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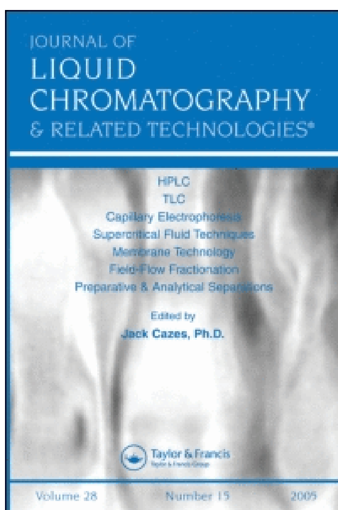

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Determination and Quantification of Amino Acids in the Urine of BALB/c Mice Infected with *Echinostoma caproni* by High-Performance Thin-Layer Chromatography-Densitometry

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Abstract: High-performance thin-layer chromatography was used to examine the amino acid profile of the urine of *Echinostoma caproni* infected mice versus that of uninfected controls from 1–35 days post infection. Amino acids were extracted from urine with ethanol-water (70:30) and determined on silica gel and cellulose layers developed with either 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26) or 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26). Separated zones were detected by postchromatographic derivitization with ninhydrin and quantified by visible mode slit-scanning densitometry at 610 nm. Taurine, alanine, threonine, and lysine were identified in mouse urine sample chromatograms. A comparison of chromatograms from the urine of infected and uninfected mice showed both qualitative and quantitative differences in alanine and taurine suggesting that these compounds may serve as biological markers for infections of mice with this trematode.

Keywords: Alanine, Amino acids, *Echinostoma caproni*, HPTLC, High-performance thin-layer chromatography, Mice, Taurine, Urine

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INTRODUCTION

Trematodiasis, a neglected disease caused by various species of trematodes, is a world-wide problem prevalent in both developed and under-developed countries. Trematodiasis can occur in human and other vertebrate hosts by cercarial penetration of the skin, or by the ingestion of tainted food products such as fish, frogs, snakes, mollusks, other invertebrates, and vegetation that are infected with the metacercarial cyst stage of the trematode. The most common diagnostic method for such diseases is the fecal smear, which uses light microscopy to detect characteristic trematode eggs in the feces of infected hosts. However, this technique is tedious and time-consuming and requires considerable skill on the part of the examiner. Improvements in the diagnosis of trematodiasis can be made by the use of metabolic profiling (also known as metabonomics or metabolomics), which uses a variety of analytical methods to characterize the metabolic phenotypes of healthy versus diseased individuals.[1]

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Metabolic profiling is best applied to trematode-mouse models that can simulate the infection in humans. One such model uses the trematode *Schistosoma mansoni*, which causes schistosomiasis in human and murine hosts. This trematode, although not an intestinal digenean, causes intestinal distress because of its residence in the mesenteric veins of humans and mice. Using nuclear magnetic resonance (NMR) spectrometry to study the *S. mansoni*-mouse model, Wang et al.[2] found a reduced level of certain tricarboxylic acid cycle intermediates in the urine of infected mice relative to the uninfected hosts. Also using NMR spectrometry, Li et al.[3] reported global metabolic changes in the amino acid, neutral lipid, and polar lipid content of a variety of mouse tissues, including the intestine, kidney, spleen, and liver correlated with *S. mansoni* infection.

Another trematode-mouse model uses *Echinostoma caproni*, an easy to maintain intestinal digenean.[4] Using NMR spectrometry to study the *E. caproni*-mouse model, Saric et al.[1,5] reported a variety of potential biological markers in the feces, urine, plasma, kidney, liver, and spleen, the most significant of which were 2-ketoisocaproate, hippurate, taurine, trimethylamine, *p*-cresol glucuronide, trimethylamine-*N*-oxide, and phenylacetylglycine in urine; propionate, butyrate, acetate, and 5-aminovalerate in feces; alanine, glutamate, aspartate, *scyllo*-inositol, and glycerophosphocholine in the renal cortex; and phosphocholine and betaine in the liver of infected mice.

