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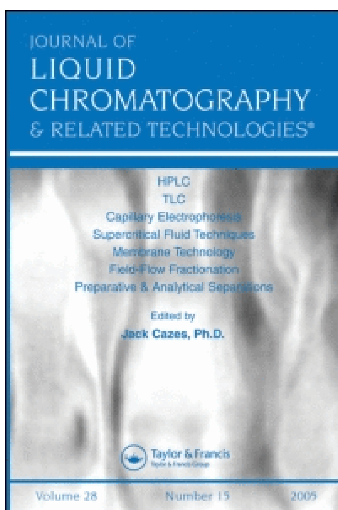

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High Performance Thin Layer Chromatographic Analysis of Neutral Lipids and Phospholipids in the Medicinal Leech *Hirudo medicinalis* and in Leech Conditioned Water

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High Performance Thin Layer Chromatographic Analysis of Neutral Lipids and Phospholipids in the Medicinal Leech *Hirudo medicinalis* and in Leech Conditioned Water

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Abstract: High performance thin layer chromatography (HPTLC) was used to determine neutral lipids and phospholipids in the medicinal leech *Hirudo medicinalis.* Whole bodies and anterior, middle, and posterior thirds were analyzed for lipids. The major neutral lipids found in the leech were free sterols and steryl esters, along with lesser amounts of free fatty acids and triacylglycerols. The major phospholipids were phosphatidylcholine and phosphatidylethanolamine. Leeches showed no significant regional differences in the mean weight percent of the major neutral lipids and phospholipids. HPTLC was used to analyze neutral lipids and phospholipids that leeches released into deionized water (referred to as leech conditioned water or LCW) during 6 or 12 h of incubation. The major neutral lipid released into the water was free sterols, and the major phospholipids were phosphatidylcholine and phosphatidylethanolamine.

Keywords: Thin layer chromatography, Neutral lipids, Phospholipids, Leech conditioned water (LCW), *Hirudo medicinalis*, Densitometry

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INTRODUCTION

The medicinal leech *Hirudo medicinalis* has been used for centuries in medical practices for bloodletting as a treatment for human illness.[1] More recently, leeches have been used in surgical and medical practices to relieve venous congestion following limb or organ implantation[2] and to provide hirudin as an alternative anticoagulant to heparin related drugs.[3]

Because of the current interest in the use of *H. medicinalis* in medical practice, we initiated basic studies on the physiology and biochemistry of this organism. Although there is no direct connection between lipid constituents and medical use of leeches, we initiated this study to get a more complete lipid profile of *H. medicinalis.* A review of the literature indicated four chromatographic studies on lipid analysis in *Hirudo medicinalis* and two studies on leeches other than *H. medicinalis.* Thus, Smiley et al.[4] used gas-liquid chromatography (GLC) to describe the fatty acid methyl esters in *H. medicinalis.* Spinedi et al.,[5] also using GLC, examined the lipids in the nervous system of *H. medicinalis* and reported the presence of cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol as the major lipids. Rabinowitz[6] used silica gel thin layer chromatography (TLC) to analyze the saliva in the medicinal leech. He found that the largest percentages of the identified lipids were phosphatidic acids and free fatty acids. Zipser et al.[7] used GLC and mass spectrometry to show that cholesterol was the major steroid in the medicinal leech.

In chromatographic studies on lipids from leeches other than *H. medicinalis*, El-Shourbagy[8] used qualitative silica gel TLC to determine the lipid profile of the leech, *Barbroni assiuit*, and reported the presence of cholesterol, free fatty acids, triacylglycerols, cholesteryl esters, gangliosides, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Hong et al.[9] examined a Korean leech, *Hirudo nipponia*, by GLC to determine the fatty acid methyl ester components of leech skin (epidermal) extracts. They also reported the lipid profile in the epidermal extracts as cholesterol, free fatty acids, triacylglycerols, glycerylether diesters, and cholesteryl esters.

