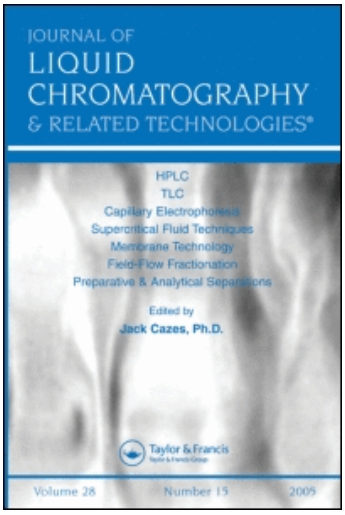

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HPTLC DETERMINATION OF AMINO ACIDS IN SNAIL-CONDITIONED WATER FROM *BIOMPHALARIA GLABRATA*, TWO STRAINS OF *HELISOMA TRIVOLVIS*, AND *LYMNAEA ELODES*

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ABSTRACT

High performance thin-layer chromatography (HPTLC) was used to analyze amino acids in water conditioned by *Biomphalaria glabrata,* a Pennsylvania and Colorado strain of *Helisoma trivolvis,* and *Lymnaea elodes.* The snail-conditioned water (SCW) samples were dried with air and reconstituted in 10% n-propanol and then applied to cellulose HPTLC plates and developed with n-propanol-water (7:3). Amino acids were detected with ninhydrin reagent and the resulting colored sample zones were compared to known standards. The amino acids present in SCW were identified as alanine, aspartic acid, leucine, phenylalanine, serine, tryptophan, and valine along with one unidentified ninhydrin positive zone. The valine zone was quantified by densitometric scanning at 610 nm, and the concentrations ranged from 1.3-2.6 $\mu g\ mL^{-1}\ snail^{-1}$ in the SCW for all four strains of snails. There were no significant differences in the concentrations of valine in water conditioned by the four strains of snails based on Student's *t*-test.

INTRODUCTION

Gastropods release substances into water that serve as chemoattractants for larval trematodes and conspecific snails.[1,2] These substances have been termed snail-conditioned water (SCW) and include urine, feces, mucous, gametes, and other products released by snails. There have been relatively few studies on the chemical components of SCW. Previous studies have analyzed the presence of neutral lipids in SCW from two strains of *Helisoma trivolvis*[3] and phospholipids from the same two strains of *H. trivolvis* and also *Biomphalaria glabrata.*[4] Chaffe et al.[5] examined neutral lipids in SCW from *B. glabrata.* These studies used HPTLC to analyze the following neutral lipids and phospholipids in SCW: free fatty acids, free sterols, hydrocarbons, methyl esters, cholesteryl esters, phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, and several unidentified components.[3,4,5] Because amino acids have been implicated as chemoattractants of larval trematodes,[6] a thin-layer chromatography study was initiated to identify and quantify these acids in the SCW of *B. glabrata,* a Pennsylvania and Colorado strain of *H. trivolvis*, and *Lymnaea elodes.* Quantification of valine in each of the samples was completed.

EXPERIMENTAL

Maintenance of Snails and Preparation of Samples

Stock cultures of both strains of *H. trivolvis, B. glabrata,* and *L. elodes* were maintained at 22-23°C in a chemically defined artificial spring water.[7] The cultures were maintained under diffuse overhead fluorescent light for 12 hr a day. The snails were fed boiled leaf lettuce with an occasional supplement of Tetramin tropical fish food.[5]

To obtain the SCW samples from both strains of *H. trivolvis* and *B. glabrata*, six snails with shell diameters of 8-15 mm were placed in a 20 mL beaker containing 2.0 mL of purified Milli-Q water (Millipore Co., Bedford, MA) for 4 hr. To obtain the SCW from *L. elodes*, six snails with shell lengths of 17-26 mm were placed in a 20 mL beaker containing Milli-Q water (2.0 mL) for 4 hr. The snails were removed with forceps and rinsed with Milli-Q water (ca 90 μL). Sample and rinse water were combined and then transferred to a sample vial with a Pasteur pipet. The samples were dried with a stream of air in a water bath at 50-60°C. The amino acid residue was reconstituted with 50.0 μL of 10% n-propanol.

Preparation of Standards

The amino acid standards (98% minimum TLC purity) were obtained from Sigma Chemical Co. (St. Louis, MO). Individual standard solutions of alanine, arginine, asparagine, aspartic acid, citrulline, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, and valine were prepared at a concentration of 0.100 μg/μL in 10% n-propanol for qualitative analysis. The valine stock solution was diluted 1:11 to yield a concentration of 0.091 μg/μL for quantitative analysis.

