Changes in Bile Acid Composition of Serum and Gallbladder Bile in Bile Duct Ligated Dogs

Tsukimi WASHIZU, Takuo ISHIDA, Makoto WASHIZU, Isamu TOMODA, and Jiro J. KANEKO

Department of Clinical Pathology, Veterinary Medical Teaching Hospital, Nippon Veterinary and Animal Science University, 1–7–1 Kyonan-cho, Musashino, Tokyo 180, Japan and Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, U.S.A.

(Received 23 June 1993/Accepted 15 November 1993)

ABSTRACT. Biliary obstruction was produced by surgical ligation of the common bile duct to observe alterations in serum bile acid composition. The percent composition of serum bile acids was found to change with time. Taurocholic acid increased on day 3 and accounted for more than 90% of the total bile acids in all dogs, however it decreased after day 7. The percentage of taurochenodeoxycholic acid (TCDC) and taurodeoxycholic acid (TDC) decreased to 4.2–6.0% and 0.2–0.7% on day 3, respectively. However, the percentage of TCDC increased after day 7 in all dogs and reached greater than 20% on day 14 in 2 dogs, whereas the percent TDC after bile duct ligation remained low in all dogs. Glycocholic acid, which was not identified in normal dog sera, was detected on day 3 and remained throughout the study in all dogs. Bile acid composition of gallbladder bile sampled on day 35 was similar to the serum bile acid composition on the same day. This indicates that the bile acids refluxed into the circulation in these dogs. In the present study, total cholic acid to chenodeoxycholic acid (C:CDCA) ratio increased to 15.5–22.3 at three days post bile duct ligation and after the day 14, the C:CDCA ratio decreased to its pre-ligation value or below. In contrast, the glycine conjugated to taurine conjugated bile acids (G:T) ratio did not change. Therefore, at this time, the G:T ratio would not be usable as an indicator of liver disease in dogs while it may be possible to use the C:CDCA ratio.—KEY WORDS: bile, bile acid, bile duct ligation, canine.

Primary bile acids are synthesized solely by the liver, excreted into the bile and stored in the gallbladder. They are released from the gallbladder into the intestines following a meal. In the intestines, these primary bile acids are converted to secondary bile acids by bacterial action [9, 25, 32]. These secondary bile acids are reabsorbed by the ileum, transported to the liver via the portal vein and extracted by the hepatocytes to form the enterohepatic circulation [12, 20]. Serum bile acid concentrations (SBA) remain constant in healthy animals and increase in various forms of hepatobiliary diseases [2, 4, 22, 28, 29, 33].

The total SBA is now widely used to detect hepatobiliary diseases because of its high sensitivity and specificity. While SBA has proven to be a sensitive liver function test in animals [2, 6–8, 17], differentiation of the various forms of liver disease cannot be made on the basis of an increase in total SBA. Recent advances in high performance liquid chromatography (HPLC) techniques now offer a rapid and accurate means to identify and quantitate individual bile acids. In humans, individual bile acids have been fractionated to elucidate the mechanisms of SBA alterations in various forms of hepatobiliary diseases to localize the type and site of the injury [21, 27]. Similarly, individual serum bile acids may be useful for the evaluation of hepatobiliary disease in the dog. The bile acid composition of serum and gallbladder bile in normal dogs has been reported [31, 35] but their changes in hepatobiliary diseases are not known. In the present study, biliary obstruction was produced by surgical ligation of the common bile duct to observe any alterations in serum bile acid composition.

MATERIALS AND METHODS

Animals: Three clinically healthy adult dogs of mixed breed weighing between 7.0 to 13.0 kg were used in this study. A complete hemogram and a serum chemistry panel, which included total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), cholesterol, urea nitrogen, and creatinine, were within normal reference ranges in these dogs. Bile ducts were ligated distal to the gallbladder under the general anesthesia using halothane. Preprandial blood samples were taken from each dog before ligation (day 0) and at 3, 7, 14, 21, 28 and 35 days after ligation for hematology, blood chemistry and bile acids. The dogs were fasted overnight for 12 hr before surgery and sampling. Gallbladder bile was taken from each dog at the surgery before ligation (day 0) and on day 35 when the dogs were euthanized and the study terminated.