Because no previous study provided quantitative data on the major neutral lipids and polar lipids in whole bodies of *H. medicinalis*, one purpose of this study was to use densitometric high performance TLC (HPTLC) analysis to determine the concentrations of these lipids in this organism. A second purpose was to determine if there are regional differences along the longitudinal axis of the leech in the quality and quantity of neutral and polar lipids in *H. medicinalis*. Rabinowitz[6] reported an unusual array of salivary lipids in *H. medicinalis.* Because the salivary glands are located in the anterior third region of the leech, we thought that this region would contain lipids different than that of the middle and posterior thirds of the organism. To test this idea, we examined the anterior, middle, and posterior thirds of the leech. The final purpose of this study was to determine if lipophilic substances were released into water in which the leeches were maintained. For these

studies, leeches were incubated in deionized water for 6 or 12 hr and the water was analyzed to determine the presence of neutral and polar lipid compounds. By analogy to work done on snail conditioned water or SCW,[10] the water containing leech excretory-secretory products was referred to as leech conditioned water (LCW). The rationale for this last study was based on the fact that lipophilic substances released by gastropods into the water serve as pheromones or carriers of pheromones to help mediate intraspecific and interspecific snail chemoattraction.[10]

EXPERIMENTAL

Sample Preparation

Hirudo medicinalis leeches were purchased from Carolina Biological Supply Company (Burlington, NC) and maintained in aerated mason jars that contained 1–5 leeches and 600 mL of artificial spring water (ASW) at 22 ± 1°C. The ASW was prepared according to methods described by Ulmer,[11] and the physical and chemical characteristics of the water were described in that paper. The leeches were maintained without the addition of exogenous food and used within 1–7 days of receipt. The water in each culture was changed every two days.

For HPTLC analysis of whole leeches, seven leeches (n = 7) were prepared for the neutral lipids analysis and three leeches (n = 3) for analysis of the phospholipids. The blotted wet weight of the leeches ranged from 244–647 mg. Each sample consisted of a single leech, and samples were prepared in a tissue grinder (15 mL, KT885300-0015, VWR International, Inc., West Chester, PA) using a volume of chloroform–methanol (2 : 1) that was twenty times the weight of the sample.[12] After homogenization, the supernatant of each sample was filtered through a cotton filter and treated with Folch wash (0.88% KCl, 1 mL) in a volume 1/4 that of chloroform–methanol (2 : 1) used for lipid extraction. The upper, hydrophilic layer was removed using a Pasteur pipet and discarded; the lower, lipophilic layer was dried under nitrogen gas in a water bath (40–60°C). Residues were reconstituted with 500–1200 μL of chloroform–methanol (2 : 1), the exact volume being chosen to yield at least one sample zone that had a densitometric scan area bracketed within the scan areas of the standards during HPTLC analysis.

For HPTLC analysis of the anterior, middle, and posterior thirds, each leech was cut into three approximately equal segments. This study used three leeches (n = 3), each of which was partitioned into anterior, middle, and posterior segments. Each segment was 13–17 mm in length by 3–5 mm in width. Each sample of the anterior third (n = 3), middle third (n = 3), and posterior third (n = 3) was prepared for both neutral and phospholipids analysis. The blotted wet weight of the anterior samples ranged from

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66.7–252 mg, the middle samples from 92.3–267 mg, and the posterior samples from 57.2–198 mg. The extraction of lipids followed the same procedure as described for the whole leech. The samples were reconstituted in 100–850 μL of chloroform–methanol (2 : 1).

To collect LCW, each of six leeches ranging in blotted wet weight from 244–839 mg was placed in a 16 × 50 mm glass vial (VWR International, Inc.) containing 5 mL of deionized (DI) water. Of the six leeches used in collecting LCW samples, three were used for the phospholipid analysis and three for neutral lipid analysis. The leeches were reused for analysis at different incubation times. The vials containing leeches were placed in a low temperature incubator (Model 2005, VWR International, Inc.) at 28°C for 6 or 12 hr. For the 12 hr incubation period, four samples of LCW (n = 4) were prepared for neutral lipid analysis and three samples (n = 3) for phospholipid analysis; for the 6 hr neutral lipid analysis, three samples of LCW (n = 3) were prepared. For controls, 6 mL of DI water was placed in a glass vial and incubated for either 6 or 12 hr. Each sample was extracted using chloroform–methanol (2 : 1) with a volume that was twice the volume of water in the glass vial (10 mL or 12 mL). The top, hydrophilic layer was removed and discarded, and the lower, lipophilic layer was evaporated to dryness using nitrogen gas and a water bath (40–60°C). The residues were reconstituted in 25.0–30.0 μL of chloroform–methanol (2 : 1) prior to HPTLC analysis.

