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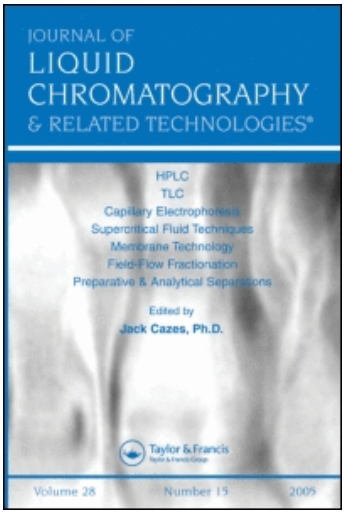

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Characterization and Quantification of the Polar Lipids in the Lizard *Uta stansburiana* by HPTLC-Densitometry

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Abstract: Silica gel high-performance thin-layer chromatography (HPTLC) with chloroform-methanol-water (65:25:4) mobile phase and cupric sulfate-phosphoric acid detection reagent was used to characterize and quantify various polar lipid classes in organs (livers, abdominal fat bodies, and tails) of 6 male and 6 female side-blotched lizards (*Uta stansburiana*) with a range of body sizes (from young-of-the-year to adult). The percentage of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin, sulfatides, and cerebrosides (C) in each organ was determined. No significant statistical differences were detected between the sexes. Across all three organs, PC was the primary polar lipid class, and the liver contained the most polar lipids. C was the most prevalent polar lipid class in the liver, PC in the tail, and PC and PE in the abdominal fat body. This is the first report of the use of TLC or HPTLC with densitometry to analyze polar lipids in lizard samples.

Keywords: High-performance thin-layer chromatography, HPTLC, Lipids, Organs, Side-blotched lizards

INTRODUCTION

Numerous studies of lipid storage and utilization in squamate reptiles (especially lizards) have been performed in a long history that extends

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well over five decades,[1–3] many of which have examined the role that fat stores play in reptilian hibernation[4–9] and reproduction.[10–14] Previous research has also compared the lipid content of various organs in diverse lizard lineages[15–18] and has consistently found that large amounts of energy are stored in the liver, abdominal fat bodies, and proximal portions of the tail. To quantify lipid content, many of the studies cited above used gravimetric techniques, while a few other studies have used gas chromatography or thin-layer chromatography (TLC) to quantify lipid classes in reptiles.[10,16–17] Within the past few decades, advancements in analytical chemistry have allowed for increasingly precise characterization and quantification of various lipid classes in animal organs, yet these most recent techniques have yet to be applied to polar lipid content in lizard organs and to comparative lizard physiology.

Previously, our laboratory investigated the neutral lipid content in the liver, abdominal fat body, and tail of the same samples used in this study by high-performance TLC (HPTLC).[19] We found that regardless of lizard sex or body size, the greatest concentrations of neutral lipids in all organs examined were triacylglycerols. Statistical analyses further revealed that the organs of females contained roughly twice the percentage of triacylglycerols compared to males. No other neutral lipid amounts were significantly different between the sexes. We also detected one significant relationship based on size: larger lizards contained more steryl esters in their livers than smaller lizards. These results are consistent with previous studies showing that females contain more energy reserves (particularly triacylglycerols) than males.

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Relatively little information is available on polar lipids in lizards; most of the work done on this topic has examined the phospholipids and sphingolipids in lung surfactant and skin of lizards. For instance, surfactant lipids were determined in several species of lizards. In these lizards, phosphatidylcholine [(PC); lecithin] was the dominant phospholipid (60 to 80%), and phosphatidylserine, phosphatidylinositol, and sphingomyelin represented the other phospholipids.[20] Relative to skin lipids, polar lipids were examined by TLC in 23 species of lizards from 13 families.[21] Most species contained PC and phosphatidylethanolamine (PE), but the bulk of the polar lipids consisted of compounds less polar than PE. Densitometric TLC studies on polar lipids in the liver, fat bodies, and tail of lizards appear not to be available. As a complement to our work on depot lipids in these organs,[19] densitometric HPTLC studies on polar lipids in these organs have now been carried out.

The purpose of this study was to examine the phospholipid and sphingolipid content in the liver, abdominal fat body, and tail of male versus female *Uta* lizards. Statistical analyses were done to examine differences between body sizes as well.

