PHARMACOLOGICAL STUDIES ON IRIDOID COMPOUNDS. III. THE
CHOLERETIC MECHANISM OF IRIDOID COMPOUNDS

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We made a study on choleretic property and mechanism of action of iridoid compounds as well as dehydrocholate (DHC), cholate (CA), and salicylate (SA), examining their effects on factors such as bile flow, bile acids, electrolytes (Na⁺, K⁺, Cl⁻, and HCO₃⁻), and their metabolites. Each sample showed a characteristic property, respectively. Genipin and patrinoside decreased biliary concentrations of bile acids, Na⁺, Cl⁻, and HCO₃⁻, corresponding to their rapid choleretic actions which were due to bile acids-independent fraction. The choleretic action of DHC is approximately twice as potent as that of CA. Their actions were due to bile acids-dependent fraction. CA gave a marked increase in Na⁺ concentration but DHC did not. And both compounds gave a marked diminution in Cl⁻ concentration and weakly decreased HCO₃⁻ concentration. SA showed a weak and durable choleretic action and also gave a marked increase in HCO₃⁻ concentration. The main metabolite detected from the bile given genipin was genipin-l-O-glucuronic acid (GGA). The periodical pattern of GGA level in bile was in agreement with that of genipin-induced choleretic action, and quantitatively cation-anion gap generated was nearly compensated by biliary concentration of GGA. From our various results, the choleretic mechanism of iridoid compounds is considered to be as follows: The hemiacetal moiety of them undergoes conjugation in the liver to give glucuronide. Glucuronide thus formed is secreted into the biliary tree being coupled mainly with Na⁺ and water is passively excreted.

Keywords—choleretic mechanism; iridoid compounds; genipin; patrinoside; dehydrocholate; cholate; salicylate; bile acids; electrolytes (Na⁺, K⁺, Cl⁻, and HCO₃⁻); cation-anion gap

INTRODUCTION

We previously carried out pharmacological studies of Gardenia fruit and reported that geniposide, an iridoid glucoside regarded as a principle component, showed no choleretic action by itself but that genipin, its aglycone produced by hydrolysis in the intestinal tract, exerted the action at the same degree as sodium dehydrocholate. Pharmacological actions of patrinoside, an iridoid glucoside widely distributed in various plants of Valerianaceae, and its aglycone were examined and facilitatory effect on bile secretion was found in both compounds. The efficacy of patrinoside was especially marked. Thus, it was found that there exist substances which show a marked choleretic action in the form of aglycone like genipin and those which give the same effect in the form of glucoside like patrinoside among iridoid compounds. Continuously, the relationship between structures and choleretic actions on various iridoid compounds (16 species) and in vivo behavior of patrinoside were investigated and it was suggested that the hemiacetal moiety of them plays an important role in exerting a choleretic action in the liver. Iridoid compounds seem to be the substance which possesses an exceeding choleretic action and whose toxicity is relatively weak in comparison with choleretics which have been known at the present time. Up to now, various investigations have been carried out on bile secretion. However, the studies on choleretic mechanism of sub-
stances except bile acids are not sufficient.

In the present study, we made a comparative study on the choleric properties of iridoid compounds (genipin and patrinoside), sodium dehydrocholate, sodium cholate, and sodium salicylate using a parameter such as electrolytes (\(\text{Na}^+, \text{K}^+, \text{Cl}^-, \text{and HCO}_3^-\)), cation-anion gap, and total bile acids in bile and moreover investigated choleric mechanism of iridoid compounds.

MATERIALS AND METHODS

Animals — Male Wistar strain rats weighing 220 ± 20 g were used.

Materials — Iridoid compounds: Genipin (iridoid aglycone) was obtained after hydrolysis using \(\beta\)-glucosidase (\(\beta\)-D-glucoside glucohydrolase, EC 3.2.1.21) of geniposide, a main component of Gardenia fruit. Patrinoside (iridoid glucoside) was isolated from roots of \textit{Patria gibbosa} MAXIM according to the procedure reported in a previous paper (yield ca. 3.2%).

