

Biliary, Fecal and Plasma Deoxycholic Acid in Rabbit, Hamster, Guinea Pig, and Rat: Comparative Study and Implication in Colon Cancer

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Bile acids are believed to play a role in the etiology of colorectal cancer, and high fecal excretion of secondary bile acids was correlated with increased incidence of colon cancer. Recently, it was also reported that there is an increase in plasma of the secondary bile acid, deoxycholic acid in men with colorectal adenomas. Since deoxycholic acid is formed in the colon and absorbed into the portal systemic circulation, it was suggested that the blood concentration of this bile acid reflects the level of exposure of colonic cells to deoxycholic acid. The objective of this study was to investigate whether plasma deoxycholic acid level represents the fecal content of this bile acid in several animal species with different bile acid composition and deoxycholic acid contribution to the bile acid pool. Eight rabbits, hamsters, guinea pigs, and rats were used in this study. Blood samples and feces were collected on days 1, 3, 5 and 7. Bile samples were obtained only on day 7. The plasma, fecal and biliary bile acids were analyzed by gas chromatography-mass spectrometry. Bile acid composition and deoxycholic acid content varied greatly between the animal species studied. There was a variation in the concentration of total bile acids in the plasma and feces obtained at different times during the experiments, however, the bile acids profile remained constant throughout the study. The data obtained shows that although plasma bile acid profile was not similar to fecal bile acids profile, however, there was a significant correlation between the level of plasma and fecal deoxycholic acid. Plasma deoxycholic acid concentration might be a reliable biomarker for the degree of exposure of colon cells to this bile acid, and may be useful in further studies on the role of secondary bile acids in colon carcinogenesis.

Key words deoxycholic acid; feces; plasma; bile; colon cancer; gas chromatography mass spectrometry

Bile acids are the main by-product of cholesterol and play a major role in maintaining bile formation. An enzymatic cascade converts cholesterol to the primary bile acids, (cholic and chenodeoxycholic acid) in the liver. During the enterohepatic circulation, primary bile acids undergo biochemical transformation such as dehydroxylation by the action of intestinal bacteria and produce secondary bile acids (deoxycholic acid and lithocholic acid).^{1,2} Secondary bile acids have long been implicated in colorectal cancer as co-carcinogens, and some people with colorectal cancer are shown to excrete higher levels of secondary bile acids in their stool. High concentration of the secondary bile acid, in particular deoxycholic acid, may damage colonic epithelium to accelerate carcinogenesis.^{3–8} Since deoxycholic acid is formed in the colon and absorbed into the portal systemic circulation, it was suggested that blood level of deoxycholic acid might reflect the degree of exposure of colon cells to this bile acid during its absorption.

Although it appears likely that secondary bile acids present in the feces stimulate the proliferation of the human colonic mucosa, no study so far has proven the correlation between the serum deoxycholic acid level and deoxycholic acid in feces. This prompted us to analyse the bile acid and deoxycholic acid compositions in bile, feces, and plasma of different species (rabbit, hamster, guinea pig and rat) with different bile acid profile and to correlate the plasma deoxycholic acid to the fecal content of the same bile acid.

MATERIALS AND METHODS

Reagents Bile acids standards (cholic acid, chenodeoxy-

cholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid and 5 β -cholanic acid) were obtained from Calbiochem, San Diego, CA, U.S.A. and were at least 98% pure. All other solvents and chemical were of either HPLC grade of a known analytical purity and obtained from Sigma and Aldrich Chemical Co., U.S.A.

Animals Eight male adults of Sprague drawly rats (350–400 g); Syrian hamsters (110–120 g); New Zealand rabbits (1.5–2 kg); and guinea pig (400–500 g) were used in this study. Each animal was kept in separate metabolic cage. All animals were fed Purina chow *ad libitum* and had free access to drinking water. Serum was collected on days 1, 3, 5, and 7 under anaesthesia. Feces were collected over a period of 24 h on days 1, 3, 5, and 7 and fecal water was separated from total feces. In rats, hepatic bile was collected after bile duct cannulation (for 30 min) under sodium Phenobarbital anaesthesia.⁹ The bile from the rabbit, hamster and guinea pig was taken from the gall bladder. Bile acids were extracted from all the samples (bile, plasma, fecal water, and fecal pellet) and bile acids were determined by gas chromatography mass-spectrometry.