Hematology and serum chemistry: The complete hemogram and serum chemistry panel were performed by using Kodak Ektachem DT60 analyzer (Nagase, Tokyo, Japan). Total SBA was determined using a commercially available kit (Enzabile 2, Daiichi Kagaku, Tokyo, Japan). 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-hydroxysteroid dehydrogenase (3a-HSD). The coloured product, formazan, is formed when NADH is transferred to the nitroblue tetrazolium in the presence of the enzyme, diaphorase.

Fractionation of bile acids: The HPLC system for bile
acid fractionation was as previously reported [34, 35]. Serum was first diluted with 9 volumes of 0.1 M NaOH solution. The diluted serum sample was then extracted using a SEP-PAK C18 cartridge (Waters Associates, Inc., Massachusetts, U.S.A.) and then eluted with methanol.

The gallbladder bile was diluted with methanol and injected onto the column without the extraction step. A Tosoh HPLC system (Tosoh, Ayase, Japan) which included a computerized system controller and TSK gel ODS-80TM (4.6 mm × 15 cm) reverse phase column.

![Fig. 1. Changes in serum bile acid concentration, enzyme activities, and other parameters after bile duct ligation.](image)

Table 1. Changes in serum bile acid composition (%) of bile duct ligated dogs

<table>
<thead>
<tr>
<th>Bile acida)</th>
<th>days after ligation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>TC</td>
<td>60.6±4.0b)</td>
</tr>
<tr>
<td></td>
<td>(56.1–63.7)c)</td>
</tr>
<tr>
<td>C</td>
<td>2.2±2.3</td>
</tr>
<tr>
<td></td>
<td>(0–4.6)</td>
</tr>
<tr>
<td>TCDC</td>
<td>10.0±1.01</td>
</tr>
<tr>
<td></td>
<td>(9.5–10.7)</td>
</tr>
<tr>
<td>CDC</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td></td>
<td>(0–0.3)</td>
</tr>
<tr>
<td>TDC</td>
<td>23.7±1.5</td>
</tr>
<tr>
<td></td>
<td>(22.0–24.8)</td>
</tr>
<tr>
<td>DC</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td></td>
<td>(0.7–1.5)</td>
</tr>
<tr>
<td>TLC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>GLC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>TUDC</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td></td>
<td>(0.5–0.7)</td>
</tr>
<tr>
<td>UDC</td>
<td>1.7±0.8</td>
</tr>
<tr>
<td></td>
<td>(0.9–2.5)</td>
</tr>
</tbody>
</table>

a) TC, taurocholic acid; C, cholic acid; TCDC, taurochenodeoxycholic acid; CDC, chenodeoxycholic acid; TDC, taurodeoxycholic acid; DC, deoxycholic acid; TLC, taurolithocholic acid; GLC, glycolithocholic acid; TUDC, tauroursodeoxycholic acid; UDC, ursodeoxycholic acid.
b) Mean±standard deviation.
c) Ranges.
(Tosoh, Ayase, Japan) was used for fractionation. After injection, the samples were eluted by a stepwise gradient method. β-nicotinamide-adenine dinucleotide (β-NAD) was added to the eluates before passing through an immobilized 3α-HSD column. The NADH formed by the reaction was then measured fluorometrically with the detector set at a wavelength of 345 nm for excitation and 450 nm for emission.

Fifteen bile acid standards [cholic acid (C), chenodeoxycholic acid (CDCA), deoxycholic acid (DC), lithocholic acid (LC), ursodeoxycholic acid (UDCA), taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), tauroursodeoxycholic acid (TUDC), glycocholic acid (GC), glycodeoxycholic acid (CDCA), glycodeoxycholic acid (GDC), glycolithocholic acid (GLC), glycochenodeoxycholic acid (GUDC)] were obtained from Sigma Chemical Co., St. Louis, and used as external standards.