Enzymes and Chemicals — \(\beta\)-Glucuronidase (\(\beta\)-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) was purchased from Miles Laboratories, sterognost-3\(\alpha\) (reagent for the determination of total bile acids) from Nyegaard Co.AS., sodium dehydrocholate from Dainippon Pharmaceutical Co., sodium cholate from Sigma Chemical Co., sodium salicylate from Kanro Chemical Co., and urethan (ethyl carbamate) from Wako Pure Chemical Ind.

Each compound was dissolved in physiological saline solution just before the administration. The structures of genipin and patrinoside are shown in Fig. 1.

Methods — I. Effects of Iridoid Compounds, Sodium Dehydrocholate, Sodium Cholate, and Sodium Salicylate on Flow, Total Bile Acids, Electrolytes, and Cation-Anion Gap in Bile

Rats were laparoromized under anesthesia with subcutaneous administration of urethan (1.5 g/kg) and a polyethylene tubing (PE-10) was inserted into the common bile duct to drain bile off freely and after 10 min the bile was collected for 30 min. The test chemical solution was then administered intravenously, and the bile was collected at 30-min intervals up to 2 h. Volume and concentration of total bile acids and electrolytes of each collected bile sample were measured.

Determination: Total bile acids were measured using sterognost-3\(\alpha\) (enzymatic method). Concentration of electrolytes (\(\text{Na}^+, \text{K}^+, \text{Cl}^-, \text{and HCO}_3^-\)) was measured according to the ion selective electrode method (Stat/Ion II, Technicon Instruments Co., New York), and cation-anion gap was calculated from the following formula: (\(\text{Na}^+ + \text{K}^+\)) - (\(\text{Cl}^- + \text{HCO}_3^-\)).

II. Studies on Metabolites of Genipin

1) Thin Layer Chromatography (TLC): The bile collected for 1 hr after intravenous administration of genipin (50 mg/kg) was lyophilized and the dried product was extracted with MeOH. This MeOH solution was concentrated and submitted to TLC plate analysis (Kieselgel F\(_{254}\), Type 60, Merck). After development with CHCl\(_3\)-MeOH (3:1), the bands of genipin and its metabolites were qualitatively examined under an ultraviolet lamp (2536 Å).

2) Separation and Purification of a Main Metabolite of Genipin: After treatment of the bile as in II-1), the major band (\(R_f 0.08\)) on a TLC plate was scraped off and extracted with MeOH. This MeOH solution was concentrated to give a fraction rich in a main metabolite (GM-1) of genipin. GM-1 was purified from this fraction with high performance liquid chromatography (HPLC) under the following conditions: Column, Semiprep \(\mu\)-Bondapak C\(_{18}\) (8 mm i.d. \(\times\) 30 cm); solvent, MeOH-1 % AcOH.

FIG. 1. Structures of Genipin (I) and Patrinoside (II)
(3:8); flow rate, 3 ml/min; detection, UV$_{254}$ nm (UVIDEC 100-II); apparatus, TRIROTAR-II (JASCO, Japan). And the structure of GM-1 was determined.

3) Determination of GM-1 from Bile after the Administration of Genipin: Rats were treated as in I. Fifty mg/kg of genipin was injected into the femoral vein and the bile was collected at 30- min intervals to measure the amount of GM-1 excreted into bile up to 2h. Each collected bile was submitted to HPLC. HPLC was carried out with TRIROTAR-II under the following conditions: Column, μ-Bondapak C$_{18}$ (4 mm i.d. × 30 cm); solvent, MeOH-1 % AcOH (3:8); detection, UV$_{240}$ nm; flow rate, 1 ml/min. The quantity of GM-1 was obtained from the peak area in HPLC, h×half width, and calculated from the calibration curve.

III. Investigation of Metabolites of Sodium Salicylate

The bile was collected at 30- min intervals for 2 h after intravenous administration of sodium salicylate (100 mg/kg) according to the procedure described in I. The bile was submitted to HPLC and the metabolite of sodium salicylate was examined under the following conditions: Column, Zorbax ODS (4.6 mm i.d. × 25 cm); solvent, MeOH-1 % AcOH (3:8); detection, UV$_{254}$ nm; flow rate, 1 ml/min; apparatus, LC-3A (Shimadzu, Japan).

