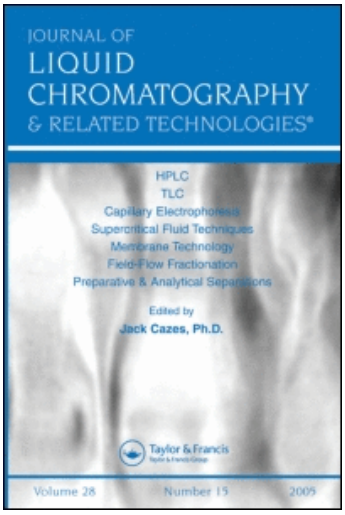

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ABSTRACT

High performance thin layer chromatography (HPTLC) was used to analyze neutral lipids and phospholipids in the cercariae of a Puerto Rican strain of *Schistosoma mansoni*. The cercariae were obtained following isolation of *Biomphalaria glabrata* snails (NMRI strain) experimentally infected with *S. mansoni* miracidia. Cercariae were pelleted to contain from 1000 to 10,000 organisms per 1.5 mL of artificial spring water and then extracted in chloroform–methanol (2 : 1). The lipid extract was analyzed by previously published silica gel HPTLC methods, using a petroleum ether–diethyl ether–acetic acid (80 : 20 : 1) mobile phase and detection with phosphomolybdic

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acid spray reagent for neutral lipids. Visual observations of the chromatograms showed that the most abundant neutral lipids in the cercariae were free fatty acids and free sterols along with two additional faster moving zones that did not match the methyl oleate and cholesteryl oleate standard zones. Quantification of the free fatty acid and free sterol fractions by scanning densitometry revealed mean weights ± standard deviation of 4.62 ± 1.9 ng and 3.51 ± 0.70 ng per cercaria, respectively.

Phospholipids were separated on silica gel using a mobile phase of chloroform–methanol–water (65 : 25 : 4), and the plates were sprayed with a detection reagent of 10% cupric sulfate in 8% phosphoric acid. Visual observations of the chromatograms showed that the most abundant phospholipids were phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Quantification of PC and PE by scanning densitometry revealed mean weights ± standard deviation of 67.0 ± 18 ng and 38.7 ± 8.8 ng per cercaria, respectively. This is the first study to provide quantitative data on neutral lipids and phospholipids on a per cercaria basis for *S. mansoni*. The possible functions of lipids and phospholipids in cercariae are discussed.

Key Words: Neutral lipids; Phospholipids; Cercariae; *Schistosoma mansoni*; HPTLC

INTRODUCTION

Recent studies in our laboratory have used high performance thin layer chromatography (HPTLC) to examine neutral lipids in cercariae of various species of hermaphroditic digeneans, i.e., *Echinoparyhium* sp. by Muller et al.,[1] *Zygocotyle lunata* by Marsit et al.,[2] and *Echinostoma caproni* by Marsit et al.[3] The first study to use thin layer chromatography (TLC) to examine lipids in a cercarial stage was carried out by Smith et al.[4] on *Schistosoma mansoni*. They reported that phospholipids, free sterols, and steryl esters were the major components of *S. mansoni* cercariae and also noted the presence of triacylglycerols, partial glycerols, and free fatty acids as minor fractions. They did not identify specific classes of phospholipids in their study. Because their TLC analyses were done 35 years ago, before the availability of efficient HPTLC plates and automated scanning densitometers, we decided to reexamine the presence of neutral lipids and phospholipids in the cercariae of a Puerto Rican strain of *S. mansoni* cercariae.

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EXPERIMENTAL

Sample Preparation for Neutral Lipids and Phospholipids

Biomphalaria glabrata snails (NMRI strain), experimentally infected with *S. mansoni* (PR1 strain), were maintained in the laboratory as described in Fried et al.[5] Snails were isolated for 2 h, 10–15 per finger bowl, in 40–60 mL of artificial spring water (ASW) that was prepared as described by Ulmer.[6] The snails were isolated periodically from 6 to 16 wk postinfection to obtain the thousands of cercariae used in this study. Cercariae with ASW were removed by use of a Pasteur pipet, usually about 1000 to 10,000 cercariae per bowl, within 2 h post-cercarial emergence into 15 mL calibrated centrifuge tubes. The cercariae were allowed to settle to the bottom of the tubes overnight in a refrigerator at 4°C. The tubes were removed from the refrigerator, and the top layers of ASW were removed. The cercarial pellets were combined into samples corresponding with the original finger bowls, and the combined samples were again refrigerated at 4°C, this time for 2 h. After the tubes were removed from the refrigerator, the top layer of ASW was removed until the total volume in each tube was 1.50 mL. A 10.0 μL aliquot of cercarial suspension was removed form the tube using a 10 μL Drummond (Broomall, Pennsylvania, USA) digital microdispenser and placed on a depression slide, and the number of cercariae in the aliquot was counted under a compound microscope at 100 diameters. The total number of cercariae in the 1.50 mL sample was then calculated by multiplying the number in 10.0 μL by a factor of 150. Cercarial counts between 1000 and 10,000 were obtained for each sample. During the course of this study, 17 samples were used for qualitative and quantitative analyses.