**RESULTS**

ALT (3855–6293 U/l) and cholesterol (480–730 mg/dl) increased to a maximum 7 days after bile duct ligation. AST (570–996 U/l), ALP (1779–3954 U/l) and TB (3.1–6.9 mg/dl) increased on day 3 and remained elevated. SBA increased above 300 μM/l (535–732 μM/l) on day 3 in all dogs and remained high although it decreased after day 28 in one dog (Fig. 1).

Among the individual bile acids, TC increased on day 3 and accounted for more than 90% (90.3–93.4%) of the total bile acids in all dogs on this day. This was followed by a gradual decrease in TC after day 7. Although the concentration of both TCDC and TDC also increased, their percentage of the total had decreased to 4.2–6.0% and 0.2–0.7% on day 3, respectively. The percentage of TCDC increased after day 7 in all dogs and reached greater than 20% on day 14 in 2 dogs. In contrast, the percent TDC remained low in all dogs throughout the study. Glycolithocholic acid, which was not identified in normal dog sera, detected on day 3 and was identified in the serum throughout the study in all dogs (Table 1). The C: CDC ratio increased to 15.5–22.3 on day 3 and decreased to its pre-ligation value or below after day 14 (Fig. 2).

The bile acid composition of the gallbladder bile on day 35 differed from that on day 0. The TC, TCDC, and TDC constituted 72.8%, 6.2% and 20.3%, respectively on day 0 whereas 80.2%, 16.3%, and 3.6%, respectively on day 35. Similar tendency was observed in bile acid composition of serum and gallbladder bile obtained on day 35 (Fig. 3).

**DISCUSSION**

The mechanism for the increase in total bile acids after biliary obstruction is thought to be first, the result of increased synthesis and second, the result of a refluxing of bile acids into the serum under conditions where their normal excretory route is obstructed [18]. After total SBA increased to a maximum on day 3, it decreased in some degree in all dogs in this study. It has been observed that there is increased urinary excretion of bile acids and increased sulfated bile acids in serum and urine in patients with extrahepatic cholestasis and biliary atresia [19, 23, 24]. The sulfation of bile acids is thought to be the protective mechanism by the liver to facilitate the excretion of bile acids into the urine [19, 23, 24]. It is possible that the sulfation and/or excretion of bile acids into the urine had occurred in the present study as in human patients.

The relationship between total bile acids and ALP in bile duct obstruction has been previously studied in rats [26, 30, 31]. Righetti and Kaplan [30] suggested that the high concentration of bile acids in the hepatocytes of animals with a biliary obstruction may have a detergent effect which could enhance the solubilization of ALP. It has also been suggested that bile acids could induce de novo ALP synthesis, because in cultured rat hepatocytes,
ALP concentration increased at 6 hr after bile acids were added to the culture media and ALP induction was completely inhibited by cyclohexamide [15, 16]. Each individual bile acid appears to have a different effect on ALP induction. Taurocholic acid induces ALP synthesis in cultured rat hepatocytes at concentrations similar to those seen in the serum of cholestatic animals and it does so in a dose dependent manner [15]. Taurochenodeoxycholic acid, TDC, GC, GCD and GDC also stimulate ALP synthesis. Among these, TCDC and TDC have almost the same stimulatory effect as TC but GC, GCD and GDC have a lesser effect as compared to TC [15]. In the present study, those bile acids (TC, TCDC, TDC) which strongly induce and solubilize ALP were the ones which increased significantly in serum after bile duct ligation. If dog hepatocytes respond to bile acids in a manner similar to rat hepatocytes, a similar mechanism could explain the increased ALP in biliary obstruction in the dog.