RESULTS

1. Effects on Bile Flow

Intravenous administration (i.v.) of genipin
(25 and 50 mg/kg), patrinose (50 and 100 mg/kg), sodium dehydrocholate (50 mg/kg), and sodium cholate (50 mg/kg) showed a rapid facilitatory effect on bile secretion. On the other

FIG. 4. Relationship between Bile Flow and Bile Acids Excretion Rate in Rats
Equation of regression line is: for control, $Y=15.86X + 84.14$ (r: 0.863); for genipin 50 mg/kg, i.v., $Y=13.08X + 229.18$ (r: 0.951); for patrinose 100 mg/kg, i.v., $Y=17.08X + 190.34$ (r: 0.928); for sodium dehydrocholate 50 mg/kg, i.v., $Y=19.91X + 68.41$ (r: 0.955); for sodium cholate 50 mg/kg, i.v., $Y=10.93X + 100.98$ (r: 0.801).
hand, sodium salicylate weakly induced a prolonged choleretic action at 100 mg/kg, i.v. The choleretic intensity of genipin was the same degree as that of sodium dehydrocholate in this experiment, too. The results are shown in Fig. 2.

2. Effects on Biliary Concentration of Total Bile Acids

As seen in Fig. 3, the control group given physiological saline solution provided a gradual diminution in biliary concentration of total bile acids along with the progress during the experimental period. Genipin (25 and 50 mg/kg, i.v.) and patrinoside (50 and 100 mg/kg, i.v.) markedly decreased it, while sodium dehydrocholate and sodium cholate gave a marked increase of it at 50 mg/kg, i.v. One hundred mg/kg, i.v. of sodium salicylate gave no significant change.

3. Relation between Bile Flow and Bile Acids

Relationships between bile flow and bile acids excretion rate for a period of 30 min just

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**FIG. 5. Effects on Biliary Ionic Concentration**

- ● - control (n=16), - ○ - -: genipin 25 mg/kg (n=6), - ○ - -: genipin 50 mg/kg (n=15), - ■ - -: patrinoside 50 mg/kg (n=11), - ■ - -: patrinoside 100 mg/kg (n=10), - ▲ - -: sodium dehydrocholate 50 mg/kg (n=11), - ▲ - -: sodium cholate 50 mg/kg (n=6), - ○ - -: sodium salicylate 100 mg/kg (n=7).

i.v.: intravenous administration. Data are expressed as the mean.
after the administration of genipin (50 mg/kg, i.v.), patrinoside (100 mg/kg, i.v.), sodium dehydrocholate (50 mg/kg, i.v.), and sodium cholate (50 mg/kg, i.v.) are shown in Fig. 4.

Genipin and patrinoside showed a significant choleretic effect and yet hardly changed bile acids excretion. In contrast, the choleretic effects of sodium dehydrocholate and sodium cholate were accompanied with the increase of bile acids excretion. In every case, there was a linear relationship between bile flow and bile acids excretion rate. The equation of each regression line was as follows: For control, \( y = 15.86x + 84.14 \) \((r = 0.863)\); for genipin, \( y = 13.08x + 229.18 \) \((r = 0.951)\); for patrinoside, \( y = 17.08x + 190.54 \) \((r = 0.928)\); for sodium dehydrocholate, \( y = 19.91x + 68.41 \) \((r = 0.955)\); for sodium cholate, \( y = 10.93x + 100.98 \) \((r = 0.801)\). Regression lines of iridoid compounds were parallelly shifted upward against control. The slope of regression line from sodium dehydrocholate treated group was significantly different from that of sodium cholate treated group.

4. Effects on Biliary Concentration of Electrolytes (Na\(^+\), K\(^+\), Cl\(^-\), and HCO\(_3\)^-\)

The results are shown in Fig. 5. i) On Na\(^+\) concentration; iridoid compounds (25 and 50 mg/kg, i.v. of genipin and 50 and 100 mg/kg, i.v. of patrinoside) gave a decrease, while sodium cholate (50 mg/kg, i.v.) gave a marked increase. Sodium dehydrocholate (50 mg/kg, i.v.) and sodium salicylate (100 mg/kg, i.v.) gave no significant change. ii) On K\(^+\) concentration; each sample showed no marked change. iii) On Cl\(^-\) concentration; iridoid compounds and cholic acids (sodium dehydrocholate and sodium cholate) gave a marked decrease and also sodium salicylate a weak decrease. iv) On HCO\(_3\)^- concentration; iridoid compounds and cholic acids gave a rapid decrease, while sodium salicylate produced a marked and persistent increase. The variation induced by iridoid compounds was far greater than that by cholic acids.

