Appearance of Compound K, a Major Metabolite of Ginsenoside Rb1 by Intestinal Bacteria, in Rat Plasma after Oral Administration
―Measurement of Compound K by Enzyme Immunoassay―

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Enzyme immunoassay (EIA) for the determination of compound K (C-K), a major metabolite of ginsenoside Rb1 (G-Rb1) from Panax ginseng root by intestinal bacterial flora, was explored. Bovine serum albumin (BSA) was coupled to the C-26 position on the unsaturated side chain of C-K. β-D-Galactosidase was introduced at the C-26 position of the saturated side chain. Antiserum, obtained by immunization of rabbits with C-K–BSA conjugate, possessed high affinity and specificity toward C-K. The EIA for C-K by the double antibody method was established in the range of 0.1–100 ng/tube.

Plasma C-K after the oral administration of C-K and G-Rb1 to rats was determined by the established EIA. C-K was rapidly absorbed from the gastrointestinal tract after the administration, then slowly decreased. On the other hand, C-K appeared late and was retained for a long period of time in the plasma after the administration of G-Rb1, which itself is hardly absorbed.

Key words compound K; ginsenoside Rb1; enzyme immunoassay; absorption; rat plasma; Panax ginseng

Ginseng, the root of Panax ginseng, has been an important drug in Oriental Medicines, and contains many kinds of glucosides of dammarene type triterpenes, protopanaxadiol and protopanaxatriol, as main constituents.1–3. Although the pharmacological and biological activities of ginsenosides have been reported,3,4) poor absorption of these saponins, such as with ginsenosides Rg1 (G-Rg1) and Rb1, has been observed.5–9) Specially, when orally administered to rats, ginsenoside Rb1 (G-Rb1), the most abundant constituent of ginsenosides, has not been found in their sera by thin layer chromatography (TLC), and only an extremely low amount (0.05%) of it has been excreted in urine within 24 h.10) Also, in human beings, G-Rb1 has not been found in plasma, after the intake of red ginseng powder, by either high performance liquid chromatography (HPLC) or a sensitive method of the enzyme immunoassay (EIA) for G-Rb1.6) These ginsenosides are transformed to deglucosylated metabolites by intestinal bacteria in vitro and in vivo.7–9) G-Rb1 is also hydrolyzed into compound K (C-K), a main metabolite, via ginsenosides Rd (G-Rd) and F2 (G-F2) by rat and human intestinal flora (Chart 1).7,10) Our series of studies on the metabolism and absorption of plant glycosides indicated that aglycones hydrolyzed by intestinal bacteria were absorbed after the oral administration of natural glycosides and exhibited many pharmacological effects.11) Therefore, in the case of G-Rb1, its metabolites such as C-K can be absorbed. However, pharmacokinetic studies of metabolites such as C-K after the oral administration of G-Rb1 have not been reported.

We established a highly sensitive and specific EIA method for the determination of C-K in biological fluids and detected an appreciable amount of C-K in rat plasma after the oral administration of G-Rb1 using this newly established EIA method.

MATERIALS AND METHODS

Apparatus All melting points were taken on a microscopic hot stage (Yanagimoto Co., Kyoto) and optical rotation was measured with a Jasco polarimeter (Tokyo). 1H- and 13C-NMR spectra were obtained on JNM-GX270 and 400 spectrometers (Jeol, Tokyo). Fluorometry was performed on a Shimadzu RF-503 spectrofluorophotometer (Kyoto).

Chemicals Bovine serum albumin (BSA, Cohen fraction V) and naringinase from Penicillium decumbens were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-Gal, EC 3.2.1.23) from Escherichia coli was a product of Boehringer Mannheim Co. (Mannheim, Germany). Goat anti-rabbit IgG(H+L) IgG whole was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). G-Rb1 was kindly supplied from Japan Korea Red Ginseng Co., Ltd. (Kobe). Buffer A was 20 mM phosphate buffered saline (pH 6.8) containing 0.1% BSA, 0.1% NaN3 and 0.001% MgCl2.

