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Claire B. Andreasen, *Oregon State University*
Erwin G. Pearson, *Oregon State University*
Brad B. Smith, *Oregon State University*
Terry C. Gerros, *Oregon State University*
Duane Lassen, *Oregon State University*

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What is This?

Normal reference intervals and the effects of time and feeding on serum bile acid concentrations in llamas

Claire B. Andreasen, Erwin G. Pearson, Brad B. Smith, Terry C. Gerros, E. Duane Lassen

Abstract. Fifty clinically healthy llamas, 0.5–13 years of age (22 intact males, 10 neutered males, 18 females), with no biochemical evidence of liver disease or hematologic abnormalities, were selected to establish serum bile acid reference intervals. Serum samples submitted to the clinical pathology laboratory were analyzed using a colorimetric enzymatic assay to establish bile acid reference intervals. A nonparametric distribution of llama bile acid concentrations was 1–23 µmol/liter for llamas >1 year of age and 10–44 µmol/liter for llamas ≤1 year of age. A significant difference was found between these 2 age groups. No correlation was detected between gender and bile acid concentrations. The reference intervals were 1.1–22.9 µmol/liter for llamas >1 year of age and 1.8–49.8 µmol/liter for llamas ≤1 year of age. Additionally, a separate group of 10 healthy adult llamas (5 males, 5 females, 5–11 years of age) without biochemical or hematologic abnormalities was selected to assess the effects of feeding and time intervals on serum bile acid concentrations. These 10 llamas were provided fresh water and hay ad libitum, and serum samples were obtained via an indwelling jugular catheter hourly for 11 hours. Llamas were then kept from food overnight (12 hours), and subsequent samples were taken prior to feeding (fasting baseline time, 23 hours after trial initiation) and postprandially at 0.5, 1, 2, 4, and 8 hours. In feeding trials, there was no consistent interaction between bile acid concentrations and time, feeding, or 12-hour fasting. Prior feeding or time of day did not result in serum bile acid concentrations outside the reference interval, but concentrations from individual llamas varied within this interval over time.

Bile acids have been used to evaluate enterohepatic circulation and, therefore, functional hepatic mass, in several species, including dogs,[3] cats,[4] cattle,[6,11] sheep,[1,13] goats,[8] and horses.[2,9,10,16] Primary bile acids are synthesized from cholesterol in the liver, conjugated, and then excreted in bile. Some conversion of unabsorbed primary bile acids to secondary bile acids occurs by intestinal bacteria. Conjugated bile acids form micelles with fat, are reabsorbed in the ileum, and are carried via portal circulation to the liver for hepatic uptake and continuation of the cycle. From 90% to 95% of bile acids are removed by the liver via the portal system, with a small concentration of bile acids detectable in serum or plasma. This cycle provides a maximum concentration of bile acids in the proximal small intestine where fat digestion and absorption occurs.[14] In monogastric carnivores with gallbladders, such as dogs and cats, there is a postprandial increase in serum bile acid concentrations with a defined narrow normal reference interval.[3,4] Increases in serum bile acid concentrations above reference intervals are a sensitive indicator of liver disease but do not define a specific hepatopathy. Bile acid concentra-

tions may be decreased as a result of ileal malabsorption.[14]

Bile acid concentrations have been utilized to a lesser degree in the evaluation of ruminant hepatobiliary disease based on individual variation in bile acid values. Although this variation broadens the reference interval, values above the reference limit are useful for the diagnosis of hepatobiliary disease. Bile acid concentrations have been evaluated in herbivores with gallbladders, such as cattle. There is a continual flow of ingesta from the abomasum into the duodenum, with periodic interruptions, that stimulates a similar pattern in the flow of bile acids.[11] These interruptions result in variability of serum bile concentrations by 60 µmol/liter between hourly samples.

Because horses do not have gallbladders and the sphincter of the common bile duct is apparently weak, continuous secretion of bile into the intestine has been documented, and enterohepatic circulation occurs approximately 38 times/day.[2,10] There is no statistical difference in bile acid concentration between ponies and horses, and no effect of feeding on serum bile acid concentrations was seen after a 14-hour fast.[9] Additionally, no diurnal variation and no effects of age or gender were found for equine bile acid concentrations.[16] Bile acid concentrations in horses did increase 2–3-fold over baseline values after 3 and 4 days of fasting to maximum values of 20–25 µmol/liter.[9]

Camelids, such as the llama, are herbivores with a

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unique digestive system consisting of three compartments. The first compartment (C1), holding approximately 83% of the stomach contents, is analogous to the rumen of cattle. Both C1 and the second compartment (C2) contain glandular saccule compartments, with C2 containing 6% of the stomach ingesta. A muscular sphincter exists between C2 and the third compartment (C3). C3 contains the remaining ingesta and secretes hydrochloric acid and digestive enzymes, analogous to the stomach or abomasum.[15] The llama, like the horse, does not have a gallbladder.