5. Effects on Biliary Cation-Anion Gap

Iridoid compounds and cholic acids transiently increased biliary cation-anion gap, on the contrary sodium salicylate showed a prolonged decrease of the gap. The results are shown in Fig. 6.

6. Studies of Metabolites of Genipin

Thin-layer chromatogram (TLC) and high performance liquid chromatogram (HPLC) of the bile collected after injection of genipin are shown in Figs. 7 and 8, respectively. Although two bands \((Rf 0.08\) and 0.37) regarded as the metabolites of genipin were detected on TLC, genipin was not. On HPLC, the peaks of its metabolites were detected at the following retention time \((tR; 8.5, 9.9, \text{and} 18 \text{min})\). The band of \(Rf 0.08\) on TLC and the peak of \(tR 8.5\) min on HPLC were regarded as the major metabolite (GM-1) of genipin. The form of purified GM-1 was an amorphous powder. GM-1 was converted into genipin after hydrolysis by \(β\)-glucuronidase in vitro. From the following experimental data, it was confirmed that GM-1 is genipin-1-O-glucuronic acid. \(IR\nu^\mathrm{KBr}\text{ cm}^{-1}\):
3400, 1710, 1630. UVλ_{max}^{MeOH} nm (log ε): 237 (3.95). \([\alpha]_{D}^{23}: -7.7^\circ\ (c = 1.04,\ MeOH)\). \(^1\)H-NMR (δ in CD₃OD): 3.75 (3H, s, OCH₃), 4.30 (2H, m, C₁₀-H), 5.32 (1H, d, J = 7 Hz, C₁-H), 5.83 (1H, m, C₇-H), 7.63 (1H, brs, C₃-H). \(^{13}\)C-NMR (δ in CD₃OD): 36.2 (C-5), 39.6 (C-6), 47.1 (C-9), 51.7 (OCH₃), 61.2 (C-10), 73.4, 74.5, 76.1, 77.5 (C-2'~5'), 98.2 (C-1), 100.3 (C-1'), 112.6 (C-4), 128.3 (C-7), 144.8 (C-8), 153.3 (C-3), 169.5 (C-11), 176.5 (C-6). FD-MS m/z: 403 (M+H)^+, 425 (M+Na)^+, 441 (M+K)^+

The structure of GM-1 is shown in Fig. 9.

**FIG. 7. Thin-Layer Chromatogram of the Bile Given Genipin**

1: control bile, 2: genipin treated bile.

**FIG. 8. High Performance Liquid Chromatogram of the Bile Given Genipin**

**FIG. 9. Structure of GM-1 (Genipin-1-O-Glucuronic Acid)**
7. **Determination of the Main Metabolite (GM-1) of Genipin in Bile**

The periodical variation of biliary concentration of GM-1 is shown in Fig. 10. GM-1 level in the bile reached a peak (14 mM) at the first collection period (for 30 min) followed by a gradual decrease.

8. **Investigation of Metabolites of Sodium Salicylate**

The metabolites of sodium salicylate (tR 4.6 and 6.9 min) were gradually excreted into bile little by little. This pattern was entirely different from that of genipin or cholic acids. HPLC of the bile (from 30 min to 60 min after sodium salicylate injection) is shown in Fig. 11.