Extraction of Bile Acids from Fecal Pellet and Fecal Water Fecal samples were lyophilized and kept at –20 °C until required for analysis. One ml of cold distilled water was added to 250 mg of lyophilized feces and homogenized for 3–5 min using a polytron homogenizer then incubate for 1 h at 37 °C. The homogenized samples were centrifuged at 4000 rpm for 10 min. The supernatant (fecal water) was separated from fecal pellet. The fecal pellet was refluxed for 1 h at 80 °C in 5 ml of ethanol containing 0.01 N NaOH (0.4 ml) and 100 μ g of internal standards (5 β -cholanic acid). The

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Table 1. Biliary Bile Acid (%) Profile and Total Bile Acids of Different Animal Species

Species	LCA	DOC	CDC	CA	Muri	URSO	Total bile acid (nmol/100 g bwt)
Rabbit	0.91±0.25	89.63±6.42	1.22±0.90	8.23±6.45	ND	ND	816.19±299.01
Hamster	0.88±0.53	14.82±12.32	12.28±3.11	72±10.52	ND	ND	1498.31±1465.63
Guinea pig	0.85±1.39	0.32±0.054	81.85±5.60	4.63±3.55	ND	12.33±2.89	440.38±269.81
Rat	1.99±5.25	5.02±3.56	6.48±4.27	76.42±18.40	6.68±7.45	5.24±6.94	59.88±48.19

Eight rabbits, hamsters, guinea pigs and rats were used. Values are mean±S.D. of the total and individual bile acids. LCA, lithocholic acid; DOC, deoxycholic acid; CDC, chenodeoxycholic acid; CA, cholic acid; Muri, muricholic acid; URSO, ursodeoxycholic acid. ND=not detected.

Table 2. Plasma Bile Acid (%) Profile in Different Animal Species

Species	% LCA (Range)	% DCA (Range)	% CDCA (Range)	% CA (Range)	% URSO (Range)	% MURI (Range)	Total bile acid (nmol/100 g bwt) (Range)
Rabbit	3.13±3.0 (0.97—7.58)	43.8±6.8 (34.12—49.89)	7.05±6.1 (2.2—17.39)	44.57±12.3 (33.4—61.64)	1.43±0.9 (0.37—2.16)	ND	1.08±0.44 (0.54—1.51)
Hamster	1.39±0.41 (0.87—1.85)	12.34±4.1 (7.21—16.14)	10.85±6.7 (4.68—20.09)	73.3±9.82 (65.04—86.42)	2.1±2.04 (0.35—4.96)	ND	9.56±4.61 (1.97—4.12)
Guinea pig	3.64±1.8 (1.25—5.25)	4.89±1.93 (2.4—7.13)	46.3±17.89 (29.56—67.69)	39.45±17.89 (20.15—55.02)	5.67±2.67 (2.28—8.5)	ND	14.6±8.3 (7.44—26.19)
Rat	1.18±0.8 (0.61—2.34)	10.74±3.1 (6.53—13.39)	10.1±3.5 (6.87—14.87)	40.46±11.5 (31.64—57.19)	8.38±1.83 (7.53—11.06)	30.46±5.28 (23.17—34.96)	8.26±6.13 (3.33—17.23)

Eight rabbits, hamsters, guinea pigs and rats were used. Values are mean±S.D. of the total and individual bile acids. LCA, lithocholic acid; DOC, deoxycholic acid; CDC, chenodeoxycholic acid; CA, cholic acid; Muri, muricholic acid; URSO, ursodeoxycholic acid. The range is the % from day 1 to day 7. ND=not detected.

samples were cooled and centrifuged at 4000 rpm for 10 min and the supernatant was dried under nitrogen. Fecal water samples were also analysed in the similar manner as fecal pellet.

Extraction of Bile Acids from Bile and Plasma One hundred micrograms of internal standards (5 β -cholanic acid) was added in each sample of bile and plasma. 5 ml of ethanol:methanol (95:5) was added to each samples and heated at 64 °C for 10 min. These samples were then centrifuged at 4000 rpm and the supernatant was separated and dried under nitrogen.

Gas Chromatography-Mass Spectrometry (GC-MS) The samples were then hydrolysed, methylated and acetylated as previously described by our laboratory.¹⁰ Identification and quantification of the bile acids were achieved by GC/MS using a Hewlett-Packard 5890 gas chromatography equipped with a Hewlett-Packard 5971A mass selective detector (MSD) employing the selected ion-monitoring mode (SIM). In this method the selected ions for the different bile acids were for cholic acid (*m/z* 253, 368), chenodeoxycholic acid (*m/z* 255, 370), deoxycholic acid (*m/z* 255, 370), ursodeoxycholic acid (255, 370), lithocholic acid, (*m/z* 257, 372) and 5 β -cholanic acids, (*m/z* 217, 374). Quantification was carried out by a correction factor obtained using 5 β -cholanic acid as internal standard. Bile acid standards were processed and analyzed in a similar manner.