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Thin Layer Chromatography

For neutral lipid analysis, the standard was TLC Reference Standard 18-4 A (Nu-Chek-Prep, Inc., Elysian, MN), which contained equal amounts of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate. The standard zones were used as markers for free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl esters, respectively. The solid standard was weighed on an analytical balance (42.7 mg) and diluted with chloroform–methanol (2 : 1) in a 25 mL volumetric flask to prepare a neutral lipid standard with 0.342 mg/mL of each compound.

HPTLC analysis was performed on Whatman (Clifton, NJ) LHPKDF high performance silica gel plates, 10 × 20 cm, with a preadsorbent zone and 19 scored lanes. Plates were prewashed by development to the top with dichloromethane–methanol (1 : 1). Standards of neutral lipids were applied with a 10.0 μL Drummond (Broomall, PA) digital microdispenser onto the separate preabsorbent lanes in aliquots of 2.00 μL, 4.00 μL, 8.00 μL, and 16.0 μL, and reconstituted samples were applied in 2.00 μL–16.0 μL aliquots. Initial zones were allowed to dry for 30 s before development of the plate in a rectangular Camag (Wilmington, NC) HPTLC twin-trough chamber. The chamber was lined with a saturation pad (Analtch, Newark, DE) and allowed to equilibrate for 15 min before plate development. The mobile phase was petroleum ether-diethyl ether-glacial acetic acid (80 : 20 : 1), as described by Mangold,[13] to determine free sterol, free fatty

acid, triacylglycerol, and methyl ester fractions in the samples. The Smith et al.[14] mobile phase, hexane-petroleum ether-diethyl ether-glacial acetic acid (50 : 25 : 5 : 1), was used to determine steryl esters. The plates were developed to a distance 8 cm past the preadsorbent-silica gel interface. The development time was approximately 9 min for both mobile phases. Developed plates were dried in a fume hood with cool air from a hairdryer for 5 min, sprayed with 5% ethanolic phosphomolybdic acid (PMA) solution, and heated for 10 min at 115°C on a Camag plate heater until blue neutral lipid bands appeared on a yellow background.

The standard for polar lipid analysis, Polar Lipid Mix (Matreya, Inc., Pleasant Gap, PA), contained 25.0% each of cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) and a total lipid concentration of 25.0 mg/mL. The standard (1.00 mL) was placed in a 25 mL volumetric flask and diluted with chloroform to prepare a solution containing 0.0250 μg/μL of each component. HPTLC analysis was done on the same plates and with the same standard and samples volumes as described for the neutral lipids. Plates were developed with the mobile phase described by Wagner et al.,[15] chloroform–methanol–water (65 : 25 : 4). The development time was approximately 20 min. Developed plates were dried in a fume hood for 5 min, sprayed with a 10% cupric sulfate solution, and heated for 10 min at 140°C to form brown polar lipid bands on a white background.

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For quantification, densitometry of the sample and standard zones was performed using a Camag TLC Scanner II with the tungsten light source set at 610 nm for neutral lipids, and the deuterium light source at 370 nm for polar lipids. Other scanner settings were slit width 4, slit length 4, and scanning speed 4 mm/s. The CATS-3 software was used to create a linear regression calibration plot relating the weights of the standard zones to their peak areas. From the calibration plot, the weights of the sample zones were automatically interpolated based on their peak areas. If the area of more than one aliquot of a single sample was bracketed within the calibration curve, the weight corresponding to the sample area closest to the mean of the two middle standard areas was used for the calculation of lipid percent. The weight percentages of neutral and polar lipids in the whole leech and regional parts were calculated using the following equation:

$$\%\ \text{Lipid} = \frac{(\text{w})(\text{R})(\text{dilution factor})(100)}{\mu\text{g sample}}$$

where w = μg interpolated from the plot and R = [reconstituted volume (μL)]/[spotted volume (μL)].

The concentrations (μg/mL DI) of neutral and polar lipids in LCW samples were calculated using the following equation:

$$\text{Lipid concentration} = \frac{(\text{w})(\text{R})}{(\text{v})}$$

where v = 5.00 mL.

For quantification of some sample solutions, dilution or concentration was necessary in order to have scan areas bracketed within the calibration plot. These factors were included in the calculations, as necessary.