EXPERIMENTAL

Lizard Collection and Maintenance

Lizards were collected from a large population (Wright's Point) 20 km south of Burns, OR (1318 m elevation, 43.44°N Lat., 118.93°W Long.) as part of a long-term study of the life-history evolution of this species by P. Zani. All animals were collected during the fall of 2007 (6–10 October), approximately one month prior to the onset of the winter inactive period (daily high temperatures <8°C). Lizards were shipped via overnight mail to Lafayette College and maintained in 1 m diameter circular cages made of aluminum flashing. The bottom of the cage was filled with 10 cm of masonry sand. A cluster of rocks in the center of the cage provided ample retreats. Animals were provided with 40 W fluorescent shop lights suspended 0.5 m above the surface of the sand (14:10 light: dark) and 120 W mercury vapor spot lights suspended 0.4 m above one end of the cage (10:14 light: dark). This provided both light and heat. The sand temperature under the heat lamp regularly reached 40°C, while the room temperature only reached 25°C. Over the course of the fall we attempted to mimic the onset of winter by reducing both photoperiod (~15 min) and temperature (~1–2°C) each week. Lizards were fed crickets two or three times per week. However, since they were fall acclimatized, most of the animals did not eat.

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Euthanasia and Dissection

After approximately two months of captive care, 12 lizards were killed by decapitation. For each lizard the following measurements were recorded: snout-vent length (SVL) to the nearest mm using a linear ruler, mass to 0.1 g using an Acculab model PP2060D electronic balance (Fisher Educational, Pittsburgh, PA, USA), and sex as determined by presence of enlarged post-anal scales on males. The liver, abdominal fat body, and a portion of the tail were then dissected out for lipid extraction. Each organ was rinsed using a general reptile ringer solution, blotted dry, and weighed using a Mettler College 150 electronic balance (Mettler Toledo, Columbus, OH, USA). Organs were immediately frozen at −80°C to await lipid extraction, which occurred within two weeks.

Phospholipid and Sphingolipid Extraction

Tissue samples of less than 100 mg in blotted wet mass were homogenized in 2 mL of chloroform-methanol (2:1) in a 7 mL Wheaton (Millville, NJ, USA) glass homogenizer. This solvent-sample ratio assured complete extraction of the lipids.[22] The Folch wash (0.88% KCl, w/v, in deionized

water) was used in a ratio of 4 parts to 1 part salt solution to remove non-lipophilic material. Samples were vortex mixed for 30s, and the top (aqueous) layer removed and discarded. The samples were dried in a warm water bath (40–60°C) using a stream of nitrogen gas and stored at −20°C until use. To obtain appropriate densitometry scan areas within the calibration curves, tissue samples were reconstituted prior to HPTLC analysis in 1.5–10 mL of chloroform-methanol (2:1), as necessary.

HPTLC Analysis

The standard for phospholipid analysis was Polar Lipid Mix no. 1127 (Matreya Inc., Pleasant Gap, PA, USA), containing 25% each of cholesterol, PC, PE, and lysolecithin; it was dissolved in chloroform-methanol (2:1) to give a concentration of 0.125 mg mL^{-1} of each component. The standard used for the sphingolipid analysis was Matreya Sphingolipid Mix no. 1128, containing equal percentages of cerebrosides (C), sulfatides (S), and sphingomyelin (SM); it was dissolved in chloroform-methanol (2:1) to give a concentration of 0.333 mg mL^{-1} of each component.

HPTLC analysis was performed for phospholipids on 10×20 cm HPTLC-HLF silica gel plates (Analtech, Inc., Newark, DE, USA), which contained 19 scored lanes of 9 mm width and a preadsorbent (concentration zone) spotting area, and for sphingolipids on 10×20 cm No. 13728/6 silica gel plates (EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany), which were unscored and contained a preadsorbent spotting area. Before use, plates were pre-cleaned by development to the top with dichloromethane-methanol (1:1), dried with a stream of air, and activated for 30 min on a Camag (Wilmington, NC, USA) plate heater at 120°C. Standard and reconstituted sample solutions were applied in aliquots of 2.00, 4.00, 8.00, and 16.0 μL to the preadsorbent of the HPTLC plates using a 10 μL Drummond (Broomall, PA, USA) digital microdispenser.

