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High-performance thin-layer chromatographic analysis of phospholipids in *Biomphalaria glabrata* snails infected with *Schistosoma mansoni*

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ABSTRACT

Biomphalaria glabrata serves as the vector for the medically important digenean *Schistosoma mansoni*. In a previous study, using two dimensional thin layer chromatography with area normalization densitometry based on relative scan areas of zones in sample chromatograms, Thompson reported that phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were the major phospholipids in the digestive gland-gonad complex of *B. glabrata* and that there were no significant differences in their content 6 weeks postinfection with *S. mansoni*. In this paper we report results of a repeated phospholipid metabolic study 2-8 weeks postinfection using truly quantitative one dimensional high performance thin layer chromatography-densitometry with Analtech laned preadsorbent silica gel plates, chloroform-methanol-water (65:25:4.5 *v/v/v*) mobile phase and cupric sulfate-phosphoric acid detection reagent. Phospholipid identification was based on comparison of chromatographic migration of zones in samples and standards on adjacent lanes, and densitometry of each identified phospholipid was carried out on an absolute percentage (w/w) basis against individual standard calibration curves. Our qualitative and quantitative findings are compared with those of Thompson.

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

INTRODUCTION

Biomphalaria glabrata serves as the vector snail for the medically important platyhelminthe *Schistosoma mansoni*. Only one study has been reported on the effects of parasitism by *S. mansoni* on the phospholipid content of the digestive gland-gonad complex (DGG) of *B. glabrata*. In that earlier study, Thompson [1] using two dimensional (2D) silica gel thin layer chromatography (TLC) identified phosphatidylethanoamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) in the DGG of *B. glabrata* maintained at 26 ^{o}C on a diet of lettuce supplemented with Tetramin fish food. Densitometry with densitometric scan area normalization found that the relative percentage composition was PC>>PS>PE. There was no significant difference between the amounts of PC, PS or PE in control and infected snails. Thompson also found low levels of the phospholipid ceramide-aminoethyl ethyl phosphonate in only a few samples but did not give quantitative data. The purpose of this study was to use modern quantitative one dimensional (1D) high performance TLC (HPTLC) to examine the effects of *S. mansoni* infection on the phospholipid profile of *B. glabrata* maintained at 23 ^{o}C for 2, 4, 6 and 8 weeks on a lettuce diet and compare the results to those of Thompson just discussed.

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). Cultures of control and infected *B. glabrata* were maintained at 23+/-1 ^{o}C in aerated glass jars containing 10 to 20 snails per 800 mL of artificial spring water (ASW) under diffuse overhead fluorescent light for 12 h/day. The ASW was prepared as described by Ulmer [2]. 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 2, 4, 6 and 8 weeks post infection (PI), the shell of each snail was crushed gently with a hammer and the snail body was removed with forceps. The DGG was dissected from the snail body, the gut tissue was removed, and the DGG was blotted on a paper towel and weighed on an analytical balance. The DGG 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. Then, the mixture was filtered through a glass wool filter 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 vortex-mixed, and the top aqueous layer was then removed and discarded. The extract was evaporated to dryness in a warm water bath (40-60 ^{o}C) under nitrogen gas, and then the residue was reconstituted in chloroform-methanol (2:1). The reconstitution volume was chosen 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 and ranged from 100-300 uL.

HPTLC analysis

The standard for phospholipid analysis was Polar Lipid Mix No. 117 (Matreya, Pleasant Gap, PA, USA) containing 25% each of cholesterol, PE, PS and lysophosphatidylcholine in a total of 25 mg in 1.00 mL. The standard solution was prepared at 0.500 µg/uL of each component by dissolving the 1.00 mL standard with 12.5 mL chloroform-methanol (2:1). A PS standard (No. 1048, Matreya) was prepared at a concentration of 0.500 ug/uL in chloroform-methanol (2:1). The two standards were mixed in equal volume to create a solution with concentration of 0.250 ug/uL for each phospholipid marker.

