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High-performance thin-layer chromatographic analysis of the neutral lipid content of urine and feces in mice experimentally infected with *Schistosoma mansoni*

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ABSTRACT

The purpose of this study was to identify and quantify neutral lipids in the urine and feces of mice experimentally infected with *Schistosoma mansoni*. Four infected and three control mice were used in this study. The samples were collected weekly for eight weeks. Samples from the infected mice were compared to those from the uninfected ones. The analyzed lipids in both feces and urine samples were free sterols, free fatty acids, triacylglycerols, and methyl esters. Steryl esters were also analyzed in fecal samples. The neutral lipids were separated on channeled preadsorbant Analtech HPTLC-HLF 20 x 10-cm silica gel plates with petroleum ether-diethyl ether-glacial acetic acid (80:20:1) mobile phase and detected with 5% ethanolic phosphomolybdic acid spray reagent. Quantification was carried out by use of a CAMAG Scanner 3 in the visible mode. Significant differences between infected and uninfected samples were found in methyl esters of feces after four weeks, and steryl esters of feces after five weeks. Also, triacylglycerols in urine samples were statistically different at eight weeks in infected versus uninfected samples. The results of our study suggest that certain neutral lipids in the urine and feces of mice may serve as indicators of infection with *S. mansoni*.

KEYWORDS: *Schistosoma mansoni*, thin-layer chromatography, mice, neutral lipids

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INTRODUCTION

The medically important trematode, *Schistosoma mansoni*, is one of the major agents of the disease schistosomiasis in humans and animals. This parasitic disease affects the liver, spleen, intestine, and mesenteric and portal blood vessels of the definitive host, and it is a serious and often fatal disease in humans. It affects approximately 200 million people in 74 countries [1].

Relatively few reported studies used high-performance thin-layer chromatography (HPTLC) to metabolically profile the feces and urine of mice infected with *S. mansoni*. Most of the HPTLC studies on metabolic profiling of these waste products in hosts infected with parasites have been done on the foodborne intestinal trematode *Echinostoma caproni*. As these studies on *E.caproni* have relevance to our work on *S. mansoni*, they are reviewed briefly in the next two paragraphs.

Massa *et al.* [2] examined the phospholipid profiles in the urine of *E. caproni* infected mice, uninfected mice and humans. They concluded that urinary phospholipids do not serve as biological markers for trematode infection in mice [2]. Moreover, they found differences between lipid profiles of mouse and human urine, which suggests that mice may not be useful models for humans in studying the effects of infectious diseases on polar lipids [2]. Vasta *et al.* [3] used HPTLC to study the composition of neutral lipids in the urine of humans and BALB/c mice. They discovered higher concentrations of neutral lipids in mouse urine than

in the human urine, which is probably due to reduced bladder capacity and higher rate of metabolism in mice. More importantly, they found that the major neutral lipids of humans and mice are the same, and that mice can probably be used as an effective model for comparative studies on humans [3].

Murray *et al.* [4] compared the phospholipids and sphingolipids in the feces of BALB/c mice infected with *E. caproni* and uninfected control mice. They found no significant differences during the seven weeks of that study. Furthermore, their study suggests that fecal polar lipids are not useful biological markers to distinguish *E. caproni* infection from uninfected controls [4]. Bandstra *et al.* [5] examined neutral lipids in the feces of BALB/c mice infected with *E. caproni*. They found an increase in triacylglycerols at three weeks postinfection, and a significant increase in free sterols at two weeks post infection relative to uninfected mice. Therefore, they concluded that the neutral lipid profile of feces could serve as an indicator of intestinal trematode infection in animals and humans [5].

Finally, we studied the effects of *S. mansoni* on the neutral and polar lipids in the liver, spleen, and small intestine of mice [6]. We found no significant differences between neutral lipids of infected and uninfected mice organs. However, we found a significantly higher phosphatidylcholine content in the liver and small intestine of the uninfected mice compared with infected mice [6]. The purpose of the current study was to use HPTLC to compare the neutral lipid profiles in urine and feces of uninfected mice and mice infected with *S. mansoni*.

MATERIALS AND METHODS

Mice maintenance and infection

Seven outbred Swiss Webster female mice, 6-8 weeks old and weighing 20-25 g, were obtained courtesy of Dr. Fred A. Lewis, Head, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, MD USA). Four mice were infected with *S.mansoni* cercariae by Dr. Fred A. Lewis as described earlier [7]. Infected and uninfected mice were kept separately, three or four of each type in plastic mouse containers (15 x 27 x 14 cm); they were fed Mazuri rodent food (PMI Nutrition, Henderson, CO, USA) and water *ad libitum*.