To isolate both the neutral lipids and phospholipids from fresh cercariae obtained as described above, each sample was extracted by adding 2–4 mL of chloroform–methanol (2 : 1) to each 1.5 mL sample of cercariae and ASW. To facilitate extraction, each tube was vortexed on high for 1 min and then refrigerated overnight at 4°C to obtain a biphasic mixture. The top, hydrophilic layer was removed with a Pasteur pipet and discarded. The lipophilic layer was evaporated to dryness under a stream of nitrogen gas at 40°C, and the lipid residue was reconstituted with 25.0–150 μL of chloroform–methanol (2 : 1) measured with either a 25 μL or 100 μL Drummond digital microdispenser.

Because Smith et al.[4] used frozen samples in their TLC studies on neutral lipids of *S. mansoni* cercariae, we prepared some samples as described above, and then froze them overnight at −20°C. These samples were thawed at 22°C and extracted with chloroform–methanol (2 : 1) as described above.

HPTLC Analysis of Neutral Lipids

HPTLC analysis of neutral lipids was performed on Whatman (Clifton, New Jersey, USA) 10×20 cm LHPKDF silica gel plates containing 19 channels and a preadsorbent sample application zone. The plates were pre-cleaned by development to the top with dichloromethane–methanol (1 : 1) and air-dried under a fume hood. For qualitative and quantitative analysis, the standard used was a neutral lipid standard 18-4A (Matreya, Pleasant Gap, Pennsylvania, USA), which was diluted in chloroform–methanol (2 : 1) to contain 0.200 $\mu g\,\mu L^{-1}$ each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol. These compounds were used as markers for qualitative and quantitative determination of the steryl ester, methyl ester, triacylglycerol, free fatty acid, and sterol fractions, respectively. Volumes of 1.00, 2.00, 4.00, and 8.00 μL of the standard and 20.0 μL of the reconstituted sample were spotted on a plate using a 25 μL Drummond digital microdispenser. The standard was also diluted 1 : 1 and 1 : 3, and a 1.0 mL aliquot of each of the dilutions was applied to the plates so that the lowest standard weight would bracket the lowest lipid weights in the samples.

Plates were developed in Camag (Wilmington, North Carolina, USA) twin trough HPTLC chambers, lined with a saturation pad (Analtech, Newark, Delaware, USA), with the Mangold solvent system (petroleum ether–diethyl ether-acetic acid, 80 : 20 : 1) to a distance of 6.5 cm past the preadsorbent-silica gel interface. In addition to the Mangold mobile phase, plates were developed with the Smith et al.[7] modified Mangold mobile phase (*n*-hexane–petroleum ether–ethyl ether–glacial acetic acid, 50 : 20 : 5 : 1) to increase resolution of the fast moving zones on the plate, and a ferric chloride spray reagent, as described by Muller et al.,[1] was used to confirm the identity of cholesterol and cholesteryl ester zones. After development, the plate was air-dried under a fume hood, sprayed with 5% ethanolic phosphomolybdic acid (PMA), and heated at 110°C on a Camag Plate Heater for 15 min. Neutral lipid zones appeared as dark blue spots against a yellow background.

Densitometry of the free sterol zones was performed using a Camag TLC Scanner II with the tungsten light source set at 700 nm, slit width 4, slit length 4, and scanning rate 4 $mm\,sec^{-1}$. The scanner was controlled by a CATS-3 software package, which produces a calibration curve relating to the weights of the standard zones and their peak areas. The weights of free sterol and free fatty acid in the sample zones were determined automatically from their areas by interpolation from the calibration curve. The weights of each lipid per cercaria were calculated as described in Muller et al.[1]

