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A study on the influence of bile acid chemical structure on dissolution of insoluble calcium salts: An *in vitro* study of the use of bile acid-phosphatidylcholine-cholesterol model bile solution

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Summary: The influence of bile acid chemical structure on dissolution of insoluble calcium salts and the reducing effect of ionized calcium was studied. Various bile acids were used to compound model bile acid-phosphatidylcholine-cholesterol model bile solutions. After $CaCO_3$ was added to these solutions, both total calcium solubility and ionized calcium concentration in the solutions were measured. Dihydroxy bile acid is more effective than trihydroxy bile acid and 7α-hydroxy bile acid is more effective than 7β-hydroxy bile acid, with regard to calcium solubility and the reducing effect of ionized calcium in model bile solution. Glutamic or asparaginic acid conjugates are more effective than glycine or taurine conjugates. Therefore, calcium solubility and the reducing effect of ionized calcium in model bile solutions are dependent on the number and orientation of hydroxy groups on the steroid nucleus as well as electrical charge of conjugating amino acid of bile acid. Chenodeoxycholic acid conjugated with glutamic or asparaginic acid possesses high calcium solubility and large binding capacity with ionized calcium. *Gastroenterol Jpn 1990;25:383–387*

Key words: *bile acid; calcium solubility; model bile solution*

Introduction

Through many attempts to dissolve cholesterol gallstones by treatment with chenodeoxycholic acid (CDCA) and/or ursodeoxycholic acid (UDCA) have been made since the effect of CDCA and UDCA on dissolution of cholesterol gallstone was reported[1,2], the complete dissolution rate is estimated at less than about 20%. Various factors have been noted as important in predicting response to the dissolution therapy. Calcium content in gallstones has proved to be one of the most important factors affecting the success of dissolution[3], but no effective method to promote dissolution of insoluble calcium salts has been established. On the other hand, calcification of radiolucent gallstones during treatment with UDCA has recently been reported[4]. Ionized calcium (Ca^{2+}) is thought to play a large role in this phenomenon[5]. Thus it seems critically important to reduce the Ca^{2+} concentration in bile to prevent this calcium precipitation on gallstones.

The purpose of this study, therefore, was to examine the influence of bile acids on not only the dissolution of insoluble calcium salts but also the effect of reduction of Ca^{2+} concentrations in bile acid-phosphatidylcholine-cholesterol model bile solutions, and to extend these studies to also determine the differences among various amino acid conjugates of tri- and di-hydroxy bile acids in this respect.

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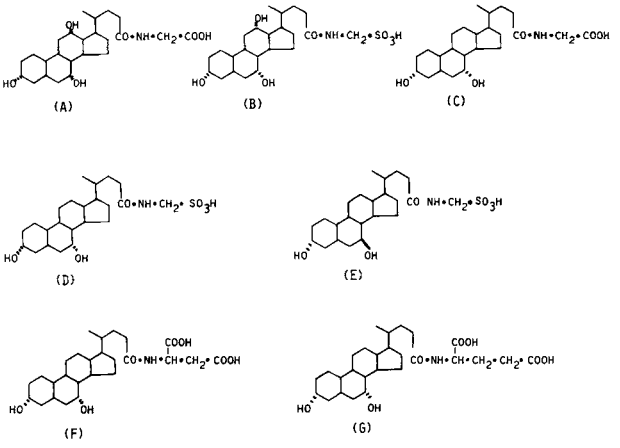

Fig. 1 Chemical structures of bile acid used for preparation of bile acid-phosphatidylcholine-cholesterol solutions.
(A) glycocholic acid, (B) taurocholic acid, (C) glycochenodeoxycholic acid, (D) taurochenodeoxy cholic acid, (E) tauroursochenodeoxycholic acid, (F) chenodeoxycholic acid conjugated with asparaginic acid, (G) chenodeoxycholic acid conjugated with glutamic acid.