Sample collection and preparation

Fecal and urine samples were collected once a week from week one to eight postinfection, from both infected and uninfected mice. The method used for collecting mouse urine was described by Massa *et al.* [2] For each sample collection, urine was taken from every mouse and combined to make two pools (one from infected and one from uninfected mice), each containing 300-900 μL of urine. Each pool was divided into three replicate samples. Samples were collected early to mid-afternoon and, if necessary, refrigerated at 2°C for up to 24 hours.

After collection, 70-600 μL of mouse urine was used for lipid extraction with 2:1 chloroform-methanol in the ratio of 20 parts solvent to 1 part urine, followed by gravity filtration of the solution through a Whatman (Florham Park, NJ, USA) No. 4 filter paper held in a glass funnel in order to remove any insoluble protein. Folch wash [8] (0.88%, *w/v*, KCl in deionized water) was added to the vial with a ratio four parts sample to one part solution (*v/v*), and vortex mixed for 30 seconds. The aqueous layer was removed with a Pasteur pipet and discarded, while the lipid layer was evaporated to dryness under nitrogen gas in a warm water bath (40-60°C). The samples were reconstituted in a volume of 2:1 chloroform-methanol that would enable bracketing scan areas of samples within the scan areas of standard zones in calibration curves, which was typically one-fifth to one-tenth of the original volume of mouse urine.

For collecting fecal samples, the method described by Bandstra *et al.* [5] was used. Sample collections were made every week from each infected and uninfected mouse. Mice were separated and placed in plastic circular containers with the filter paper at the bottom. Approximately 120 mg of feces per sample was collected after 30 minutes of mice isolation.

Samples were then homogenized in a 7 mL Wheaton (Millville, NJ, USA) glass homogenizer and extracted in the same way as urine with 2:1 chloroform-methanol in the ratio of 20 parts of solvent to 1 part feces. For complete lipid extraction, the feces were first homogenized in approximately

4 mL of 2:1 chloroform-methanol and filtered through cotton into a glass vial. Then, the extract was again filtered using the same procedure, but with only 2 mL of 2:1 chloroform-methanol. Folch wash was then added to the vial following the same procedure as for urine samples. The reconstitution volume was determined in the same way, and it was typically 200-400 μL.

HPTLC analysis

HPTLC analysis was performed on 20 x 10 cm Analtech, Inc. (Newark, DE, USA) channeled plates with preadsorbent application zone and 19 scored channels (Catalog No. 61927). Plates were prewashed by development to the top with 1:1 dichloromethane-methanol and dried with the stream of air on a CAMAG (Wilmington, NC, USA) plate heater at 120°C for approximately 30 minutes in a fume hood.

The neutral lipid standard used (Nonpolar Lipid Mix B, Matreya, Pleasant Gap, PA, USA) was the same as that described by Bolstridge *et al.* [9]. It contained 20% each of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate and served for identification and quantification of free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl ester fractions in samples, respectively. Standards and reconstituted samples were applied with a gentle air stream flowing across the preadsorbent area using a 10-mL Drummond (Broomall, PA, USA) microdispenser in amounts of 2.00, 4.00, 8.00, and 16.0 μL; these four standard zones contained 0.400, 0.800, 1.60, and 3.20 μg of each compound, respectively.

The plates were developed with Mangold mobile phase, i.e., petroleum ether (36-68°C)-diethyl ether-glacial acetic acid (80:20:1), in a vapor saturated CAMAG HPTLC twin through chamber with an Analtech saturation pad placed in the rear compartment. Plates were developed 9 cm past the preadsorbent zone-silica gel layer interface, which took about 10-15 minutes.

Plates were then dried under a stream of cool air from a hair drier for approximately 10 minutes and then sprayed with 5% ethanolic phospomolybdic acid solution and heated on a CAMAG plate heater at 120°C for approximately 5 minutes. Neutral lipids appeared as blue zones on a yellow background.