Using high-performance thin-layer chromatography (HPTLC) and inductively coupled plasma atomic emission spectrometry to study the *E. caproni*-mouse model, metabolic changes in the neutral lipid profile of infected mouse feces,[6,7] urine,[8] intestinal mucosa,[9,10] and serum;[10] the polar lipid profile of the intestinal mucosa;[11] and the metallic ion

profile of the intestinal mucosa[12] have been determined. The purpose of this study is to use our recently optimized HPTLC systems for the determination of amino acids (AAs)[13] to confirm the presence of the urinary amino acid biological markers described by Saric et al.[1,5] using the *E. caproni*-mouse model.

EXPERIMENTAL

Mouse Maintenance and Infection

Mice were maintained as described in Bandstra et al.[4] Male BALB/c mice (age six to eight weeks) were used as hosts for *E. caproni* infection. Six mice were exposed individually per os to 75 ± 25 metacercarial cysts of *E. caproni* and identified by a unique ear punch pattern. Infected mice were housed in a plastic mouse cage ($15 \times 27 \times 14$ cm) and fed Mazuri rodent food (PMI Nutrition, Henderson, CO, USA) and water ad libitum. Confirmation by light microscopy of patent infections was made on all six exposed mice by fecal smears and recognition of characteristic *E. caproni* eggs at 2 weeks post infection (PI).[1] Six mice comprising a control group were handled and treated identically except they were not exposed to *E. caproni* metacercariae and thus remained parasite free throughout the study.

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Sample Collection and Preparation

The method of urine collection employed in this study was that of Vasta et al.[8] Urine samples were collected at 1, 2, 3, 5, 7, 8, 9, 12, 13, 14, 15, 19, 20, 21, 23, 26, 27, 28, 29 33, 34, and 35 days post infection (DPI), data points that include many significant events in the developmental biology of *E. caproni* in the mouse host.[1,5] For each collection, urine was taken from every mouse and combined to make two pools, one from infected mice and one from control mice, ranging in volume from 300 to 900 μL. Urine pools were stored at 2°C in a refrigerator until use within 48 hours of collection. Three 50 μL aliquots were removed from each pool to prepare three replicate samples of infected and control urine for each day.

Each 50 μL sample was placed into a microcentrifuge tube with 550 μL of ethanol-water (70:30) and centrifuged at 8,000 g for 5 min. The supernatant was quantitatively transferred to a glass vial and evaporated under a stream of air in a 60°C water bath. Sample residues were reconstituted in 800 μL of ethanol-water (70:30) and stored at 2°C prior to HPTLC analysis. Following this initial reconstitution, samples were diluted or concentrated as necessary for quantification of specific AAs.

Thin Layer Chromatography

HPTLC methodology for the determination of amino acids was adapted from Vasta et al.[13] Standards of alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), citrulline (Cit), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val) were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions of each acid were prepared in ethanol-water (70:30) at a concentration of $1.00\,\mu g\,\mu L^{-1}$. Amino acid HPTLC standards were prepared at concentrations of 0.025 and $0.100\,\mu g\ \mu L^{-1}$ for each acid by dilution of the appropriate stock solution with ethanol-water (70:30).

Both silica gel and cellulose layers were employed for the identification and quantification of the AAs in mouse urine. The silica gel layer was 20×10 cm HPTLC silica gel with concentration zone (CZ), no. 13728-6 (EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany) and the cellulose layer was 20×10 cm HPTLC cellulose F, no. 15036-6 (EMD Chemicals, Inc.). All layers were precleaned by development to the top with dichloromethane-methanol (1:1) and dried before use. In qualitative AA determinations and spiking analyses, the above layers were developed with either of the following two mobile phases: 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26; mobile phase A) or 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26; mobile phase B). Quantification of Tau was performed on the silica gel layer developed with mobile phase A and quantification of Ala was performed on the silica gel layer developed with mobile phase B.

