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Short communication

Liquid chromatographic determination of the sphinganine/sphingosine ratio in serum

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Abstract

The fumonisin mycotoxins, which are worldwide contaminants of corn, inhibit de novo sphingolipid biosynthesis leading to elevation in the ratio of the sphingoid bases, sphinganine and sphingosine, in the serum of animals exposed to fumonisins. A new HPLC method for the determination of the ratio of these bases in serum has been developed involving lipid extraction, clean-up on a silica minicolumn and alkaline hydrolysis prior to precolumn *o*-phthaldialdehyde derivatisation and HPLC separation and quantification by fluorescence detection. Based on serum from both normal and fumonisin-exposed vervet monkeys, the method was shown to be reproducible (R.S.D.<10%).

Keywords: Sphinganine; Sphingosine; Fumonisin; Mycotoxin

1. Introduction

The fumonisins are mycotoxins produced by the fungus *Fusarium moniliforme* Sheldon and occur as contaminants in corn around the world [1]. Their ingestion produces a range of syndromes in animals, including leukoencephalomalacia in horses [2], pulmonary oedema in pigs [3] and liver cancer in rats [4]. In addition, they have been associated with human oesophageal cancer in South Africa [5] and China [6]. They have been shown to be potent inhibitors of de novo sphingolipid biosynthesis via inhibition of the enzyme, ceramide synthase [7]. This enzyme catalyses the formation of dihydroceramide from the sphingoid base, sphinganine (Sa). This inhibition has been observed in a number of cell cultures, including cultured renal cells [8], cultured cerebellar neurons [9] and rat primary hepatocytes [10]. A similar disruption has been observed in various animal species consuming fumonisin-contaminated feed. Elevations in Sa and particularly in the ratio sphinganine/sphingosine (Sa/So) were noted in the sera and urine of vervet monkeys [11], ponies [12], chickens [13], rabbits [14] and rats [15]. Because the changes in free sphingoid bases occur before other biochemical markers of fumonisin-induced cellular injury, such as elevation of liver enzymes, it has been proposed that the Sa/So ratio could be used as a biomarker of consumption of fumonisin-contaminated feed [16].

The determination of the ratio, Sa/So, has generally been based on the original method published for the determination of free sphingosine in liver tissue

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by HPLC which involved lipid extraction with chloroform/methanol, alkaline hydrolysis to cleave acylglycerolipids and to hydrolyse lysosphingolipids and determination of the sphingoid bases by HPLC of their preformed *o*-phthaldialdehyde (OPA) derivatives using fluorescence detection [17]. This method for tissues has been generally applied to both So and Sa in serum, urine and cell cultures, but has the disadvantage of being both time-consuming and labour intensive [16]. More recently, these issues have been addressed in the development of shorter methods for the determination of the Sa/So ratio in urine using either ethyl acetate extraction [18] or a silica minicolumn for clean-up of the chloroform extract [19].

Recent work on the changes induced in the ratio by the exposure of vervet monkeys to fumonisins has indicated that in primates, measurement of the ratio in serum may be a more sensitive biomarker than that of urine [11]. The new method described here provides improved chromatography with less chromatographic interferences and better recovery than the previous methods for serum [16,17] and extends a recently described rapid method for urinary levels to the analysis of the Sa/So ratio in serum.

2. Experimental

2.1. Samples and reagents

Serum samples were obtained during a recent study of the effects of long term fumonisin exposure in vervet monkeys [11]. Sa and So standards were purchased from Sigma (St Louis, MO, USA). All reagents and solvents were analytical grade from Merck (Darmstadt, Germany).

2.2. Determination of Sa/So in serum

Serum (500 μl) was deproteinised with methanol (2 ml) and the protein precipitate was centrifuged down at 1200 *g* for 10 min at 10°C. An aliquot of the supernatant (2 ml) was mixed with water (1.9 ml) and 0.35 *M* ammonium hydroxide (1.2 ml) and extracted with chloroform (4 ml). After thorough mixing on a vortex mixer, the layers were separated by centrifugation at 1200 *g* for 10 min at 10°C and a portion of the lower (chloroform) layer (3 ml) was transferred to a silica minicolumn, prepared in a polypropylene column (15 mm I.D.) with a lower layer of 0.5 g of silica 60 (40–63 μm particle size) and an upper layer of anhydrous sodium sulphate (5 g).

The minicolumn was conditioned prior to use with chloroform (4 ml). After loading the sample, the minicolumn was washed with chloroform (1 ml) and the sphingoid bases were eluted with chloroform–methanol–concentrated ammonium hydroxide (20:20:0.8, v/v; 5 ml). The eluate was dried under nitrogen at approximately 38°C. The dried residue was redissolved by ultrasonication in 0.125 *M* potassium hydroxide (1 ml) in methanol–chloroform (4:1, v/v) and incubated at 37°C for 1.5 h. After cooling on ice, chloroform (1 ml) was added and the solution was washed with alkaline water (dilute ammonium hydroxide, approximate pH 8; 5 ml). The phases were separated by centrifugation at 1200 *g* for 10 min at 10°C and the lower (chloroform) layer was dried under nitrogen at approximately 38°C.