Statistical Analysis Data were analyzed statistically using analysis of variance (ANOVA) test and variation and correlation coefficient was made between the percent contribution of deoxycholic acid level in plasma and fecal pellet or fecal water.

RESULTS

Table 1 shows the total and percent biliary bile acid composition of rabbit, hamster, guinea pig and rat. In rabbit the

total bile acid was 816.19 nmol/100 g body weight (bwt). Deoxycholic acid (89.63%) was the major bile acid followed by cholic acid (8.23%), chenodeoxycholic acid (1.22%), and lithocholic acid (0.91%). In hamster the total bile acid was 1498.31 nmol/100 g bwt. Cholic acid (72%) was the major bile acid followed by deoxycholic acid (14.82%), chenodeoxycholic acid (12.28%), and lithocholic acid (0.88%). In guinea pig the total bile acid was 440.38 nmol/100 g bwt. Chenodeoxycholic acid (81.85%) was the major bile acid followed by ursodeoxycholic acid (12.33%), cholic acid (4.63%), lithocholic acid (0.85%), and deoxycholic acid (0.32%). In rat the total bile acid was 59.88 nmol/100 g bwt. Cholic acid (76.42%) was the major bile acid followed by muricholic acid (6.68%), chenodeoxycholic acid (6.48%), ursodeoxycholic acid (5.24%), deoxycholic acid (5.02%), and lithocholic acid (1.99%).

Bile Acid Composition in the Plasma of Rabbit, Hamster, Guinea Pig and Rats Table 2 depicts the analysis of bile acids in plasma of rabbit, hamster, guinea pig and rats. Deoxycholic acid and cholic acid were the major bile acids in the plasma of rabbit. In hamster the major bile acid was cholic acid followed by deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and lithocholic acid. Chenodeoxycholic acid and cholic acid were the major bile acids in plasma of the guinea pig. However, other bile acids including deoxycholic acid, ursodeoxycholic acid, and lithocholic acids were also present in the plasma of guinea pig. In rat, muricholic acid and cholic acid were the major bile acid in plasma; however chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and lithocholic acids were also present. There was no significant difference in the plasma bile acids profile of all the species (rabbit, hamster, guinea pig and rat) studied at different days (days 1, 3, 5, 7) despite the variation in the concentration of total bile acids.

Bile Acid Composition in the Fecal Pellet of Rabbit, Hamster, Guinea Pig and Rats Table 3 gives the analysis

Table 3. Fecal Pellet Bile Acid (%) Profile in Different Animal Species

Species	% KETO (Range)	% LCA (Range)	% DCA (Range)	% MONO (Range)	% CDCA (Range)	% MURI (Range)	% CA (Range)	% URSO (Range)	Total bile acid (mg/g feces) (Range)
Rabbit	42.21±2.4 (39.73—44.88)	10.14±2.25 (7.44—12.37)	36±0.1 (34.96—36.83)	11.59±0.9 (0.37—2.16)	ND	ND	ND	ND	0.6±0.2 (0.44—0.91)
Hamster	46.3±5.6 (40.7—53)	22.5±1.6 (21—24.5)	16.6±4.4 (10.2—19.5)	16.6±1.2 (15.6—18.1)	ND	ND	ND	ND	3.2±0.1 (1.97—4.12)
Guinea pig	55.3±1.4 (53.5—56.6)	27.8±1.7 (25.6—29.5)	ND	16.8±1.9 (14.7—18.9)	0.4±0.2 (0.24—0.53)	ND	ND	ND	1±0.24 (0.78—1.33)
Rat	5.3±2.7 (2.11—8.25)	6.23±1.23 (4.7—7.7)	39.5±3.7 (35.4—43.7)	ND	1.1±0.23 (0.9—1.42)	35.6±6.2 (28.9—41.91)	4.8±0.54 (4.5—5.6)	1.5±0.22 (1.2—1.72)	11.53±8.8 (5.6—24.6)

Eight rabbits, hamsters, guinea pigs and rats were used. Values are mean±S.D. of the total and individual bile acids. LCA, lithocholic acid; DOC, deoxycholic acid; CDC, chenodeoxycholic acid; CA, cholic acid; Muri, muricholic acid; URSO, ursodeoxycholic acid. KETO-monohydroxylated bile acid. The range is the % from day 1 to day 7. ND=not detected.

