The Effect of Bile Salts on the Oral Mucosal Absorption of Human Calcitonin in Rats

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The relationship between the inhibition of the degradation of human calcitonin (HCT) in the supernatant of the rat’s oral mucosa homogenate and the promoting effect on the rat’s oral mucosal absorption of HCT was investigated by using ten kinds of sodium bile salts. The promoting effects and the inhibition of the degradation by the dihydroxy bile salts were related to the hydrophobicity, respectively. The inhibition of the degradation of HCT was related to the promoting effect on the oral mucosal absorption of HCT in the presence of the dihydroxy bile salts and to the concentration of the bile salts. Furthermore, the micelle formed by the bile salts would be concerned with the inhibition of the degradation of HCT.

Keywords — human calcitonin; oral mucosal absorption; promoter; bile salt; inhibition; degradation; micelle

Introduction

It is well known that protein and peptides are degraded by proteases and peptidases on the surface and in the cells of the oral mucosa in the permeable processes. It was reported that surfactants, such as bile salts, appeared to increase the permeability of insulin on the nasal mucosa and a possible explanation was the decrease of the proteolytic enzyme activity in the nasal mucosa by their surfactants. And FK-448 which was a potent and specific inhibitor of chymotrypsin enhanced the intestinal absorption of insulin in rats and dogs. Furthermore, we have reported that bile salts have a promoting effect on the oral mucosal absorption of human calcitonin (HCT) in rats and inhibit the degradation of HCT in the oral mucosa supernatant.

The aim of this study is to compare the influence of ten kinds of bile salts on the inhibition of the degradation of HCT in the rat’s oral mucosa, and to find a relation between their promoting effect on the oral mucosal absorption of HCT in rats and the inhibition of the degradation of HCT.

Materials and Methods

Chemicals — HCT was obtained from Peptides Institute (Minou, Japan). Ten sodium bile salts were used in this study (Table 1). Sodium glycodeoxycholate (GDCANa), sodium taurodeoxycholate (TDCANa), sodium glycodeoxycholate (GCDCANa), sodium taurochenodeoxycholate (TCDCANa), sodium glycocholate (GCANa) and sodium taurocholate (TCANa) were obtained from Sigma Chemical Co. (St Louis, U.S.A.). Sodium deoxycholate (DCANa) and sodium cholate (CANa) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and sodium tauroglycocholate (TGCANa) was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Chenodeoxycholic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and was transformed to the sodium salts by reaction with an equimolar amount of sodium hydroxide in a small volume of deionized water with subsequent dilution to a desired final concentration in the buffer solution used in present studies. Other reagents used were of reagent grade obtained commercially.

HCT Preparations — HCT solution with or without bile salts was prepared by dissolving 0.51 mg of HCT in 1 ml of distilled water. HCT preparations were prepared by mixing equal volumes of the HCT solution and pH 7.4 buffer solution which was prepared with 0.2 M tris (hy-
droxymethyl) aminomethane and hydrochloride.

**Animals** — Wistar male rats (4 weeks old) were fasted for 20 h prior to experimentation. During the experiment, rats were anesthetized with sodium pentobarbital (40 mg/kg i.p.) and kept on a warm surface at 38 °C.

**Preparation of the Supernatant of the Rat’s Oral Mucosa Homogenate** — Preparation of the supernatant of mucosa was carried out according to the previous method. Wistar male rats (4 weeks old) were killed by diethylether. Mucosa in the labium inferius and gingiva was removed immediately with a blade. Mucosal specimens were rinsed twice in isotonic KCl, and then homogenized in 5 volumes of isotonic KCl in a Potter’s homogenizer at 4 °C. The homogenate was centrifuged at 9000 × g in a refrigerated (4 °C) centrifuge for 10 min. The protein concentration in the resulting supernatant was determined using Lowry’s method with serum bovine albumin as a standard.

**Determination of HCT by Means of High Performance Liquid Chromatography (HPLC)** — A HPLC assay was carried out according to the previous method. The HPLC system used consisted of Waters model 510 HPLC pumps, model U6K sample injector and UNISIL PACK 5C18 reverse phase ODS column, 15 cm × 4.6 mm (Gaskuro Kogyo Inc., Japan). The mobile phase was a mixture of MeOH and water (70:30, v/v) containing 3 ml of PIC-A LOW UV (Waters, U.S.A.) per one liter. The flow rate was 1.0 ml/min at 30 °C. HCT in the eluate was monitored spectrophotometrically at 214 nm using Waters Model 481 UV detector.

