Serum Bile Acid Composition of the Dog, Cow, Horse and Human

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ABSTRACT. The fractionation of serum bile acids was performed in the dog, cow, horse, and human by high performance liquid chromatography equipped with an immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column. There were significant differences in the bile acid compositions, conjugation patterns and quantities of each bile acid among these animals. Cholic acid was the major primary bile acid in the dog and cow, which constituted 62.9% and 83.5%, respectively, whereas chenodeoxycholic acid was the major acid in the horse and human, which constituted 68.4% and 46.3%, respectively. Taurine conjugates were predominant in the dog and horse, which constituted 94.4% and 85.3%, respectively, whereas glycine conjugates were predominant in the cow and human, which constituted 31.0% and 49.4%, respectively. Although there were several unidentified peaks, it was confirmed that they had a hydroxy group at the C-3α position by chromatographing the samples without the 3α-HSD column.—KEY WORDS: composition, cow, dog, horse, serum bile acid.


The bile acids are synthesized in the liver and excreted into the bile. They are released after a meal and are reabsorbed in the ilium after completing their functions in the intestine [15, 20]. The reabsorbed bile acids are transported to the liver via the portal vein where they are extracted, conjugated and re-excreted. The bile acids are involved in hepatic function in a number of physiologically important ways. First, bile acids constitute the major metabolic pathway for hepatic cholesterol excretion [17]. Second, bile acids are involved in gastro-intestinal function by stimulating hepatic bile flow [12, 17]. Third, bile acids enhance lipid absorption from the gut by the formation of mixed micelles. From a pharmacological point of view, chenodeoxycholic acid, ursodeoxycholic acid and hydoxycholic acid have been found to be useful for the dissolution of gallstones in man and prairie dogs [4, 10, 29]. Under physiological conditions, the total serum bile acids (SBA) level is a balance between intestinal absorption, hepatic uptake and recycling. Therefore, in healthy animals, SBA generally remains constant [3, 27, 33]. SBA increases in the following situations: 1) insufficient extraction by the hepatocytes; 2) leakage or regurgitation from the hepatocytes; and 3) portosystemic shunting [5, 22, 26].

Recently, SBA as a liver function test has been investigated in veterinary medicine in both experimental and clinical situations [3, 6–8, 18]. However, SBA levels increase in a variety of hepatobiliary diseases, so that differentiation of the various forms of liver disease cannot be made simply by measuring SBA. The fractionation and identification of the individual serum bile acids offers a new approach to differentiate the forms of liver disease and has been applied in man using high performance liquid chromatography (HPLC) [21, 25]. Similarly, the evaluation of the fractionated individual bile acids may potentially be a useful and definitive test for the differential diagnosis of various forms of liver disease in the dog. The purpose of the present study was to establish the methods for HPLC assay of serum bile acids in dog serum and fractionate the serum of cow, horse, and human in this method for comparative purposes.

MATERIALS AND METHODS

Animals: Ten adult mixed breed dogs weighing between 8.0 to 11.3 kg were used. A complete blood count and standard serum chemistry tests, which included total protein, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, cholesterol, urea nitrogen and creatinine, were all within normal range. Five clinically healthy adult Holstein cattle and thoroughbred horses were used. All animals were fasted overnight for 12 hr before sampling. Human samples were taken from three healthy volunteers after an overnight fast.

Measurement of total SBA: The concentration of SBA was measured using a commercially available kit (Enzabile 2, Daiichi Kagaku, Tokyo, Japan).
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 (3α-HSD). The nitroblue tetrazolium (NBT) is converted to a coloured product, formazan, when NADH is oxidized in the presence of the enzyme, diaphorase.

**Equipment and mobile phase for SBA fractionation:** A Tosoh HPLC system (Tosoh, Ayase, Japan) which included a computerized system controller (SC-8010) and a TSK gel ODS-80 Tm (4.6 mm x 15 cm) reverse phase column was used. Fifty μl of each sample was applied to this column, which was eluted at 0.6 ml/min with A) CH$_3$OH: 20 mM Na$_2$HPO$_4$, pH 3.5 (65:35, v/v) for 6.5 min B) CH$_3$OH: 20 mM Na$_2$HPO$_4$, pH 6.5 (67:33, v/v) for 22.5 min, C) CH$_3$OH: Na$_2$HPO$_4$, pH 6.0 (72:28, v/v) for 11 min. Seven hundred μmol/l of β-nicotinamide-adenine dinucleotide (β-NAD) solution prepared in 200 mM K$_2$HPO$_4$ and 1 mM ethylenediamine tetraacetic acid-4H was added to the eluates at a flow rate of 0.3 ml/min before passing through an immobilized 3α-HSD column. The NADH formed by the reaction was determined fluorometrically with the detector (FS-8010) set at a wave length of 345 nm for excitation, and 450 nm for emission.