Preparation of C-K (1) G-Rb1 (500 mg) was treated with naringinase (250 mg) for 3 d at 40 ℃ in 32 ml of phosphate–citrate buffer (pH 4.0) containing 10% EtOH. The precipitate formed was dissolved in 3.5 ml of EtOH and then mixed with the mother solution and the phosphate–citrate buffer (31.5 ml). The mixture was further treated for 7 d with naringinase (250 mg). The reaction mixture was extracted successively with EtO (100 ml, 20 ml×2) and n-ButOH (30 ml×3). The combined EtO extract was evaporated in vacuo to give C-K without further purification, and preparative TLC of the BuOH extract was carried out using 20% MeOH–CHCl3 as the developing solvent to purify C-K. 253 mg (86%) of C-K was obtained from both the EtO and BuOH extracts.

Hexaacetyl C-K (2) C-K (1) (249 mg, 0.4 mmol) was acetylated with acetic anhydride (2 ml) and pyridine (2 ml) at 80 ℃ for 2 h and then kept at room temperature overnight. MeOH (10 ml) was added to the reaction mixture, the solution was evaporated to dryness, and the MeOH treatment was repeated twice. The dried residue was dissolved in AcOEt (80 ml) and washed with 1 N HCl, brine, and 10% Na2CO3, dried over anhydrous MgSO4 and evaporated in vacuo. The residue was recrystallized from MeOH to give 2 (293 mg, 35% recovery).

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83.6%) as colorless needles, mp 174—175 °C, [α]D 26 +9.3° (c=1, CHCl₃). 1H-NMR δ 5.01 (1H, H-24).

**Hexaacetyl 24,25-Dihydro-C-K-24,25-diol (3)** OsO₄ (48 mg) was added to a stirred solution of 2 (154 mg, 0.18 mmol) in pyridine (2 ml) under ice cooling. After being kept overnight, 0.7% sodium bisulfite (7 ml) was added, and the reaction mixture was stirred at room temperature for 2 h, then poured into ice water (10 ml). The aqueous solution was extracted with CHCl₃ (70 ml), and the CHCl₃ extract was washed with 1 N HCl, brine and 10% Na₂CO₃, dried over anhydrous MgSO₄, and evaporated in vacuo. The dried residue contained a mixture probably due to 24S and 24R of 3 (155 mg, 97%). The mixture was separated by preparative TLC using 3% MeOH—CHCl₃ to give more polar and less polar compounds. 1H-NMR δ 3.17 (1H, br d, J=9.4 Hz, H-24 less polar compound) and 3.26 (1H, br d, J=9.9 Hz, H-24 more polar compound).

**Hexaacetyl 24,25,26,27-Tetranor-C-K-23-carbaldehyde (4)** A 10% HIO₄ solution (600 μl) was added to a solution of 3 (118 mg, 0.13 mmol) in dioxane (2 ml) under ice cooling. After being stirred for 2 h, the reaction mixture was poured into ice water (10 ml). The aqueous solution was extracted with CHCl₃ (50 ml), and the CHCl₃ extract was washed with brine, dried over anhydrous MgSO₄, and evaporated in vacuo. The residue was recrystallized from CH₃Cl₂—n-hexane to give 4 (104 mg, 95%) as colorless needles, mp 136—138 °C. 1H-NMR δ 9.76 (1H, s, H-24).

**Hexaacetyl 26,27-Dinor-C-K-25-carboxylic Acid Methyl Ester (5)** A mixture of 4 (110 mg, 0.13 mmol), methyl (triphenylphosphonyl)acetate (44 mg, 0.13 mmol) and dimethyl sulfoxide (DMSO) (1 ml) was heated at 110 °C for 7 h and then allowed to stand at room temperature. The reaction mixture was poured into ice water (15 ml) and extracted with CHCl₃ (30 ml). The CHCl₃ extract was washed with brine, dried over anhydrous MgSO₄, and evaporated in vacuo. The residue was purified by preparative TLC using 3% MeOH—CHCl₃ as the developing solvent and was then recrystallized from MeOH to give 5 (104 mg, 89%) as colorless needles, mp 183—185 °C. [α]D 26 +4.8° (c=1, CHCl₃). 1H-NMR δ 6.09 (1H, d, J=15.8, 6.8 Hz, H-24) and 5.83 (1H, d, J=15.8 Hz, H-25) due to trans olefinic protons.