**Determination of Hydrophobicity (HPLC Retention Factor: k’)** of Bile Salts — The HPLC retention factor (k’) of the bile salts was calculated by the following equation according to the methods of Armstrong and Carey:

$$k' = \frac{t_r - t_o}{t_o}$$

where $t_o$ = retention time of the solvent front and $t_r$ = retention time of the bile salt elution profile measured at 214 nm. The HPLC was performed on an isocratic liquid chromatography (Waters, U.S.A.) with a Model 510 HPLC pump, a Model U6K sample injector and a Model 481 UV detector. The reverse-phase column was Inertsil ODS column, 25 cm × 4.6 mm (Gaskuro Kogyo Inc., Japan). The flow rates were set at 1.0 ml/min at 30 °C. The mobile phase consisted of MeOH and water buffered with 0.005 M of each: KH$_2$PO$_4$ and H$_3$PO$_4$ (75:25 v/v). The apparent pH of the final solution was 5.0 ± 0.1.

**Absorption Study** — The oral mucosal absorption study was carried out according to the previous method. At first, a cotton ball (1 mg) was placed between the rat’s labium inferius and gingiva. Then 50 μl of HCT preparation was administered to the cotton ball. After the administration, blood samples (about 0.2 ml) were withdrawn periodically from the jugular vein. Plasma was obtained by centrifugation at 15000 × g for 3 min.

**Analytical Method** — Plasma calcium level was determined by the previous method to evaluate the oral mucosal absorption of HCT by Calcium C Test Wako. The decrement of the plasma calcium level (ΔA, total decrease) from 0 to 4 h after dosing of HCT was calculated from the following equation according to the method of Hirai et al.:

$$\Delta A \ (\%) = \left( \frac{AUC_C - AUC_H}{AUC_C} \right) \times 100$$

where $AUC_C$ is the area under the plasma calcium level versus time curve from 0 to 4 h after administration of additives only, and $AUC_H$ is the area under the plasma calcium level versus time curve from 0 to 4 h after administration of HCT with additives.

**Effect of Bile Salts on the Stability of HCT in the Supernatant of the Oral Mucosa Homogenate** — The kinetics of degradation of HCT in the supernatant of the homogenate with bile salts were studied by incubating, in triplicate: 25 μl of HCT preparation (510 μg/ml), 25 μl of homogenate supernatant and 50 μl bile salts solution (prepared in pH 7.4 buffer solution) at 37 °C. Residual HCT was measured by HPLC at regular time intervals.
TABLE I. Chemical Structures and Hydrophobicity of Bile Salts

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>log k'</th>
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<tbody>
<tr>
<td>Dihydroxy bile salts</td>
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<tr>
<td>Sodium deoxycholate</td>
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<td>Sodium glycodeoxycholate</td>
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<td>Sodium taurodeoxycholate</td>
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<td>Sodium chenodeoxycholate</td>
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<td>Sodium taurochenodeoxycholate</td>
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<tr>
<td>Trihydroxy bile salts</td>
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<tr>
<td>Sodium cholate</td>
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<td>Sodium glycocholate</td>
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<td>Sodium taurocholate</td>
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<td>Sodium tauroglycocholate</td>
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Treatment of the Glass Vessel Surface — The surface of all glass materials was treated with 3% (w/v) trimethylchlorosilane in benzene to prevent the adsorption of HCT. After treatment, the glass surface was washed 5 times with 5 ml of MeOH, and air-dried at room temperature.

Statistics — Results are expressed as the mean ± S.E. Student’s t-test was used for the comparison of the results. Statistical significance was indicated at a level α = 0.05.