**Standards:** Bile acids for use as standards were obtained from the Sigma Chemical Co., St. Louis. A mixture of fifteen bile acid standards (choleic acid(C), chenodeoxycholic acid (CDCA), deoxycholic acid (DC), lithocholic acid (LC), ursodeoxycholic acid (UDCA), taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), tauroliothocholic acid (TLC), tauroursodeoxycholic acid (TUDC), glycocholic acid (GC), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDC), glycolithocholic acid (GLC), and glycoursoxycholic acid (GUDC)) were used as external standard. Each ml of standard solution contained 5 μg of each bile acid.

**Serum preparation:** One ml of serum was diluted with 9 ml of 0.1 M NaOH solution and applied to a SEP-PAK C$_{18}$ cartridge (Waters Associates, Inc., Massachusetts, U.S.A.). After washing with 20 ml of distilled water, the bile acids were eluted with 4 ml of 95% ethanol. The effluent was evaporated to dryness under vacuum after which it was dissolved in 0.1 ml of 65% methanol and 50 μl was then injected onto the HPLC column.

**RESULTS**

The mean total SBA concentrations of the dog, cow, horse and human as measured by the 3α-HSD method were 2.45±0.34 μmol/l, 93.44±15.48 μmol/l, 3.64±0.96 μmol/l, 4.87±0.96 μmol/l, respectively. Nine bile acids (TUDC, TC, UDC, C, TCDC, TDC, CDC, DC, and TLC) were identified in the dog sera using HPLC (Fig. 2A) and the average total concentration of these bile acids was 791.2±849.42 ng/ml. While TC, TCDC and TDC were consistently present in all dog sera, one or more of the other 6 bile acids were absent in some dogs. These three major bile acids, TC, TDC and TCDC, constituted 55.1%, 26.2% and 11.3%, respectively. The primary to secondary bile acid ratio was 7:3 and C:CDCA ratio was 4.7. Taurine
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Fig. 2. Serum bile acid chromatogram of the dog (A), cow (B), horse (C), and human (D). Peaks without identifying initials are unidentified bile acids. Peaks with retention times of less than 9 are solvent impurities. TUDC, tauroursodeoxycholic acid; GUDC, glycocholic acid; GC, glycocholic acid; UDC, ursodeoxycholic acid; C, cholic acid; TCDC, taurochenodeoxycholic acid; GCDC, glycochenodeoxycholic acid; TDC, taurocheno- cholic acid; GDC, glycodeoxycholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; TLC, tauro lithocholic acid; GLC, glycolithocholic acid.

Table 1. Serum bile acid composition of the dog, cow, horse and human

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>Dog</th>
<th>Cow</th>
<th>Horse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUDC</td>
<td>0.8±1.6</td>
<td>-</td>
<td>-</td>
<td>9.6±8.3</td>
</tr>
<tr>
<td>GUDC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TC</td>
<td>55.1±8.6</td>
<td>21.6±5.9</td>
<td>23.9±6.1</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td>GC</td>
<td>-</td>
<td>20.5±7.0</td>
<td>-</td>
<td>4.7±3.2</td>
</tr>
<tr>
<td>UDC</td>
<td>0.9±1.5</td>
<td>-</td>
<td>6.3±1.2</td>
<td>7.0±3.8</td>
</tr>
<tr>
<td>C</td>
<td>2.9±3.3</td>
<td>41.4±10.3</td>
<td>1.4±0.4</td>
<td>9.2±2.5</td>
</tr>
<tr>
<td>TCDC</td>
<td>11.3±5.0</td>
<td>0.3±0.7</td>
<td>61.4±3.7</td>
<td>3.1±0.9</td>
</tr>
<tr>
<td>GCDC</td>
<td>-</td>
<td>2.4±0.8</td>
<td>7.0±3.8</td>
<td>27.1±5.3</td>
</tr>
<tr>
<td>TDC</td>
<td>26.2±6.2</td>
<td>2.3±1.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GDC</td>
<td>-</td>
<td>7.4±2.2</td>
<td>-</td>
<td>8.1±7.3</td>
</tr>
<tr>
<td>DC</td>
<td>0.6±0.7</td>
<td>2.7±1.4</td>
<td>-</td>
<td>11.7±9.8</td>
</tr>
<tr>
<td>TLC</td>
<td>1.0±1.7</td>
<td>0.2±0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GLC</td>
<td>-</td>
<td>0.7±0.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a) TUDC, tauroursodeoxycholic acid; GUDC, glycocholic acid; TC, taurocholic acid; GC, glycocholic acid; UDC, ursodeoxycholic acid; C, cholic acid; TCDC, taurochenodeoxycholic acid; GCDC, glycochenodeoxycholic acid; TDC, taurocheno- cholic acid; GDC, glycodeoxycholic acid; DC, deoxycholic acid; TLC, tauro lithocholic acid; GLC, glycolithocholic acid.

b) Mean±standard deviation.

conjugates accounted for 94.4% of the total bile acids.

