Postprandial Changes in Serum Bile Acids Concentration and Fractionation of Individual Bile Acid by High Performance Liquid Chromatography in Normal Dogs

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(Received 9 October 1986/Accepted 31 March 1987)

ABSTRACT. Serial determination of serum bile acids (SBA) concentration and its fractionation after feeding were performed in 8 healthy dogs. The mean preprandial SBA was 1.07±0.91μM/l. The mean SBA reached a maximum value of 10.35±7.32 μM/l at 6 hour with very rapid rise at 1 hour after feeding. There were no significant differences in any of the mean SBA values during the 1 to 8 hour postprandial period. The mean preprandial triglycerides (TG) concentration was 37.07±13.51mg/dl. The mean TG increased rapidly after feeding and reached a maximum value of 73.53±12.01mg/dl at 2 hour. It would appear that postprandial sample for SBA can be taken at 1 hour after feeding. Unfortunately, none of the peaks was identified in the fractionation study. An unidentified peak with a retention time near 6.4min. increased significantly in the 2 hour sample. A new unidentified peak with a retention time near 9.0min. appeared in the 2 hour sample.---KEY WORDS: dog, high performance liquid chromatography, serum bile acid, 3-alpha-hydroxysteroid dehydrogenase.

Bile acids are synthesized from cholesterol solely in the liver and play physiologically important roles; stimulate hepatic bile flow and enhance lipid absorption [16, 25, 42]. Bile acids released into the duodenum following a meal are reabsorbed either unchanged or after bacterial transformation mainly in the ileum [22, 32]. These reabsorbed bile acids are transported to the liver, extracted, conjugated and reexcreted. This circuit is called as the enterohepatic circulation of bile acids.

In the physiological state, total serum bile acids (SBA) levels are a balance between intestinal absorption and hepatic uptake. Hepatic extraction of bile acids is believed to be constant and its Vmax is far greater than the physiological load of bile acids [17, 38] so that the fraction of bile acids which are spilled into the systemic circulation is constant [3, 37, 44]. Therefore, the intestinal input [33] and the hepatic blood flow [44] are the major determinants of SBA in healthy individuals. If intestinal absorption is within the normal physiological range, an elevated SBA is to be expected in the presence of the following situations: 1) portosystemic shunting; 2) damaged hepatocytes which can not extract bile acids efficiently; 3) bile acid leakage from the hepatocytes into the systemic circulation [6, 34, 36].

SBA as a liver function test was first reported when Sherlock and Walsh (1948) measured blood cholate in healthy individuals and patients with liver diseases [27]. In veterinary medicine its usefulness has been postulated in both experimental and clinical situations [3, 7–9, 16, 17, 28]. There are limited number of reports regarding SBA and its significance in liver disease in

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the dog. Anwer et al. (1976) reported that SBA increased significantly following carbon tetrachloride administration and SBA concentration was correlated with sorbitol dehydrogenase and alanine aminotransferase (ALT) [4]. In canine dirofilariasis, SBA was significantly increased in patients with serious clinical signs and in patients with hemoglobinuria. SBA level was highly directly correlated with cholesterol and bilirubin, and inversely correlated with lecithin cholesterol acyltransferase [30]. In a study of 18 dogs with portosystemic venous anomalies (PSVA), SBA was found to be more sensitive than conventional liver tests or BSP retention, and equal in sensitivity to the ammonia tolerance test [12]. The diagnostic value of SBA was examined in 150 dogs which suspected of having liver disease and the specificity of SBA for the diagnosis of liver disease was found to be more than 90% at values >30 μM/l and 100% at >50 μM/l. Recently, the measurement of SBA by radioimmunoassay and the enzymatic method were validated for use in dogs and cats [8, 13]. Fasting and two hour postprandial sampling are now the standard methods for evaluating liver function using SBA. Although two hour postprandial SBA value has been preferentially used over the fasting SBA [1, 2, 4, 6, 11, 12, 19, 21, 23, 31], recent studies demonstrate that fasting SBA is as sensitive as postprandial SBA [13, 14, 25, 26, 29, 32].

The purpose of this study was to evaluate the time course of SBA concentration changes after feeding and follow time course to elucidate the effect of food intake on the SBA concentration and the best timing to obtain the postprandial sample in the dog. Triglycerides (TG) was measured simultaneously to confirm absorption. Also, pre- and postprandial SBA were fractionated by high performance liquid chromatography (HPLC) to establish the types of bile acids seen in dog serum and develop the methods for HPLC assay of bile acids in dog serum.