TLC Analysis

TLC analysis was performed on HPTLC cellulose plates (Merck, 10 x 20 cm, catalog no. 15035). For qualitative analysis, 1.00 μL of each amino acid standard solution and 3.00 μL of each reconstituted sample were spotted, 2.0 cm from the bottom of the plate and 1.0 cm apart, by means of a 10 μL Drummond (Broomall, PA) digital microdispenser. The spots were dried with a hair drier.

The plate was developed in a glass, paper-lined, Camag (Wilmington, NC) twin-trough, solvent equilibrated, HPTLC chamber with n-propanol-water (7:3) to a distance of 7.5 cm past the origin. The plate was air dried in the fumehood, sprayed with ninhydrin detection reagent (0.3 g ninhydrin in 100 mL of n-butanol and 3 mL of glacial acetic acid), air dried for 10 sec, resprayed, and heated in a 110°C oven for 10 min.

For quantitative analysis of valine, 1.00, 2.00, 4.00, 6.00, and 8.00 μL of the 0.091 μg/μL standard solution and 5.00 μL of the reconstituted sample were spotted. After development and detection, the zones were scanned in the single beam, reflection mode at 610 nm using a Shimadzu (Columbia, MD) Model 930 densitometer.

A calibration curve was generated from the scan areas and weights of the standard zones using a PC linear regression program, and the weight of valine in the sample zone was obtained by interpolation from the calibration curve. The amount of valine in the original SCW sample was calculated from the following equation:

$$\text{weight of valine in 2.00 mL SCW} = (W \times R)/S$$

where W is the interpolated weight of valine in the sample from the calibration curve (μg), R is the reconstitution volume of the sample (50.0 μL), and S is the volume of sample spotted (5.00 μL). The linearity correlation (r) values for calibration curves determined on each plate used for quantitative analysis of samples ranged from 0.987 to 0.994.

RESULTS AND DISCUSSION

Preliminary tests of silica gel, chemically bonded reversed phase, and cellulose layers[8] indicated that cellulose layers gave the best resolution of the amino acids in SCW. Therefore, all subsequent analyses were done on cellulose layers.

Qualitative identification was accomplished by comparing the hR_f ($R_f \times 100$) values of the samples and standards and the ninhydrin colors of the detected zones. TLC analysis revealed seven identified amino acids and one unidentified ninhydrin-positive zone in both strains of *H. trivolvis* and in *B. glabrata*. The *L. elodes* samples contained six identified amino acids and one unidentified ninhydrin-positive zone. Table 1 summarizes the amino acids identified, the hR_f values, and the zone colors.

Visual observations of zone intensities for all of the samples showed leucine, valine, and aspartic acid to have the darkest colors, indicating that these amino acids had higher concentrations than the others. Of these three major amino acids, valine was the only one quantified. The valine zone could be scanned reliably because it was compact and well resolved from the other amino acids, and its hR_f value was in the optimum range at the center of the chromatogram.

The data for valine demonstrate the ability of the HPTLC system to provide precise quantitative analysis of amino acids present in SCW. Quantification of other amino acids could be done by determining a calibration curve for each amino acid, i.e., by scanning a comparable series of standard zones and adjusting the reconstitution and spotted volumes of the samples so that the scan area of the analyte zone in the sample chromatogram was bracketed by that of the standards.

The concentrations of valine ($\mu g\ mL^{-1}\ snail^{-1}$) in the SCW based on incubation of six snails for four hours in 2.0 mL of Milli-Q water ($n = 4$ for each sample) were as follow: 1.9 ± 0.35 for *B. glabrata*, 2.1 ± 0.48 for the CO

Table 1

hR_f Values of the Amino Acids and Color of Zones Detected with Ninhydrin in the Four Strains of Snails Analyzed

Amino Acid	hR_f	Color
Leucine	69	purple
Phenylalanine*	64	light blue
Valine	58	purple/orange
Tryptophan	51	purple
Alanine	40	purple
Serine	29	purple
Aspartic acid	21	purple
Unknown	7	purple

* Phenylalanine was not detected in *Lymnaea elodes.*

strain of *H. trivolvis,* 1.31 ± 0.095 for the PA strain of *H. trivolvis*, and 2.6 ± 0.45 for *L. elodes.* On the basis of Student's *t*-test (with $P<0.05$ being considered significant), there were no significant differences in the concentrations of valine in water conditioned by the four strains.

Several studies on amino acid analyses in snail hemolymph and the digestive gland-gonad complex of *B. glabrata* indicated the presence of approximately 18 amino acids in these samples.[8] This study is the first to report qualitative and quantitative data on the presence of amino acids in SCW from any snail. Of the seven amino acids detected in SCW in this study, five (alanine, aspartic acid, leucine, serine, and valine) were tested previously in agar plug assays and served as chemoattractants for the larval (cercarial) stages of *Echinostoma trivolvis* and *E. caproni.*[9]

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