**Hexaacetyl 26,27-Dinor-24,25-dihydro-C-K-25-carboxylic Acid Methyl Ester (7)** A stirred solution of 5 (38 mg, 0.04 mmol) in AcOEt (2 ml) was catalytically hydrogenated over 10% Pd—carbon (50 mg) at atmospheric pressure overnight. The catalyst was filtered off and the filtrate was concentrated in vacuo. The residue was recrystallized from CH₃Cl₂—n-hexane to give 7 (40 mg, 98%) as colorless plates, mp 85—87 °C. 1H-NMR δ 2.31 (2H, t, J=7.5 Hz, H-25) due to the methylene protons adjacent to the carbonyl group.

**Preparation of C-K—BSA Conjugate (9)** A mixture of carboxylic acid methyl ester (5) (15 mg, 0.017 mmol), hydrazide hydrate (0.6 ml) and MeOH (0.2 ml) was stirred at 70 °C for 7 h. MeOH (10 ml) was added to the reaction mixture and the whole was concentrated in vacuo. The MeOH treatment was repeated three times. The residue was washed with AcOEt (3 ml) and then dissolved in a small amount of MeOH, and n-hexane was added to give 26,27-dinorC-K-25-carboxyhydrzone (6) (8 mg, 54.5%). This crude product was used for the next step without purification. A stirred solution of 6 (11 mg, 0.017 mmol) in dimethyl formamide (DMF) (0.3 ml) was treated with 10% NaNO₂ (30 μl) and kept at pH 2 by adding 1N HCl at 0 °C. After 10 min, BSA (31 mg, 0.46 μmol) and Na phosphate buffered saline (pH 7.2, 2 ml) were added to the reaction mixture, which was stirred at 0 °C for 3 d. The resulting solution was dialyzed against distilled water with one change a day for 5 d. The dialysate was lyophilized to afford C-K—BSA conjugate (29 mg).

**Preparation of C-K—β-Gal Conjugate (10)** A mixture
of dihydrocarboxylic acid methyl ester (7) (19 mg, 0.021 mmol), hydrizine hydrate (0.5 ml) and MeOH (1.0 ml) was treated at 50 °C for 2 h and allowed to stand at room temperature overnight. The MeOH (5 ml) treatment was repeated 5 times in the same manner described above. The residue was purified by preparative TLC using 20% MeOH–CHCl₃ as the developing solvent to give 26,27-dinor-24,25-dihydro-C-K-25-carboxyhydrizine (8) (14 mg, 0.021 mmol). A stirred solution of 8 (63 µg, 0.097 µmol) in DMF–H₂O (5:55, 20 µl) was treated with 0.1% NaNO₂ (6.7 µl) and 0.1% HCl (9.7 µl) at 0 °C. The reaction mixture was added to a solution of β-Gal (2.5 mg, 4.83 mmol) in 20 mM Na phosphate buffered saline (pH 7.2, 2 ml). The mixture was kept at pH 8 by adding 10% K₂CO₃, and was stirred at 0 °C overnight. The reaction mixture was applied to a Sephadex 6B column (2.7×35 cm) using buffer A as the eluent. Fractions containing peak enzyme activity were collected, pooled and stored at 4 °C until use.

**Determination of the Number of C-K Molecules Linked to One BSA Molecule in C-K–BSA Conjugate**

Aliquots of 40 and 80 µl of BSA solution and C-K–BSA conjugate solution (1 mg/ml), respectively, were used. The number of free amino groups in BSA of the standard and C-K–BSA conjugate samples was determined using trinitrobenzenesulfonyl acid according to Habeeb. The differences in these numbers in the BSA molecules, the number of C-K molecules linked to the BSA molecule of the conjugate was calculated.

**Preparation of Antiserum for C-K**

Three domestic female albino rabbits weighing 2.0 kg were used for immunization. The C-K–BSA conjugate (9, 3 mg) was dissolved in sterile saline (1.5 ml) and emulsified with complete Freund's adjuvant and then injected into the rabbits subcutaneously at multiple sites on the back. Half the initial dose of immunogen was given as a booster once every 2 weeks for 2 months and then once a month for 2 months. The sera were obtained by centrifugation after standing at room temperature for 2 h, and were then stored at −20 °C until use.