Serum bile acid concentrations have not been evaluated in llamas. The objectives of the present study were to establish reference intervals for serum bile acid concentrations in healthy llamas, to determine whether feed consumption affected these concentrations, to examine the influence of a prolonged time period on serum bile acid concentrations, and to determine if there were any significant variations in bile acid concentrations over time.

Materials and methods

Llamas selected for reference interval analysis. Serum samples were obtained from llamas that were clinically healthy with no history of liver disease. The llamas ranged in age from 0.5 to 13 years; there were 22 intact males, 10 neutered males, and 18 females. These llamas originated from the northwestern United States, and the majority were receiving health screening prior to admission into the Oregon State College of Veterinary Medicine camelid herd. The remaining llamas were receiving clinical pathology testing for clinical health screens, prepurchase examinations, or presurgical examinations. Complete blood count (CBC) values, serum concentrations of blood urea nitrogen (BUN), total bilirubin, total protein, and albumin, and serum activities of gamma-glutamyltranspeptidase (GGT) and aspartate transaminase (AST) were within adult llama reference intervals for the laboratory. Alkaline phosphatase (ALP) serum activities for all llamas were within laboratory reference intervals, except for 3 llamas that were ≤1 year of age. Values for these llamas were 159–525 IU/liter, which is within the reference range reported for llamas ≤18 months of age.[7] All other parameters for these 3 llamas were within normal limits.

Llamas selected for time and feeding trials. Ten clinically healthy llamas maintained in the Oregon State Veterinary College herd were selected for the study. These llamas had been routinely dewormed and maintained on pasture with hay supplementation. Llamas ranged in age from 5 to 11 years; there were 5 males and 5 females. Forty-eight hours prior to the study, the llamas were transported to an animal isolation facility and given grass hay and water ad libitum. Blood samples for CBCs and chemistry analysis, including BUN, total protein, albumin, total bilirubin, ALP, GGT, AST, bile acids, and sorbitol dehydrogenase, were obtained. No abnormalities were found. Twenty-four hours prior to the study, indwelling jugular catheters[a] were placed to obtain samples for bile acid analysis. Water and feed were available

during the 11-hr study period during which blood samples were obtained at hourly intervals (sample times: 0–11 hr). Food was then removed for 12 hr; however, water was provided. Blood samples were taken 23 hr after the initiation of the trial (12 hr after food removal). Grass hay was then fed to the llamas, and blood samples were obtained 30 min after feeding, at sample time 23.5 hr. The remaining samples were collected at sample times 24, 25, 27, and 31 hr, which corresponded to 1, 2, 4 and 8 hr postprandial. After the trial ended, catheters were removed and llamas were observed for 24 hr and then returned to the herd.

Bile acid analysis. Serum bile acid concentrations were determined by a modified colorimetric enzymatic method[b] on a random access analyzer[c]. In the assay, 3α-hydroxysteroid dehydrogenase converts 3α-hydroxy bile acids and NAD$^+$ to 3-oxo bile acids and NADH. Then diaphorase converts NADH and nitro blue tetrazolium to formazan and NAD$^+$. The formazan color is proportional to the bile acid concentration measured at a primary wavelength of 540 nm. Calibration was done using calibration standards.[b] The within-analysis precision expressed as a coefficient of variation (CV) was 4.2%. The day-to-day precision (CV values) at 3 C and at −16 C was 7.2% and 7.3%, respectively. Bile acid concentrations were stable at 3 C for a minimum of 10 days and 30 days at −70 C (data not shown). Serum samples collected to establish reference intervals were frozen at −16 C, thawed, and analyzed within 5 days of receipt. Blood samples collected during the feeding trial over time were placed in serum tubes[d] and centrifuged, serum was removed, and samples were frozen at −70 C, thawed, and then analyzed within 14 days. Pooled llama serum was used as an internal control during each analysis.