In qualitiative AA determinations, 2.00–8.00 μL aliquots of HPTLC standard or reconstituted sample were applied to the layer (cellulose) or CZ (silica gel) using a Linomat IV (Camag, Wilmington, NC, USA) fitted with a 100 uL syringe and operated with the following settings: band length 6 mm, application rate $15\,s\,uL^{-1}$, table speed $10\,mm\ s^{-1}$, distance between bands 4 mm, distance from plate side edge 7 mm, and distance from the bottom of the plate 1.0 cm. Plates were developed with the appropriate mobile phase in a solvent vapor equilibrated Camag HPLTC twin-trough chamber with a saturation pad (Analtech, Newark, DE, USA) for a distance of 7 cm beyond the origin. Development required about 1 h for silica gel layers and about 2 h for cellulose layers. Plates were air dried in a fumehood, sprayed to saturation with ninhydrin solution (0.3 g ninhydrin in 100 mL of *n*-butanol plus 3 mL of glacial acetic acid), and heated on a Camag plate heater at 110°C for 10 min to detect the amino acids as spots of various colors on a white to pink background.

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Spiking analyses using the over-spotting technique[14] were used to confirm the identities of certain ninhydrin-positive zones and test the susceptibility of the HPTLC method to matrix effects. For each AA determined by spiking, 4.00–6.00 μL aliqouts of HPTLC standard of the AA was spotted, followed by two replicate 4.00 μL aliquots of one reconstituted urine sample. Then, 4.00–6.00 μL of TLC standard was spotted over the second replicate aliquot of reconstituted urine sample. Plate development and postchromatographic derivatization were performed identically as described above for qualitative determinations.

In quantitative AA determinations, HPTLC standards of Tau (0.100 $\mu g\,\mu L^{-1}$) and Ala (0.025 $\mu g\,\mu L^{-1}$) were spotted in 2.00, 4.00, 6.00, and 8.00 μL aliquots (200, 400, 600, and 800 ng for Tau and 50, 100, 150, and 200 ng for Ala) to create a calibration curve, followed by 3.00 μL of reconstituted sample for Tau and 4.00–8.00 μL of reconstituted sample (eight times concentrated compared to the initial reconstitution) for Ala chosen so that the scan areas of Tau and Ala zones in sample chromatograms would be bracketed within the standard areas of the calibration curve. The limit of quantification (LOQ) for Tau was 200 ng and the LOQ for Ala was 50 ng. The areas of the bands were measured using a Camag TLC Scanner II with the tungsten light source set at 610 nm. The settings were slit width 4, slit length 4, and scanning speed 4 mm s^{-1}. The CATS-3 software was used to create a linear regression calibration curve relating the weights of the standard zones to their peak areas. The correlation coefficient (r-value) for each calibration curve was at least 0.99 in all analyses.

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The equation used for calculation of the concentration ($\mu g\,\mu L^{-1}$) of AA in urine was

$$\mu g \ \mu L^{-1} = (w * R/1000)/(V)$$

where w = ng interpolated from calibration curve, R = [reconstitution volume (μL)]/[spotted volume (μL)], and V = urine volume (μL). If a dilution or concentration step was required in the quantification, the final concentration calculated using the above equation was modified by multiplication using a dilution factor (DF), where the DF was calculated by dividing the original sample volume by the diluted or concentrated sample volume.

Statistical Significance and Trend Analysis

Microsoft EXCEL's version of the Student's *t*-test (two-tailed) was used to determine the statistical significance of the quantitative data based on the mean ± standard deviation of the concentration of lipids in mouse urine samples, with $P < 0.05$ considered significant.

To determine trends in biomarker excretion over the course of the infection, the concentration data for each metabolite (both control and infected) was first smoothed using a centered moving average method[15] where each data point was averaged with four adjacent data points (two on either side). The first two and last two data points were used without smoothing because of the lack of the appropriate number of adjacent data points. After smoothing, the difference between the metabolite concentration for the infected and control samples was calculated, and the resulting differences were plotted graphically against the course of infection (DPI).

