Postprandial changes in serum unconjugated bile acid concentrations in healthy Beagles

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Objective—To investigate postprandial changes in serum concentrations of unconjugated bile acids in healthy Beagles.

Animals—7 healthy Beagles.

Procedure—Blood samples were obtained from dogs at regular intervals up to 8 hours after consumption of a meal. Serum concentrations of 5 unconjugated bile acids were determined at each time point, using gas chromatography-mass spectrometry with selected ion monitoring.

Results—Total serum unconjugated bile acid concentration was significantly increased, relative to baseline values, at 360, 420, and 480 minutes after feeding. Unconjugated cholic acid was significantly increased at 360, 420, and 480 minutes. The proportion of total unconjugated bile acids represented by cholic acid was significantly increased at 240 to 480 minutes. Deoxycholic acid was significantly increased at 360 and 420 minutes. Chenodeoxycholic acid was significantly increased at 360 to 480 minutes. Lithocholic acid was significantly increased at 180 minutes, whereas no significant changes in ursodeoxycholic acid were detected at any time point.

Conclusions and Clinical Relevance—Healthy Beagles had significant increases in serum concentrations and changes in the profile of unconjugated bile acids after a meal. These increases persisted > 8 hours, indicating that prolonged withholding of food is necessary when to avoid the risk of a false-positive diagnosis when assessing serum unconjugated bile acid concentrations in dogs. (*Am J Vet Res* 2002;63:789–793

Primary bile acids, such as cholic and chenodeoxycholic acid, are produced by hepatic synthesis from cholesterol. After synthesis, the liver conjugates most primary bile acids to amino acids. These conjugated bile acids are subsequently secreted into the duodenum in the bile. In dogs, the primary bile acids are virtually exclusively conjugated to taurine.[1] Dogs differ from humans, in which primary bile acids are conjugated to either taurine or glycine before biliary secretion.

Bile acids are absorbed in the small intestine and undergo enterohepatic circulation, with highly efficient first-pass hepatic extraction of bile acids from portal blood. Postprandial entry of bile into the small intestine leads to an increase in bile acid uptake, leading to a small postprandial increase in serum bile acid concentration in animals with normal hepatic function.

In the small intestinal lumen, bile acids may undergo post-secretory structural modification attributable to bacterial action, producing a variety of secondary bile acids. The first step in the modification of bile acids is usually deconjugation; during this step the amino acid moiety is removed from the bile acid core by bacterial deconjugase enzymes. This deconjugase activity is unique to bacterial cells; mammalian cells lack the necessary deconjugase enzymes to carry out this reaction. Studies of rats with surgically induced intestinal blind loops and bacterial overgrowth reveal that bile acids within the intestine remain conjugated in bacteria-free rats.[2]

It has been estimated that approximately one third of the bile acid pool undergoes bacterial deconjugation each day in humans.[3] In dogs, the proportion of bile acids that undergo deconjugation is apparently much lower; this likely reflects a greater resistance to bacterial deconjugation of the taurine-conjugated bile acids.[4]

Because deconjugation of bile acids is exclusively a bacterial metabolic activity, unconjugated bile acid formation in the proximal portion of the small intestine is considered an index of intestinal bacterial activity.[5] Measurement of serum concentrations of unconjugated bile acids has been found to have diagnostic value in human patients with suspected small intestinal bacterial overgrowth.[6-8] Studies in our laboratory of dogs with small intestinal bacterial overgrowth, documented by quantitative bacteriologic culture of duodenal fluid, have revealed an increase in total unconjugated bile acid concentration. In these dogs the proportion of total circulating bile acids in the unconjugated form and the absolute value of serum unconjugated cholic acid concentration were increased, compared with healthy control dogs.[4] These findings suggest that measurement of serum concentrations of unconjugated bile acids has potential as a diagnostic test for small intestinal bacterial overgrowth in dogs.

