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Analytical method for the determination of sphinganine and sphingosine in serum as a potential biomarker for fumonisin exposure

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Abstract

The toxins produced by *Fusarium moniliforme*, which include fumonisins, are possible human carcinogens. Fumonisins are inhibitors of de novo sphingolipid biosynthesis. Alterations of the ratio of sphinganine (Sa) to sphingosine (So) in urine and serum has been proposed as a possible biomarker of exposure to this toxin. A new method was developed for their analysis in tissues and urine. This work describes the further adaptation of the method to the analysis of Sa and So in serum and its validation in sera of untreated and fumonisin B_1 (FB_1) treated rats and mice. No significant differences in the Sa/So ratios were observed in the FB_1 treated rats. In mice, the increase was only of marginal statistical significance. Determination of Sa/So ratios in human sera could readily be made in small volumes (from 0.3 to 0.5 ml) of serum.

Keywords: Biomarker; Sphinganine; Sphingosine; Fumonisins

1. Introduction

Fumonisins are mycotoxins produced by the fungus, *Fusarium moniliforme* and several other *Fusarium* species [1,2]. The mycotoxins were purified from cultured extracts of the *F. moniliforme* [3] and the chemical structures determined by Bezuidenhout et al. [4]. Contamination of animal feed and human foods by *F. moniliforme* is widespread, the main substrate being corn (*Zea mays*) in which high levels of fumonisins have been detected [5,6] but also other foods may be contaminated e.g. beans [7]. Fumonisin B_1 (FB_1) induces leukoencephalomalacia in horses [8] and pulmonary oedema in pigs [9]. There is no evidence that fumonisins are genotoxic (for review, see [5]). FB_1 was shown to be hepatocarcinogenic in rats, [10] although most car-

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cinogenicity testing was with *F. moniliforme* cultures known to contain significant amounts of fumonisins. Based on these data an IARC working group concluded that there was sufficient evidence for the carcinogenicity of these cultures in animals [5].

A prominent biological effect of the fumonisins is the disruption of sphingolipid biosynthesis via the inhibition of sphinganine and sphingosine *N*-acyltransferase [11]. The interruption of sphingolipid biosynthesis was suggested to be an important mechanism through which the fumonisins could disrupt the regulation of cell growth and differentiation, subsequently leading to neoplastic transformation [12]. This effect was demonstrated in ponies [13], pigs [14], chickens [15], rabbits [16], rats [17] catfish [18], monkeys [19] and mink [20]. Various organs are affected including the liver, lung and kidney. The main target in rats is the kidney which is reflected by an altered urinary ratio of sphinganine to sphingosine (Sa/So) with a similar alteration in the target tissues themselves and in blood [17,21]. A method to analyse sphingolipid bases has been reported [22,23] but involves numerous steps and consequently not ideally suited to analysis of large numbers of samples, as is often required in epidemiological studies. New methods have therefore been developed for the analysis of the Sa/So ratio in tissues as well as human and rat urine [24,25]. Ecological studies showed a positive correlation between dietary fumonisin levels and oesophageal cancer incidence rates in China and Southern Africa (for review see [5]). Epidemiological studies to further evaluate this possible relationship would be facilitated by specific biomarkers to monitor exposure of a population to the fumonisins at an individual level. The altered Sa/So ratio in the urine has been suggested as an appropriate biomarker of exposure to fumonisins. However, one problem encountered with the analysis of human urine was the extremely low level of Sa in males [24,25]. This would necessitate the use of large volumes of urine for exposure assessment in human populations. Shephard et al. [19] suggested that measurement of the Sa/So ratio in serum may be a more sensitive biomarker and therefore in the current study the method of Castegnaro et al. [24] was adapted to the analysis of the sphingoid bases in serum.

2. Experimental

2.1. Animal treatments

Eight-week-old male BDIV rats were treated by gavage with a daily dose (5 days a week) of 1 mg/kg body weight FB_1 over a period of 5 weeks. On the last day of the treatment the animals were killed, blood collected, the serum prepared and frozen immediately in liquid nitrogen followed by storage at −80°C until analysis.

Hepatitis B virus (HBV) transgenic [26] and non-transgenic mice (C57 black) were treated by gavage with 16.8 mg FB_1/kg body weight 3 times a week for 63 weeks. These mice are part of a long-term study of the interaction between FB_1 and HBV that will be published elsewhere. The mice were bred in the IARC animal facility as described for previous studies [27]. Control animals from each group of mice received distilled water. The last day of the treatment, the animals were sacrificed and the sera prepared and stored as described above.

