DL-*erythro*-dihydrosphingosine) and D-sphingosine (Sigma, St. Louis, MO, USA) in methanol–water (9:1) were diluted to obtain standard solutions at concentrations of 0.06 μg/ml, 0.3 μg/ml and 0.6 μg/ml, these solutions were then used to perform the calibration curves as well as the spiking experiments. A methanol–water (9:1) solution (0.3 μg/ml) of C:20 sphinganine, not available commercially, was prepared synthetically as described by Nimkar et al. [13] and provided by Dr. A.H. Merrill Jr. (Emory University, Atlanta, GA, USA). C:20 sphinganine was used to check the reliability of this compound as internal standard.

o-phthaldialdehyde (OPA) reagent solution was prepared according to Riley et al. [11]: 5 mg OPA was dissolved in 100 μl methanol, and 5 μl of 2-mercaptoethanol and 10 ml of borate buffer (3% boric acid in water, pH adjusted to 10.5 with KOH)

were added. The solution was stable for 1 week at ambient temperature in the dark.

The analytical procedure employed a silica gel minicolumn clean-up with HPLC as the end-determination step of the long-chain (sphingoid) base OPA derivatives as following. Urine (2 ml) was diluted with methanol (2 ml), alkalinized with NH_4OH 0.35 *M* (1.2 ml) and extracted with 4 ml chloroform (previously dehydrated with sodium sulphate anhydrous powder) by vortexing. After centrifugation at 2800 rpm for 10 min, an aliquot of chloroform extract was cleaned-up through a minicolumn consisting of 5 g of sodium sulphate anhydrous crystal (Baker, Deventer, Netherlands) packed on top of 0.2 g silica gel 60 (15–40 μm) (Merck, Darmstadt, Germany) in a 12 mm diameter polypropylene column. The minicolumn was conditioned with 3 ml chloroform before use, maintaining flow-rate below 2 ml/min. The column was loaded with 3 ml chloroform extract and washed with 1 ml chloroform. The long-chain (sphingoid) bases were eluted with 4 ml of $CHCl_3$–MeOH–NH_4OH (50:50:2). After evaporation of the solvent at 60°C under a stream of nitrogen, samples were redissolved in 250 μl methanol–water (9:1) and analyzed by HPLC. Spontaneous urine samples were collected from healthy humans (male and female 28–45 years old) stored at 4°C and analyzed within 2 days.

2.1. Animal experiment

Male Wistar rat (Morini, Polo D'Enza, Italy), 8 weeks of age was housed with free access to diet and water. The experimental diet was prepared by mixing a standard commercial diet (Mucedola, Settimo Milanese, Italy) with appropriate amounts of maize contaminated with fumonisins in order to obtain a diet containing 15 μg/g of fumonisins (fumonisin B_1 plus fumonisin B_2). Control diet did not contain fumonisins. The fumonisin levels in the diets were confirmed by HPLC analysis performed as described by Visconti and Doko [14]. Control and positive urine samples (24 h) were collected by placing the rat in a polycarbonate metabolism cage (Tecniplast Gazzada, Buguggiate, Italy) before and after feeding for 1 week with the contaminated diet. Urine samples were stored at 4°C and analyzed within 2 days.

2.2. HPLC determination

The purified extract was derivatized with 50 μl OPA reagent mixed by vortexing for 30 s, and 50 μl (equivalent to 263 μl of urine) were injected into the HPLC system 1 h after adding OPA reagent. The HPLC system consisted of a Series 3B liquid chromatograph connected to a MPF-44B fluorimetric detector (λ_{ex}=335, λ_{em}=440 nm) and a Turbochrom 4.0 data system (Perkin Elmer, Norwalk, CT, USA). The separations were performed on a C_{18} reversed-phase Supelcosil LC-ABZ+plus column (15 cm×4.6 mm, 5 μm particles), preceded by a guard column (20×4.6 mm) of the same packing material (Supelco, Bellefonte, PA, USA). The mobile phase consisted of 13 min methanol–water (9:1) followed by 5 min with methanol, at a flow-rate of 1 ml/min. Equilibration of the column was reached by pumping methanol–water (9:1) for 7 min between each chromatographic run. SO and SA were quantified by measuring peak areas, and comparing them with the relevant calibration curve. Recovery experiments for SO and SA were performed on urine samples containing no detectable level of SA and a low level of SO by spiking urine samples at 15 ng/ml, 7.5 ng/ml and 1.5 ng/ml, respectively.