HPTLC Analysis of Phospholipids

For qualitative and quantitative analysis, the standard used was a Matreya Polar Lipid Mix containing 6.25 $\mu g\,\mu L^{-1}$ each of cholesterol, PE, PC, and lysophosphatidylcholine. This standard was then diluted 1 : 100 in chloroform–methanol (2 : 1) to make a working standard solution of 0.0625 $\mu g\,\mu L^{-1}$. Aliquots of the standard solution (2.0, 4.0, 8.0, 16.0, and 32.0 μL) and reconstituted sample solution (10.0, 15.0, and 20.0 μL) were applied to the prewashed Whatman plates described above. The mobile phase was chloroform–methanol–water (65 : 25 : 4), and phospholipids were detected as black zones on a white background by spraying with 10% cupric sulfate in 8% phosphoric acid reagent, and heating at 140°C for 15 min. The spray reagent was prepared as described in Aloisi et al.[8] The zones were scanned at 370 nm using the deuterium source of the Camag scanner. Other procedures and calculations were performed as described above for the neutral lipids.

RESULTS

Neutral Lipids

The R_f values of the neutral lipid standards in the Mangold mobile phase were as follow: cholesterol, 0.27; oleic acid, 0.32; triolein, 0.64; methyl oleate, 0.75; and cholesteryl oleate, 0.85. Qualitative analysis of lipids in five samples of *S. mansoni* cercariae containing between 4000 and 10,000 cercariae resulted in six PMA-positive zones. The major identifiable neutral lipids in all trials were free fatty acids (R_f 0.32) and free sterols (R_f 0.27). Unidentified zones migrated below the free sterols (R_f 0.11) and above the free fatty acids (R_f 0.44). The most intense zones were near the mobile phase front and did not line up with either the cholesteryl oleate or methyl oleate standard zones. The fastest moving zone, probably containing hydrocarbons, had an R_f value of 0.88, and a second zone migrated just below this one but was not completely resolved. Neither of these upper zones reacted with ferric chloride detection reagent, proving that they did not contain cholesteryl oleate. Only free sterol zones in the sample lanes appeared red-violet against a red background, as did the cholesterol and cholesteryl oleate standard zones. Development of sample extracts with the modified Mangold mobile phase devised by Smith et al.[7] also confirmed that cholesteryl esters were not present. This mobile phase is known to resolve cholesteryl esters from methyl esters and from other fast moving PMA-positive non-lipid zones.

Densitometric quantification data of the free sterol and free fatty acid zones in the *S. mansoni* cercarial fresh samples gave mean values ± standard deviation of 3.51 ± 0.70 ng and 4.62 ± 1.9 ng per cercaria, respectively.

Frozen samples were used in three trials, and the sterol content was found to be 0.790 ± 0.090 ng per cercariae. Free fatty acid zones in sample chromatograms were visually detected, but their content was too low to be quantified exactly, because their scan areas were below that of the lowest standard zone in the calibration curve. The limit of quantification was calculated as 0.000360 ng per cercariae for free fatty acids, so the content in samples was below this value. Qualitative TLC results for the frozen samples were as described for the fresh samples.

Phospholipids

Visual observations of the chromatograms used to detect phospholipids revealed that the major fractions in the cercarial samples corresponded to PC (R_f 0.44) and PE (R_f 0.61) standard zones. Quantification data of the PC and PE zones by scanning densitometry revealed that mean weights ± standard deviation of PC and PE were 67.0 ± 18 ng and 38.7 ± 8.8 ng per cercaria, respectively. There were other zones migrating above the cholesterol standard that corresponded to the neutral lipids reported above. Another zone that appeared as a faint pink line between the cholesterol and PE standards on the plate was unidentifiable.

DISCUSSION

Smith et al.[4] performed qualitative TLC on neutral lipids from frozen cercarial pellets and noted that the free fatty acid fraction was a minor one. In our analysis of cercarial samples frozen prior to HPTLC, the free fatty acid content was significantly less than the free sterol fraction. Reddy et al.[9] demonstrated that samples frozen and thawed prior to lipid TLC gave spurious results, compared to TLC lipid analysis on fresh material extracted with chloroform–methanol.

Smith et al.[4] alluded to a large, fast moving lipid zone, and that zone is visible clearly in the chromatograms shown in Fig. 2, lanes A and C, in their paper. They identified that zone as steryl esters, based on comigration with a cholesteryl ester standard and colors formed with their *o*-phosphoric acid detection reagent. With our more advanced HPTLC techniques, it is clear that this zone does not contain steryl esters, because it did not comigrate with the steryl oleate standard in the modified Mangold mobile phase, nor react with the specific ferric chloride detection reagent. Further studies using additional chromatographic techniques are needed to positively identify the fast moving fractions in *S. mansoni*.