Statistical analysis. Bile acid concentrations were used to calculate ranges, 95% confidence intervals, and 99% confidence intervals. Although, confidence intervals could be calculated as parametric based on the central limit theorem, reference intervals for this study also were calculated as the central 95% interval bounded by the 2.5 and 97.5 percentiles using a nonparametric rank sum determination.[12] Data were log transformed to compare bile acid concentrations across gender or age groups using an analysis of variance. When significant differences ($P < 0.05$) were detected, these were located using Tukey's studentized range test. A comparison among males, females, and neutered males was made for gender. Age groups were compared by using the following categories: group 1, 0–1 yr (4 females, 2 males); group 2, 1–5 yr (7 females, 5 males, 6 neutered males); group 3, 5–10 yr (5 females, 13 males, 4 neutered males); 4, >10 yr (2 females, 2 males). For the feeding and time trial, paired *t*-tests ($P < 0.05$) were used to compare mean preprandial and postprandial values for llama bile acid concentrations over the 31-hr time period.

Results

Bile acid concentration ranges and intervals. Bile acid concentrations were 10–44, 1–23, 2–19, and 9–14 μmol/liter for age groups 1, 2, 3, and 4, respectively. The parametric 95% confidence intervals were 13.0–42.6, 9.0–15.0, 6.8–11.0, and 7.6–15.9 μmol/li-

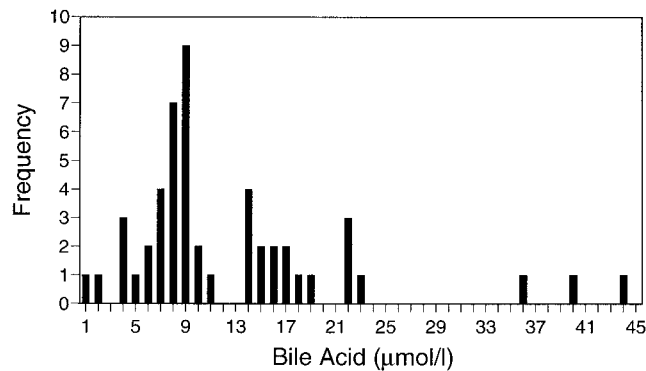**Figure 1.** Frequency histogram of bile acid concentrations in llamas ranging in age from 0.5 to 13 years. Values ≥36 μmol/liter were found in llamas ≤1 year of age.

ter for age groups 1, 2, 3, and 4, respectively. The mean concentration for bile acids was skewed, primarily because of bile acid concentrations of 36–44 μmol/liter in 3 llamas that were ≤1 year old (Figs. 1, 2). The remaining 3 llamas in age group 1 had bile acid concentrations of 10, 15, and 22 μmol/liter. The mean for age group 1 was significantly different from those of age groups 2, 3, and 4, but the means for age groups 2, 3 and 4 were not significantly different from each other (Fig. 2); therefore, confidence intervals were calculated for bile acid concentrations in llamas ≤1 year of age and llamas >1 year of age. The 95% and 99% parametric confidence intervals were 13.0–42.6 and 4.6–51.0 μmol/liter, respectively, for llamas ≤1 year of age and 9.2–12.3 and 8.7–12.8 μmol/liter, respectively, for llamas >1 year of age. Nonparametric
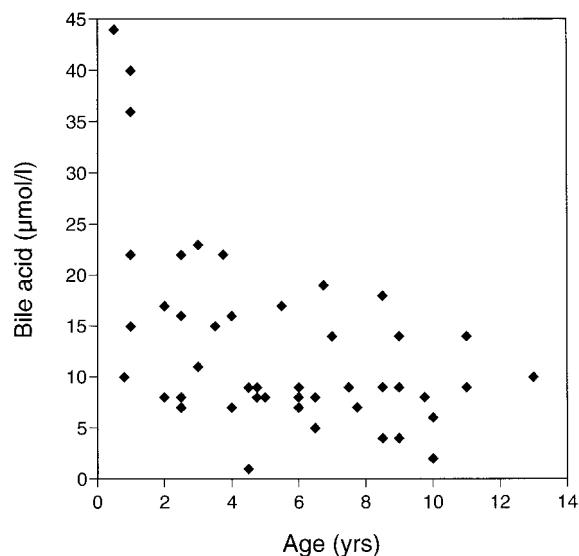