Quantitative densitometric analysis was performed using a CAMAG TLC Scanner 3 with the tungsten light source set at 610 nm and the following settings: slit width 0.45 mm Micro, slit length 4 mm, data resolution 100 μm $step^{-1}$, and scanning rate 4 mm s^{-1}. Linear calibration curves for the individual lipids were created using the winCATS software, which plotted the standard zone weights versus the corresponding peak areas. The percentages of lipids were calculated using the equation:

$$\%\,\text{Lipid} = \frac{(w)(R)(100)}{\mu\text{g tissue}}$$

where w = μg sample interpolated from the calibration curve and R = [(reconstituted volume (μL)/(spotted volume (μL)]. In some cases, in order to obtain sample zone scan areas within the calibration curve, a dilution or concentration of the sample solution was performed, and a correction factor was included in the equation. If the scan area of more than one aliquot of a single sample was within the calibration curve, the value closest to the mean of the two middle standard zone scan areas was used for the calculations.

Multiple Microsoft Excel's *Student's t*-tests were performed to determine the statistical significance of the obtained data. The mean ± standard deviation for every neutral lipid fraction for each week was determined. The data obtained from infected and uninfected mice were then compared every week from week 1 to week 8. Results were considered statistically significant when the *P* value was less than 0.05.

RESULTS

Necropsy of three control mice and four mice exposed to *S. mansoni* cercariae was performed 8 weeks postinfection. Exact worm counts were not made, but every host contained male and female worm pairs, as well as uncoupled male and female worms. All four exposed mice showed hepatomegaly and splenomegaly, characteristic of patent *S. mansoni* infection. The control mice did not contain any worms and showed no sign of the infection.

The peaks in sample densitograms were identified based on the correspondence between their R_f values and those of peaks in standard chromatograms.

Table 1. Percent by mass (mean ± standard error) of neutral lipids in the urine samples of three uninfected and four *S.mansoni* infected mice.

Week	Free Sterols		Free Fatty Acids		Triacylglycerols		Methyl Esters	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
1	1.1± 0.1	1.1 ± 0.4	4 ± 2	4 ± 1	2.0 ± 0.2	2.7 ± 0.4	1.2 ± 0.2	0.8 ± 0.4
2	0.9 ± 0.1	1.2 ± 0.4	3.0 ± 0.5	3 ± 1	1.5 ± 0.1	1.5 ± 0.1	ND	ND
3	1.0 ± 0.2	1.2 ± 0.4	3 ± 1	2.4 ± 0.9	4 ± 1	4.1 ± 0.1	0.6 ± 0.2	0.8 ± 0.4
4	1.0 ± 0.2	0.8 ± 0.2	5 ± 1	6 ± 1	2.1 ± 0.4	2.5 ± 0.3	0.6 ± 0.3	0.8 ± 0.4
5	1.4 ± 0.3	1.3 ± 0.4	3 ± 1	2.1 ± 0.7	2.0 ± 0.4	1.8 ± 0.1	0.48 ± 0.08	0.5 ± 0.2
6	0.5 ± 0.1	0.7 ± 0.2	4.5 ± 0.8	7 ± 2	1.4 ± 0.4	0.8 ± 0.1	1.1 ± 0.2	1.1 ± 0.4
7	1.0 ± 0.4	1.2 ± 0.1	1.7 ± 0.6	1.1 ± 0.8	4.6 ± 0.8	4.4 ± 0.5	1.0 ± 0.3	1.1 ± 0.2
8	0.8 ± 0.1	1.0 ± 0.3	0.8± 0.2	0.9 ± 0.9	4.0 ± 0.4*	2.9 ± 0.4*	ND	ND

Table 2. Percent by mass (mean ± standard error) of neutral lipids in the feces samples of three uninfected and four *S.mansoni* infected mice.