Fig. 1. The Relationship between the Degradation Rate Constants of HCT in the Supernatant of the Rat’s Oral Mucosa Homogenate with Various Bile Salts (1.0 mg/ml) at 37 °C (pH 7.4) and the Decrements of Plasma Calcium Levels Following Oral Mucosal Administration of HCT with Various Bile Salts (22.5 mg/ml) in Rats at pH 7.4

Dose of HCT: 12.75 μg/head. Each value is expressed as the mean ± S.E. of 3 or 4 experiments. (a) Dihydroxy bile salts, \( Y = -1.92X + 26.35, r = -0.81 \ (n = 6), 0.01 < p < 0.05 \). (b) Trihydroxy bile salts, \( Y = 0.77X + 8.65, r = 0.71 \ (n = 4), p > 0.05 \).
Fig. 2. The Relationship between log $k'$ of the Bile Salts and the Decrements of Plasma Calcium Levels Following Oral Mucosal Administration of HCT with Various Bile Salts (22.5 mg/ml) in Rats at pH 7.4. Dose of HCT: 12.75 μg/head. Each value is expressed as the mean ± S.E. of 3 or 4 experiments. (a) Dihydroxy bile salts, $Y = 36.78X - 14.02$, $r = 0.95$ ($n = 6$), $p < 0.01$. (b) Trihydroxy bile salts, $Y = 30.54X + 8.98$, $r = 0.82$ ($n = 4$), $p > 0.05$.

Results

Hydrophobicity of Bile Salts

It was well known that the hydrophilic-hydrophobic balance of a bile salt can be predicted on the basis of its retention factor ($k'$) in the reverse-phase HPLC. The log $k'$ values of bile salts used in the present study are listed in Table I. The log $k'$ values decreased in the following order, unconjugated > glycoconjugated > tauroconjugated bile salts.

Effects of Bile Salts on the Stability of HCT

The relationship between the effect of bile salts on the degradation rate constants of HCT in the supernatant of the oral mucosa homogenate and the enhancement on the oral mucosal absorption of HCT is shown in Fig. 1. The dihydroxy bile salts decreased the degradation rate constants of HCT comparing with that in the absence of any bile salts ($13.9 ± 0.4$ h⁻¹/mg protein). Furthermore, the effect of the dihydroxy bile salts on the oral mucosal absorption of HCT resulted in an increase in $\Delta A$, as the degradation rate constants were lowered (Fig. 1(a)). There was the correlation between the degradation rate constants and the $\Delta A$ ($r = 0.81$, $n = 6$, $0.01 < p < 0.05$). On the other hand, all of four trihydroxy bile salts increased the $\Delta A$, but they prompted the degradation of HCT in the oral mucosal preparations, except TGCANa. There was no significant correlation between the $\Delta A$ and the degradation rate constants of HCT as shown in Fig. 1(b).

Relationship between Hydrophobicity of Bile Salts and Promoting Effect of Bile Salts on the Oral Mucosal Absorption of HCT

Figure 2 demonstrates that oral mucosal absorption of HCT correlates positively with the log $k'$ values which is considered as an indication of the hydrophobicity of the bile salts. The $\Delta A$ of unconjugated bile salts are larger than the $\Delta A$ of conjugated bile salts. In the case of the dihydroxy bile salts, there was the correlation between the log $k'$ value and the $\Delta A$ ($r = 0.95$, $n = 6$, $p < 0.01$), but there was not the correlation in the case of the trihydroxy bile salts ($r = 0.82$, $n = 4$, $p > 0.05$).

Relationship between Hydrophobicity of Bile Salts and Effect of Bile Salts on the Stability of HCT

Figure 3 shows the relationship between the hydrophobicity (log $k'$) of the bile salts and the degradation rate constants of HCT in the supernatant of the oral mucosa homogenate with the
Fig. 3. The Relationship between log $k'$ of the Bile Salt and the Degradation Rate Constants of HCT in the Supernatant of the Rat's Oral Mucosa Homogenate with the Various Bile Salts at 37 °C (pH 7.4)

Bile salt concentration was 1.0 mg/ml. Each value is expressed as the mean ± S.E. of 3 or 4 experiments. (a) Dihydroxy bile salts, $Y = -12.60X + 16.33$, $r = -0.78$ ($n = 6$), 0.01 $< p < 0.05$. (b) Trihydroxy bile salts, $Y = 18.16X + 8.39$, $r = 0.53$ ($n = 4$), $p > 0.05$.