RESULTS AND DISCUSSION

E. caproni Infection

Necropsies of infected mice were performed at 36 DPI. Worm recovery (mean $\pm$ standard deviation) was 22 ± 16 worms per mouse, which represents a mean recovery of 29% compared to the initial inoculum. This recovery was well within the general range of 20–80% for *E. caproni* reported from various strains of mice.[4]

Qualitative Determinations

Four HPTLC systems were used in the determination of the AAs in mouse urine. AAs were identified in sample chromatograms based upon comigration of their zones with corresponding standard zones. In all four HPTLC systems, a matrix effect was discovered that caused a slight retardation of most ninhydrin-positive zones in samples that were eight times concentrated compared to the initial reconstitution. Therefore, spiking (described above) was used to determine AAs that became detectable at the aforementioned sample concentration. Table 1 provides identification data for all AAs detected in urine based upon comigration, color similarity, and spiking analyses.

Tau was found to be the principal ninhydrin-positive compound (NPC) in mouse urine and was immediately detectable at the initial reconstitution volume. All other AAs and unidentified NPCs (UNPCs) were considerably below the LOQs found in this study, and they required concentration of the sample before identification and quantification (if applicable) could be achieved. Our identifications of Tau and Ala in urine agree with the findings of Saric et al,[5] who also reported Tau and Ala in ICR mouse urine. However, we also report in this study the presence of Lys and Thr in mouse urine. This discrepancy in our findings versus those of Saric et al.[5] is possibly due to both differences in the sensitivities of

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Table 1. hR_F[a] values for the amino acids determined in mouse urine

Amino acid	System 1[b] retention values	System 2[c] retention values	System 3[d] retention values	System 4[e] retention values
Ala	26	35	34	22
Lys	15	15	15	3
Tau	29	40	36	37
Thr	27	38	32	31

[a] $hR_F = R_F \times 100$.
[b] System 1 = cellulose layer developed with mobile phase A.
[c] System 2 = cellulose layer developed with mobile phase B.
[d] System 3 = silica gel layer developed with mobile phase A.
[e] System 4 = silica gel layer developed with mobile phase B.

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the analytical methods used in biomarker discovery (NMR spectrometry versus HPTLC) and to the different strains of mice used (ICR versus BALB/c).

In addition to the AAs identified, urine sample chromatograms contained a variety of additional UNPCs (Table 2). Because these bands were not identifiable among the amino acids used in this study, they were not further characterized.

Qualitative and Time Course Analyses

Among the AAs that were identified in mouse urine, only Tau and Ala were separated enough from surrounding zones for quantification.

Table 2. hR_F values of additional, unidentified, ninhydrin-positive zones found in mouse urine in each HPTLC system[a]

System 1 retention values	System 2 retention values	System 3 retention values	System 4 retention values
9	3	19	0
12	6	23	6
19	12	63	11
33	21		15
40	25		43
50	28		45
62	31		52
68	54		55
	60		66
	63		

[a] See Table 1 for definitions of the systems.

Table 3 presents concentration ($\mu g\ \mu L^{-1}$) data for Tau and Ala quantified using the silica gel layer developed with mobile phase A and B, respectively. These data show that the Tau concentration in urine was significantly increased on days 1, 2, 3, 15, and 33 PI and significantly decreased on days 12, 14, 19, 20, 23, 27, 28, and 29 PI, and that the Ala concentration was significantly increased on days 7, 8, 9, 12, 15, and 21 PI, and significantly decreased on days 1, 2, 19, 20, 27, 28, 29, and 34 PI. Figure 1 provides chromatograms displaying the typical amino acid profile of infected and uninfected BALB/c mouse urine.

Figure 2 provides a time course analysis of the effects of the *E. caproni* infection on the excretion of urinary Tau and Ala. Figure 2 shows that the concentration of Tau in infected mouse urine was higher

Table 3. Concentration (mean ± standard deviation) in $\mu g\ \mu L^{-1}$ of alanine and taurine in the urine of BALB/c mice infected with *E. caproni* at days 1 to 35 PI