**Procedure of EIA**

Unless otherwise stated, dilution was carried out with buffer A. A sample or standard solution of C-K (100 µl) was added to 1000-fold diluted anti-C-K antiserum (100 µl) and C-K–β-Gal conjugate (50 µl), and the mixture was incubated at room temperature for 2 h. Then, 4-fold diluted goat anti-rabbit IgG anti IgG (50 µl) and 100-fold diluted normal rabbit serum (20 µl) were added to the incubation mixture, and the new mixture was allowed to stand at 4 °C overnight. After the addition of buffer A (1 ml), the mixture was centrifuged at 3000 rpm for 20 min, and the supernatant was removed. The immune precipitate was washed twice with buffer A (1 ml) and used for measuring enzyme activity. The immune precipitate was incubated with 0.1 mM 7β-d-galactopyranosylxy-4-methylcoumarin (150 µl) at 30 °C for 30 min. After incubation, 0.1 M glycine–NaOH (pH 10.3, 2 ml) was added to the reactin mixture, and the fluorescence intensity of 7-hydroxy-4-methylcoumarin was measured using 365 nm as excitation and 448 nm as emission.

**Specificity of the Antiserum**

The cross reaction of anti-C-K antiserum with G-Rb, and its intestinal bacterial metabolites, such as G-Rd, G-F₃, and 20(S)-protopanaxadiol (20(S)Ppd), were examined using C-K–β-Gal conjugate according to the assay procedure described above.

**Animals, Treatments and Sampling**

Male Wistar rats (6 weeks old, SLC Co., Hamamatsu, Japan) were purchased. Animals were fed standard laboratory chow with water ad libitum, maintained for one week, and then fasted overnight before the experiments.

G-Rb dissolved in pure water was given orally to the rats (n = 3 at each point) at a dose of 200 mg/kg. Blood samples were obtained from the tail vein using a heparinized capillary at 40 min, 2, 4, 7, 15 and 24 h after the administration. Moreover, C-K dissolved in pure water was given orally to the rats (n = 3) at a dose of 56.2 mg (equal mole to 100 mg G-Rb)/kg. Blood samples were taken at 15, 30 min, 2, 4, 7, 10 and 24 h after the administration. Plasma samples immediately separated by centrifugation were kept at −20 °C until use. Plasma (50 µl) was extracted with MeOH (200 µl, ×2). The MeOH extract was dried in vacuo and dissolved with MeOH (20 µl) followed by the addition of buffer A (80 µl) to measure the C-K concentration by the EIA method as described above.

**RESULTS AND DISCUSSION**

**EIA for C-K**

BSA- and β-Gal conjugates with C-K were prepared as immunogen and labeled antigen, respectively, according to procedures similar to the RIA method for G-Rg₁ and the EIA method for G-Rb (Chart 2).

Compound 2 was oxidized with osmium tetroxide to give 3, which was further cleaved by periodic acid oxidation to give 4. The tetranor-aldehyde was converted into an unsaturated ester (5) with methyl(triphenylphosphoryl)dieneacetate by the Wittig-Horner reaction and then hydrogenated over palladium carbon to give a saturated ester (7) for bridge heterologous EIA. Each acetyl C-K-carboxylic acid methyl ester (5 and 7) was allowed to react with hydrizine hydrate to afford the corresponding 6 and 8, which were deacetylated. These hydrazides were used without purification to be coupled to BSA and β-Gal. The former hydrazide (6) was converted with nitrous acid to the azide, which was immediately coupled with BSA to give 9. In these reactions, the hydrazide and BSA were used in a mole ratio of 37:1. This conjugate was used for immunization after dialysis. From the difference between the number of free amino groups of BSA and that of the 9, the number of C-K molecules bound to the 9 was calculated to be 8.4. Antisera to C-K were elicited in three rabbits immunized with 9. The optimum dilution of anti-C-K antiserum was 10000-fold.

The other hydrazide (8) was similarly converted to an azide and coupled with β-Gal to give 10, which was used as a labeled antigen after purification on a Sepharose 6B column. The bound and free 10 were separated by a double antibody method with an anti-rabbit IgG goat IgG, and the enzyme activity of the immune precipitate was determined fluorometrically with 7β-d-galactopyranosylxy-4-methylcoumarin as a substrate. A typical standard curve for C-K was obtained with a measurable range at 0.1—100 ng/tube (Fig. 1). Although C-K in plasma was less reactive with the antiserum, probably due to the interference of some components in the plasma, a similar curve was obtained by extraction with MeOH with the same measurable range (Fig. 1). In the presence of plasma, the coefficients of variance at 1—100
Chart 2. Preparation of Immunogen and Labeled Antigen for Enzyme Immunoassay of C-K

Fig. 1. Typical Standard Curves of C-K
C-K diluted with A buffer (○) and with rat control plasma (●) was used at a log dose for the measurement of bound enzyme activities. Data were the means in triplicate.