Fig. 4. Effect of the Concentration of CDCANa on the Stability of HCT in the Supernatant of the Rat's Oral Mucosa Homogenate at 37 °C (pH 7.4)

Each value is expressed as the mean ± S.E. of 3 or 4 experiments.

Fig. 5. Effect of the Concentration of CANa on the Stability of HCT in the Supernatant of the Rat's Oral Mucosa Homogenate at 37 °C (pH 7.4)

Each value is expressed as the mean ± S.E. of 3 or 4 experiments.

bile salts. The stability of HCT with the dihydroxy bile salts was increased according to the increasing of the log $k'$ values and there was the correlation between the log $k'$ values and the degradation rate constants ($r = -0.78$, $n = 6$, $0.01 < p < 0.05$), but there was not the correlation in the case of the trihydroxy bile salts ($r = 0.53$, $n = 4$, $p > 0.05$).

Effects of the Concentration of Bile Salts on the Stability of HCT

Figures 4 and 5 show the effects of the concentration of sodium chenodeoxycholate (CDCANa), and CANa, on the stability of HCT in the supernatant of the oral mucosa homoge-
TABLE II. Effect of Lecithin on the Degradation of HCT in the Supernatant of the Rat's Oral Mucosa Homogenate with the Dihydoxy Bile Salts at 37 °C (pH 7.4)

<table>
<thead>
<tr>
<th>Bile saltsa)</th>
<th>Concentration of lecithin (mg/ml)</th>
<th>( k^{b)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>13.9 ± 0.5c)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>13.4 ± 0.8d)</td>
</tr>
<tr>
<td>DCANa</td>
<td>0</td>
<td>6.2 ± 0.5e)</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>7.2 ± 0.4f)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>9.9 ± 0.2g)</td>
</tr>
<tr>
<td>CDCANa</td>
<td>0</td>
<td>2.2 ± 0.2h)</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3.0 ± 0.2i)</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>4.8 ± 0.9j)</td>
</tr>
</tbody>
</table>

a) Bile salts concentration was 1.0 mg/ml. b) Results are expressed as the degradation rate constants per mg protein contained in the supernatant (h⁻¹/mg protein) and as the mean ± S.E. with 3 experiments. Significant differences: c) - e(-j), d) - e(-j), e) - g), \( p < 0.01; h) - i), h) - j), 0.01 < \( p < 0.05.\)

nate, respectively. The degradation rate constants of HCT in the presence of 0.1 mg/ml of CDCANa (17.1 ± 1.0 h⁻¹/mg protein) and 1.0 mg/ml of CANa (20.6 ± 0.2 h⁻¹/mg protein) were larger than control (13.9 ± 0.4 h⁻¹/mg protein). The degradation rate constants of HCT in 0.6 mg/ml of CDCANa (7.0 ± 0.4 h⁻¹/mg protein) was slightly smaller than control, and that of HCT in 1 and 10 mg/ml were 2.2 ± 0.2 and 0.8 ± 0.1 h⁻¹/mg protein, respectively. In the case of CANa, the degradation rate constants of HCT in 10 and 50 mg/ml were 7.8 ± 0.3 and 3.6 ± 0.5 h⁻¹/mg protein, respectively.

Effects of Lecithin on the Stability of HCT

It was reported that the lowering of the critical micelle concentration (CMC) and the change of the micellar molecular weights (MMW) were observed in bile salts-lecithin systems.7) Table II shows the effect of the concentration of lecithin on the degradation of HCT in the supernatant of the oral mucosa homogenate with the dihydroxy bile salts. The stability of HCT without sodium bile salts was not influenced by the addition of lecithin. But the stability of HCT in the supernatant of the oral mucosa homogenate with DCANa or CDCANa was decreased according to the increasing of content of lecithin.

Discussion

It has been reported that the leucine aminopeptidase activity exists on the surface of the cell.8) Peptides, such as HCT, will be degraded by proteases and peptidases on the surface, and in the permeable processes of the mucosa. We have already reported that DCANa and TGCANa have a promoting effect on the oral mucosal absorption of HCT3) and these additives inhibited the degradation of HCT in the oral mucosa supernatant.4) In the present study, the relationship between the inhibition of degradation of HCT and the promoting effect was investigated by using ten kinds of sodium bile salts which were available commercially. The effect of these bile salts on the degradation of HCT was also investigated in detail.