The serum concentration of unconjugated bile acids represents a balance between input by intestinal absorption and hepatic extraction from the portal and systemic circulations. The hepatic extraction efficiency for unconjugated bile acids is lower than for conjugated bile acids, which may be attributable to a difference in the extent of binding of unconjugated bile acids to albumin.[9] Because the extraction efficiency of unconjugated bile acids is lower, spillover into the systemic circulation is facilitated for the unconjugated bile acids.

The purpose of the study reported here was to assess postprandial alterations in the serum concentrations of unconjugated bile acids in dogs. We hypothesized that serum unconjugated bile acid concentrations

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would be altered significantly after feeding in dogs from which food had been withheld, reflecting increased uptake of unconjugated bile acids resulting from bacterial metabolism of bile released in response to feeding.

Materials and Methods

Animals—Seven adult (age range, 2.5 to 3.0 years) laboratory Beagles (3 male and 4 female) were selected from a colony of laboratory dogs, owned by and housed at the Hill's Science and Technology Center in Topeka, Kan. Dogs were selected from the pool of available dogs on the basis of the animal care technician's subjective impression that the selected dogs would consume a meal rapidly; this would decrease the between-dog variation in timing of food consumption. No abnormalities were detected in any dog via physical examination. Serum chemistry panels and CBC revealed no substantial abnormalities. Serum trypsin-like immunoreactivity, cobalamin, and folate concentrations were within our laboratory reference range for all dogs. Venous access was established in all dogs with indwelling 18.5-gauge 12-in jugular catheters.[a] Dogs were fed at 10 AM on the day preceding the study, then all bowls and any residual food were removed from the dogs' enclosures at 3 PM on the day prior to the study. Following collection of the baseline serum sample at 8 AM, all dogs were fed a commercial dry canine maintenance diet.[b] All dogs consumed the diet readily and rapidly.

Serum collection—Blood samples were collected from all dogs at baseline, every 15 to 120 minutes after feeding, every 30 to 240 minutes after feeding, and then hourly until 480 minutes after feeding. A volume of blood equal to the catheter dead space was withdrawn and discarded before each sample, and the catheter was flushed with heparinized saline (0.9 NaCl) solution after each sample was obtained. Serum was immediately separated by centrifugation and stored at –80 C until further processing.

Analysis of serum unconjugated bile acids—Serum concentrations of unconjugated bile acids were measured as described,[4] with some modifications. After liquid-solid extraction of bile acids from serum and separation of unconjugated bile acids by use of lipophilic anion exchange chromatography, the unconjugated bile acid fraction was converted to the methyl ester form by reaction with 2,2-dimethoxypropane[c] in acidic methanol. This step replaced a reaction with ethereal diazomethane in the originally published method. Subsequent generation of trimethylsilyl ethers and sample clean up were performed as described.[4]

Chromatographic separation and quantitation were performed in a gas chromatograph[d] coupled to a mass spectrometer.[e] The mass spectrometer was operated in selected-ion monitoring, electron-impact mode. Ions monitored were mass-charge 368, 370, and 372, representing relatively strong, specific ions generated by monohydroxy- (cholic acid), dihydroxy- (deoxycholic, chenodeoxycholic and ursodeoxycholic acid) and trihydroxycholanoates (lithocholic acid), respectively.[4] The trimethylsilyl ethers were chromatographically separated using a 30 m × 0.25- mm column.[f] The temperature program was modified from the original description. The program began with a temperature of 220 C and a 3-minute isothermal period; column temperature was then raised to 250 C at 25 C/min, followed by an isothermal period of 2.5 minutes. The unconjugated bile acids were eluted by use of a subsequent temperature gradient between 250 and 300 C at 5 C/min, with a final isothermal period of 2.5 minutes. Carrier gas (ultrapure helium)[g] flow rate was maintained at 1.8 ml/min with a 50:1 split ratio; however, the injection was made in splitless mode for 30 seconds. Quantification of the unconjugated bile acids in serum was achieved by generation of calibration curves with mixtures of pure standards. Total serum unconjugated bile acid concentration was reported as the sum of the 5 individual bile acids, with concentrations expressed in nanomoles per liter.

