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High-performance thin-layer chromatographic analysis of phospholipids and neutral lipids in different organ systems of *Biomphalaria glabrata* snails infected with *Schistosoma mansoni*

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ABSTRACT

Silica gel high-performance thin-layer chromatography-densitometry was used to examine neutral lipid (NL) and phospholipid content in different organ systems of *Biomphalaria glabrata* snails infected with the parasite *Schistosoma mansoni*. NLs were determined using petroleum ether-diethyl ether-glacial acetic acid (80:20:1) mobile phase, phosphomolybdic acid detection reagent, and scanning at 610 nm, and phospholipids with chloroform-methanol-water (65:25:4) mobile phase, cupric sulfate-phosphoric acid detection reagent, and scanning at 370 nm. Considerable differences were found in both the NL and phospholipid fractions in the organ systems examined (visceral mass, digestive-gonad gland (DGG), and head-foot region) as a function of the *S. mansoni* infection. Reasons for these findings probably relate to the metabolic needs of the infected snails.

KEYWORDS: thin layer chromatography, TLC, phospholipids, neutral lipids, digestive gland-gonad complex, *Biomphalaria glabrata*, *Schistosoma mansoni*, trematoda

INTRODUCTION

Biomphalaria glabrata serves as the vector for the medically important digenean *Schistosoma mansoni*. In a previous study [1], it was shown that infection of *B. glabrata* with *S. mansoni* caused several changes in the phospholipid profile of the *B. glabrata* digestive gland-gonad (DGG) complex. These changes varied as the infection progressed from 2 to 8 weeks post-infection. In the present study, we expanded the scope of the investigation to neutral lipids as well as phospholipids and considered different organ systems of the snails (DGG, visceral mass, and head-foot region) as illustrated in a photograph in the review by Fried and Sherma (1990) [2]. Since our previous study showed that most differences occurred between 2 and 4 weeks post-infection, in the present study we collected samples at 3 weeks post-infection. The only previous work on this topic examined the differences in neutral lipid profiles that were induced by different diets using electron and light microscopy and visual semiquantitative thin layer chromatography [3]. In that study, kidney, intestine, and heart were analyzed separately. In this study, we grouped these organs and referred to them as the visceral mass, and modern instrumental high-performance TLC (HPTLC) was used for lipid quantification.

MATERIALS AND METHODS

Maintenance of snails and preparation of test samples

Both uninfected and infected snails were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD, USA). Cultures of control and infected *B. glabrata* were maintained at 23 +/- 1 °C in aerated glass jars containing 10 to 20 snails/800 mL of artificial

spring water (ASW) under diffuse overhead fluorescent light for 12 h/day. The ASW was prepared as described by Ulmer [4]. Snails were fed the leafy portion of boiled Romaine lettuce, and food and water were changed three times a week.

To prepare samples for HPTLC analysis at 3-week post infection, the shell of each snail was crushed gently with a hammer, and the snail body was removed with forceps. The DGG, visceral mass, and head-foot region were then dissected from the snail body, the gut tissue was removed, and the tissues were blotted on a paper towel and weighed on an analytical balance. Due to the small size of each snail, tissue from the same region of 3 snails were pooled together to make up one sample. Each sample was homogenized using a 15 mL capacity Wheaton glass tissue grinder (Fisher Scientific, Pittsburg, PA, USA), and the lipids were extracted in chloroform-methanol (2:1) in a ratio of 20 parts of solvent to one part tissue. The mixture was then filtered through a glass wool and treated with Folch wash (0.88% KCl, w/v, in deionized water) with a ratio of four parts sample volume to one part salt solution. The mixture was mixed by vortexing, and the top aqueous layer was then removed and discarded. The extract was evaporated to dryness in a warm water bath (40-60 °C) under nitrogen gas, and then the residue was reconstituted in chloroform-methanol (2:1). The reconstitution volume was chosen between 100-300 µL so that the densitometry scan areas of zones in sample chromatograms were bracketed within the scan areas of the standard zones comprising each calibration curve.

HPTLC analysis

The standard for phospholipid analysis was Polar Lipid Mix No. 117 (Matreya, Pleasant Gap, PA, USA) containing 25% each of cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine at a total of 25.0 mg in 1.00 mL. The standard solution was prepared at 0.500 µg/µL of each component by diluting the 1.00 mL standard with 12.5 mL chloroform-methanol (2:1). A phosphatidylserine (PS) standard (No. 1048, Matreya) was prepared at a concentration of 0.500 µg/µL in chloroform-methanol (2:1). The two standards were mixed in equal volume to create a solution with concentration of 0.250 µg/µL for each phospholipid marker. The standard for neutral lipid analysis was Matrteya Polar Lipid mixture B, containing cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate as marker compounds for free sterol, free fatty acid (FFA), triacylglycerol (TAG), methyl ester, and cholesteryl ester fractions, respectively. The standard, which contains 25.0 mg total lipid in 1.00 mL of chloroform-methanol (2:1), was diluted in a 25 mL volumetric flask with the same solvent to give a 0.200 µg/uL concentration of each neutral lipid.