**MATERIALS AND METHODS**

**Animals:** Seven male and one female clinically healthy adult mixed breed dogs in weighing between 19.1 to 29.9 kg were used for this study. They were housed in the animal care facility (Animal resource Service) of the University of California, Davis. Their hematological and blood chemistry values prior to the study were all within the normal range. A complete blood count was performed by the Clinical Hematology Laboratory at the Veterinary Medical Teaching Hospital (University of California, Davis). The standard chemistry tests including total protein, albumin, ALT, aspartate aminotransferase, lactate dehydrogenase, alkaline phosphatase (ALP), total bilirubin (T. Bil.), cholesterol and blood urea nitrogen were run on SMA 12/60 Auto analyzer (Technicon Instruments Corporation, Tarrytown, N. Y.). The dogs were fasted overnight for 12 hr prior to initiation of the study. The preprandial samples were taken from the jugular vein, and the each dog was fed 2/3 can (280g) of regular commercial canned dog food (Kal Kan M. P. S. Chunks, Kal Kan Foods, INC. Vernon, Ca.) and postprandial samples were taken at 1, 2, 4, 6, 8, 10, 12 and 24 hr. This study was started at 8 o'clock in the morning.

**Measurement of total serum bile acids and triglycerides:** Five ml of blood samples were obtained by jugular vein puncture and placed in a plain tube, allowed to clot and centrifuged at 4°C at 3000rpm. for 15 min. Collected serum was stored at 4°C until analyzed. The concentration of SBA was measured using a commercially available kit (Enzabile, Nyegaard & Co, Oslo, Norway). In this method, 3-alpha-hydroxy bile acids are oxidized to the corresponding 3-keto derivatives with a simultaneous generation of NADH from NAD by 3-alpha-hydroxy-
steroid dehydrogenase. The coloured product, formazan, is formed when NADH is transferred to the tetrazolium salt, NBT, by the enzyme diaphorase. The concentration of TG was measured using a commercially available kit (No. 335-UV, Sigma Diagnostics, St. Louis, Mo.) based on the ultraviolet method upon Bucolo and David [5]. After TG are hydrolyzed to glycerol and fatty acids by lipase, the glycerol is quantitated by a kinetic assay based on NADH disappearance resulting from a series of coupled enzyme reactions.

_Serum bile acids fractionation:_ The equipment used for this study was a Varian Model 5060 HPLC equipped with a Varian Model UV-100 ultraviolet detector monitoring the absorbance at 210 nm and a Varian VISTA 402 chromatography data system (Varian Inc. Walnutcreek, CA.). A Micropak MCH-5-n-cap (4mm×15cm) reverse phase column was employed.

0.3% ammonium carbonate (A) and acetonitrile (B) were used as the mobile phase at a flow rate of 1ml/min. The composition of mobile phase was 76% A, 24% B at the beginning and then changed to 72% A, 28% B or 73% A, 27% B.

Bile acid standards were obtained from Sigma Chemical Co., St. Louis. A working standard solution of seven conjugated bile acids (taurodehydrocholic acid (TDHC), glycocholic acid (GC), taurocholic acid (TC), glychenodeoxycholic acid (GCDOC), taurochenodeoxycholic acid (TCDOC), glyc deoxycholic acid (GDOC), taurodeoxycholic acid (TDOC)) was prepared with equimolar amounts of each bile acid standard and used daily as an external standard to calculate retention time and response factors based on relative peak areas.

Amberlite XAD-2 resin was placed in a column (2.5×45 cm) and successively washed with eight volumes of distilled water, methanol, acetone and finally distilled water and stored in 3 volumes of distilled water.

1 ml of serum was diluted with 9 ml of 0.1 N NaOH prepared in 0.85% NaCl solution and placed on the Amberlite XAD-2 column. After washing with distilled water to pH 6.8, the bile acid fraction was eluted with 50 ml 95% ethanol. The effluent was evaporated to dryness under vacumn. The flask was washed with 10 ml of ethanol and the wash was reevaporated under vacuum to dryness after which it was resolved in ethanol with 1/4 volume of the original serum sample volume.

_Statistical analysis:_ Samples taken before feeding, which were signified as 0 hour, were used as controls. Significance of differences between mean values was determined using Student’s _t_ test.

RESULTS

In fig. 1, pre and postprandial SBA concentration changes during 24 hr are given. The mean preprandial SBA was 1.07±0.91 μM/l. The mean SBA was significantly increased at 1 hr postprandially (P<0.05) and reached
a maximum value of 10.35±7.32 μM/l at 6 hr, after which it gradually decreased. There were no significant differences between any of the mean SBA values during the 1 to 8 hr postprandial period. In individual dogs, two or three peaks in SBA were seen between 1 and 8 hr postprandially. The first peak was seen at 1 or 2 hr postprandially and the second peak was seen at 6 or 8 hr postprandially (Fig. 1).

The mean preprandial TG concentration was 37.07±13.51 mg/dl. The mean TG value was increased significantly at 1 hour postprandially (P<0.05) and reached a maximum value of 73.53±12.01 mg/dl at 2 hr postprandially after which it gradually decreased (Fig. 2).