3. Results and discussion

The use of the increased SA/SO ratio as a biomarker of fumonisin exposure was proposed by Riley et al. [11] based on the inhibition by fumonisins of de novo SO biosynthesis which leads to the accumulation of free SA, a precursor of SO. The same authors found that a significant increase in SA/SO ratio in kidney and serum of pigs fed with a diet containing 5 ppm of fumonisins occurred long before any other serum biochemical parameters became elevated. The sensitivity of this biomarker to fumonisins has been demonstrated in different animal species, e.g., rat, pig, equine, poultry, catfish and rabbit [11,12,15,16]. The determination of the SA/SO ratio in serum, lung, liver and kidney after exposure to fumonisins provided evidence that kidney is the most sensitive organ [11]. Moreover, elevated ratios were observed in the rat urine before serum ratios were elevated [17]. These data promp-

ted us to use urine as a matrix for the determination of SA/SO ratio in humans. The method proposed here for the determination of SO and SA in human and animal urine was satisfactory in terms of accuracy and precision. The maximum HPLC response of the SO and SA OPA derivatives was reached after 1 h and remained stable for 24 h. Calibration curves of SO and SA were linear and stable in the range 0.5–10 ng (absolute injected amount). The within-day repeatability and between-day repeatability of the injection of calibrants gave coefficient of variation (C.V.) lower than 3% and 5%, respectively. Retention time for SO and SA was 7.2 min and 9.2 min, respectively. Fig. 2 shows the chromatogram relevant to a urine sample of a 38 years old healthy female person not exposed to fumonisin. In Fig. 3 the chromatograms relevant to the urine samples

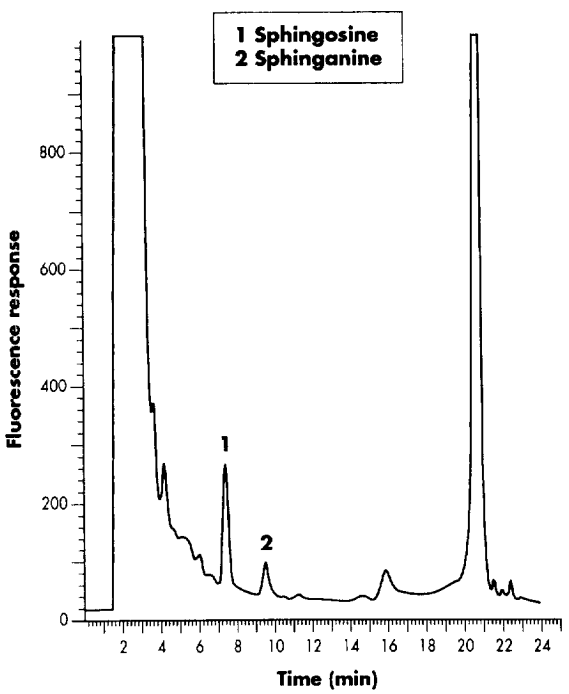

Fig. 2. Chromatogram of a human urine sample containing 5.1 ng/ml sphingosine and 1.6 ng/ml sphinganine. Urine extract derivatized with OPA reagent. Volume of derivatized extract injected, 50 μl (=263 μl urine); column, C_{18} Supelcosil LC-ABZ+plus, (150×4.6 mm I.D., 5 μm); precolumn with the same stationary phase (20×4.6 mm I.D.); mobile phase, methanol–water (90:10), followed by 5 min methanol only; flow-rate, 1 ml/min; detection, fluorescence at 335 nm excitation and 440 nm emission.