Analyses were performed on 20 x 10 cm HPTLC silica gel 60 F_{254} glass plates (EMD Millipore Corp., Billerica, MA, USA, a division of Merck KGaA, Darmstadt, Germany, Catalog No. 1.95642.0001). Before use, plates were precleaned by development to the top with dichloromethane-methanol (1:1), dried with a stream of air from a hair dryer in a fume hood, and activated for 30 min on a CAMAG (Wilmington, NC, USA) plate heater at 120 °C.

Standard and reconstituted sample solutions were applied in 2.00, 4.00, 8.00, and 16.0 µL aliquots (0.400 to 3.20 µg standard for neutral lipids and 0.500 to 4.00 µg standard for phospholipids) to the HPTLC plates using a CAMAG Linomat 4 spray-on applicator (band length 6 mm, application rare 4 s/uL, table speed 10 mm/s, distance between bands 4 mm, distance from the left edge of the plate 17 mm, and distance from the bottom of the plate 1 cm). For phospholipid analysis, the Wagner mobile phase was used, chloroform-methanol-water (65:25:4.5). For neutral lipid analysis, the Mangold mobile phase was used, petroleum ether-diethyl ether-glacial acetic acid (40:10:0.5). One dimensional ascending development was carried out in an HPTLC twin trough chamber (CAMAG) containing a saturation pad (Miles Scientific Corp., Newark, DE, USA). Prior to insertion of the spotted plate for development, the chamber was equilibrated with the mobile phase vapors for 30 min. Development to within 1 cm of the top of the plates required approximately 10-15 min.

After development, plates were dried with a stream of cool air from a hair dryer in a fume hood for about 10 min. For phospholipids analysis, plates were sprayed with 10% cupric sulfate in 8% phosphoric acid and heated at 140 °C for approximately 30 min to detect brown-black bands

on a white background. For neutral lipids, plates were sprayed with 5% phosphomolybdic acid (PMA) in ethanol and heated on the hotplate at 120 °C for approximately 30 min to detect blue bands on a yellow background.

Both types of lipids were quantified by slit-scanning densitometry in the absorbance-reflectance mode using a CAMAG TLC Scanner 3 with slit dimensions 4.00 x 0.45 mm Micro and scanning rate 20 mm/s. The deuterium light source was set at 370 nm for phospholipid scanning and the halogen-tungsten lamp at 610 nm for neutral lipids. The winCATS software automatically generated polynomial regression calibration curves (standard zone weights versus peak areas) and interpolated sample zone weights based on their peak areas. The percentage by weight of lipid in each wet sample was calculated using the equation:

Percent lipid = 100*(w)*(R)/sample weight in µg

where w = lipid mass (µg) of sample interpolated from the calibration curve and R = reconstitution volume (µL)/spotted volume (µL). If the area of more than one sample aliquot was bracketed within the calibration curve, the weight of the aliquot giving a scan area closest to the average area of the two middle standards was used for calculations.

Student's *t*-test was used to determine significance of data based on mean values of the lipids of a sample population, with $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

Separations of the neutral lipids and phospholipids in Mangold and Wagner silica gel HPTLC systems used for analysis are illustrated in a recent review [5]. All of the target compounds are well separated in compact symmetrical bands that are ideal for densitometric scanning.

As expected from our previous work, there were considerable changes in the lipid profiles of each organ region in infected versus uninfected samples (Table 1). Overall, there was a significant increase (Table 2) in the level of TAG in all three organ systems as the result of the infection. This observation was consistent with the findings of Thompson [6], in which, the level of TAG in the DGG was elevated as the result of the infection. The current study, therefore, showed that similar biological mechanisms that caused elevated TAG level in DGG in the Thompson [6] study also occurred in the visceral mass and head-foot region of our snails.

The head-foot region showed changes with significant differences in both neutral lipids and phospholipids except for the PS fraction. However, while the level of TAG increased, the level of other lipid fractions decreased as the result of the infection. This trend was interesting since it showed that a combination of changes could also be seen in both the DGG and visceral mass group.

