Supplemental Data

A Blood Spot Method for Detecting Fumonisin-Induced Changes in Putative Sphingolipid Biomarkers in LM/Bc Mice and Humans

Ronald T. Riley ^{a*}, Jency L. Showker ^a, Christine M. Lee ^a, Cody E. Zipperer ^a, Trevor R. Mitchell ^a, Kenneth A. Voss ^a, Nicholas C. Zitomer ^a, Olga Torres ^{b,c}, Jorge Matute ^b, Simon G. Gregory ^d, Allison E. Ashley-Koch ^d, Joyce R. Maddox ^e, Nicole Gardner^e and Janee B. Gelineau-van Waes ^e

^a *Toxicology and Mycotoxin Research Unit, R.B. Russell Research Center, USDA - ARS, Athens, GA, USA;*

^b *Laboratorio Diagnostico Molecular S.A., Guatemala City, Guatemala;*

^c *Centro de Investigaciones en Nutrición y Salud, Guatemala City, Guatemala;*

^d *Department of Medicine, Duke University Medical Center, Durham, NC, USA,*

^e *Department of Pharmacology, Creighton University, School of Medicine, Omaha, NE, USA;*

*Corresponding author:

Tel: +706 546-3377

Fax: +706 546-3116

E-mail: ron.riley@ars.usda.gov

Collection of blood spots on absorbent paper from finger sticks has many advantages over collection of whole blood via venipuncture. In addition to being less invasive, the amount of equipment, materials and personnel required on site is much reduced. Once the spots are dry they can be stored, transported, and shipped at room temperature.

The FTA™ Elute Micro Cards were specifically chosen for use in our biomarker study using human blood spots because they offer several advantages over the Whatman 903® Specimen Collection Paper which we have used previously (Gelineau van Waes et al., 2012) and continue to use in our mouse studies. One specific advantage is that the FTA™ Elute Micro Cards are impregnated with chemicals that denature proteins and lyse cells on contact. In addition, according to the manufacturer, hemoglobin and proteins are more tightly bound to the FTA™ Elute Micro Card matrix resulting in a cleaner extract preparation which can be easily seen in the greater retention of red pigment in the extracted FTA™ paper and the reduced red pigmentation in the sample vials and more importantly reduced sample residues when cleaning the spray cone of the mass spectrometer after running sequences with many samples (>20).

In order to simplify the spotting of various volumes of mouse blood, the blood was collected in tubes. In order to ensure that the addition of anticoagulants did not interfere with the analysis, blood collected using tubes containing K2 EDTA or spotted directly were compared. The results showed that the ratio of Sa 1-P/So 1-P in the male LM/Bc mouse blood spots (n=8) with or without K2 EDTA treatment collected on FTA Elute Micro Cards did not differ significantly (t-test: $t = -1.046$; d.f.=4; $p=0.355$); nor did they differ in A_{270} (t-test: $t=0.759$; d.f.=4; $p=0.490$) or protein content (t-test: $t = -1.40$; d.f.=4; $p=0.234$). The ratio, A_{270} , and protein content in mouse also did not differ between CO₂ and isoflurane methods of euthanasia (Mann-Whitney; $p=0.418$, $p=0.777$, $p=0.940$ respectively). The ratio of Sa 1-P/So 1-P (t-test, $t=0.9$;

d.f.=12; $p=0.386$) in mouse did not differ when comparing the two types of collection cards used (Whatman 903® Specimen Collection Paper vs. FTA™ Elute Micro Cards); however, the card types did differ in A_{270} (t-test: $t=2.941$; d.f.=13; $p=0.011$) and protein content (Mann-Whitney; $p<0.001$).

Table 1S. Extraction efficiency (mean $\pm$ SD) from FTATM Elute Micro Cards spiked with a standard mixture of sphingoid bases and sphingoid base 1-phosphates.^a

	C₁₇ So	So 1-P	Sa 1-P	So	Sa	deoxySa	C₂₀ So
Mean	62%	61%	74%	73%	72%	73%	57%
SD	$\pm 7\%$	$\pm 5\%$	$\pm 4\%$	$\pm 10\%$	$\pm 6\%$	$\pm 7\%$	$\pm 4\%$

^aTwo cards (4 spots/card) were spotted with eight drops of human blood and then allowed to dry. A total of 300 pmols of a standard mixture of C₁₇ sphingosine 1-phosphate (C₁₇ So 1-P), sphingosine 1-phosphate (So 1-P), sphinganine 1-phosphate (Sa 1-P), sphingosine (So), sphinganine (Sa), deoxysphinganine (deoxySa), and C₂₀ sphingosine (C₂₀ So) were added (10 μ l) to four spots and vehicle only to the remaining four spots and all were left overnight in a desiccator to dry. An 8 mm biopsy punch was used to remove discs of blood spots which were placed in individual 2 ml polypropylene tubes (total of eight tubes). The same standard mix (10 μ l) was also added to three 2 ml polypropylene tubes (without blood spots). All samples were extracted as described in the Methods. Recoveries were calculated using the areas of the peaks for each analyte in the standard mixture added to the polypropylene tubes without blood spots as 100% recovery. The extracts of blood spots spiked with the standard mixture were corrected for the area of each analyte in the blood spot extracts to which the standard mixture had not been added.