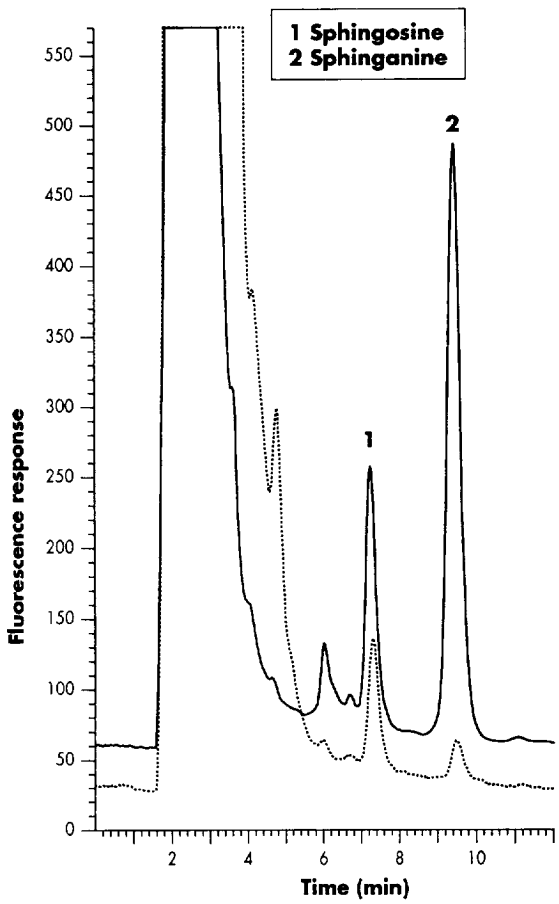

Fig. 3. Chromatograms of rat urine with (solid line) and without (dashed line) fumonisins in the diet at a level of 15 μg/g. Volume of derivatized extract injected, 50 μl (=263 μl urine); chromatographic conditions as in Fig. 2.

collected from a rat not exposed to fumonisins (dashed line) and exposed to fumonisins (solid line) are reported . The conspicuous increase of free SA in urine of rat exposed to fumonisins brought the SA/SO ratio from 0.42 (control) to 2.55.

The use of methanol as mobile phase after elution of SA was required in order to elute compounds that can interfere in the successive run (see peak at 20 min in Fig. 2). The use of a strong deactivated C_{18} column resulted in particularly efficient analysis of the OPA derivatives considered herein, providing low backpressure, good stability and high efficiency. The choice of such a column was determined by the successful results experienced in the author's labora-

tory with the separation of OPA-fumonisin derivatives.

The use of a synthetic internal standard, C:20 sphinganine (C:20-SA), was proposed by Riley et al. [11], due to the low precision and accuracy in the determination of SO and SA in tissues and biological fluids. However, this internal standard was inadequate for urine analysis because some samples exhibited a peak at the same retention time (13.6 min) as the C:20-SA (see Fig. 4). This peak appears using both the present method or the one proposed by Riley et al. [11]. This evidence suggests more studies in order to understand if this peak is the C:20-SA or an interfering compound which, in HPLC, with precolumn OPA reagent derivatization, coelutes with C:20-SA. This problem and the good recoveries for SO and SA obtained in this study prompted us for not using C:20-SA as internal standard.

The method described herein provided recoveries for SO and SA, spiked at concentration ranging from 1.5 ng/ml to 15 ng/ml, consistently higher than 80% and coefficient of variation lower than 10% (Table 1). Recoveries of the two compounds, tested on twelve different urine samples at 15 ng/ml spiking level, were similar to those obtained with replicate analyses of the same urine sample, indicating the lack of matrix effect. The anhydrous sodium sulphate for preparing the SPE column, was used as crystals in order to avoid analyte losses occurring due to using the powder. Recovery losses of SA and SO by using anhydrous sodium sulphate powder was first noted by Riley (pers. comm.) and confirmed in our laboratory where up to 0–5% recoveries for SA and SO were obtained by using the powder of anhydrous sodium sulphate.

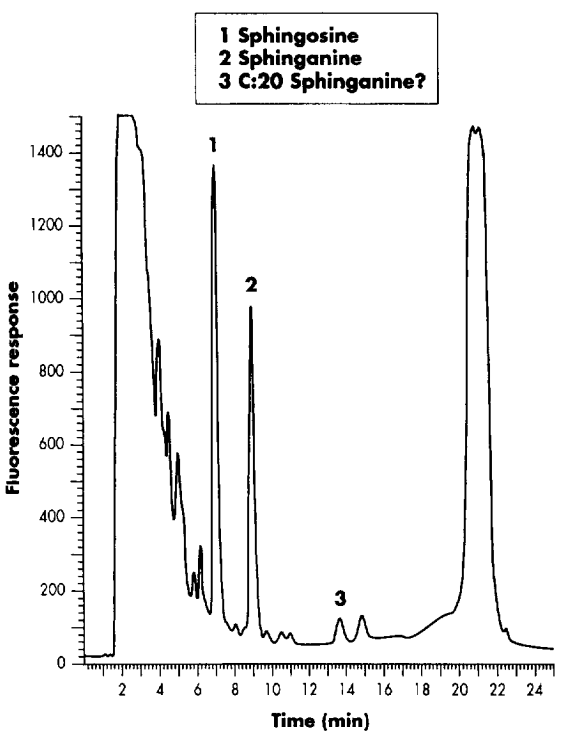

Fig. 4. Chromatogram of OPA derivatized extract of urine sample from a person (female, 28 years old) bearing inflammation of the genital apparatus; chromatographic conditions as in Fig. 2.