Variations in the efficiency of solid-phase recovery of the unconjugated bile acids were corrected by use of an internal standard. The internal standard, nordeoxycholic acid, was added to the serum in known quantity (final concentration, 500 nmol/L) prior to extraction of the unconjugated bile acids from serum, with the nordeoxycholic acid monitored at mass-charge 521.

Statistical analyses—Data were analyzed with a statistical software package.[h] Variations in serum concentration of each unconjugated bile acid were analyzed by time point using 1-way repeated measures ANOVA, followed by the Dunnett multiple comparison test to compare each time point with the baseline sample. The null hypotheses tested were that there was no overall effect of time-from-feeding on serum concentration of each unconjugated bile acid, and there was no significant difference between each time point and the unconjugated bile acid concentration at the pre feeding time point. The null hypotheses were rejected and significance was assigned when values of $P < 0.05$ were calculated.

Results

Unconjugated bile acids were readily detectable in serum at all time-points in all dogs. Cholic acid was quantitatively the most prominent unconjugated bile acid in most samples. Total serum unconjugated bile acid concentration was increased from 240 minutes after feeding, with significant ($P < 0.001$) increases observed at 360, 420, and 480 minutes (**Fig 1**). The profile of unconjugated bile acids in the serum changed significantly over time (**Fig 2**). The proportion of total unconjugated bile acids represented by

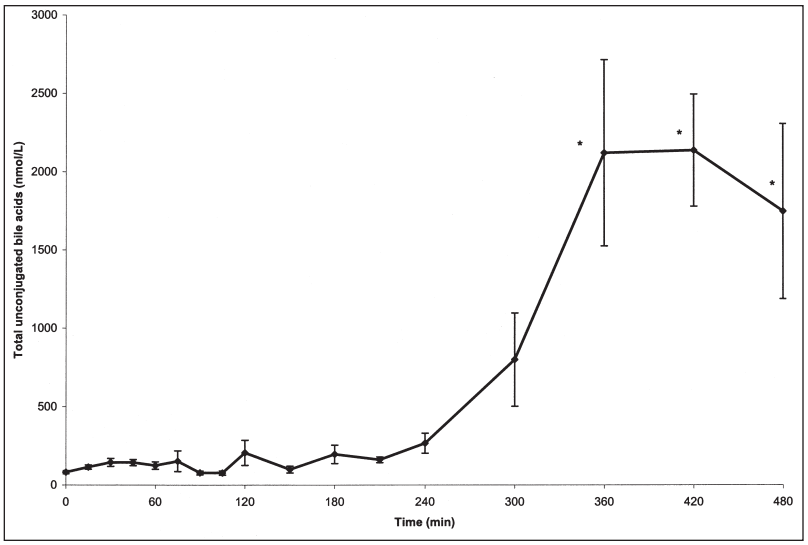

Figure 1—Serum concentrations (mean ± SEM) of total unconjugated bile acids in 7 healthy dogs at various time points after feeding. *Significant ($P < 0.01$) increase, compared with baseline value.