2.2. Human subjects

One set of 16 blood samples was collected by the 'Centre de Transfusion Sanguine, Lyon, France' from healthy donors (10 females and eight males).

A second set of 17 samples was obtained from South Africa. Four were from patients hospitalised with oesophageal cancer and 13 were healthy controls.

2.3. Chemicals

Dr. J.D. Miller (Carleton University, Canada) kindly provided fumonisin B_1. C-20 sphinganine (C20 Sa) was a generous gift from Dr. A.H. Merrill Jr. (Emory University School of Medicine, Atlanta, GA). Sphinganine (Sa), sphingosine (So), mercaptoethanol, *o*-phthaldialdehyde (OPA) and boric acid were purchased from Sigma (Saint-Quentin-Fallavier, France). Ethyl acetate (Merck LiChrosolv), potassium hydroxide, potassium hydrogen phosphate (analytical grade) were from Merck (Darmstadt, Germany). HPLC grade methanol was from S.D.S. (Peypin, France) and pronase from Calbiochem (La Jolla CA, USA).

Stock solutions of Sa and So for HPLC analyses were prepared in ethanol at a concentration of 1 m*M*. The working standards were prepared by diluting the stock solution to 10 μ*M* in ethanol. All the solutions were kept at −20 °C in the dark.

2.4. Equipment

An HPLC liquid chromatographic system (Perkin Elmer series 4) was coupled to an automatic injector (Spectra system AS 3000, Thermo Separation Products) maintained at 15°C, a fluorescence detector (Perkin Elmer LS40) and a data computing system (Borwin V1.21). Separation of sphingolipids was on an Ultrabase column (Kromasil) C18, 5 μm (25 cm long×4.6 mm) maintained at 35°C.

A shaker with turning movement at a speed of 18 rpm (Amilabo AO 226244, Paris, France) was used for sphingolipid base extraction from the sera.

2.5. Determination of sphingosine (So) and sphinganine (Sa) in serum

2.5.1. Extraction procedures

Ethyl acetate (as used by Castegnaro et al. [24]) and a mixture chloroform–methanol (3:1) (as used by Merrill et al. [22]) were compared as extraction solvents. In general ethyl acetate gave higher recoveries of Sa and So from the sera. In order to optimise the time of extraction, a series of experiments were performed by extracting the serum for 5, 20, 30 and 60 min with ethyl acetate.

In addition, several techniques were investigated in order to yield the purest sphingolipid preparation for HPLC analysis. These included deproteinisation with a 10% salicylic acid solution, pronase to digest serum proteins, albumin extraction, and a pre-extraction with hexane. However, none of these approaches improved the quality of the sample preparations for HPLC analysis.

2.5.2. Detailed description of the optimised methodology

2.5.2.1. Sphingolipid extraction. Potassium chloride solution (1.5 ml, 0.8%), 50 μl 1 *M* potassium hydroxide and 5 μl of the C20 Sa standard solution were added to 0.5 ml of serum. The mixture was extracted with 4 ml of ethyl acetate by gentle rotation for 20 min and the phases were separated by centrifugation at 1100 *g*. for 15 min. The organic phase was evaporated to complete dryness at 55°C under nitrogen.

2.5.2.2. OPA-derivatisation. The derivatisation mixture consisted of 12.5 mg OPA in 250 μl of ethanol containing 12.5 μl of mercaptoethanol and 3% boric acid solution adjusted to pH 10.5 with potassium hydroxide to obtain a final volume of 12.5 ml. This solution can be stored for a maximum of a week in a refrigerator at 4°C in the dark. Care has to be taken to protect the mixture from light during derivatisation and to minimise the time kept at room temperature.

Following the ethyl acetate extraction the dried samples were dissolved by vortex shaking in 275 μl of a 0.07 *M* K_2HPO_4–methanol (1:9) solution and derivatised for at least 30 min by addition of 25 μl of the above OPA mixture.

2.5.2.3. HPLC analysis of the derivatives. The derivatives were analysed by HPLC with fluorescence detection (excitation wavelength of 340 nm, emission wavelength of 455 nm). The column was kept in an oven at 35°C and the flow-rate maintained at 1 ml/min using the solvent gradient systems that are summarised in Table 1.