Table 1
Recoveries and coefficients of variation (C.V.) of LC determinations of sphingosine and sphinganine in human urine samples

Spiking level (ng/ml)	Mean recovery ($n=3$) (%)	C.V. (%)
Sphingosine		
1.5	95.9	3.0
7.5	84.4	4.2
15	84.8[a]	6.8
Sphinganine		
1.5	86.4	2.9
7.5	81.9	4.9
15	85.4[a]	7.4

[a] $n=12$, from different urine samples.

Detection limit for SO and SA was 0.1 ng/ml (S/N 3:1) with the detector used in the present study.

No difference was observed in chromatograms obtained using a mobile phase with and without potassium phosphate buffer. On the other hand, the potassium phosphate buffer (used by Riley et al.) is not required because no ionizable groups are present in the molecule of SO and SA after derivatization with OPA reagent. A great advantage offered by the present method, compared to the method of Riley et al. [11] which is labor intensive and time consuming, is the drastic reduction of the number of steps during extraction and clean-up. The Riley method, tested on three human urine samples spiked with C:20-SA at 90 ng/ml (three replicates for each sample), gave recoveries of 64–73% and R.S.D.$_r$ (within laboratory R.S.D.) values ranging from 11 to 48%. The extraction and purification procedure took about 9 h as

compared with 2 h required by the method proposed herein. Moreover the base hydrolysis step used by Riley et al. [11] was not required as no interfering peaks appear in the chromatograms of urine extracts processed as described herein both in human and rat urine samples with and without fumonisins in the diet as shown in Figs. 2 and 3.

The results of the analysis of some few urine samples from humans, measuring the concentration of SO, SA and the SA/SO ratio, are reported in Table 2. Values for SO and SA were higher in urine samples of the female than those of the male. The values reported in Table 2 are relevant to healthy humans not exposed to fumonisins. It was not possible to establish a value for the SA/SO ratio in the case of male urine samples because SA could not be detected below 0.1 ng/ml in any of the examined samples. Yet, in urine of humans exposed to fumonisins SA would accumulate as a result of the inhibition of the de novo sphingolipid biosynthesis rendering this compound detectable with our method.

Levels of SA and SO in human urine can vary considerably in relation to the number of cells released in urine, but the ratio SA/SO remains roughly the same if the person has not been exposed to fumonisins. Urine analysis of a 28-year-old person (female) not exposed to fumonisins, tested in different periods with and without inflammation of the genital apparatus showed a great difference in the absolute concentration of SO and SA, but the same difference was not observed with the SA/SO ratio. The level of SO in urine varied from 4.0±0.4 ng/ml (n=3) to 39.9±2.1 ng/ml (n=6) before and during the inflammation evolution, and the level of SA varied from 1.2±0.15 ng/ml (n=3) to 21.4±1.1 ng/ml (n=6), whereas the ratio SA/SO remained roughly the same before and during the inflammatory process, 0.30±0.01 (n=3) and 0.54±0.01 (n=6) respectively. The chromatogram of the urine sample collected from this person during the inflammatory evolution is shown in Fig. 4. The method described in this paper was also successfully applied to the analysis of SO and SA in urine samples of rats fed with a diet containing maize contaminated with fumonisins as shown in Fig. 3.

Table 2
Levels of sphingosine (SO), sphinganine (SA) and SA/SO ratio in urine samples of healthy humans from southern Italy

Sphingosine (ng/ml) Median (range)	Sphinganine (ng/ml) Median (range)	SA/SO Median (range)
Female (n=6)		
4.60 (2.36–7.60)	1.15 (0.51–3.60)	0.29 (0.17–0.53)
Male (n=9)		
0.25 (0–1.58)	N.D.	–

N.D.=not detected.

The rapidity of the proposed method and the specificity of the SA/SO biomarker make this method suitable to epidemiological studies aimed to ascertain if populations exposed to fumonisins result in significantly increased SA/SO ratios in urine.

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