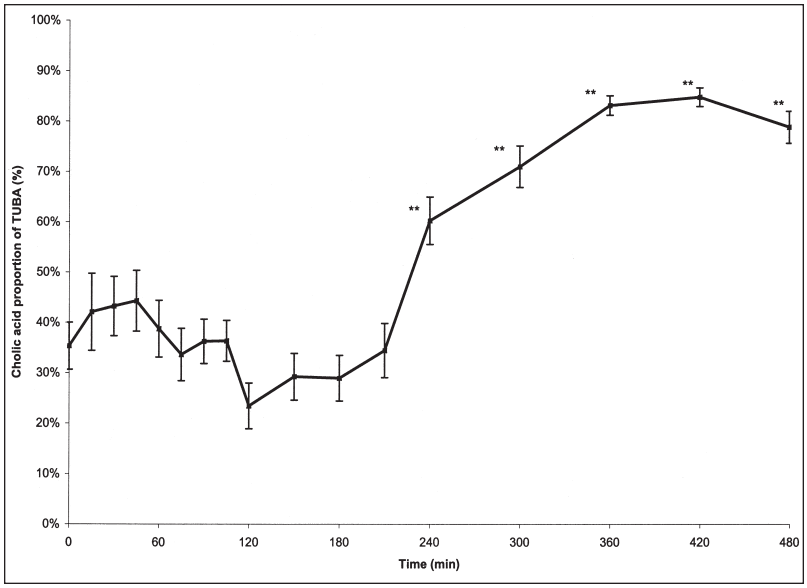

Figure 2—Proportions (mean ± SEM) of serum total unconjugated bile acids (TUBA) represented by unconjugated cholic acid in 7 healthy dogs at various time points after feeding. **Significant ($P < 0.01$) increase, compared with baseline value.

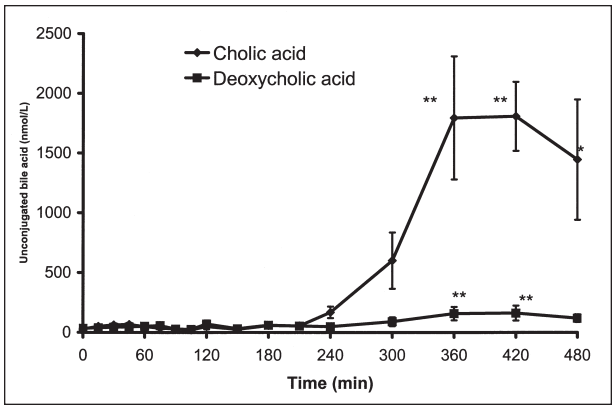

Figure 3—Serum concentrations (mean ± SEM) of unconjugated cholic and deoxycholic acid in 7 healthy dogs at various time points after feeding. *Significant ($P < 0.05$) increase, compared with baseline value. **Significant ($P < 0.01$) increase, compared with baseline value.

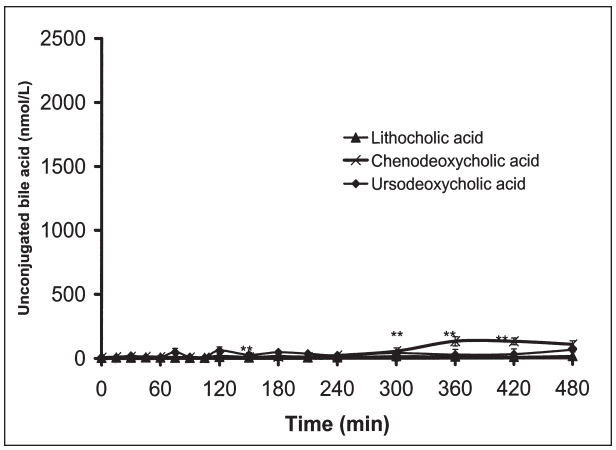

Figure 4—Serum concentrations (mean ± SEM) of unconjugated chenodeoxycholic acid, lithocholic acid and ursodeoxycholic acid in 7 healthy dogs at various time points after feeding. **Significant ($P < 0.01$) increase, compared with baseline value.

cholic acid increased significantly ($P < 0.001$), compared with the baseline sample at all time points after 240 minutes. Mean serum unconjugated cholic acid concentrations from the 7 dogs at each time point were determined (**Fig 3**). Serum unconjugated cholic acid concentrations were typically increased 240 minutes after feeding. A significant increase of unconjugated cholic acid over baseline concentration was detected at 360, 420, and 480 minutes after feeding ($P < 0.001$, 0.01, and 0.05, respectively).

Deoxycholic acid, a secondary bile acid derived from cholic acid, was increased significantly ($P < 0.001$) in serum concentration relative to baseline at 360 and 420 minutes after feeding.