	Alanine		Taurine	
Day PI	Uninfected	Infected	Uninfected	Infected
1	0.077 ± 0.005	0.05 ± 0.01[b]	2.9 ± 0.3	3.5 ± 0.2[a]
2	0.059 ± 0.003	0.041 ± 0.002[b]	3.9 ± 0.3	4.7 ± 0.2[a]
3	0.054 ± 0.001	0.054 ± 0.001	3.3 ± 0.2	5.9 ± 0.3[a]
5	0.035 ± 0.006	0.027 ± 0.003	3.9 ± 0.2	4 ± 1
7	0.033 ± 0.003	0.0632 ± 0.0004[a]	6 ± 1	5.9 ± 0.1
8	0.06 ± 0.01	0.094 ± 0.003[a]	3.2 ± 0.4	6 ± 2
9	0.063 ± 0.007	0.118 ± 0.003[a]	3.8 ± 0.6	5.3 ± 0.1
12	0.075 ± 0.007	0.092 ± 0.005[a]	3.1 ± 0.4	5.2 ± 0.3[b]
13	0.066 ± 0.003	0.071 ± 0.004	3.7 ± 0.5	4.1 ± 0.4
14	0.064 ± 0.001	0.063 ± 0.004	5.2 ± 0.3	3.8 ± 0.5[b]
15	0.0558 ± 0.0007	0.0600 ± 0.0001[a]	3.81 ± 0.08	4.3 ± 0.1[a]
19	0.067 ± 0.004	0.0508 ± 0.0006[b]	4.5 ± 0.4	3.3 ± 0.4[b]
20	0.080 ± 0.002	0.0508 ± 0.0007[b]	5.3 ± 0.1	2.8 ± 0.1[b]
21	0.059 ± 0.001	0.075 ± 0.003[a]	4.6 ± 0.4	4.4 ± 0.2
23	0.078 ± 0.003	0.073 ± 0.005	5.91 ± 0.01	4.87 ± 0.08[b]
26	0.043 ± 0.002	0.041 ± 0.004	4.8 ± 0.4	4.6 ± 0.1
27	0.033 ± 0.001	0.0275 ± 0.0004[b]	4.12 ± 0.02	3.2 ± 0.1[b]
28	0.064 ± 0.004	0.049 ± 0.006[b]	4.8 ± 0.01	3.8 ± 0.2[b]
29	0.055 ± 0.002	0.033 ± 0.002[b]	4.7 ± 0.3	3.2 ± 0.1[b]
33	0.061 ± 0.001	0.0608 ± 0.0004	4.3 ± 0.2	5.9 ± 0.2[a]
34	0.068 ± 0.001	0.0626 ± 0.0006[b]	5.0 ± 0.7	5.5 ± 0.4
35	0.0743 ± 0.0007	0.076 ± 0.008	4.4 ± 0.3	4.7 ± 0.2

[a]Value significantly increased compared to uninfected samples (Student's *t*-test, $P < 0.05$).
[b]Value significantly decreased compared to uninfected samples (Student's *t*-test, $P < 0.05$).

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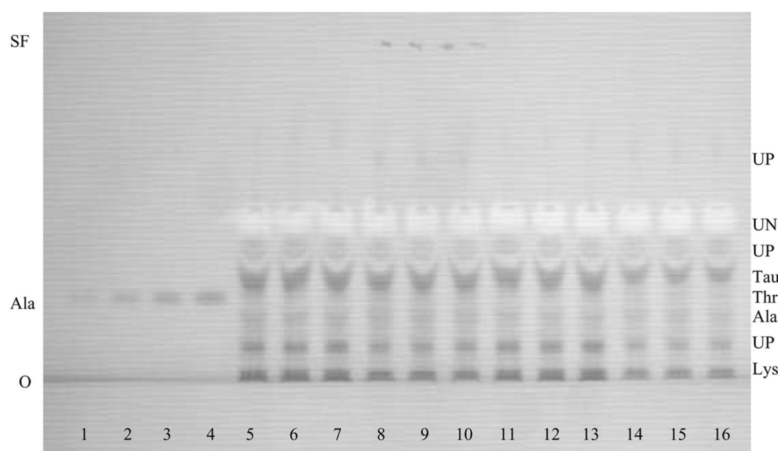