Eleven bile acids (TC, GC, C, TCDC, GCDC, TDC, GDC, CDC, DC, TLC, and GLC) were identified in cow sera and there was one unidentified peak immediately after the GC peak (Fig. 2B). TC, GC and C were the major bile acids and constituted 21.6%, 20.5% and 41.4%, respectively. The C:CDC ratio was 26.1. The unconjugated fractions, which accounted for 47.7%, predominated in this species.

Five bile acids (TC, UDC, C, TCDC, and GCDC) were identified in horse sera and there were
two unidentified peaks with retention times near 26 and 28 min (Fig. 2C). TC, TCDC and GCDC were the major bile acids and constituted 23.9%, 61.4% and 7.0%, respectively. The C:CD ratio was 0.37. Serum bile acids of this species were conjugated predominantly with taurine and which constituted 85.3% of the total.

Ten bile acids (GUDC, TC, GC, UDC, C, TCDC, GCDC, GDC, CDC, and DC) were identified in human sera and all peaks were identifiable (Fig. 2D). Glycine conjugates were the major components in human sera and the taurine conjugated bile acids were the minor components. CDC was the major bile acids and constituted 46.3%. The C:CD ratio was 0.38 in human sera.

DISCUSSION

The method used in this study employed an immobilized 3α-HSD column has been shown to be sufficiently sensitive to detect serum bile acids of domestic animals as well as in the human. It has been reported that in healthy persons, CDC was the predominant bile acid and that the bile acids are mainly conjugated to glycine [1, 13, 31, 32]. Goto reported [16] that CDC constituted 54% of the SBA and C:CD ratio was 0.34. Similar fractionation pattern was observed in the present study, therefore, the results obtained using this method will be applicable to compare with the results of others.

In the first step of bile acid synthesis, cholesterol is metabolized primarily to 7α-hydroxycholesterol and to 26-hydroxycholesterol to a lesser extent. Both of these metabolites undergo further enzymatic modification to either C or CDC. In humans, a greater proportion of 26-hydroxycholesterol is converted to CDC than to 7α-hydroxycholesterol [2]. In order to form C, 12α-hydroxylation is necessary whereas CDC does not require 12α-hydroxylation. One explanation for the high C concentration in the dog is that the degree of conversion of cholesterol to 26-hydroxycholesterol is less and that the 12α-hydroxylase activity is higher in the dog as compared to humans. In the horse, TCDC was the major component and its C:CD ratio was the most similar to humans.

SBA of the dog was conjugated mainly with taurine and glycine conjugates were not observed in the present study. However, it is possible that their concentration was below the detection limits of this system because a small amount of glycine conjugated bile acids were found in gall-bladder bile of the dog [35]. Conjugation of bile acids with either glycine or taurine is catalyzed by a common enzyme, the N-acyltransferase which is present in the soluble fraction of the liver cell [19, 34]. The affinity of the bovine N-acyltransferase for glycine and taurine is reported [30] to be quite similar with a Km for glycine of 8.8 mM and a Km for taurine of 6.7 mM. However, there was a difference in the rates of conjugation for the different CoA adducts in the presence of the same concentration of glycine [11].

In humans, oral administration of taurine resulted in a G:T ratio less than one whereas glycine administration did not change this proportion [30]. Furthermore, it is known in humans that G:T ratio changes significantly in liver diseases, in bowel disorders and under bile acid therapy [1, 9, 13, 14, 23, 31]. In the dog, even though the taurine pool was completely depleted, glycine could not be used for conjugation with cholic acid [24]. Similarly, conjugation of bile acids with taurine in the cat fed a taurine deficient diet was only moderately affected, though plasma and retinal taurine pools were largely depleted [28]. It would appear to be an inherent property of the N-acyltransferase of an animal species whether glycine or taurine can be used preferentially or interchangeably as a substrate. Dogs appear to have an N-acyltransferase with a very high affinity for taurine, whereas the enzyme of cows and humans appears to have similar affinities to both of amino acids though glycine is preferred.

There were several unidentified peaks in the present study. When these samples were chromatographed without the immobilized 3α-HSD column, none of these unidentified peaks appeared indicating that these peaks represented compounds with a hydroxy group at the C-3α position. They may be allo-bile acids which are the 5α-isomers of bile acids, the bile acids whose C-6, C-7 and C-12 are substituted with other groups to give different bile acids or isomers of ordinary primary and secondary bile acids.

In the present study, the fractionation of SBA in the dog, cow, horse, and human was performed using HPLC. There were significant differences in the major bile acids, conjugation patterns and concentrations of the individual bile acids among these species. This indicates that there are considerable interspecific differences in the specificity, affinity and activity of the enzymes which involve bile acids synthesis. Therefore, a corollary to the use of
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SBA fractionation for the understanding of the pathophysiological processes underlying hepatobiliary diseases of animals is a thorough knowledge of species specific patterns of bile acid metabolism.

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REFERENCES