The ursodeoxycholate was the more potent pepsin inhibitor than other conjugated bile salts such as sodium glycocholate and sodium taurocholate.9) It has also been reported that the hydrophobic properties of bile acids correspond to the gradation of enzyme inhibition measured.10) The promoting effects of the dihydroxy bile salts were related to the log \( k' \) values of bile salts (Fig. 2). The gradations of the hydrophobic properties of the dihydroxy bile salts, the log \( k' \) values, correspond to that of the inhibition of the degradation (Fig. 3). On using the dihydroxy bile salts, the inhibition of the degradation of HCT related to the promoting effect on the oral mucosal absorption of HCT as shown in Fig. 1.

The hydrophobic properties of bile salts are relative to the micelle formation.11) The CMC of CDCANa is reported as 3.72 × 10⁻³ mol dm⁻³ (1.54 mg/ml).12) The inhibition of the degradation of HCT was considerably reduced at above 1.0 mg/ml concentration of CDCANa as shown in Fig. 4. This result showed that the observed inhibition of the degradation of HCT by the dihydroxy bile salts would be related to the formation of the micelle. The micelle of CDCANa consists of 8 molecules and its molecular weight is about 330013) which is comparable with that of HCT (about 3200). The other dihydroxy bile salts, DCANa and TDCANa, consist of 4—10 molecules.14) Furthermore, it has been reported
that the horseradish peroxidase MW about 40000) penetrated the oral mucosa. Therefore, there would be the possibility that the micelle of the dihydroxy bile salts penetrated the oral mucosa and HCT was protected from proteases and peptidases by the micelle of the dihydroxy bile salts.

As to the trihydroxy bile salts, the inhibition of the degradation of HCT didn't relate to the promoting effect. And the inhibition of the degradation of HCT and the promoting effect didn't relate to the log k' value. The degradation of HCT in the supernatant of the oral mucosa homogenate was accelerated compared to the control by adding the trihydroxy bile salts except TGCANa as shown in Fig. 1. But as shown in Fig. 5, the inhibition of the degradation of HCT was admitted on using CANa of which concentrations were 10 and 50 mg/ml (degradation rate constant; 7.8 ± 0.3, 3.6 ± 0.5 h⁻¹/mg protein, respectively) and the acceleration of the degradation of HCT was admitted on using the dihydroxy bile salt, such as CDCANa, of which concentration was 0.1 mg/ml (degradation rate constant; 17.1 ± 1.0 h⁻¹/mg protein). The acceleration or inhibition of the degradation of HCT would be related to the concentration of the bile salts from these results. It is necessary to study in detail the reasons why there is a difference between the dihydroxy and the trihydroxy bile salts.

It was stated that the micellar formation would be one important mechanism of the observed inhibition of the degradation of HCT by bile salts. When lecithin was added to the bile salt solution, the CMC of the bile salts decreased and the MMW changed.⁷ If the mechanism of the inhibition of the degradation of HCT by bile salts is only micellar formation, the inhibition of the degradation does not occur when lecithin is added. But the degradation rate constant of HCT on using DCANa or CDCANa increased when lecithin was added (Table II). This result indicates that the mechanism of the inhibition of the degradation of HCT by bile salts is not only micellar formation but is also attributable to the micellar size, MMW and the micellar form. The mechanism of inhibition of pepsin by bile salts has been demonstrated to be a non-competitive one.⁶ Bile salts might also cause a nonspecific denaturation or binding of pepsin perhaps related to their detergent properties.⁹ So, it will be necessary to study the mechanism of the inhibition of the degradation of HCT by bile salts in detail. TDCANa accelerates the release of phospholipids from isolated everted rat small intestinal sac and the effect occurs at concentrations above the CMC.¹⁷ It will be necessary to study the effect of the released phospholipid on the inhibition of the degradation of HCT by bile salts in tissue.

In conclusion, the inhibition of the degradation of HCT by the dihydroxy bile salts related to their promoting effect on the oral mucosal absorption of HCT. Furthermore, the micelle formed by the bile salts will be concerned with the inhibition of the degradation of HCT.

References