The percent composition of serum bile acids was also found to change with time in this study. The total C (C + TC) was 92.5% on the 3rd day, 85.4% on day 7, and 77.0% on day 14. The total CDC (CDC + TCDC) which was 4.9% on day 3, 6.5% on day 7, 18.8% on day 14, and reached 23.7% on day 21 after bile duct ligation. The serum bile acid composition in bile duct obstructed rats is different from that in intact animals and it could be the result of an alternate bile acid synthetic pathway in cholestatic animals [18]. In normal rat liver, the trihydroxy to dihydroxy bile acids ratio is 3:2, however, trihydroxy bile acids were 85% at 3 days after bile duct ligation and reached 97% at 8 to 10 days after ligation [14]. Most of the trihydroxy bile acids in rats is β-muricholic acid which is hydroxylated CDC on the 6β position. It has been suggested that the increased production of trihydroxy bile acid is a protective mechanism of hepatocytes, because CDC is more cytotoxic than to β-muricholic acid [1, 14]. Greim et al. [13], who observed increased C in human livers with biliary obstruction, also suggested that the increased synthesis of C was a protective mechanism to prevent the formation of the other primary bile acid, CDC which is known to be hepatotoxic at high concentration. In human patients with protracted extrahepatic biliary obstruction, it was demonstrated that the concentration of CDC correlated with the histological change, feathery degeneration [13]. An increase in total C concentration during early bile duct ligation in the dog may similarly be a protective mechanism in a manner similar to β-muricholic acid formation in the rat and C formation in humans. This would be an important protective mechanism if CDC was also a hepatotoxic agent in dogs. In the present study, total C decreased with time and total CDC increased after the 14th day post-ligation. The decrease in total C could be the result of the impairment of one or more enzymatic reactions which are directly involved in C synthesis. It is unlikely to be due to the impairment of 7a-hydroxylase, the rate limiting enzyme in bile acid synthesis, because total CDC, the other primary bile acid, had increased. The late increase of total CDC in prolonged cholestasis is thought to be due to the impairment of 12α-hydroxylation of bile acids and the activation of alternative pathways [10]. The increase in total CDC after the 14th day post-ligation in the dogs in this study might be explained by a similar impairment of the 12α-hydroxylation system. Taurodeoxycholic acid composition decreased markedly on day 3 post-ligation. This can be explained by the complete cessation of the enterohepatic circulation of bile acids by the bile duct ligation. Glycolithocholic acid, the other secondary bile acid, appeared in the serum 3 days after bile duct ligation in all dogs. A possible explanation is that the peak identified as GLC could be another bile acid with a retention time similar to that of GLC. However, when these sample were chromatographed without the 3α-HSD column, this peak did not appear indicating that at least this peak represented a compound with a hydroxy group at the C-3α-position. Additionally, it might be one of the abnormal bile acids observed in patients with biliary obstruction [5]. While GLC remained in the sera throughout the study, it was not identified in gallbladder bile on day 35. This would indicate that GLC was not excreted into the bile but had refluxed into the blood. Lithocholic acid formation should have been absent in bile duct obstructed dogs because of the disruption of the enterohepatic circulation. This could mean that GLC may have been synthesized via an alternate pathway.

The bile acid composition of gallbladder bile sampled on day 35 was similar to the serum bile acid composition on the same day. This indicates that the bile acids refluxed into the circulation in these dogs. It has been reported [11] that bile acid carrier proteins move from bile canaliculi to basolateral membranes in bile duct obstructions and that these carrier proteins are still able to transport bile acid. It was suggested that the carrier proteins which move to the basolateral membrane, play an important role for the refluxing of bile acids into the circulation [11].

Virtually all the bile acids in serum and gallbladder bile were conjugated in this study, therefore, hepatocytes in bile duct obstruction retain their ability to conjugate bile acids. The G:T and C:DC ratios have been used as indicators of liver disease in humans. In human patients with cholestasis, it is known that the G:T and C:DC ratios increase. In primary biliary cirrhosis, the C:DC ratio decreases as the disease progresses with CDC predominating as is observed in hepatocellular injury [3]. In the present study, the C:DC ratio increased to 15.5-22.3 at 3 days post bile duct ligation and after day 14, the C:DC ratio decreased to its pre-ligation value or below. In contrast, the G:T ratio did not change. Therefore, at this time, the G:T ratio would not be usable as an indicator of liver disease in dogs while it may be possible to use the C:DC ratio. The use of these ratios, however, would require further study.
REFERENCES