Material and Methods

Substrate

The sources of materials used in this study were as follows: $CaCO_3$ and cholesterol from Wako Ltd. Tokyo Japan; L-α-phosphatidylcholine (type V) from Sigma, St. Louis, MO USA; glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCD), and taurochenodeoxycholic acid (TCDCA) from Sigma; tauroursodeoxycholic acid (TUDCA) from Tokyo Tanabe Co. Ltd., Tokyo Japan. CDCA conjugated with asparaginic acid (Asp.CDCA) or glutamic acid (Glu.CDCA) were kindly supplied by Dr. Yamatsu of Eisai Co. Ltd., Tokyo Japan. These two kinds of bile acid were synthesized by Dr. Yamatsu using the method of Tserng et al.[6]. The purity of the bile acids used in this study were greater than 98% after analysis on a TCL/FID analyzer (Iatroscan THE-10, Iatron Laboratory, Tokyo, Japan). Chemical structures of bile acids using in this study are summarized in **Figure 1**.

Preparation of bile acid-phosphatidylcholine-cholesterol model bile solution

Each bile acid-phosphatidylcholine-cholesterol model bile solution was prepared as follows: in case of preparation of GCA-phosphatidylcholine-cholesterol model bile solution (GCA model bile solution), 64.7 μmol of GCA dissolved in Folch solution (chloroform:methanol = 2:1), 22.2 μmol of L-α-phosphatidylcholine in chloroform and 4.7 μmol of cholesterol in Folch solution were collected together in the same test tube. The tube was sonicated and shaken, and then the solvent was evaporated under a stream of nitrogen. GCA-phosphatidylcholine-cholesterol mixed film was formed at the bottom of the tube. Then this film was dissolved in 1 ml of 50 mM Tris-HCl buffer (pH7.5) and adjusted to pH7.5 by the addition of diluted HCl or NaOH solution. The final biliary lipid concentration of the model bile solution was 4 g/dl and composition of this solution in mole percent was as follows: bile acid 70%, phosphatidylcholine 25% and cholesterol 5%. This solution was incubated at 37°C for 72 hours prior to the assay of calcium solubility in the model bile solution.

We prepared seven kinds of bile acid-phosphatidylcholine-cholesterol model bile solutions: GCA model bile solution, TCA model bile solution, GCDCA model bile solution, TCDCA model bile solution, Asp.CDCA model bile solution, Glu.CDCA model bile solution and TUDCA model bile solution. All the solutions were clear and colorless.

Assay of solubility of $CaCO_3$

Calcium carbonate was used as insoluble calcium salt in this study. 100 mg of $CaCO_3$ was added to 1 ml of each bile acid-phosphatidylcholine-cholesterol model bile solution. The test tube was capped, sonicated, inverted and shaken vigorously.

After three hours incubation at 37°C, the tube was centrifuged at 3,000 rpm for 15 minutes. The supernatant was further analyzed for calcium concentration. As a control, 100 mg of $CaCO_3$ was added to 1 ml of 50 mM Tris HCl buffer (pH7.5), and the same procedure was carried out.

Determination of calcium concentration

Total calcium concentration was determined by means of orthocresolphthalein complexone[7].