Table 4. Fecal Water Bile Acid (%) Profile in Different Animal Species

Species	% KETO (Range)	% LCA (Range)	% DCA (Range)	% MONO (Range)	% CDCA (Range)	% MURI (Range)	% CA (Range)	% URSO (Range)	Total bile acid (mg/g feces) (Range)
Rabbit	31.7±10.2 (25.4—46.9)	ND	62.68±11.9 (45.5—72.1)	5.5±2.3 (2.6—7.4)	ND	ND	ND	ND	0.01±0.001 (0.003—0.008)
Hamster	51.2±5.96 (43—56.5)	ND	29.98±1.7 (28.4—32.4)	18.42±5.95 (14.3—16.9)	ND	ND	ND	ND	0.02±0.002 (0.01—0.03))
Guinea pig	74.02±5.3 (68.98—80.83)	ND	ND	25.96±5.3 (19.2—29.03)	ND	ND	ND	ND	0.01±0.002 (0.004—0.007)
Rat	1.3±1.23 (0.9—2.7)	1.23±0.2 (1.1—1.4)	12.8±6.5 (6.2—21.5)	ND	0.62±0.7 (0.1—1.42)	78.4±8.7 (70—88.2)	4.54±1.9 (2.2—6.8)	0.3±0.3 (0.12—0.6)	0.2±0.08 (0.11—0.29)

Eight rabbits, hamsters, guinea pigs and rats were used. Values are mean±S.D. of the total and individual bile acids. LCA, lithocholic acid; DOC, deoxycholic acid; CDC, chenodeoxycholic acid; CA, cholic acid; Muri, muricholic acid; URSO, ursodeoxycholic acid. KETO-monohydroxylated bile acid. The range is the % from day 1 to day 7. ND=not detected.

of bile acids in fecal pellet of rabbit, hamster, guinea pig and rats. In rabbit, only the secondary bile acids including deoxycholic acid, unknown keto, lithocholic acid and an unknown monohydroxy bile acids were present. Fecal pellet of hamster also contains the secondary bile acids like rabbit which include deoxycholic acid, unknown keto, lithocholic acid and the unknown monohydroxylated bile acids. In guinea pig, fecal pellet contained the similar profile like rabbit and hamster except the absence of deoxycholic acid and presence of chenodeoxycholic acid. Fecal pellet of rat contains both primary and secondary bile acids including cholic acid, chenodeoxycholic acid, muricholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid and unknown keto bile acids were present. However, muricholic acid and deoxycholic acid were the major bile acids. Like the case in plasma, despite the difference in the concentration of total bile acids in all the species studied, there was no significant difference in the bile acids profile at different days.

Bile Acid Composition in the Fecal Water of Rabbit, Hamster, Guinea Pig and Rats Table 4 shows the analysis of bile acids in fecal water of rabbit, hamster, guinea pig and rats. In rabbit, the total bile acids in fecal water were very low including Keto, deoxycholic acid and monohydroxylated bile acid and account for 0.65—1.12% of the total bile acids in fecal pellet. The profile was similar to fecal pellet except the absence of lithocholic acid. The total bile acids in fecal water of hamster were also very low and account for 0.24—0.9% of the total bile acids in fecal pellet. Like in the rabbit, there was a variation in the concentration of total bile acids

in plasma, fecal pellet, and fecal water with time but the bile acids profile remains constant in the fecal water of hamster. In the guinea pig, the total bile acids in fecal water were very low and account for 0.3—0.89% of the total bile acids in fecal pellet. The profile was similar to fecal pellet except the absence of lithocholic acid and deoxycholic acid. In rats, the total bile acids in fecal water were very low and account for 0.88—3.42% of the total bile acids in fecal pellet. There was a variation in the concentration of total bile acids in plasma, fecal pellet, and fecal water of rat with time of collection but the bile acids profile remains the same.

Figures 1a—c shows the correlation between the concentration of deoxycholic acid in plasma and bile, fecal pellet and fecal water. All correlation were significant at $p < 0.01$.

DISCUSSION

The composition of bile acids greatly varied between the species studied. As shown in Table 1 the main biliary bile acid in rabbit was deoxycholic acid accounting for 89.63%, in hamster cholic acid accounting for 72%. On the other hand in guinea pig chenodeoxycholic acid was the major bile acid accounting for 81.85% and in rat cholic acid contributed to 76.42% of the bile acid profile. But in the plasma obtained from rabbits, hamsters, guinea pigs, and rats, the main bile acids were deoxycholic acid and cholic acid in rabbits, in hamster cholic acid, and Chenodeoxycholic acid were the major bile acids, in guinea pig cholic acid was the main bile acid, and however in rat muricholic acid and cholic acid were