Week	Free Sterols		Free Fatty Acids		Triacylglycerols		Methyl Esters		Steryl Esters	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
1	0.03 ± 0.01	0.03 ± 0.02	0.20 ± 0.08	0.10 ± 0.07	0.04 ± 0.03	0.02 ± 0.01	0.006	ND	0.01 ± 0.01	0.04
2	0.10 ± 0.05	0.09 ± 0.03	0.20 ± 0.06	0.30 ± 0.08	0.10 ± 0.07	0.10 ± 0.05	0.006	0.001	0.02 ± 0.01	0.06 ± 0.05
3	0.08 ± 0.03	0.06 ± 0.04	0.10 ± 0.08	0.20 ± 0.04	0.20 ± 0.08	0.2 ± 0.1	0.05 ± 0.04	ND	0.05 ± 0.01	0.04 ± 0.01
4	0.06 ± 0.01	0.10 ± 0.02	0.20 ± 0.04	0.30 ± 0.03	0.20 ± 0.04	0.2 ± 0.1	0.002 ± 0.002*	0.10 ± 0.07*	0.03 ± 0.02	0.020 ± 0.005
5	0.06 ± 0.04	0.02	0.20 ± 0.08	0.30 ± 0.5	0.30 ± 0.09	0.30 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.02 ± 0.01*	0.050 ± 0.003*
6	0.10 ± 0.03	0.2 ± 0.1	0.40 ± 0.1	0.5 ± 0.3	0.20 ± 0.03	0.10 ± 0.09	0.05 ± 0.02	0.03 ± 0.02	0.06 ± 0.01	0.06 ± 0.04
7	0.02 ± 0.01	0.07 ± 0.04	0.10 ± 0.04	0.3 ± 0.2	0.10 ± 0.05	0.07 ± 0.08	0.03 ± 0.01	0.030 ± 0.004	0.05 ± 0.03	0.2
8	0.04 ± 0.03	0.10 ± 0.07	0.3 ± 0.1	0.40 ± 0.09	0.10 ± 0.02	0.10 ± 0.06	0.01 ± 0.05	0.10 ± 0.03	0.020 ± 0.007	0.08 ± 0.09

Correlation coefficients of calibration curves were always greater than 0.98 and usually 0.99 for each lipid quantified. The HPTLC analysis of urine samples showed that the concentrations of free sterols, free fatty acids, triacylglycerols, and methyl esters were large enough to be quantified. Steryl esters were not present in quantifiable amounts in the urine samples. The results for each week are shown in Table 1. The only statistically significant difference was found in triacylglycerols in week 8 samples (labeled with an asterisk in Table 1). Data on methyl esters for both infected and uninfected mice could not be quantified for weeks 2 and 8 (labeled as ND).

The HPTLC analysis of feces samples showed that free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl esters were present in high enough concentration to be quantified. The results for each week are shown in Table 2. Statistically significant differences were found in methyl esters for samples in week 4 and steryl ester samples for week 5 (these results are labeled in Table 2 with an asterisk). Data on methyl esters for infected mice could not be quantified for weeks 1 and 3 (ND). There was only one sample to quantify for the following analytes: free sterols of infected mice for week 5, methyl esters of uninfected mice for week 1, methyl esters of both infected and uninfected mice for week 2, and steryl esters of infected mice for weeks 1 and 7.

DISCUSSION

Since neutral lipids represent a source of energy stores, they can give useful information on the effects of *S. mansoni* infection on its host. Therefore, it is important to study the neutral lipid content of the host's organs, and also that of the feces and urine. O'Sullivan *et al.* [6] studied the effects of *S. mansoni* infection on neutral and polar lipids in the liver, spleen, and small intestine of the mice host. However, they did not find any significant difference between infected versus uninfected hosts, which implies that *S. mansoni* does not affect the neutral lipid content of mouse organs. The differences that we found in this study imply that *S. mansoni* adversely affects neutral lipids of mouse urine and feces.

Bandstra *et al.* [5] studied the neutral lipids of feces of mice infected with *E. caproni*. They found statistical differences in the free sterols after two weeks and in the triacylglycerols after three weeks, which suggested that neutral lipid profiles of feces might serve as an indicator of schistosomiasis infection. Based on our results, we also suggest that neutral lipid composition of feces and urine may serve as an indicator of *S. mansoni* infection in mice.

Furthermore, it is important to mention that Vasta *et al.* [3] found that, because of similar neutral lipid urinary profiles between humans and mice, mice can probably be used as a model for studies on metabolic profiling of humans infected with schistosomes.

The detected neutral lipids in urine samples of infected and uninfected mice were free sterols, free fatty acids, triacylglycerols, and methyl esters. Additionally, steryl esters were found in the fecal samples. Statistically significant differences were found in the triacylglycerols in urine samples for week 8, in the methyl esters of feces samples for week 4, and in steryl esters of fecal samples for week 5. Therefore, we conclude that certain neutral lipids of feces and urine of mice may serve as indicators of *S. mansoni* infection at 1 to 8 weeks postinfection. Further metabolic profiling studies should be done to determine if these findings can be applied to humans.

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