Figure 1. Chromatograms on a silica gel HPTLC plate developed with 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26) photographed in white light with a Camag VideoStore documentation system showing the typical amino acid profile of BALB/c mouse urine on days 26 and 27 PI. Lanes 1–4 contain alanine HPTLC standard solution spotted in 2.00, 4.00, 6.00, and 8.00 µL aliquots, respectively. Lanes 5–7 and 8–10 contain reconstituted urine samples from control and infected hosts, respectively, at 26 DPI. Lanes 11–13 and 14–16 contain reconstituted urine samples from control and infected hosts, respectively, at 27 DPI. Retardation of the alanine zone in sample chromatograms compared to its zone in the pure HPTLC standard was caused by a matrix effect that occurred at high sample concentrations. Taurine and alanine were found to be significantly decreased ($P < 0.05$) in infected compared to uninfected samples at 27 DPI. SF = solvent front, Ala = alanine, Tau = taurine, Lys = lysine, UP = unidentified ninhydrin-positive compound, UN = unidentified ninhydrin-negative compound, O = origin.

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than in uninfected urine starting at day 1 PI. This increase in Tau in infected host urine reached a maximum at approximately 10 DPI, after which its level began to decrease rapidly. By 14 DPI, Tau excretion in infected hosts reached levels similar to that of the uninfected mouse urine. By 15 DPI, Tau excretion in infected hosts reached a minimum compared to that of the uninfected controls, after which these minimum levels stabilized. At approximately 25 DPI, Tau excretion began to rise again to levels comparable to that of the control mice.

Figure 2 also shows that the concentration of Ala in infected mouse urine was significantly decreased at days 1 and 2 PI. However, Ala excretion in infected hosts began to rise rapidly at 3 DPI, quickly exceeding that of the control hosts and reaching a maximum at approximately 10 DPI. After 10 DPI, Ala levels decreased rapidly, reaching similar levels to that of the control hosts by 14 DPI and stabilizing at levels slightly

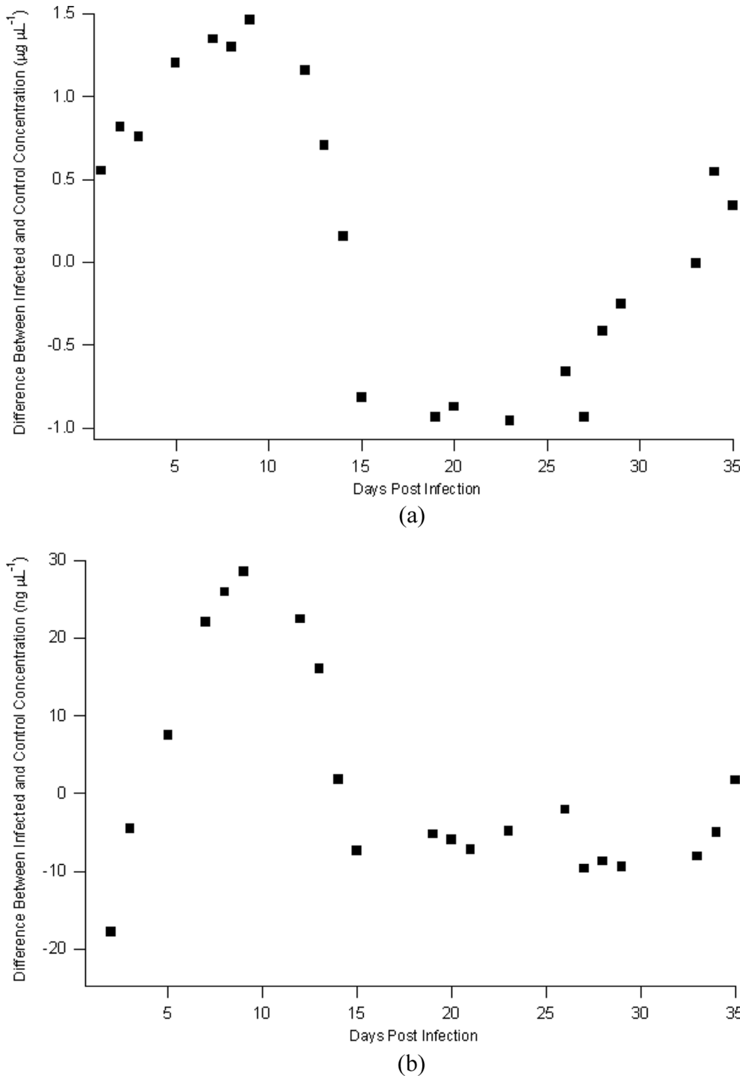

Figure 2. Effect of *E. caproni* infection on the urinary excretion of taurine (a) and alanine (b) from BALB/c mice over the course of the infection.