Figure 2. Scatter plot of bile acid concentrations (μmol/liter) versus age. Llamas with the same bile acid concentration and age appear as one data point. Note the wide variation in concentrations for llamas ≤1 year of age.
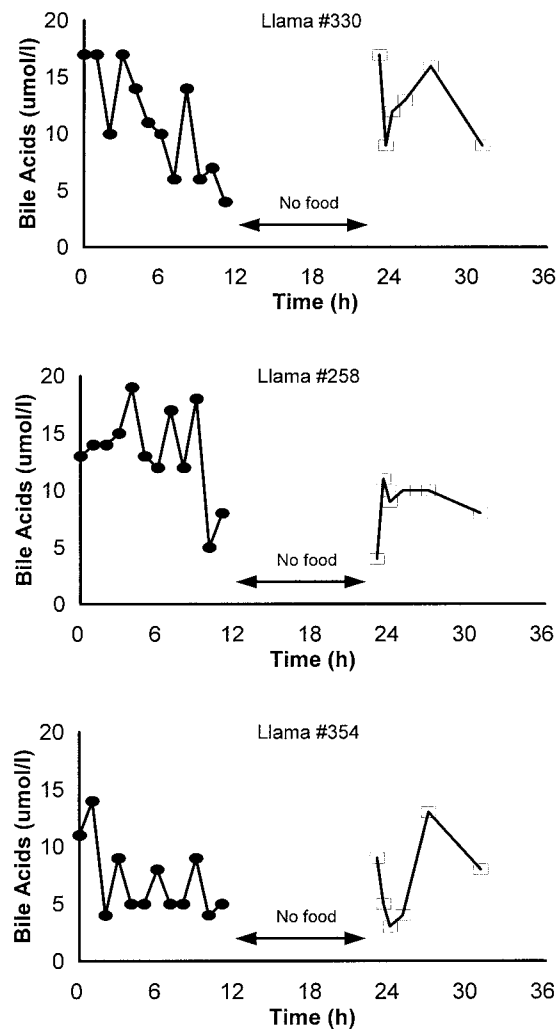

Figure 3. Representative examples of bile acid concentrations in 3 llamas over 31 hours with a 12-hour fasting interval.

2.5 and 97.5 percentiles were 1.8–49.8 μmol/liter for llamas ≤1 year of age and 1.1–22.9 μmol/liter for llamas >1 year of age. There was no correlation between gender and bile acid concentrations.

Bile acid concentrations over time and feeding trial. No significant differences were found between preprandial and postprandial bile acid concentrations or over time. The individual values were within the range of bile acid concentrations for this age group (groups 2, 3). Over 24 hours, individual llamas exhibited some variation in bile acid concentrations; variation from the mean for individual llamas ranged from 0 to 8 μmol/liter (Fig. 3). There was no predictable pattern associated with time or feeding. After the 12-hour fast was discontinued and llamas received food again, 4 llamas had bile acid concentrations that decreased from the 23-hour sample to the 23.5-hour sample, and 6 llamas had concentrations that increased during that interval.

Discussion

The bile acid concentration range of 1–23 μmol/liter in llamas >1 year of age (reference interval, 1.1–22.9 μmol/liter) includes a maximum reference concentration slightly higher than that for dogs and cats but markedly less than established limits in cattle and sheep. Reference intervals in dogs have been useful for detecting hepatobiliary disease, reaching 100% specificity at preprandial bile acid concentrations >20 μmol/liter and postprandial values >25 μmol/liter.[5] In cats, 100% specificity for hepatobiliary disease is found at preprandial bile acid concentrations ≥15 μmol/liter.[4] Variations in bile acid concentrations in cattle have been reported between dairy and beef breeds, among stages of lactation, and among feeding regimens in dairy breeds and among individual cattle over time.[6,11] Reported bile acid concentrations (5th to 95th percentile range) for beef cattle, lactating dairy cattle, and 6-month-old dairy heifers are 9–126, 15–88, and 11–64 μmol/liter, respectively.[6]

Limited studies have been performed in sheep,[1,13] and individual variation in bile acid concentrations may contribute to an overlap in reference intervals for healthy sheep and sheep with hepatobiliary disease.[13] One outlier value in the study was included in the bile acid reference interval, resulting in a stated 99% reference interval of <207 μmol/liter.[13] Graphs of bile acid concentrations from clinically healthy and subclinical pyrrolizidine alkaloid toxicity groups with minimal hepatic lesions appeared to have markedly different values as compared with sheep with acute hepatocellular necrosis.[13] Bile acid concentrations in goats were evaluated in a study utilizing bile acid loading, which determined the half-life clearance of bile acids from the circulation to be 4 ± 1 minutes.[8] The baseline bile acid concentrations in these 16 goats ranged from 1.7 to 21.6 μmol/liter, and the mean ± SD was 8.2 ± 5.8 μmol/liter (unpublished data).