The current method is easy to perform and up to 50 samples can be analysed per week when using an automatic sample injector.

Table 1
Programme of gradient elution for HPLC

Time (min)	% Solvent A[a]	% Solvent B[b]
0	80	20
30	70	30
35	0	100
45	0	100
47	80	20
60	80	20

[a] Solvent A, 0.07 M K_2HPO_4–methanol (1:9).
[b] Solvent B, methanol.

Table 2
Results from the analysis of Sa, So and C20 Sa in serum from FB_1 treated and untreated rats

Rat number	FB_1 treatment	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
1	No	179.2	144.9	59	0.81
2	No	105.3	69.1	81	0.66
3	No	51.5	25.9	63	0.50
4	Yes	104.4	36.9	41	0.35
5	Yes	91.6	38.4	78	0.42
6	Yes	147.9	61.3	62	0.41

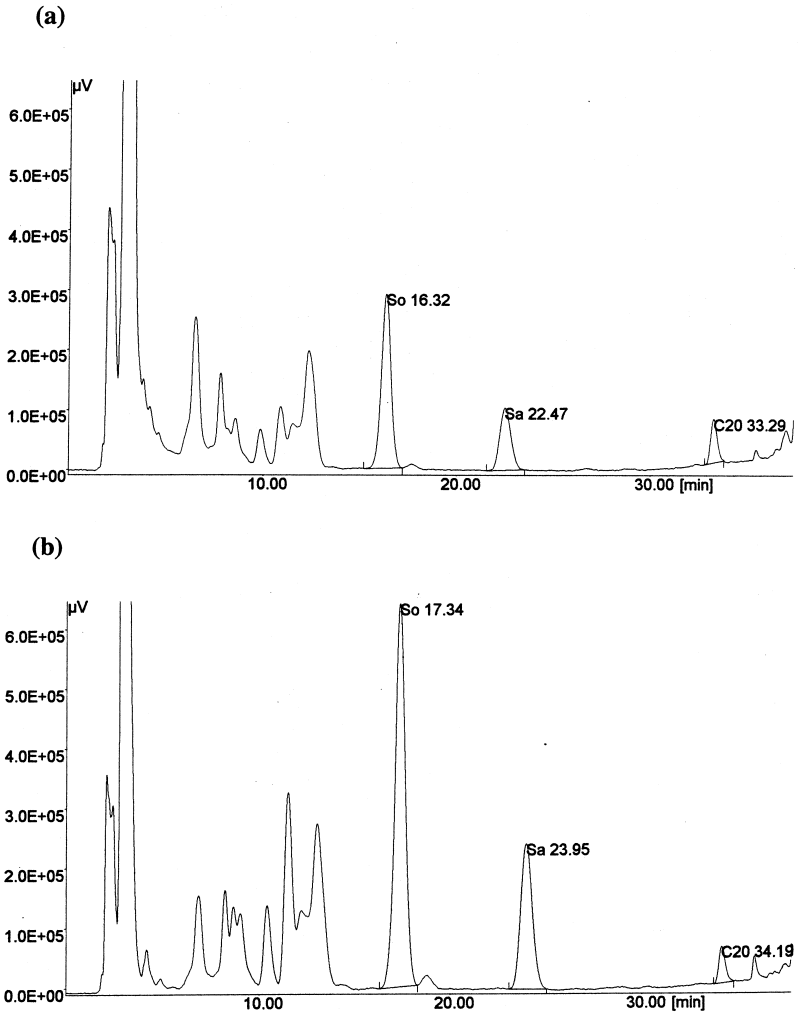

Fig. 1. Typical chromatograms of sera from a rat treated for 5 weeks, 5 days a week, with 1 mg/kg body weight of FB_1 (a) and of the corresponding control (b).

2.6. Statistical analyses

All the data were analysed statistically by the non-parametric Kruskal–Wallis Test in order to determine if the mean values for Sa/So ratios differ significantly between groups.