Analyses were performed on 10 x 20 cm HPTLC-HLF silica gel plates (Analtech Inc., Newark, DE, USA, Catalog No. 61927) that contained 19 scored lanes and a preadsorbent zone spotting area. Before use, plates were precleaned by development to the top with dichloromethane-methanol (1:1), dried with a stream of air in a fume hood, and activated for 30 min on a CAMAG (Wilmington, NC, USA) plate heater at 120 ^{o}C.

Standard and reconstituted sample solutions were applied in 2.00, 4.00, 8.00 and 16.0 µL aliquots (0.250 to 2.00 µg of each standard) to the preadsorbent zone of separate lanes on the HPTLC plates using a 10 µL Drummond (Broomall, PA, USA) digital microdispenser. A gentle stream of air was directed across the preadsorbent zone during application of the sample to quickly dry the standards and samples as they were applied. Plates were developed to a distance of 8 cm beyond the preadsorbent zone–silica gel interface with 50 mL of the Wagner mobile phase: chloroform-methanol-water (65:25:4.5 *v/v/v*). 1D development was carried out in an HPTLC twin trough chamber (CAMAG) containing a saturation pad (Analtech). Prior to insertion of the spotted plate for development, the chamber was equilibrated with the mobile phase vapors for 30 min. Development 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, sprayed with 10% cupric sulfate in 8% phosphoric acid, and heated at 140 ^{o}C for approximately 30 min to detect brown-black phospholipid bands on a white background.

Phospholipids 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, scanning rate of 20 mm/s, and distance between band centers of 10.2 mm to match the center to center distances between scored lanes. The deuterium light source was set at 370 nm. 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 cell 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.

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

Table 1 presents data for the percentage by weight of neutral lipids and phospholipids in DGGs of control snails and infected snails 2, 4, 6 and 8 weeks PI. Overall, the major phospholipid fraction in both infected and uninfected DGG samples was PC ($R_f = 0.50$). In uninfected snails, the amount of PE ($R_f = 0.30$) was relatively equal throughout the study, while the level of PS ($R_f = 0.23$) fluctuated over time. In infected snails, however, the amount of both PS and PE changed over the course of the study.

The PE content in both infected and uninfected snail had the same trend. The level was stable between weeks 2 and 4 PI. However, the level of PE went slightly up at 6 weeks PI. Samples from 8 weeks PI showed a decrease in the level of PE back to relatively the same level of PE from the starting point at 2 weeks PI. Not only did the level of PE have the same trend between the uninfected and infected snails, but the amount in both groups was also similar. At 6 weeks PI, *S. mansoni* parasite inside the snail's DGG usually develops into a fork-tailed cercariae. Even so, there was no significant difference between infected and uninfected samples at this point. This leads us to hypothesize that the level of PE in *B. glabrata* is not affected by infection of *S. mansoni*, but just by the physiological change of the snail body as it ages.

The level of PS, however, exhibited differences in both trends and numerical values between the infected and uninfected snails. In uninfected snails, the amount of PS showed a sharp decrease (almost by half) between week 2 and 4 PI samples. The level of PS then increased from week 4 to 6 PI and stayed relatively stable up to week 8 PI. In infected snail, the level of PS showed a sharp increase transitioning from weeks 2 to 4 PI and from weeks 6 to 8 PI. From week 4 to 6 PI, it had a slight decrease. The amount of PS compared between uninfected and infected snails was significantly different at 2, 4, 6 and 8 weeks PI. We believe that the difference in PS level between uninfected and infected snails is caused by the parasitism of *S. mansoni*. To explain the different in trends of PS level, we propose two explanations: 1) the changes in PS level caused by the parasite outweigh the changes that would otherwise occur naturally as the snail ages, thus skewing the overall trends of PS level; 2) the parasitism affected the metabolism of the snail, thus causing a difference in the way the snail physiology changes as it ages. Further studies are needed to address this issue.

Similar to PS, the level of PC also exhibited differences between infected and uninfected snails. However, differences were only significant in samples at 2 and 4 weeks PI. At 2 weeks PI, uninfected snails showed a significantly higher level of PC than infected snails. However, the PC level in uninfected snails dropped off significantly up to 4 weeks PI, while the amount of PC in infected snails stayed relatively the same as it was at 2 week point PI. After 4 weeks PI, both uninfected and infected snail showed no significant changes in level of PC as the study proceeded. This has lead us to believe that the infection by *S. mansoni* caused changes in the level of PC in *B. glabrata*. However, it is unknown if this change in PC level diminishes over time as the result of the development of the infection or as the snail physiology changes as it ages.