RESULTS AND DISCUSSION

Experiments have shown that extraction with a volume of chloroform-methanol (2 : 1) that is 20 times the weight of the sample, followed by washing with Folch wash (0.88% KCl, 1 mL), results in complete and reproducible recovery of the lipids.[12] This was applicable for the extraction of the whole body and the sections of the leech. However, for the extraction of lipids from LCW samples, a volume of chloroform : methanol (2 : 1) twice the volume of the LCW sample was used, as in the previous study on snail conditioned water (SCW).[10] The mobile phase of Smith et al.[14] allowed for the unequivocal quantification of steryl esters, which may comigrate with hydrocarbons in the mobile phase of Mangold.[13] Calibration plots relating the scan areas to the weights of lipid (0.683–5.46 μg) and phospholipid (0.500–4.00 μg) standards consistently gave linear regression coefficient (r) values of 0.98 or 0.99.

All samples analyzed for neutral lipids showed zones with comparable migration to the standards at R_F values of 0.18 (cholesterol), 0.23 (oleic acid), 0.58 (triolein), 0.72 (methyl oleate), and 0.83 (cholesteryl oleate) in the Mangold[13] mobile phase. The R_F value of the steryl ester zone (cholesteryl oleate) was 0.66 in the Smith et al.[14] mobile phase.

The free sterol and steryl ester zones were the major neutral lipid fractions in all six whole leech samples. Lesser amounts of free fatty acids, triacylglycerols, and methyl esters were found in the leeches. Of the six leeches analyzed for neutral lipids, four showed free fatty acids, three showed triacylglycerols, and one showed methyl esters. This variability of the minor neutral lipids in biological samples is not uncommon, as discussed in Fried.[16] The major neutral lipid classes (free sterols and steryl esters) were quantified in all samples, but only two samples had quantifiable amounts of free fatty acids and three samples had quantifiable amounts of triacylglycerols. The qualitative lipid profile of the anterior, middle, and posterior third segments of the leech was similar to that described for the whole leech.

All three leeches analyzed for polar lipids showed sample and standard zones at R_F values of 0.48 (PC) and 0.68 (PE) in the Wagner et al.[15] mobile phase. PC and PE were the major polar lipids present and were quantified in this study. LPC in the polar lipid standard was not detected with the cupric sulfate reagent at the concentrations applied, and zones that might have been LPC were not seen in the samples.

Table 1 presents data for the amounts of major neutral and polar lipids found in the whole body and the anterior, middle and posterior thirds of the *H. medicinalis* leech. For the samples that contained free fatty acids and triacyl-

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Table 1. Percent by weight (mean ± SE) of lipids in the medicinal leech, *Hirudo medicinalis*, whole body and anterior, middle, and posterior thirds

Sample	Free sterols	Steryl esters	PC	PE
Whole body	0.122 ± 0.022[a]	0.0159 ± 0.0065[b]	0.358 ± 0.032[b]	0.122 ± 0.0059[b]
Anterior third	0.240 ± 0.081[b]	0.0914 ± 0.027[b]	1.031 ± 0.17[b]	0.230 ± 0.077[b]
Middle third	0.167 ± 0.058[b]	0.151 ± 0.044[b]	0.9532 ± 0.042[b]	0.308 ± 0.089[b]
Posterior third	0.228 ± 0.080[b]	0.0651 ± 0.019[b]	0.766 ± 0.18[b]	0.301 ± 0.13[b]

[a]n = 6 leeches.
[b]n = 3 leeches.

glycerols, the mean weight percent ± standard error (SE) of triacylglycerols was 0.0935 ± 0.0337 and of free fatty acids was 0.019516 ± 0.0090. The mean concentration of free sterols in the whole leech was approximately 7.7, 6.3, and 1.3 times greater than the steryl esters, free fatty acids, and triacylglycerols, respectively. The neutral lipid fractions of the anterior third of the leech were compared to the middle third and the posterior third. The different regions did not show any significant differences (Student's *t*-test, $P > 0.05$) in the mean weight percent ± SE of the lipids. The neutral lipids of the middle third were also compared to the posterior third with a similiar result.