Mean free sterol values in ng are now available on a per cercaria basis for four digeneans as follows: 120 for *Zygocotyle lunata*;[2] 0.53 for *Echinostoma*

caproni;[3] 22 for *Echinoparyphium sp.*;[1] and 3.51 for *Schistosoma mansoni* (present study). These values reflect considerable variation in free sterol content between cercariae of various taxa. This is not surprising, considering the extreme variation in the morphology of cercariae from different taxa. Moreover, the function of the free sterols in larval trematodes is poorly understood, although these lipids undoubtedly play a role in cell and tissue structure.

HPTLC studies on the free fatty acid fraction of cercariae have been reported previously for one other digenean, *Z. lunata*,[2] at a level of 160 ng per cercaria. In the present study, we report a value of 4.6 ng per cercaria for *S. mansoni*. The cercaria of *Z. lunata* is large and robust compared to that of *S. mansoni*, and this size difference may, in part, account for the much larger amount of free fatty acids in the *Z. lunata* vs. *S. mansoni* cercaria.

The functions of free fatty acids in cercariae are still mainly speculative, although these compounds have been implicated as excretory wastes resulting from the end products of carbohydrate metabolism. Further studies are needed to elucidate the roles of neutral lipids in the biology of cercariae.

This is the first study that reports quantitative data on a per cercaria basis for PC and PE in any digenean. Furlong et al.[10] gave relative percentages of the phospholipid content in *S. mansoni* cercariae, but their results were reported on a weight percentage basis using high performance liquid chromatography (HPLC). Their relative amounts of PC and PE were consistent with the results in our study. Using HPLC, they also found relatively small amounts of phosphatidylserine and phosphatidylinositol, which we did not find using HPTLC. The functions of phospholipids in the biology of cercariae are not known, but these substances presumably play a role in the structural integrity of the cells and are major components of cell membranes.

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REFERENCES

1. Muller, E.E.; Fried, B.; Sherma, J. HPTLC Determination of Neutral Lipids in the Cercariae of *Echinostoma trivolvis* and *Echinoparyphium sp.* (Trematoda). J. Planar Chromatogr. – Mod TLC **1999**, *12*, 306–308.

2. Marsit, C.J.; Fried, B.; Sherma, J. Neutral Lipids in the Cercariae, Encysted Metacercariae, and Rediae of *Zygocotyle lunata.* J. Parasitol. **2000**, *86*, 1162–1163.
3. Marsit, C.J.; Fried, B.; Sherma, J. Neutral Lipids in Cercariae, Encysted Metacercariae, and Rediae of *Echinostoma caproni.* J. Helminthol. **2000**, *74*, 365–367.
4. Smith, T.M.; Brooks, T.J., Jr.; White, H.B., Jr. Thin Layer and Gas Liquid Chromatographic Analysis of Lipids from Cercariae of *Schistosoma mansoni.* Am. J. Trop. Med. Hyg. **1966**, *15*, 307–313.
5. Fried, B.; Muller, E.E.; Broadway, A.; Sherma, J. Effects on the Development of *Schistosoma mansoni* in *Biomphalaria glabrata* and on the Neutral Lipid Content of the Digestive Gland Complex of the Snail. J. Parasitol. **2001**, *87*, 223–225.
6. Ulmer, M.J. Notes on Rearing Snails in the Laboratory. In *Experiments and Techniques in Parasitology*; MacInnes, A.J., Voge, S.M., Eds.; Freeman W.H.: San Francisco California, 1970; 143–144.
7. Smith, M.C.; Webster, C.L.; Sherma, J.; Fried, B. Determination of Neutral Lipids in Regular and Lowfat Eggs by High Performance Thin Layer Chromatography with Densitometry. J. Liq. Chromatogr. **1995**, *18*, 527–535.
8. Aloisi, J.; Fried, B.; Sherma, J. Comparison of Mobile Phases for Separation of Phospholipids by One-dimensional TLC on Preadsorbent High Performance Silica Gel Plates. J. Liq. Chromatogr. **1991**, *14*, 3269–3275.
9. Reddy, P.; Muller, E.E.; Fried, B.; Sherma, J. Effects of Freezing and Thawing and of Formalin and Ethanol Fixation on HPTLC Analysis of Neutral Lipids in *Biomphalaria glabrata* Snails. J. Parasitol. **1999**, *12*, 397–399.
10. Furlong, S.T.; Caulfield, J.P. *Schistosoma mansoni*: Sterol and Phospholipid Composition of Cercariae, Schistosomula, and Adults. Exp. Parisitol. **1988**, *65*, 222–231.

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