Serum concentration of chenodeoxycholic acid, the other major primary bile acid of dogs, was increased significantly ($P < 0.01$) at 360, 420, and 480 minutes after feeding (**Fig 4**). Lithocholic acid was significantly increased relative to the prefeeding sample at 180 minutes after feeding ($P < 0.01$), whereas ursodeoxycholic acid was not significantly increased at any time point after feeding.

Discussion

Small intestinal bacterial overgrowth in dogs is a difficult condition to diagnose and effectively treat. No readily available diagnostic test is sensitive and specific for this disorder. Quantitative bacteriologic culture of aseptically sampled duodenal juice is considered the gold standard diagnostic test for small intestinal bacterial overgrowth, but this is an invasive, methodologically complex and expensive test that is not suited to routine practice. There is a substantial need for a sensitive and specific diagnostic test that assesses the number and metabolic activity of small intestinal bacteria in dogs in a cost-effective and less invasive manner.

Deconjugation and some structural modifications of bile acids are carried out by small intestinal bacteria, with deconjugation being carried out exclusively by deconjugase enzymes of bacterial origin. Because the liver excretes essentially 100% of bile acids in conjugated form, unconjugated bile acids in the small intestinal lumen and the portal and peripheral circulations are attributable only to bacterial metabolic action. Detection and measurement of unconjugated bile acids in the serum potentially allow the specific assessment of intestinal bacterial metabolic activity or numbers. An increased population of deconjugating bacteria in the small intestine would be expected to be associated with an increased input of unconjugated bile acids from the small intestine and, hence, an increase in the baseline serum unconjugated bile acid concentration.

The serum concentration of unconjugated bile is influenced by hepatic extraction efficiency of the unconjugated bile acids and by input from the small intestine. A small study[3] of healthy humans revealed

diurnal variations in serum unconjugated bile acids, with the maximum concentration of each bile acid generally occurring during the day between meals.

Data reported here indicate increased total unconjugated bile acid and cholic acid concentrations in the serum of healthy Beagles from 240 to 480 minutes after feeding. Significant increases were detected from 360 to 480 minutes after feeding. All of the dogs in our study had peak serum concentrations of unconjugated cholic acid markedly greater than our laboratory reference range for unconjugated cholic acid in the sera of unfed healthy dogs (0 to 76 nmol/L). Deoxycholic acid, a secondary bile acid derived from cholic acid, was increased relative to the baseline sample at 360 and 420 minutes after feeding. Chenodeoxycholic acid was increased significantly, compared with baseline value at 360, 420, and 480 minutes; however, the magnitude of the change was much less than that detected for cholic acid.

In the postprandial period, the profile of unconjugated bile acids in the serum was significantly altered. In the baseline samples, the proportion of unconjugated bile acids represented by cholic acid was 35.35 ± 4.68%. At the apparent peak of the postprandial increase in unconjugated bile acids (420 minutes), cholic acid was 84.83 ± 1.85% of total serum unconjugated bile acids. This is consistent with findings that cholic acid is quantitatively the most important unconjugated bile acid in the portal circulation and peripheral circulation of dogs.[1,4] The proportion of serum total unconjugated bile acids represented by cholic acid was significantly greater than the baseline value after 240 minutes and was still significantly increased 480 minutes after feeding. These findings suggest that the serum profile of unconjugated bile acids after withholding of food is different from the serum profile 8 hours after feeding in dogs. Higher serum total unconjugated bile acids, cholic acid, and the proportion of total unconjugated bile acids present as cholic acid were measured 8 hours after feeding in this group of healthy Beagles.

We speculate that the increase in total serum unconjugated bile acid concentration and the proportion of cholic acid in the serum unconjugated bile acid profile occurring from 240 minutes onwards represents the effect of entry of bile into the large intestine. Fujii et al[1] detected absorption of substantial quantities of unconjugated bile acids into portal blood from the distal portion of the large intestine in dogs.