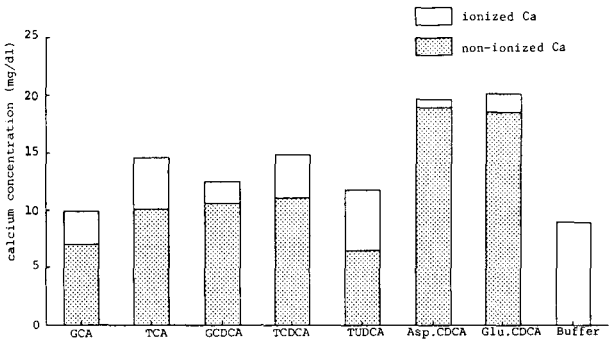

Fig. 2 Dissolved calcium concentration in bile acid-phosphatidylcholine-cholesterol solutions. The height of each column represents for dissolved calcium concentrations. Open columns are for the ionized calcium concentrations, hatched columns represent for non-ionized calcium concentrations. The number of data points in each group was five. With regard to total dissolved calcium concentration, Asp.CDCA model bile solution and Glu.CDCA model bile vs. the others ($P<0.01$), TCA model bile solution vs. GCA model bile solution ($P<0.01$), TCDCA model bile solution vs. GCDCA model bile solution ($P<0.01$), TCDCA model bile solution vs. TCA model bile solution (N.S.), GCDCA model bile solution vs. GCA model bile solution ($P<0.01$), and TUDCA model bile solution vs. the others ($P<0.01$). With regard to ionized calcium, Asp.CDCA model bile solution and Glu. CDCA model bile solution vs. the others ($P<0.01$), TCA model bile solution vs. GCA model bile solution ($P<0.01$), TCDCA model bile solution vs. GCDCA model bile solution ($P<0.01$), TCDCA model bile solution vs. TCA model bile solution ($P<0.01$), GCDCA model bile solution vs. GCA model bile solution ($P<0.01$), and TUDCA model bile solution vs. the others ($P<0.01$).

Ionized calcium concentration was determined by Ca^{2+}-selective meter (Type PW 9415, Phillips Ltd., The Netherlands). Then the solubility of $CaCO_3$ in the model bile solution was calculated as follows:

$$S = TCM - TCB$$

where S, TCM and TCB are solubility of $CaCO_3$ in the model bile solution, total calcium concentration in the model bile solution, and total calcium concentration in the buffer, respectively.

All experimental operations were carried out five times.

Statistical analysis

All data were expressed as means±SE. To compare means among groups, analysis of variance (ANOVA) and the least significant difference (LSD) were used. P values of <0.05 were considered statistically significant.

Results

The data for total calcium concentration, Ca^{2+} concentration and $CaCO_3$ solubility in each model bile solution are demonstrated in **Figure 2**.

The mean values for total calcium concentration in each model bile solution were higher than the value in the buffer. The values in model bile solution were in order; Glu.CDCA model bile solution (20.3±0.4 mg/dl) > Asp.CDCA model bile solution (19.8±0.2 mg/dl) > TCDCA model bile solution (15.0±0.4 mg/dl) > TCA model bile solution (14.7±0.3 mg/dl) > GCDCA model bile solution (12.6±0.3 mg/dl) > TUDCA model bile solution (11.9±0.4 mg/dl) > GCA model bile solution (10.0±0.3 mg/dl). The mean values for total calcium concentration in Glu.CDCA model bile solution and Asp.CDCA model bile solution were significantly higher than all the other model bile solutions ($P<0.01$). Calcium carbonate solubilities in both Glu.CDCA model bile solution and Asp.CDCA model bile solution were significantly higher (11.3±0.4 mg/dl and 10.8±0.3 mg/dl, respectively) than all the other model bile solutions ($P<0.01$). The mean values for Ca^{2+} concentration in the model bile solutions were much lower than that in the buffer. Bile acids had the ability to reduce Ca^{2+} concentration in the model bile solution. The values for Ca^{2+} in model bile solutions were in order; TUDCA model bile solution (5.4±0.1 mg/dl) > TCA model bile solution (4.5±0.3 mg/dl) > TCDCA model bile solution (3.8±0.2 mg/dl) > GCA model bile solution (2.9±0.1 mg/dl) > GCDCA model bile solution (1.9±0.1 mg/dl) > Glu.CDCA model bile solution (1.6±0.0 mg/dl) > Asp.CDCA model bile solution (0.8±0.0 mg/dl).

The percentages of Ca^{2+} to total calcium concentration were 4 and 8% for Asp.CDCA model bile solution and Glu.CDCA model bile solution, respectively. These were significantly lower than all the other model bile solutions ($P<0.01$), while in the TUDCA model bile solution, nearly half of the total calcium existed in the ionized form.

