Studies on Absorption, Distribution, Excretion and Metabolism of Ginseng Saponins. VIII.[1] Isotope Labeling of Ginsenoside Rb_2

Masami KARIKURA,* Hisayuki TANIZAWA, Takashi HIRATA, Toshio MIYASE, and Yoshio TAKINO

School of Pharmaceutical Sciences, University of Shizuoka, 52–1, Yada, Shizuoka 422, Japan. *Received February 8, 1992*

To clarify the pharmacokinetics of absorption, distribution and excretion of ginsenoside Rb_2 (Rb_2), one of the major saponins of the root of *Panax ginseng*, following oral administration to rats, a tritium (^3H) labeling of Rb_2 was examined. The C-12 position of Rb_2 was labeled with ^3H-sodium borohydride (^3H-$NaBH_4$) and 12-^3H Rb_2 and 12-^3H-epi Rb_2 was synthesized. This method of specific position labeling of Rb_2 may be applicable to other ginsenosides. In the near future, the pharmacokinetics of Rb_2 in rats may be clarified with ^3H labeled Rb_2.

Keywords isotope labeling; tritium labeling; ginsenoside Rb_2; ^{13}C-NMR; FAB-MS; ginseng saponin

We have been studying the pharmacokinetics of ginsenosides regarded as the components principally responsible for the pharmacological activities of the root of *Panax ginseng* C. A. MEYER (Araliaceae). We have found several metabolites of ginsenoside-Rg_1 (Rg_1), -Rb_1 (Rb_1) and -Rb_2 (Rb_2), in the rat gastrointestinal tract after oral administration, and also reported the absorption, distribution and excretion of Rg_1 and Rb_1 in rats. These absorption rates were very low, 0.1% and 1.9%, respectively.[2] Sankawa also reported the low absorption rate of Rg_1 of 8.6% in rats using radioimmunoassay method.[3] In view of the many important findings on the pharmacological activities of ginsenosides, their low absorption rates seem unconvincing and we, therefore, felt it necessary to determine whether or not these were correct. Tritium (^3H) labeling of a ginsenoside was seen as useful to resolve this, because the methods of determining ginsenosides and their metabolites in biological samples using high performance liquid chromatography (HPLC) or thin layer chromatography (TLC)- densitometry are not always perfect and may have inadvertently been missed. The existence of ^3H is easily found because of its high detection sensitivity.

Our previous studies revealed that the metabolites of Rg_1, Rb_1 and Rb_2 in rat gastrointestinal tract were mostly prosapogenins derived from hydrolysis of their sugar moieties. Therefore, we considered that the most suitable position for ^3H labeling might be at C-12 of dammarene type aglycones. In the present paper, we used Rb_2 as a ginsenoside, and investigated the process of ^3H labeling in detail, as there is no report on this.

Experimental

Materials and Equipment Most of the materials and equipment used were the same as described in our previous paper.[1] Sodium borohydride ($NaBH_4$) and lithium aluminum hydride ($LiAlH_4$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and sodium borodeuteride ($NaBD_4$) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). ^3H-Sodium borohydride (^3H-$NaBH_4$, 185 MBq in 500 μl of 0.01 N NaOH solution) was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Cation exchange resin (Dowex 50W × 8, 50—100 mesh) was purchased from Muromachi Kagaku Kogyo Co. (Tokyo, Japan). Tetrahydrofuran (THF) was treated with $LiAlH_4$, and distilled before using.