Essentially all of the bile acids in the terminal portion of the ileum, the large intestine, and fecal matter are unconjugated, which is attributable to bacterial activity in this region.[10] Thus, we speculate that the baseline profile of unconjugated bile acids in dogs examined in this study represent the balance of input of unconjugated bile acids from the colon and hepatic extraction of these bile acids. Cholic acid, while still the most prominent unconjugated bile acid in serum, was not as prominent as was observed at the peak of the postprandial increase in unconjugated bile acids.

The group of dogs used in this study was relatively homogeneous, because all were Beagles and the age range was narrow. Dogs were selected for the study on the basis of their willingness to rapidly consume a meal. Consequently, the results reported here were derived from dogs that had a single large meal (essentially a bolus of food) rather than frequent small meals, as may happen with client-owned dogs fed ad libitum. Laboratory-housed Beagles may have substantially different intestinal bacterial flora than client-owned pet dogs do, and the intestinal flora of laboratory Beagles alters with age.[11] Subclinical bacterial overgrowth with alterations in intestinal permeability has been reported in ostensibly healthy Beagles.[12] In Batt et al's study[12], however, serum folate concentrations were commonly increased in Beagles with small intestinal bacterial overgrowth, compared with a group of mixed-breed healthy control dogs.[12] Serum of the dogs in our study yielded values within our laboratory reference ranges for serum cobalamin, folate, and unfed unconjugated cholic acid concentrations; these ranges were determined from healthy client-owned dogs. Although we cannot totally rule out the presence of intestinal flora quantitatively or qualitatively different from client-owned dogs in our study group, we have no evidence that our study group had altered intestinal flora.

All of the dogs in the study reported here had peak serum unconjugated cholic acid concentrations markedly greater than our laboratory reference range for unfed dogs, and serum unconjugated cholic acid concentrations reached concentrations equivalent to those in dogs with small intestinal bacterial overgrowth.[4] It is important that blood sampling to determine serum concentration of unconjugated cholic acid be performed at a time when substantial quantities of bile are not in the terminal portion of the ileum or entering the large intestine. Entry of bile into the terminal portion of the ileum and large intestine may allow deconjugation and absorption of unconjugated bile acids regardless of the presence or absence of pathologic bacterial populations in the small intestine. This situation introduces the risk of a false positive diagnosis of small intestinal bacterial overgrowth in an otherwise healthy dog if an adequate food-withholding period has not been observed. The change in serum unconjugated bile acid concentration and profile in dogs with small intestinal bacterial overgrowth revealed by use of bacteriologic culture of duodenal contents is a potential avenue for further investigation. It remains possible that the peak concentrations of unconjugated bile acids, timing of the peak, or the profile of unconjugated bile may differ between dogs with small intestinal bacterial overgrowth and healthy dogs.

In this study, food had been withheld from the dogs for a minimum of 17 hours (3 PM to 8 AM), and potentially for 22 hours if the prior-day meal was consumed rapidly before the study began. Although extrapolation beyond the 8- hour time point is fraught with uncertainty, cholic acid, dexoycholic acid, chenodeoxycholic acid, and total unconjugated bile acid concentrations were all apparently in decline at this time-point. On the basis of the results of this study and clinical experience using this assay, we recommend that food be withheld at least 12 hours prior to blood collection for determination of serum concentration of unconjugated bile acids. From the data presented here,

an overnight fast of at least 17 hours appears to allow the establishment of a true baseline concentration and profile of unconjugated bile acids in healthy Beagles.

[a]Charter Medical Inc, Lakewood, NJ.
[b]Science Diet, Canine Maintenance, Hills Pet Nutrition Inc, Topeka, Kan.
[c]Sigma Chemical Co, St Louis, Mo.
[d]GC8000TOP, Thermoquest Corp, Schaumburg, Ill.
[e]Voyager MS, Thermoquest Corp, Schaumburg, Ill.
[f]DB-1MS column, J&W Scientific, Folson, Calif.
[g]Ultrapure Helium (99.999% Helium), Praxair Corp, Danbury, Conn.
[h]GraphPad Prism 3.0 for Windows, GraphPad Software, San Diego, Calif.

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