Table 1. Cross Reactivity of the Antiserum for C-K

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cross reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-K</td>
<td>100</td>
</tr>
<tr>
<td>G-F2</td>
<td>27.5</td>
</tr>
<tr>
<td>G-Rd</td>
<td>3.21</td>
</tr>
<tr>
<td>G-Rb4</td>
<td>1.53</td>
</tr>
<tr>
<td>20(S)Ppd</td>
<td>0.995</td>
</tr>
</tbody>
</table>

ng/tube were 4.1—15.2% for inter-day assay. The cross reactivities of anti-C-K antiserum with G-Rb4 and its intestinal bacterial metabolites are shown in Table 1. The antiserum cross-reacted with G-F2 (27.5%), less with G-Rd (3.21%) and G-Rb4 (1.53%), and not at all with 20(S)Ppd (0.995%). The former two have the same one glucose conformation at the C-20 position on the protopanaxadiol moiety as C-K, except for the sugar moiety at the C-3 position. It seems that the anti-C-K antiserum specifically recognizes one glucose moiety at C-20 on the protopanaxadiol. As G-F2 is a minor metabolite of G-Rb4 by intestinal bacteria, the antiserum obtained is considered to be usable for determining C-K in plasma samples after the oral administration of G-Rb4.

Determination of Plasma C-K by the Established EIA after the Oral Administration of C-K and G-Rb4

Figure 2 shows a plasma concentration curve of C-K after the oral administration of C-K to rats at a dose of 56.2 mg/kg. C-K appeared even 15 min after the administration, reached a maximum within a short time ($T_{max} = 30$ min, $C_{max} = 520$ ng/ml), and was maintained for a considerable time (until 10 h). The $AUC_{0-24h}$ was 3120 ng·h·ml$^{-1}$. These results indi-
cate that C-K is absorbed rapidly from the gastrointestinal tract. Although it is reported that G-Rg, also appears within 1 h after its oral administration, in spite of poor absorption,\(^1\) it has been eliminated more rapidly from the plasma compared with C-K. The discrepancy of retainability in rat plasma between C-K and G-Rg, may be due to the easy metabolism of G-Rg, by rat intestinal bacteria.\(^7\)

Figure 3 shows a plasma concentration curve of C-K after the oral administration of G-Rb, at a dose of 200 mg (equal mole to 112.4 mg of C-K)/kg. C-K appeared late (after 4 h), reached a maximum at 7 h, was maintained at a high concentration for a long period of time (through 15 h), and was still found 24 h after the administration. As G-Rb, is hardly absorbed from the rat gastrointestinal tract,\(^4\) where G-Rb, seems to be retained for a long time and to be slowly transformed to C-K, a major intestinal bacterial metabolite, the amount of C-K produced seems to be absorbed from the lower part of the intestinal tract. However, the \(AUC_{0-24h}\) (1160 ng·h·mL\(^{-1}\)) of C-K after the oral administration of G-Rb, was about one-sixth of that after the oral administration of C-K, suggesting that not all molecules of G-Rb, are metabolized completely to C-K; a considerable amount of G-Rb, probably remains without being metabolized and (or) some amount of metabolites other than C-K are produced.

Other ginsenosides such as G-Rb, and G-Rc, which are involved with appreciable contents in ginseng radix and have an identical protopanaxadiol structure as aglycones, are poorly absorbed\(^5,8\) and are metabolized to C-K as a main metabolite by rat intestinal bacterial flora.\(^6,8\) Accordingly, after the oral administration of ginseng radix to rats, C-K seems to be one of the major components absorbed from the gastrointestinal tract, as reported by Hasegawa et al.\(^9\)

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REFERENCES


Fig. 3. Plasma C-K Concentration after Oral Administration of G-Rb, to Rats
G-Rb, was orally administered at a dose of 200 mg/kg to rats (n=3 at each point). Each value represents the mean ± S.D. (vertical bar).