In Fig. 3, a high performance liquid chromatograph (HPLCG) of seven bile acid standards is shown. Peaks of TDHC, GC,
TC, GCDOC, TCDOC, GDOC, and TDOC are clearly seen. In the figure, the numbers over the peaks are retention times and peaks without identifying initials are unidentified peaks. Also, retention times of less than 2.000 min are solvent impurities. The HPLC of preprandial and 2 hr postprandial serum bile acids are given in Figs. 4 and 5, respectively. Unfortunately, none of the peaks could be identified as comparable to the standards in preprandial and postprandial SBA samples from normal dogs. An unidentified peak with a retention time near 6.4 min showed a significant increase in the 2 hr sample. In addition, a new unidentified peak with a retention time near 9.0 min appeared in the 2 hr sample.

DISCUSSION

There is a distinct advantage in the measurement of SBA over conventional liver enzymes in hepatobiliary disease because of its specificity. Recently the usefulness of measuring SBA concentrations has been reported in veterinary medicine [4, 10-12, 15, 23, 24]. SBA increase significantly in dogs treated with corticosteroids and hepatic duct ligation [15]. SBA was concluded to be a much more sensitive indicator than ALT or ALP to detect secondary liver involvement in dogs with heart failure or diabetes [23]. In the diagnosis of portosystemic venous anomalies SBA concentration is more sensitive than BSP test or other conventional liver enzyme tests and more readily performed than the ammonia tolerance test [12]. Among various combinations of SBA and conventional liver enzymes, SBA + T Bil + ALT appear to be the best combination for the diagnosis of hepatobiliary disease in the dog [10].

The two-hour postprandial SBA values have been preferentially used over the fasting SBA values [1, 2, 5, 9, 18, 19, 26, 28, 31, 43], because the postprandial SBA values were more frequently abnormal than the fasting SBA values when compared to conventional liver tests. The postprandial SBA was thus believed to be more sensitive than the fasting SBA by many investigators [2, 5, 18, 28]. This is based on the concept that in patients with liver disease, the fractional hepatic uptake decreases when the bile acid load increases after a meal. However, if the fractional hepatic extraction is constant, fasting and postprandial SBA should have same sensitivity [35, 44]. Thus higher sensitivity in postprandial determination of SBA may be the result of a more accurate determination of SBA when its value is elevated after the meal [35] or some extrahepatic factors, i.e. shunting during digestion [45].

SBA and TG increased very rapidly at 1 hr after feeding in this study. TG concentration decreased rapidly at 4 hr whereas SBA remained high up to 8 hr. This may be explained by the different absorption sites of bile acids and triglycerides. Although appreciable amounts of fat are absorbed in the ileum, the greatest amounts are absorbed in the proximal small intestine. Almost 50% of the total fat contained in a meal is absorbed within the duodenum [27, 46]. Passive, nonionic diffusion of bile acids is thought to occur throughout the intestine whereas the active transport is limited to the ileum. Additionally, bile acids can be supplied continuously by the enterohepatic circulation whereas triglycerides disappeared from the intestine once they are absorbed. Because of the rapid, significant rise of SBA observed at 1 hr after feeding, postprandial blood samples can be taken at 1 hr instead of at 2 hr as is used by most of the investigators [1, 2, 5, 9, 18, 19, 26, 28, 31].

In the present study the fractionation was done to establish the types of bile acids seen in dog serum and develop the methods for HPLC assay of bile acids in dog serum, and
it could be the first step of the future study to clarify the response of individual bile acids to liver injury and efficiency and effectiveness of individual bile acids as indicators of various types of liver injury in the dog. Unfortunately, none of the peaks was identified during fractionation of SBA by HPLC in this study. The unidentified peak with a retention time near 6.4 min, which increased significantly at 2 hr after feeding, was not cholic acid. A second unidentified peak appeared after feeding near 9.0 min. These unidentified peaks might be the species-specific bile acids, for example, such as β-muricholic acid [29], which is a metabolite of chenodeoxycholic acid and found only in rats and mice, and its bacterial deconjugation and/or deoxyxylation products.

REFERENCES

SERUM BILE ACIDS IN NORMAL DOGS


要約

正常犬における食餌後の血清胆汁酸濃度および高速液体クロマトグラフィーによる胆汁酸の分画：鶴巻美・
吉塚 薫・KANEKO, Jiro, J. (California 大学農医学部, 臨床病理学教室) —— 正常犬における食餌後の血清胆
汁酸濃度の推移を調べるとともに高速液体クロマトグラフィーによる血清胆汁酸の分画を試みた。食前の胆汁酸
濃度は1.07±0.91μM/1で、食後1時間で著しく増加し、6時間で最高値10.35±7.32μM/1に達した。食後1
時間から8時間までの平均血清胆汁酸濃度の間に有意差は見られなかった。このことから、食後サンプルの採取
は2時間ではなく1時間でよいと思われた。高速液体クロマトグラフィーによる血清胆汁酸の分画では、空腹時
サンプルで保持時間6.4分にピークがみられ、食後2時間のサンプルでは9.0分に新たなピークが出現したが、い
ずれのピークについても胆汁酸の種類を確認できなかった。