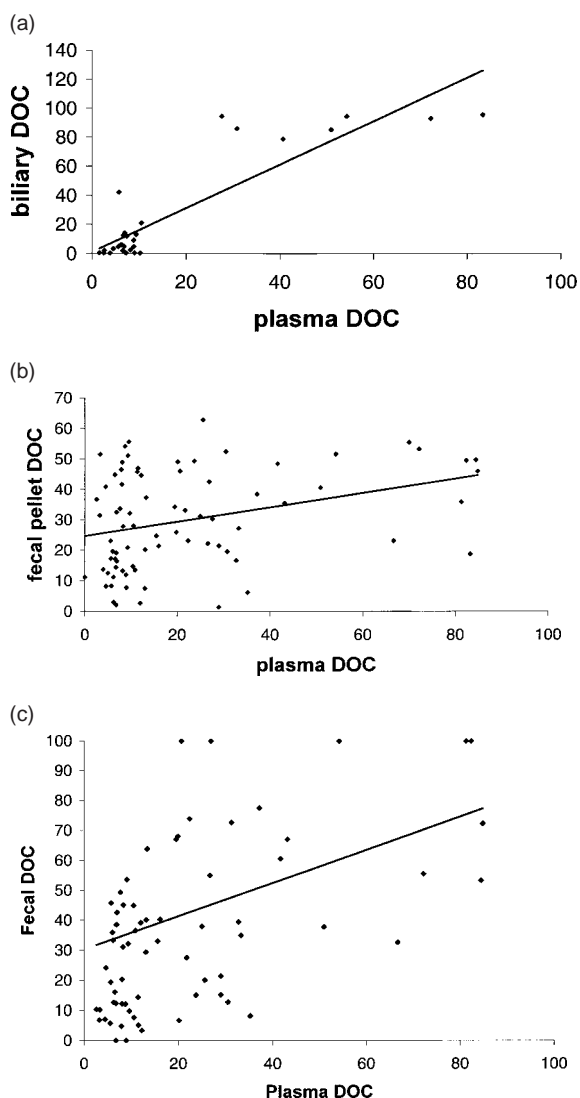


Fig. 1. (a) Correlation between Plasma DOC Level and Biliary DOC, (b) Correlation between Plasma DOC Level and DOC Level in Fecal Pellet and (c) Correlation between Plasma DOC Level and Fecal Water DOC Level

the major bile acids. A great variation in the bile acids composition of fecal pellet and fecal water of rabbit, hamster, guinea pig and rat were also observed. (Tables 2—4). This variation in the composition of bile acid between species allowed us to correlate the level of deoxycholic acid in bile with that in plasma, fecal water and fecal pellet.

One reason for the lack of published data on the correlation between the bile acids and colon cancer is the large intra-individual and day-to-day variation in bile acid excretion due to dietary and environmental factors.¹¹ To provide insight into the extent of day to day variation in the total and individual bile acids concentration in bile, plasma, fecal pellet and fecal water of rats, rabbits, hamster and guinea pig were used. No attempt was made to change their normal dietary routine. The results obtained from the present study shows that although bile acid composition varied greatly between the animal species studied, however the bile acids pro-

file in each species remains constant throughout the study (Tables 1—4). The present data also showed that despite the plasma bile acids profile does not represent the fecal bile acid profile however, there was a significant correlation observed between the level of plasma deoxycholic acid and fecal deoxycholic acid (Figs. 1b, c).

Epidemiological studies reported high amount of secondary bile acids in the population at high risk of colorectal cancer.^{3—8} In experimental studies, deoxycholic acid has been shown to damage colonocytes and lead to a cell proliferation, as well as it induces apoptosis in colonic cells, which are one of the first notable changes proceeding neoplastic transformation. Enhanced colonic mucosal proliferation is usually found in normal mucosa of patients with colorectal adenomas or colorectal carcinomas.^{12—14} Thus, secondary bile acids might be considered as major carcinogenic factor in the development of colonic carcinoma. The present data show that the secondary bile acids, lithocholic acid was absent in the fecal water except in rat suggesting that lithocholic acid may precipitate because of its very low solubility in water or may binds to fecal pellet and thus its effect on colonic cell may be questionable. Since deoxycholic acid in plasma correlate with deoxycholic acid in fecal pellet and fecal water. This support the concept that deoxycholic acid in plasma may be a biomarker for the degree of exposure of colonic cell to this bile acid and further suggests that colonic epithelium is mainly exposed to deoxycholic acid. In conclusion, the present study suggests that measuring the level of plasma deoxycholic acid in clinical studies may help to elucidate the role of secondary bile acids in colon carcinogenesis.

Acknowledgments This work was supported by a grant from the CIHR of Canada. The first three authors contributed equally to this article.

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