2.3. Chromatographic analysis

The dried sample extracts were derivatised with OPA prior to HPLC analysis. The derivatising reagent was prepared by dissolving 15 mg OPA in 0.5 ml ethanol, adding 15 μl 2-mercaptoethanol and diluting with 14.5 ml of 3% boric acid with pH adjusted to 10.5 with potassium hydroxide. The sample extract was redissolved in 250 μl methanol by ultrasonication (1 min) and incubation at room temperature (15 min). The solution was derivatised by addition of 50 μl OPA reagent, ultrasonicated (1 min) and incubated at room temperature (15 min). Aliquots (25–100 μl) of the derivatised sample were chromatographed on a Waters Assoc. (Milford, MA, USA) HPLC consisting of a model 6000 pump, U6K injector and model 470 fluorescence detector set at wavelengths of 335 nm excitation and 440 nm emission. The column was a reversed-phase Supelcosil (Supelco, Bellefonte, PA, USA) ABZ+Plus (150×4.6 mm I.D.) packed with C_{18} material of 5 μm particle size and eluted at 1.5 ml/min with methanol–water (90:10, v/v). Quantification was achieved by comparison of peak areas using an Apex AutoChrom (Milford) chromatographic data system.

3. Results and discussion

Initial efforts to determine the ratio of the sphingoid bases, Sa and So, in serum by direct application of the newly developed silica minicolumn method for the determination of the ratio in urine [19] was unsuccessful, as the use of serum without prior deproteinisation gave large chromatographic interferences. Hence, serum samples were deproteinised with methanol and, after centrifugation, the methanolic supernatant was used for the lipid extraction step with chloroform under alkaline conditions. Although the use of the supernatant eliminated major interferences, considerable interferences still remained and it was found necessary to perform an alkaline hydrolysis step after the clean-up on the silica mini-column. The alkaline hydrolysis will cleave acylglycerolipids and release any sphingosine from lysosphingolipids. This procedure yielded an extract suitable for chromatographic determination of the ratio of the sphingoid bases. The HPLC separation and quantification was based on precolumn derivatisation of the extract with OPA coupled with fluorometric detection of the resultant derivatives. Although OPA derivatives are generally labile and require injection at standardised time intervals immediately after preparation, the derivatives of the sphingoid bases prepared by this method are relatively stable and can be stored for over 24 h at 4°C. Fig. 1 shows typical chromatograms of serum sample extracts from both a normal vervet monkey (not consuming contaminated feed) and one exposed to fumonisin-contaminated feed.

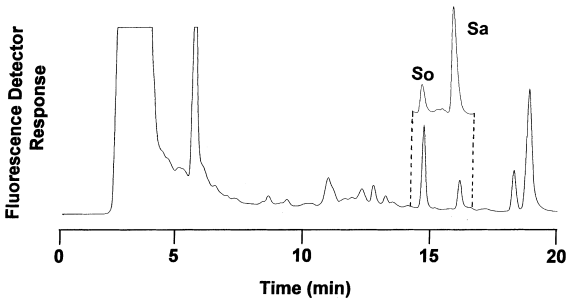

Fig. 1. Chromatogram of an extract of serum from a normal vervet monkey, showing the elution of the sphingoid bases, So and Sa. The inset shows the effect on the sphingoid bases of the consumption of contaminated feed.

The new method was reproducible. Determination of the ratio in a serum sample from a normal vervet monkey gave a ratio value of 0.67 with a relative standard deviation (R.S.D.) of 8.9% ($n=6$). The ingestion of fumonisin-contaminated feed by vervet monkeys results in the disruption of sphingolipid metabolism and an increase in the Sa/So ratio in serum due to an elevation in Sa levels. The determination of higher levels is accompanied by an increase in analytical precision. For two vervet monkeys on a fumonisin-contaminated diet, Sa/So ratios were measured by this method as 1.83 (R.S.D. 3.2%; $n=6$) and 3.67 (R.S.D. 2.9%; $n=6$).

Recoveries of Sa and So from serum were tested by spiking serum with the bases at the 100 n*M* level. Average recoveries over 5 samples were found to be 55% and 53% respectively, indicating equivalent recovery of both bases. These recoveries are better than those achieved with previous methods for serum [16]. Determination of absolute values of the bases in this type of analysis is best achieved by using an internal standard to account for analytical losses and previous workers have used C:20 Sa for this purpose [16]. However, this compound is not commercially available.

Nevertheless, detection of fumonisin-induced changes in sphingolipid metabolism can be readily detected by measurement of the Sa/So ratio, such that the absolute values are not required. Indeed, it has been noted that the ratio appears to be a better biomarker for consumption of fumonisin-contaminated feed than the absolute levels of the individual sphingoid bases themselves [16]. An advantage of this method over the other new methods [18,19] recently described for determination of the ratio in urine is that plasma levels of Sa can be readily detected, whereas the urinary methods were unable to detect Sa in normal male volunteers. Thus normal values for the ratio in urine could not be established. This method has been applied to serum analysis in various animal species and is capable of determining Sa/So ratios as low as 0.02.

Although this method was developed to have general applicability for the determination of the ratio in serum, should a suitable internal standard (such as C:20 Sa) be available, the method would allow the determination of the absolute levels of the sphingoid bases in serum. Although not relevant to

the development of a biomarker for fumonisin exposure, the method described here can also be applied to the determination of the ratio in tissue (liver and kidney) collected from experimental animals. Studies on such samples have indicated that the reproducibility of the method applied to homogenised tissue extracts, as opposed to serum, is similar to that achieved with serum (R.S.D.$<$10%).

The development of reproducible methods for the determination of the ratio of the sphingoid bases in serum will enable investigations to be conducted into the suitability of this ratio as a biomarker for fumonisin exposure. Its applicability in animal studies, where animals are exposed to heavily contaminated feed, has already been demonstrated. However, further studies are required to address its applicability in human populations that are chronically exposed to fumonisin-contaminated staple food supplies.

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