The major polar lipid in the whole leech was PC. There was approximately 2.9 times more PC than PE in the whole leech sample. Although Spinedi et al.[5] found phosphatidylinositol (PI) and phosphatidylserine (PS) as major lipids in the nervous system of the *H. medicinalis*, we did not find other major phospholipid zones on our chromatograms that could have been PI and PS. Because we did not use PI and PS standards in our study, we cannot provide further information on the presence of these phospholipids in our leech samples. The polar lipid fraction of the anterior third of the leech was compared to the middle and posterior thirds. There were no significant differences (Student's *t*-test, $P > 0.05$) in the mean weight percentages ± SE of these lipids. The polar lipids of the middle third were also compared to the posterior third with a similar result. The finding of no significant differences between the regions can be attributed to the great variation in concentrations of lipids in each regional sample. There was no difference in the mean weight percent of any lipid between the segments and the whole leech. Individual samples showed marked variation in mean lipid percent, contributing to the lack of significant differences between segments and whole leeches.

LCW was analyzed quantitatively for the neutral lipids and polar lipids that leeches released into the water. Quantitative data for lipids released into the water are also shown in Table 2. For the analysis of neutral lipids, the LCW from the incubation period of 6 hr ($n = 3$) and 12 hr ($n = 4$) contained quantifiable levels of free sterols with a similar R_F value of 0.18 compared to the standard. In the 12 hr incubation, the leech released 2.6 times more free sterols than in the 6 hr incubation. Equivalent zones of steryl esters were detected in both the control and LCW samples; therefore, it could not be assumed that the leech released them into the water (N/A in Table 2). For the analysis of phospholipids, the LCW from the incubation period of 6 hr ($n = 4$) contained quantifiable levels of PC and PE with R_F values of 0.52 and 0.63, respectively. All of the 6 hr samples showed that the major phospholipids released into the water were PC and PE, but they were not present at quantifiable levels. The LCW from the 12 hr incubation period ($n = 3$) contained quantifiable levels of PC and PE. In the 12 hr sample, the PC released into the water was 1.5 times greater than the PE.

Zipser et al.[7] reported cholesterol as the main sterol in *H. medicinalis*. Our study found that steryl esters in addition to free sterols comprise significant

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Table 2. Mean concentrations of lipids in leech conditioned water (μg/mL)

Sample	Free sterols	Steryl esters	PC	PE
LCW 12 hr	0.307 ± 0.11[a]	N/A[c]	1.43 ± 0.37[a]	0.935 ± 0.37[a]
LCW 6 hr	0.118 ± 0.021[a]	N/A[c]	0.0813[b]	0.210[b]

[a]n = 3 leeches.
[b]n = 1 leech.
[c]N/A = See explanation in the text.

neutral lipid fractions in the medicinal leech. The occurrence of these neutral lipid fractions along with significant concentrations of the phospholipids PC and PE is expected because these lipids are important in the structural integrity of cells, and they comprise the major components of cell membranes.[17]

Both free fatty acids and triacylglycerols are lipids associated with depot fats in many invertebrates.[16] These lipids occurred in quantifiable amounts in our study, even though we maintained our leeches without exogenous food. Future studies on depot lipids in fed *H. medicinalis* are planned.

Rabinowitz[6] reported an unusual lipid profile in the salivary gland secretions of *H. medicinalis*, including large concentrations of phosphatidic acid and free fatty acids. He did not describe his method for obtaining saliva. Because the salivary glands occupy a considerable portion of the anterior third of *H. medicinalis*, we examined that region with the intent of determining if its lipid profile was different from that of the whole leech or the middle- or posterior-third segments. We did not find any unusual lipids in the anterior third region of the leech and suggest that the salivary gland lipids of this organism should be reexamined.

The major lipids released into the LCW were free sterols and PC. These lipids were found in high concentrations in the whole leech. *H. medicinalis* is a complex metazoan with a well-defined epidermis and numerous body openings. Therefore, it is impossible from the design of our LCW study to determine the origin of the lipids released into the water. As in other studies on invertebrates, e.g., helminths by Haseeb and Fried[18] and gastropods by Chaffee et al.,[10] lipids released into the water may serve as pheromones or carriers of pheromones to help mediate organism to organism chemoattraction. Behavioral modalities in the leech are complex and include at least tactile and visual stimuli in addition to chemical. The role of lipophilic substances released into water by *H. medicinalis* warrants further study in regard to leech-mediated chemoattraction.

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