In the visceral mass group, significant differences occurred in FFA, TAG, and PC. Levels of TAG and PC were slightly increased within the infected group. However, FFA showed a decrease by one half in the infected group compared to the control group. It is hypothesized that the snail infection served as a competitor for resources inside the snail body, thus decreasing the FFA level available for the snails and that the infected snails

Table 1. Weight percentage +/- standard deviation of each lipid fractions in different organs.

Lipid fractions	Infected			Control		
	Head-foot	Visceral	DGG	Head-foot	Visceral	DGG
Sterol	0.08 +/- 0.04	0.086 +/- 0.007	0.04 +/- 0.02	0.19 +/- 0.07	0.12 +/- 0.04	0.06 +/- 0.01
FFA	0.04 +/- 0.02	0.09 +/- 0.03	0.04 +/- 0.01	0.10 +/- 0.02	0.17 +/- 0.05	0.16 +/- 0.03
TAG	0.033 +/- 0.006	0.11 +/- 0.02	0.13 +/- 0.03	0.054 +/- 0.008	0.07 +/- 0.01	0.07 +/- 0.01
PE	0.08 +/- 0.05	0.17+/- 0.05	0.11 +/- 0.02	0.36 +/- 0.03	0.18 +/- 0.02	0.29 +/- 0.04
PC	0.3 +/- 0.2	0.39 +/- 0.09	0.56 +/- 0.03	0.6 +/- 0.1	0.24 +/- 0.03	0.53 +/- 0.09
PS	0.11 +/- 0.04	0.099 +/- 0.006	0.09 +/- 0.01	0.11 +/- 0.02	0.12 +/- 0.02	0.21 +/- 0.02

Table 2. Two-tailed P-value comparing between infected and control samples.

	P-value		
	Head-foot	**Visceral**	**DGG**
Sterol	3.35×10^{-2}*	1.47×10^{-1}	3.53×10^{-2}*
FFA	4.03×10^{-3}*	2.90×10^{-2}*	8.31×10^{-2}
TAG	5.61×10^{-3}*	9.01×10^{-3}*	4.52×10^{-3}*
PE	1.29×10^{-4}*	6.18×10^{-1}	8.48×10^{-5}*
PC	1.05×10^{-2}*	2.45×10^{-2}*	5.82×10^{-1}
PS	2.45×10^{-1}	1.44×10^{-1}	7.53×10^{-5}*

*P-value of less than 0.05, thus, considered significantly different.

used this substrate for its growth. The increased level of TAG that was observed could also have been an adaptation to enhance the life cycle of the parasite. Since the early larval Schistosoma (sporocyst) stages would develop into mature cercariae (about 6 weeks post-infection), it would be favorable for the schistosomes to induce the host to store energy reserves such as TAG for that process.

In the DGG organ group, significant differences were observed in sterols, TAG, PE, and PS. Only the TAG fraction showed an increased level in infected snails, while the level of sterols, PE, and PS decreased with the infection. In the previous study [1], the most abundant phospholipid was PE. In the present study, for both control and infected DGG samples PC was by far the most abundant phospholipid fraction. This difference compared to the earlier study could be explained by the fact that the snail system is highly variable in terms of its lipid composition. Interestingly, although PC was the most abundant, it did not show significant changes between control and infected snails, a trend observed in our earlier study, i.e., as the infection proceeded to later stages, the level of PC remained similar whether the snails were infected or not.

CONCLUSION

For the first time it was demonstrated that HPTLC-densitometry can be used to quantify neutral lipid and phospholipid fractions in various organ systems of snails. Considerable differences were found in lipids (phospholipid and NL) in the organ systems (viscera, DGG, head-foot) of *B. glabrata* snails infected with *S. mansoni*.

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CONFLICT OF INTEREST STATEMENT

None of the authors has a conflict of interest in any aspects of this work.

REFERENCES

1. Bui, Q., Fried, B. and Sherma, J. 2016, Trends Chromatogr., 10, 7-11.
2. Fried, B. and Sherma, J. 1990, J. Planar Chromatogr. Mod. TLC, 3, 290-299.
3. Fried, B., Cahn-Hidalgo, D., Fujino, T. and Sherma, J. 1991, Trans. Am. Microsc. Soc., 110, 163-171.
4. Ulmer, M. J. 1970, Notes on rearing snails in the laboratory, In A. J. MacInnis and M. Voge (Eds.), Experiments and Techniques in Parasitology, W. H. Freeman, San Francisco, CA, 143-144.
5. Bui, Q. H. D., Fried, B. and Sherma, J. 2016, J. Planar Chromatogr. Mod. TLC, 29, 99-107.
6. Thompson, S. N. 1987, Comp. Biochem. Physiol., 87B, 357-360.