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below that of the control hosts by 15 DPI. Ala excretion in infected mouse urine remained slightly below that of the controls from 15 DPI until approximately 33 DPI when excretion began to return to levels comparable to the excretion in controls.

Saric et al.[5] also reported significant decreases in urinary Tau excretion in ICR mice infected with *E. caproni* that occurred to the highest

degree at 19 DPI. Tau is known to be involved in primary bile salt formation with cholic and chenodeoxycholic acid. Saric et al.[5] speculated that these decreases in Tau excretion most likely resulted from the higher demand for lipid digestion due to intestinal damage by adult *E. caproni* worms;[5] these worms attach to the intestinal wall by means of the acetabulum.[16] Our findings agree with those of Saric et al. in that we also observed significant decreases in urinary Tau in infected hosts around 19 DPI. However, our findings do not agree with those of Saric et al. with respect to the significant increases in urinary Tau and Ala excretion during the first two weeks PI. This discrepancy is likely attributable to both differences in the sensitivities of the analytical methods used in biomarker discovery (NMR spectrometry versus HPTLC) and possibly to the different strains of mice used (ICR versus BALB/c).

It has been speculated previously[1] that many of the discovered biological markers of *E. caproni* infection could be related to changes in enteric bacterial activity or composition, especially because many of the discovered biological markers, such as *p*-cresol glucuronide, 2-ketoisocaproate, acetate, hippurate, phenylacetylglycine, and trimethylamine, are compounds synthesized by the enteric microbial flora. It is possible that the metabolic changes in urinary Tau and Ala excretion that we observed in urine could also be related to disturbances in the enteric bacteria caused by the presence of juvenile and adult worms of *E. caproni*. Indeed, the significant increases in the excretion of these two metabolites occurred mainly during the first two weeks of the infection, a time when *E. caproni* juvenile worms are achieving sexual maturity and preparing for egg production beginning at about 9–12 DPI.[16] Parasite development and egg release during this time could have a greater affect on the enteric bacteria than that of the older worms recovered beyond two weeks PI; this could possibly help to explain the rapid decline in both Tau and Ala metabolism from 10–14 DPI.

The changes observed in Ala and Tau excretion could also possibly be linked to the metabolism of enteric bacteria, especially during the first 14 DPI with *E. caproni*. *Bilophila wadsworthia*, a gram-negative anaerobic bacterium, is known to inhabit the lower gastrointestinal tract of mice.[17] Excess Tau is eliminated by *B. wadsworthia* by conversion to ammonia, acetate, and sulfide through a metabolic pathway that begins with taurine-pyruvate amino transferase.[18] This enzyme is responsible for the transfer of an amino group from taurine to pyruvate, yielding the products Ala and sulfoacetaldehyde. We speculate that increased Tau levels in the mouse host, such as those that occurred during the first three days of *E. caproni* infection, could have resulted in the production of Ala though the previous metabolic pathway, which would explain the apparent similarities in the trends observed for Ala and Tau in infected host urine during the first 15 DPI. However, such speculations need to be tested experimentally.

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CONCLUSIONS

Worm infections of about 22 per host induced significant changes in Tau and Ala excretion in the urine of BALB/c mice infected with *E. caproni* from 1–35 DPI. Thus, examination of the AA profile of urine by HPTLC may be helpful as a screen to supplement fecal examinations to detect echinostome infections in mice. These findings agree with those of Saric et al,[5] who also reported that Tau may serve as a urinary biological marker for mice infected with *E. caproni*.

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