Equine bile acid concentration intervals appear to be similar to those of dogs and cats. Unlike dogs and cats, there is no significant postprandial effect in horses, similar to observations in this llama study. In horses, the absence of a gallbladder and the documented frequent enterohepatic circulation indicate that bile acids are not stored for excretion into the intestine after ingestion of food but are cycled continuously.[9] This also may the case in llamas, but to date no physiologic studies have been done to monitor bile acid cycling. Horses and llamas appear to have less variability in serum bile acid concentrations than do cattle.[11,16] Bile acid concentrations were determined in 51 clinically healthy horses (no evidence of hepatobiliary disease) as 5.3 ± 6.5 μmol/liter, with no value >20 μmol/liter. In horses confirmed to have chronic liver disease, bile acid concentrations were ≥24 μmol/liter.[10] Other investigators have reported reference values of 5.94 ± 2.72 μmol/liter.[9] Like horses,[16] no correlation for gender and bile acid concentrations was found in llamas.

In llamas ≤1 year of age (group 1), 3 bile acid values (36, 40, 44 μmol/liter) contributed to the skewed distribution of the concentrations (Fig. 1). The remaining values in this age group (10, 15, 22 μmol/liter) contributed to a wide distribution of values (95% confidence interval of 13.0–42.6 μmol/liter). The 3 llamas also had increased ALP concentrations, which were within the reference interval for their age group and were thought to be due to production of the ALP bone isoenzyme. Spurious elevated bile acid concentrations, which qualify as statistical outliers, have been observed in ruminants without evidence of liver disease.[13] These values tend to increase the reference limits and decrease the diagnostic utility of reference intervals. Bile acid concentrations in calves <6 weeks old and in 6-month-old heifers were lower than concentrations in lactating dairy cows but were within the reference interval of beef cattle.[6] Young age was not correlated with higher bile acid concentrations in horses.[16] In monogastric dogs and cats, preprandial serum concentrations may sometimes exceed postprandial concentrations or false increases in bile acid concentrations may be seen.[5] These findings in dogs and cats may be due to spontaneous gallbladder contraction or differences in gastric emptying rates, gastrointestinal transit time, and gastrointestinal flora.[5] These factors, except gallbladder contraction, also may contribute to differences in llama bile acid concentrations. There may be differences in the C1 bacterial flora of young llamas and mature llamas; however, this has not been determined. For llamas ≤1 year of age, all bile acid concentrations were used to determine the confidence intervals because no hepatic disease could be documented and the 3 llamas have remained clinically healthy.

Hourly mean and individual animal bile acid fluctuations were less in llamas than in cattle or sheep. In dairy cattle, hourly bile acid fluctuations were as large as 65 μmol/liter for an individual animal[11] versus a maximum fluctuation of 8 μmol/liter for individual llamas. No consistent interaction was found between feeding or short-term fasting and serum bile acid concentrations in llamas.

Further examination of bile acid concentrations in llamas ≤1 year of age versus llamas >1 year of age is warranted because significant differences were found, and the range of concentrations in llamas ≤1 year were widely distributed (Fig. 2). Parametric 95% confidence intervals are sometimes used for reference intervals, but in llamas >1 year of age, the large number of concentrations between 5 and 11 μmol/liter

(Fig. 1) in age groups 2 and 3 probably made this interval narrower than would be desired for clinical assessment. Using the 95% confidence interval for this age group would probably result in the false diagnosis of hepatobiliary disease. Even though the data could be analyzed using parametric confidence intervals, the nonparametric percentiles of bile acid concentrations, 1.1–22.9 μmol/liter, in llamas >1 year of age is probably more appropriate as an adult reference interval. For llamas ≤1 year of age, the 95% and 99% confidence intervals were broad, but because of the small sample number ($n = 6$) and distribution of bile acid concentrations, the nonparametric percentiles (1.8–49.8 μmol/liter) also are a more representative reference interval. Because individual llama bile acid concentration fluctuations over time and with feeding were minimal and within the determined ranges, single serum samples should reflect baseline serum bile acid concentrations. Increased serum bile acid concentrations have been seen in llamas with hepatobiliary disease (personal observations) and could be useful for diagnosis of this disease.

Sources and manufacturers

a. Angiocath, Becton Dickinson, Sandy, UT.
b. Sigma Diagnostics, St. Louis, MO.
c. Express 550, Ciba Corning, Oberlin, OH.
d. Vacutainer, Becton Dickinson, Franklin Lakes, NJ.

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