3. Results and discussion

3.1. Determination of sphingosine (So) and sphinganine (Sa) in serum

3.1.1. Preliminary investigations

As mentioned in Section 2 several methods were evaluated for the extraction of Sa and So from serum and the purification of the extracts prior to HPLC. A mixture of chloroform–methanol (3:1) as extraction solvent resulted in lower recoveries of the sphingoid bases as compared to ethyl acetate (data not shown). With ethyl acetate, vigorous shaking led to formation of an emulsion from which it was difficult to separate sphingolipids. However, an alternative mild rotation on a shaker with a speed of 18 rpm resulted in an extraction without formation of an emulsion. Ethyl acetate was therefore selected for optimisation of the extraction.

3.1.2. Sphingoid bases recovery

When using the optimised method described above the recoveries of C20 Sa were generally between 50 and 70% but with an overall range of 20–100% (Tables 2–4). It was therefore important to determine whether the variation in C20 Sa recovery was associated with a parallel variability in Sa and So recoveries.

Comparative recoveries of Sa, So and C20 Sa were performed 6 times on the same spiked sample within the same experiment. The corresponding values, presented in Table 5A, indicated that the three sphingolipids are recovered to the same extent (96.5±6.7; 90±6.9; 96.5±7.1). In addition, a series of different spiked samples were analysed whose results are presented in Table 5B. It would therefore appear that, although some variability in the recovery occurs between experiments this will have a minor impact on the Sa/So ratio and the Sa and So values which can be corrected by the C20 Sa recovery.

3.2. Sphingolipid levels and Sa So ratios in sera from rats, mice and humans

Typical chromatograms of serum extracts from a rat treated with 1 mg FB_1/kg body weight for 5 weeks and a corresponding untreated control are

Table 3
Results from the analysis of Sa, So and C20 Sa in serum from FB_1 treated and untreated mice

Type of mice	FB_1 Treatment	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
C57 BL	No	45	15	45	0.33
		40	13	50	0.33
		41	14	42	0.34
		10	6	98	0.6
C57BL/-AlbHBV		27	7	47	0.26
		112	51	67	0.46
		143	48	91	0.34
		93	26	97	0.28
C57 BL	Yes	91	75	67	0.82
		52	20	82	0.38
		101	21	100	0.21
		94	53	100	0.56
C57BL/-AlbHBV		16	13	51	0.81
		62	19	100	0.31
		22	16	97	0.73
		66	25	65	0.39

Table 4
Results from the analysis of Sa, So and C20 Sa in human serum

Individuals	Sex	So (ng/ml)	Sa (ng/ml)	C20 Sa recovery (%)	Sa/So
Healthy	F	7	4	95	0.57
controls		13	7	47	0.54
from		14	11	59	0.78
France		18	7	68	0.39
		13	7	65	0.54
		32	11	37	0.34
		25	7	55	0.28
		22	4	35	0.18
		14	4	24	0.29
		2.5	n.d.	99	0
	M	14	4	58	0.29
		14	3	76	0.21
		18	8	69	0.57
		40	11	70	0.28
		14	4	74	0.29
		41	14	52	0.34
		22	8	76	0.36
		5.6	0.6	92	0.11
Healthy	F	54	24	54	0.44
controls		153	29	47	0.19
from S.A.		63	9	62	0.14
		36	8	67	0.22
		136	24	51	0.18
		54	12	39	0.22
		16	7	62	0.44
		41	7	52	0.17
		116	10	51	0.09
		65	10	34	0.15
		90	16	24	0.18
		160	22	44	0.14
		18	5	53	0.28
Cancer	M	11	4	34	0.36
patients		22	4	69	0.18
		15	3	58	0.20
		55	9	53	0.16

illustrated in Fig. 1. No significant differences in Sa, So and the Sa/So ratio were detected between the control animals and the animals sacrificed immediately after the gavage treatments with FB_1 although the small numbers of animals should be noted (Table 2).

Fig. 2 shows typical chromatograms of serum extracts from C57 BL and C57BL/-AlbHBV mice after gavage treatment for 63 weeks (16.8 mg FB_1/kg body weight 3 times weekly) and of the respective untreated controls that received distilled water. An increase in Sa/So ratio is observed after treatment with FB_1 when compared to the control values; 0.53 ± 0.24 vs 0.37 ± 0.11 although this is only of borderline statistical significance ($P=0.09$) (Table 3). No significant changes were observed in the So concentration in the serum while the Sa concentration was slightly increased in the treated mice. The HBV status did not affect the Sa/So ratio.