Plate development was performed in a Camag HPTLC rectangular twin-trough chamber that contained an Analtech saturation pad in the trough opposite to the one used for development. Before development, the closed chamber was left to equilibrate for 25 min with the mobile phase. The mobile phase used was chloroform-methanol-deionized water (65:25:4) as described by Wagner et al.[23] The mobile phase development time was 18–20 min. Laboratory temperature during development was typically 21 ± 1°C, and the relative humidity was typically 57%. Developed plates were dried in a fume hood for 5 min using a stream of cool air from a hair dryer, sprayed with 10% cupric sulfate in 8% phosphoric acid, and heated on the plate heater at 140°C for 30 min until polar lipids were detected as brown-black spots on a white background.

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Quantitative densitometric analysis was done with a Camag TLC Scanner II using the deuterium light source set at 370 nm, slit width 4, slit length 4, and scanning speed 4 mm s^{-1}. The CATS-3 software automatically generated linear calibration curves (standard zone masses versus peak areas) and interpolated sample masses based on their peak areas. The percentage by mass of lipid in each tissue sample was calculated using the equation:

$$\text{percent polar lipid} = \frac{\text{W} \cdot \text{R} \cdot \text{dilution factor} \cdot 100}{\text{initial tissue sample mass } (\mu\text{g})}$$

where W = lipid mass (μg) of sample interpolated from calibration curve and R = reconstituted volume (μL)/spotted volume (μL). For samples that were diluted or concentrated to obtain bracketed scan areas within the calibration curve, an appropriate dilution factor was included in the calculation of percent polar lipid.

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Statistical Comparisons

Differences in the amount of polar lipid classes present in the different organs were compared by conducting a separate one-factor analysis of variance on each organ in which sex (male/female) was included as the factor. In addition, relationships between lipid classes in each organ and body size were evaluated by conducting regression analysis using lizard body mass. All statistical analyses were conducted using JMPv 5.1[24] for Macintosh computer.

RESULTS AND DISCUSSION

Multiple extractions with chloroform-methanol (2:1) followed by the use of the Folch procedure gave complete extraction of the lipids from the organ samples, and the extracts were pure enough for HPTLC analysis. Whatman (Florham Park, NJ, USA) silica gel LHPKDF plates with 19 scored lanes and preadsorbent were also tested, but this plate did not give as good resolution of the polar lipids as the Analtech and Merck plates for the analyses reported in this paper. The Wagner et al. mobile phase used has been shown in a comprehensive comparative study[25] to be optimum for polar lipid separations on silica gel preadsorbent plates in terms of simplicity, resolution, speed, and scan peak asymmetry. In the same study,[25] cupric sulfate-phosphoric acid was shown to be the best general polar lipid detection reagent; selective detection reagents can be used to identify certain classes of phospholipids, e.g., ninhydrin for amino lipids and α-napthal for glycolipids. The detection and quantification limits of the

cupric sulfate reagent for the analytes studied were approximately 100 ng. Although the detected zones were colored, it was found that scanning in the ultraviolet mode at 370 nm gave better results than the visible mode of the scanner.

Polar lipids were identified based upon comigration of standard zones with corresponding zones in liver, abdominal fat body, and tail samples of lizards (Figure 1). Phospholipid classes (PC and PE) were identified in all organs and samples and gave retention factor (R_F) values of 0.40 and 0.56, respectively. Both PC and PE were most prevalent in the liver, yet were found in the abdominal fat body and tail as well. These phospholipids migrated identically with the phospholipid standard. Sphingolipid classes (SM; $R_F = 0.39$, S; $R_F = 0.51$, and C; $R_F = 0.71$) were identified in the liver and tail, while only trace amounts of C was found in the abdominal fat body of males (Figure 1). Neither SM nor S were found in either male or female abdominal fat body organs. Like the phospholipids, SM, S, and C were most prevalent in the liver. These sphingolipids migrated almost identically with the sphingolipid standard.