Table 2S. Teratology Data in Pregnant LM/Bc Mice Dosed Orally with FB₁.^a

DOSE (oral gavage)	Implants	Resorptions	Delayed	Exencephaly	Normal (E9.5)	# NTD positive litters	% NTD positive litters
Control	13	1	0	0	12	0/4	0
	11	0	0	0	11		
	9	0	1	0	8		
	12	0	0	0	12		
5 mg/kg	10	1	5	0	4	0/2	0
	14	1	6	0	7		
10 mg/kg	13	1	0	0	12	1/2	50%
	13	1	6	1	5		
15 mg/kg	8	2	5	1	0	2/3	67%
	13	0	0	0	13		
	10	0	0	1	9		
25 mg/kg	11	3	1	3	4	3/4	75%
	10	2	2	1	5		
	14	0	0	0	14		
	13	2	0	1	10		
50 mg/kg	13	1	1	3	8	2/2	100%
	13	2	2	2	7		

^a Sphingolipid analysis was only conducted on tissues from two dams/group.

Table 3S. The calculated concentration (mean pmol/ml $\pm$ SD), range (minimum and maximum), and “n” of sphingosine 1-phosphate (So 1-P), sphinganine 1-phosphate, the Ratio (Sa 1-P/So 1-P), urinary fumonisin B₁ (UFB₁), and the predicted volume of blood (ml) collected from men in two low fumonisin exposure communities (Chimaltenango and Escuintla) and spotted on FTA™ Elute Micro Cards. The predictive model was from the regression analysis of the absorbance at 270 nm (A_{270}) and the actual volume of blood spotted (ml) shown in the insets of Figure 3B^a.

	Sa 1-P pmol/ml	So 1-P pmol/ml	Ratio	UFB ₁ ^b	Predicted ml spotted
Chimaltenango	452 $\pm$ 161 180-824 n=53	1414 $\pm$ 419 747-2387 n=53	0.32 $\pm$ 0.04* 0.23-0.42 n=53	0.23 $\pm$ 0.34 0.0-1.53 n=53	0.014 $\pm$ 0.003* 0.007-0.023 n=53
Escuintla	528 $\pm$ 222 152-1187 n=47	1822 $\pm$ 676* 591-3337 n=47	0.29 $\pm$ 0.04 0.22-0.40 n=47	0.57 $\pm$ 0.74* 0.0-4.08 n=47	0.010 $\pm$ 0.002 0.007-0.017 n=47

^a The predicted relationship between the spotted blood volume and the absorbance at 270 nm for human blood spots from Figures 3B (inset) where blood volume human (ml) = 9.406(A_{270})-6.805e-4 (df=19, r^2 =0.96). The concentration of sphingoid base 1-phosphates were calculated based on the areas of the So 1-P or Sa 1-P under the peak divided by the area of the internal standard (C_{17} So 1-P) from the LCMS analysis of the extract multiplied by 60 pmol and divided by the predicted volume based on the A_{270} . An * indicates a significant difference between departments based on a t-test or Rank Sum Test.

^b Urinary FB1 data from Torres et al. (2014a).

Figure 1S. Change in concentration over time of sphingosine 1-phosphate (solid circles, So 1-P), sphinganine 1-phosphate (open circles, Sa 1-P) and the ratio of Sa 1-P/So 1-P (inverted triangles). Three FTA™ Elute Micro Cards (4 spots/card) were spotted with 12 drops of blood and then allowed to dry at which time they were placed in air tight plastic bags and stored at -20°C and desiccated. All samples were extracted as described in the Methods and the results were normalized to the area of the C₁₇ So 1-P added at extraction and estimated volume spotted based on the absorbance at 270 nm. Inset is the results of the Pearson product correlation analysis. Only eleven of the blood spots were used for the study.

Figure 2S: The correlation between the volume of mouse blood spotted (n=8) on Whatman 903® Specimen Collection Paper and the pmoles of So 1-P (A) and Sa 1-P (B) detected in the blood spot extracts. Inset in (A) is the relationship between the total Bradford protein content of the extracts (Absorbance at 595 nm) and the ultraviolet absorbance at 270 nm. Inset in (B) is the relationship between the volume of blood (ml) spotted and the A_{270} (ml spotted = $0.0140(A_{270}) + 0.00103$). The coefficient of determination (r^2) is shown in each inset figure.

Figure 3S. Examples of chromatograms of extracts of mouse blood spots from a control dam (A) and a dam dosed orally with 10 mg/kg bw/day (B) as described in Figure 4 in the main text. The blood was collected on Whatman 903® Specimen Collection Paper.

Figure 4S. Dose-response increase in Sa concentration (A, B) and deoxySa concentration (C, D) in liver (A, C) and kidney (B, D) of pregnant LM/Bc mice orally dosed with FB₁ at 0, 5, 10, 15, 25 and 50 mg/kg bw/day (GD 6.5 to 8.5). Inset in “D” is the increase in Sa 1-P concentration in kidney. The correlation coefficient (r) and p-value (p) for the Pearson product moment correlation analysis is inset in each panel.

Figure 5S. Dose-dependent upward trend in the Sa/So ratio in the heads of E9.5 LM/Bc embryos exposed *in utero* to FB₁ (n = number of embryo heads analyzed from each dose group). Pregnant LM/Bc dams were dosed with FB₁ (0, 5, 10, 15, 25, and 50 mg/kg bw/day) on GD 6.5 – 8.5 by oral gavage. The Sa/So ratio in embryo heads exposed to the 50 mg/kg dose was statistically higher (p < 0.001) based on ANOVA using an all pairwise multiple comparison (Holms-Sidak method). The result of the Pearson product moment correlation is inset.