Fig. 3 shows chromatograms from a healthy blood donor and a patient with oesophageal cancer while the Sa, So values and C20 Sa recoveries are pre-

sented in Table 4. No significant difference was detected for the Sa/So ratio between the healthy South African control subjects and patients with oesophageal cancer. However the number of cancer patients is small ($n=4$). The patients are males and the controls female and in addition, data on the duration of hospitalisation prior to blood sampling is unknown. In rats, we have demonstrated that the altered Sa/So ratio returns to normal 2 weeks after cessation of the FB_1 treatment (IARC, unpublished data). One notable result is that the healthy females from South Africa have much higher serum So levels and lower serum Sa/So ratios than healthy female subjects from France; all the South African healthy female donors were of Asian origin. It is also worth noting the large interindividual variation in Sa/So ratios that is of a similar order of magnitude to that seen in human urine samples [24]. Clearly there are many factors, both environmental and genetic, which could influence this ratio in a given individual.

The above method provides a fast and sensitive tool for the analysis of the Sa/So ratio in serum of

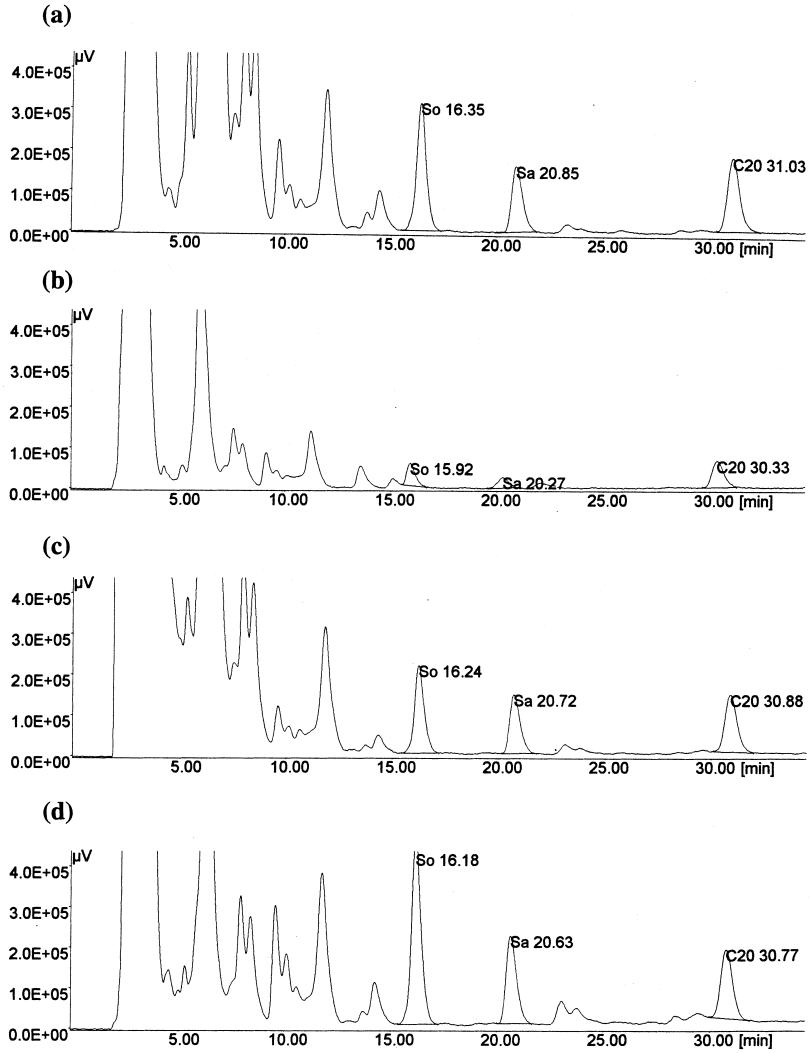

Fig. 2. Typical chromatograms of sera from C57BL and C57BL/-AlbHBV mice treated for 68 weeks, 3 days a week, with 16.8 mg/kg body weight of FB_1 (a, c) and of the respective controls that received distilled water (b, d).