Discussion

In the present study, it was demonstrated that some amount of $CaCO_3$ could be dissolved in model mixtures of bile acid-phosphatidylcholine-cholesterol. As can be seen from the data, the total calcium concentration for taurine conjugates bile acid model bile solution was in the order of chenodeoxycholic model bile solution > cholic model bile solution > ursodeoxycholic model bile solution, and that of glycine conjugates was in the order of CDCA model bile solution > CA model bile solution. Therefore, $CaCO_3$ solubility of dihydroxy bile acid was superior to trihydroxy bile acid in case of similar orientation of hydroxy groups, and 7α-hydroxy bile acid was superior to 7β-hydroxy bile acid in case of an identical number of hydroxy groups. Thus, dissolution ability of $CaCO_3$ could be affected by both the number and the orientation of hydroxy groups on the steroid nucleus. On the other hand, it is of interest that differences in $CaCO_3$ solubility were also induced by the nature of the conjugating amino acid. The taurine conjugates of the dihydroxy and trihydroxy bile acids could dissolve $CaCO_3$ more effectively than the corresponding glycine conjugates. Furthermore, the conjugates of either asparaginic or glutaminic acid dissolved more insoluble calcium salts than taurine conjugates. These findings suggest that the amino acid with two carboxyl terminal ions such as asparaginic and glutamic acid might promote dissolution of $CaCO_3$ owing to a role as a metal chelating agent.

In human bile the majority of biliary calcium is bound to micellar and non-micellar bile acids[8,9]. It has been suggested that these complexes could increase the solubility of calcium in bile. However, Ca^{2+} is physiologically active, and the ability to form insoluble calcium salts may be related to the activity of Ca^{2+}[5]. Thus, bile acid is an important buffer for Ca^{2+} in bile and would serve to protect against calcium precipitation[10].

As shown in **Figure 2**, the order of Ca^{2+} ion concentrtion in model bile solution was as follows: TUDCA model bile solution > TCA model bile solution > TCDCA model bile solution > GCA model bile solution > GCDCA model bile solution > Glu.CDCA model bile solution > Asp.CDCA model bile solution. Because the content of bile acid in each model bile solution was identical, it appears that the extent of binding capacity of Ca^{2+} is determined by not only the number and orientation of hydroxyl group on the steroid nucleus but also the nature of the conjugating amino acid. The degree of binding to calcium is likely; dihydroxy > trihydroxy bile acid, 7α-hydroxy > 7β-hydroxy bile acid, and glutamic acid conjugates = asparaginic acid conjugates > glycine conjugates > taurine conjugates.

Beteson et al. reported occasional calcification of radiolucent gallstones in patients during UDCA therapy[4]. This evidence suggests a close relationship between the calcification and UDCA rich bile, but the detailed mechanism is still unclear.

In the present study, we found that the $CaCO_3$ solubility of TUDCA was extremely low and binding of Ca^{2+} to this bile acid was markedly weak, so that calcification of gallstones during UDCA therapy could be due to these factors.

Recently, Freilich and coworkers postulated that the calcium content of the gallstone outer rim was one of the most important factors that determined whether dissolution of gallstones by CDCA therapy was successful or not, this was done by analyzing the composition of gallstones obtained by cholecystectomy during the National Cooperative Gallstone Study[3]. Accordingly, it may be important to reduce Ca^{2+} concentration in bile and to dissolve insoluble calcium salt in order to prevent nucleating of gallstones and to increase the success rate of CDCA or UDCA therapy.

From our data, it was demonstrated that Glu.CDCA and Asp.CDCA possessed high $CaCO_3$ solubility and large binding capacities of Ca^{2+} ion. However, the findings in model bile solutions may not be identical to *in vivo* findings. For the application of these bile acids to dissolution therapy of gallstones, the absorption from intestine, the enterohepatic circulation and effect on biliary lipid composition of these two bile acids should be investigated further.

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