We identified PE, PS and PC as the three major phospholipids in our samples as did Thompson [1], but we did not find ceramide-amino ethyl phosphonate in any of our samples. Like Thompson, we found that PE had the greatest concentration among the three phospholipids in our samples. However, our quantitative results cannot be compared directly to those of Thompson because he used a higher temperature, he fed his snails with both Tetramin and lettuce rather than our diet of lettuce only, and he infected reproductively active snails with a ca. 1 cm shell diameter whereas our snails were smaller juveniles. Most importantly, Thompson

Table 1. Weight percentage +/- standard deviation of phospholipid in snail DGG at 2, 4, 6 and 8 weeks PI.

	Week 2			**Week 4**			**Week 6**			**Week 8**		
	PC	**PE**	**PS**	**PC**	**PE**	**PS**	**PC**	**PE**	**PS**	**PC**	**PE**	**PS**
Uninfected	0.47 +/- 0.06	0.26 +/- 0.04	0.28 +/- 0.04	0.27 +/- 0.07	0.23 +/- 0.04	0.14 +/- 0.06	0.48 +/- 0.06	0.29 +/- 0.03	0.21 +/- 0.02	0.47 +/- 0.08	0.24 +/- 0.06	0.22 +/- 0.02
Infected	0.37 +/- 0.04	0.24 +/- 0.04	0.22 +/- 0.03	0.39 +/- 0.05	0.24 +/- 0.09	0.27 +/- 0.05	0.52 +/- 0.07	0.29 +/- 0.05	0.25 +/- 0.05	0.46 +/- 0.07	0.22 +/- 0.05	0.31 +/- 0.04
P-value ($n = 10$)	3.15E-04*	4.24E-01	7.31E-04*	6.40E-03*	8.33E-01	3.26E-04*	1.95E-01	9.24E-01	2.76E-02*	7.43E-01	5.32E-01	6.87E-05*

*****significantly different at $P < 0.05$.

performed his analyses by 2D TLC with development of a layer containing a single sample in a corner with two mobile phases at right angles, in contrast to the current study in which we used 1D HPTLC with development of multiple standards and samples on one plate with a single mobile phase. Poole [3] explained that slit scanning densitometers of the type used both in our study and Thompson's are designed for evaluation of separations contained in a single track on a plate, as obtained in 1D development, and not for evaluation of zones spread over the entire layer surface on plates developed two dimensionally. Sample zones in 1D chromatograms are quantified against standards developed identically in parallel to produce individual calibration curves. Thompson did not quantify against standards but used area normalization whereby the three phospholipids were scanned assuming equal area/weight (which is not true for these compounds), their areas were added together, and relative percentage composition was calculated by dividing each individual area by the total area. Our densitometry gives absolute percentages (w/w) for each compound in the DGG by interpolating sample zone weights based on their areas from individual calibration curves (scan areas versus corresponding weights of standards). In addition, our qualitative analysis is more reliable because sample and standard R_f values are compared on the same plate run under identical experimental conditions for each track, not samples and standards developed by 2D TLC on separate plates one at a time. HPTLC plates afford better resolution and more sensitive detection of zones than do TLC plates; a separation of PE, PC and PS is shown on an HPTLC silica gel plate in a review article by Bui *et al*. [4].

CONCLUSION

A widely cited 2D TLC study by Thompson [1] on the effect of *S. mansoni* infection on the phospholipid content of *B. glabrata* DGG was repeated using more quantitative 1D HPTLC-densitometry. Our investigations found several differences in phospholipid profile of *B. glabrata* DGG induced by the parasitism. The study provides preliminary information on the biochemical aspects of *S. mansoni* parasitism by looking at the final morphological changes that it induces in the host snail. This information might be of interest for further investigation that focus on the mechanisms in which *S. mansoni* affects its snail host, since, to our best knowledge, no work has been done to investigate the said subject.

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CONFLICT OF INTEREST STATEMENT

None of the authors has a conflict of interest relative to this work.

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