Table 5
Comparative recovery of Sa, So and C20 Sa in serum samples

Sample number	So recovery (%)	Sa recovery (%)	C20 Sa recovery (%)
A			
1a	89	83	89
1b	96	89	98
1c	106	100	104
1d	104	96	105
1e	88	83	89
1f	96	89	94
Mean±S.D.	96.5±6.7	90.0±6.9	96.5±7.1
B			
2	76	76	69
3	67	66	63
4	68	72	70
5	72	77	67
6	46	45	37
7	45	39	45
8	72	77	67

animals and humans. It will allow the investigation of the health effects of fumonisins in human populations and possibly other toxins that can modify sphingolipid biosynthesis such as AAL toxins produced by *Alternaria alternata* [12]. The method has the advantage of only requiring small volumes of serum (0.3–0.5 ml), but clearly the sensitivity of the method in the case of human exposures needs to be established.

In the present study no increase of the Sa/So ratio has been detected in serum of rats following FB_1 treatment, while an alteration was detected in kidney, urine and liver of the same rats [21]. A recent study by Riley et al. [17] indicated that rats administered feed containing 150 μg FB_1/g for 4 weeks had significantly altered Sa/So ratios in the serum while those administered 50 μg FB_1/g had no alteration in ratio. As one rat consumes about 10 g feed/250g b.w./per day [28] the equivalent doses administered by Riley et al. [17] were about 6 and 2 mg/kg

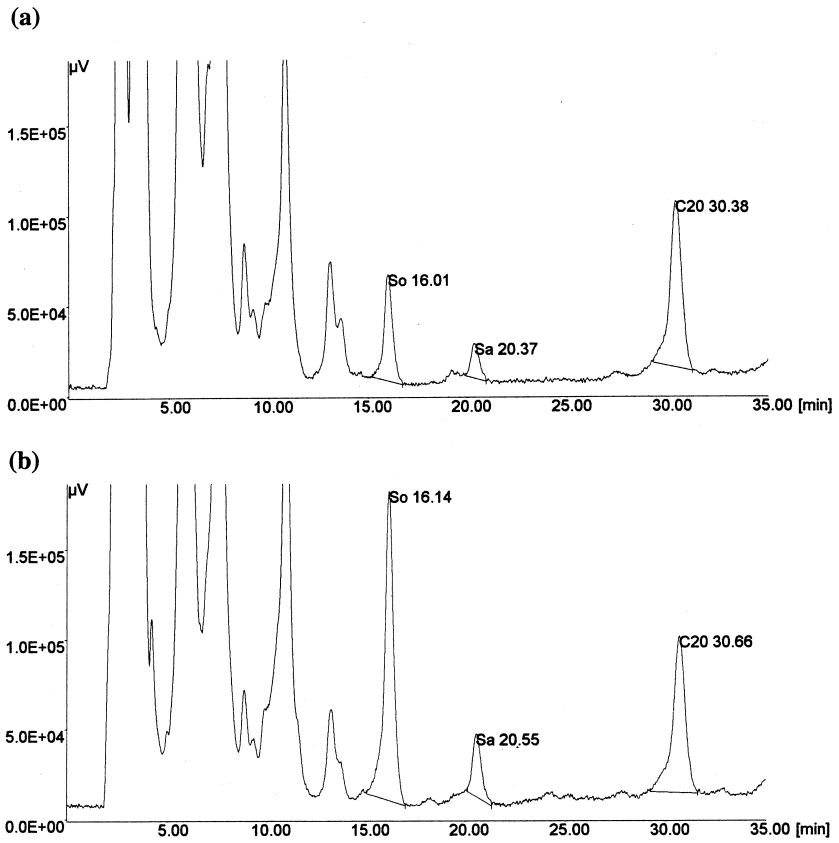

Fig. 3. Typical HPLC chromatograms obtained from human blood: (a) healthy donor and (b) patient with oesophageal cancer.

b.w./day for 4 weeks. Their data are therefore in agreement with our current observations since at a dose of 1 mg/kg b.w. for 5 weeks we did not see any significant changes.

A marginal significant ($P=0.09$) increase of the Sa/So ratio was detected when comparing FB_1 treated and untreated mice with a treatment dose >15-fold higher than in the rat study and a 12-fold longer treatment period.

While the methodology described above provides a convenient way of assaying changes in the Sa/So ratio, a considerable amount of research has to be performed to validate it as a possible marker of fumonisin exposure in human populations. Field studies in humans will be directed to investigate changes in the Sa/So ratios as a function of fumonisin exposure determined at an individual level. As mentioned above, it is currently unknown how sensitive the Sa/So ratio will be in human populations exposed environmentally to fumonisins.

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