**DISCUSSION**

Bile, depending on its formation region, was classified into canalicual bile and distal ductule bile with the clearance method using an inert substance such as erythritol or mannitol by Schanker et al.\(^5\) and Forker.\(^6\) The formation of canalicual bile is thought to result from transport of solutes across the canalicual membrane of the hepatocyte accompanied by osmotic water flow.\(^6\) Canalicual bile consists of at least two fractions,\(^7-10\) one of which (bile acids-dependent fraction) is related to a secretion of bile acids.\(^10-12\) The other fraction (bile acids-independent fraction) is believed to result from active transport of Na\(^+\) into the bile canalicuals.\(^8,10\) and this formation is regulated by the enzyme Na\(^+\), K\(^+\)-stimulated adenosinetriphosphatase (Na\(^+\), K\(^+\)-ATP\(_{ase}\)).\(^13\) The existence of the latter mechanism has been supported by observation on a closed relationship between bile flow and Na\(^+\), K\(^+\)-ATP\(_{ase}\) activity in the canalicual enriched liver plasma membrane fractions in several studies.\(^14-16\) Other authors, however, failed to demonstrate such a relationship.\(^17,18\) Blitzer and Boyer concluded that Na\(^+\), K\(^+\)-ATP\(_{ase}\) exists in the sinusoidal and lateral membrane but not in the canalicual membrane.\(^19\) These discrepancies have yet been unexplained. Secretin is believed to act on the ductual part of biliary system and stimulate the generation of bicarbonate rich bile.\(^20,21\) Forker\(^22\) suggested that secretin promotes distal fluid secretion or inhibits distal fluid reabsorption and that distal alterations in fluid transfer are associated with increased bicarbonate production or suppression of its reabsorption from the result of electrolyte composition in the bile. A few reports on cation-anion gap observed in rat bile are found.\(^23,24\) Klos et al. suggested that bile contains anions which increase bile flow in a dose-dependent
fashion without affecting the permeability of the biliary tree besides 3-hydroxy bile acids and that bile acids-independent bile flow partly depends on excretion of a currently undefined anions. From these findings, it is conceivable that transportation of bile acids, inorganic electrolytes, and solutes (especially organic anions) across the canalicular membrane is important on bile secretion.

We made a comparative study on choleretic property and mechanism of action of iridoid compounds as well as dehydrocholate, cholate, and salicylate which have been well known as choleretics, examining their effects on factors such as bile flow, bile acids, and electrolytes and their metabolites. In the present study, biliary concentrations of Na⁺, K⁺, Cl⁻, and HCO₃⁻ were measured. Although bile contains Ca²⁺, Mg²⁺, HPO₄²⁻, SO₄²⁻, etc. as minor inorganic ions, it seems that these ions may be neglected since their amounts are very small and their physiological meanings have not been clarified. Each sample affected bile flow, bile acids concentration, the relationship between bile flow and bile acids excretion rate, concentrations of Na⁺, Cl⁻, and HCO₃⁻, and cation-anion gap except concentration of K⁺. Both genipin and patrinoside decreased biliary concentrations of bile acids, Na⁺, Cl⁻, and HCO₃⁻ and increased cation-anion gap corresponding to their rapid choleretic action. In the relationship between bile flow and bile acids excretion rate at the 1st 30-min period after injection, plots from genipin treated group and patrinoside treated group and those from the respective control group were distributed approximately in parallel and the slope of regression lines obtained from each iridoid compound treated group was not significantly different from that of the respective control group. The slope of regression line calculated from plots of bile flow to bile acids excretion rate indirectly expresses the osmotic pressure. One μmole of bile acids creates about 16, 13, and 17 μl of biliary moisture (bile flow) in groups given physiological saline solution, genipin, and patrinoside, respectively. The periodical pattern of cation-anion gap after injection of each iridoid compound was similar to that of its choleretic effect. These findings suggest that genipin, iridoid aglycone, and patrinoside, iridoid glucoside, possess the same choleretic property and their effects are due to bile acids-independent fraction and that factors offsetting cation-anion gap should exist. The choleretic effect of sodium dehydrocholate is approximately twice as potent as that of sodium cholate. Both compounds gave a marked decline in Cl⁻ concentration and weakly decreased HCO₃⁻ concentration. Sodium cholate markedly increased Na⁺ concentration, whereas sodium dehydrocholate showed no marked change. Cation-anion gap and bile acids concentration were increased by both compounds and the change in both parameters induced by sodium cholate was significantly large in comparison with that induced by sodium dehydrocholate. Though concentration of dehydrocholate can not be measured with sterognost-3α since it does not possess a hydroxyl moiety at 3α-position, it undergoes metabolism in the liver and is mainly converted into 3α, 7α-dihydroxy-12-keto-5β-cholanic acid, a measurable metabolite, and is rapidly excreted into bile. The slope of regression line between bile flow and bile acids excretion rate in the sodium dehydrocholate- treated group is different from that in the sodium cholate- treated group. This fact shows that choleretic action of both compounds depends on bile acids and that the volume of water generated from 1 mol of bile acids is different. The volume of canalicular bile created from 1 μmol of bile acids by sodium dehydrocholate and sodium cholate is calculated to be about 20 and 11 μl, respectively, from the slope of their regression line. Biliary bile acids of normal animals exist mostly forming a complex micelle with phospholipid and cholesterol. Choleretic action of taurocholate is thought to be due to osmotic gradient of Na⁺ as a conjugated cation. Since a metabolite of dehydrocholate hardly forms a micelle, it is explained that its efficacy to create canalicular bile is better than
other cholic acids. Sodium salicylate showed a weak and durable choleretic effect and also gave a weak decrease in Cl⁻ concentration as well as a marked increase in HCO₃⁻ concentration. As a result, its cation-anion gap persistently decreased. This compound produced no marked change on bile acids concentration. These ionic data and choleretic pattern indicate that mechanism of action of sodium salicylate is entirely different from that of iridoid compounds and cholic acids. These results support the view that salicylate promotes a release of secretin followed by stimulation of secretion of bile rich in HCO₃⁻ from the hepatic canaliculi and yet are contradictory to the reports made on a dog by Stone et al. After the administration of salicylate, only a small amount of metabolites was excreted into bile for a long period. It seems that the biliary metabolites of salicylate do not quantitatively affect its choleretic action, although those of iridoid compounds closely relate to their choleretic action as mentioned below.