Calibration graphs typically had very high linearity correlation coefficients ($r = 0.99$). The percentage of polar lipids in the liver, abdominal fat body, and tail of *Uta stansburiana* were determined (Table 1). Sample

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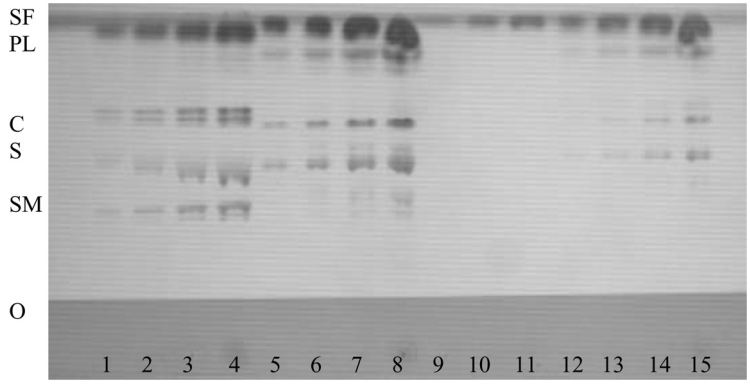

Figure 1. Chromatograms on a No. 13728/6 EMD Chemicals, Inc. HPTLC plate photographed in white light with a Camag VideoStore Documentation System showing the sphingolipid profiles of liver, fat body, and tail samples from a single lizard. Lanes 1–4 contain the sphingolipid standard spotted in 2.00, 4.00, 8.00, and 16.0 μL aliquots, respectively. Lanes 5–8 contain liver sample spotted in 2.00, 4.00, 8.00, and 16.0 μL aliquots, respectively. Lanes 9–11 contain fat body sample spotted in 4.00, 8.00, and 16.0 μL aliquots, respectively. Lanes 12–15 contain tail sample spotted in 2.00, 4.00, 8.00, and 16.0 μL aliquots, respectively. SF = solvent front, PL = polar lipids, C = cerebrosides, S = sulfatides, SM = sphingomyelin, O = origin.

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Table 1. Percent by weight (mean ± standard error of mean) of polar lipids in organs of six male and six female *Uta stansburiana* lizards

	Liver (% of organ mass)		Fat body (% of organ mass)		Tail (% of organ mass)		Sum of lipids in organs (% of total lizard mass)	
Polar lipid	Male	Female	Male	Female	Male	Female	Male	Female
Phosphatidylcholine	5 ± 1	5 ± 2	1.0 ± 0.5	0.20 ± 0.06	1.0 ± 0.3	1.4 ± 0.3	0.15 ± 0.05	0.15 ± 0.04
Phosphatidylethanolamine	1.6 ± 0.4	1.6 ± 0.6	1.0 ± 0.4	0.10 ± 0.05	0.36 ± 0.08	0.32 ± 0.07	0.05 ± 0.02	0.04 ± 0.01
Sphingomyelin	1.0 ± 0.2	1.0 ± 0.5	N.D.[a]	N.D.	0.36 ± 0.08	0.3 ± 0.1	0.04 ± 0.01	0.03 ± 0.02
Sulfatides	1.8 ± 0.6	2.4 ± 0.7	N.D.	N.D.	0.6 ± 0.1	0.7 ± 0.2	0.06 ± 0.01	0.07 ± 0.02
Cerebrosides	8 ± 1	6 ± 1	3 ± 2	N.Q.[b]	0.40 ± 0.09	0.44 ± 0.07	0.08 ± 0.04	0.11 ± 0.04

[a]N.D. = Not detected (no visible chromatographic zone).
[b]N.Q. = Below limits of quantification.

zones with scan areas below the area of the lowest standard were considered to be not quantifiable.

No statistical differences were detected between the sexes (all P values >0.10) or the variations of body size. Across all three organs PC was the primary polar lipid class, and the liver contained the most polar lipids. C was the most prevalent polar lipid class in the liver, PC in the tail, and PC and PE in the abdominal fat body (Table 1).

In summary, silica gel HPTLC is an essential and modern technique for quantifying lipid classes, and this is the first paper to employ this technique to quantify polar lipids in squamate reptiles. Polar lipids play an integral role in the study of lipid storage and utilization, and they remain an important aspect to our understanding of physiological and life-historical variation.

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