Concerning genipin which shows the strongest efficacy among the iridoid compounds, the relation between the amount of its metabolite and cation-anion gap in bile was examined in order to investigate what kind of compound may offset the cation-anion gap. Numerous organic substances which are very efficaciously excreted into bile from the liver are known and their excretory process was generally thought to be a carrier-mediated active transport although only a few substances have been proved to do so. Three peaks were detected as a metabolite from bile given genipin on HPLC. The main peak (tᵣ 8.5 min) of them was genipin-1-O-glucuronic acid. Biliary concentration of genipin-1-O-glucuronic acid reached a maximum (14 mM) at the first collection period (for 30 min) after the administration of genipin and then gradually decreased. The periodical pattern of genipin-1-O-glucuronic acid level in bile was in agreement with that of the genipin-induced choleretic effect, and quantitatively cation-anion gap produced was nearly compensated by biliary concentration of genipin-1-O-glucuronic acid. Genipin-1-O-glucuronic acid is considered to be of anion form in bile. From these findings, it was suggested that genipin-1-O-glucuronic acid plays an important role in a choleretic action of genipin. Sperber indicated that secretion of biliary moisture is due to osmotic gradient of solutes (chiefly bile acids in physiological condition) which is actively transported into bile. Although movement of water in tissues is caused either by hydrostatic pressure or by osmotic pressure, movement of water due to the former in the biliary tree is hardly expected in view of the hepatic physiology. In fact, the biliary secretory pressure is always greater than the pressure of the sinusoid or the portal vein. The polarity of bile acids increases by conjugation with glycine or taurine. All of conjugated bile acids are actively secreted into bile in a completely dissociated form from the hepatocyte being accompanied by Na⁺. On the genipin-treated group, it was found that genipin-1-O-glucuronic acid is substituted for bile acids as an organic anion.

We previously examined the effect of genipin on erythritol clearance and reported that its choleretic action is due to the facilitation of canaliculare bile formation in the same fashion as bile acids.

From our various experimental results, we consider the choleretic mechanism of iridoid compounds to be as follows: In exerting the choleretic action of iridoid compounds such as genipin and patrinoside, the hemiacetal moiety is important. C₆-Hydroxy group of iridoid compounds undergoes conjugation in the liver to give glucuronide. Glucuronide thus formed is secreted into the biliary tree being coupled mainly with Na⁺, and water is passively excreted.

Such mechanism may apply to other bile acids-independent choleretics which act at the site of the bile canaliculi. Although, up to now, contribution of Na⁺, K⁺-ATPase or cyclic AMP to bile acids-independent mechanism is considered to be important, we also consider that the mechanism described above occupies an important part in the movement of Na⁺.
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