Partial Acetylation and Oxidation of Rb_2 Rb_2 (2 g) was stirred for 24 h at room temperature in a mixture of acetic anhydride (10 ml) and pyridine (10 ml). After dilution with water (100 ml), the reaction mixture was extracted with ethyl acetate (AcOEt, 500 ml). After washing the AcOEt layer with 10% HCl, saturated aqueous $NaHCO_3$ and water, the AcOEt solution was dried with $MgSO_4$, and concentrated to dryness *in vacuo*. The residue was purified by column chromatography [silica gel 200 g, benzene–acetone (5 : 1)] to yield trideca-acetyl Rb_2 (I, 2 g, white powder). The proton nuclear magnetic resonance (^1H-NMR) spectral data of I showed 21 methyl signals. To a solution of I (1.5 g) in pyridine (5 ml), a mixture of chromic acid (1.5 g) and pyridine (10 ml) was added dropwise and the mixture was stirred for 5 h at room temperature, then diluted with 2-propanol (2-PrOH, 20 ml) and 10% HCl (100 ml) and extracted with AcOEt (200 ml). After washing the AcOEt layer with 10% HCl, saturated aqueous $NaHCO_3$ and water, the AcOEt solution was dried with $MgSO_4$, and concentrated to dryness *in vacuo*. The residue was purified by preparative HPLC (YMC-packed column SH-343-5, 82% aqueous acetonitrile, 5 ml/min, 205 nm) to yield trideca-acetyl 12-keto derivative (II, 1.1 g, white powder). The identification of II was performed by comparing the carbon-13 nuclear magnetic resonance (^{13}C-NMR) and ^1H-NMR spectral data of deacetylated II with that of Rb_2 and chikusetsusaponin LN_4.[4]

Deacetylated II: A white powder. ^1H-NMR (pyridine-d_5) δ: 0.80, 0.90, 1.10, 1.28, 1.29, 1.56 (3H each, all s, *tert*-CH_3 × 6), 1.68 (6H, s, vinyl. CH_3 × 2), 4.92, 4.96, 5.03, 5.36 (1H each, all d, J=7.7, 5.8, 7.3, 7.6 Hz, respectively, anomeric H × 4). ^{13}C-NMR (pyridine-d_5) δ: 105.1 (C-3 glc), 106.1 (C-2′ glc), 98.5 (C-20 glc), 104.9 (C-6‴ arap) (anomeric C × 4), 40.7 (C-1), 26.6 (C-2), 88.6 (C-3), 39.7 (C-4), 56.2 (C-5), 18.5 (C-6), 34.8 (C-7), 40.9 (C-8), 56.4 (C-9), 37.4 (C-10), 40.1 (C-11), 211.3 (C-12), 54.8 (C-13), 56.3 (C-14), 32.3 (C-15), 24.6 (C-16), 42.6 (C-17), 17.0 (C-18), 17.9 (C-19), 81.5 (C-20), 22.3 (C-21), 38.8 (C-22), 24.1 (C-23), 125.9 (C-24), 130.9 (C-25), 25.8 (C-26), 16.5 (C-27), 28.0 (C-28), 16.3 (C-29), 16.0 (C-30).

Reduction of II 1) Isolation of Rb_2 and 12-Epi Rb_2 II (100 mg) was refluxed for 1 h in THF (10 ml) with $LiAlH_4$ (300 mg). AcOEt (10 ml) and methanol (20 ml) were added to the reaction mixture, and refluxed for 30 min. A clear solution was evaporated to dryness *in vacuo*, the residue, was suspended in water (20 ml) and 10% HCl (50 ml) was added to the suspension, extracted with 1-butanol (100 ml). After washing the 1-butanol layer with water, saturated aqueous $NaHCO_3$ and water, the 1-butanol solution was concentrated to dryness *in vacuo*. The residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield Rb_2 (8 mg, t_R 35 min) and 12-epi Rb_2 (40 mg, t_R 60 min). The identification of 12-epi Rb_2 was performed by comparing the ^1H- and ^{13}C-NMR spectral data with that of Rb_2.

12-Epi Rb_2: A white powder. ^1H-NMR (pyridine-d_5) δ: 0.85, 0.96, 1.14, 1.30, 1.41, 1.73 (3H each, all s, *tert*-CH_3 × 6), 1.59, 1.65 (3H each, both s, vinyl. CH_3 × 2), 4.90, 4.99, 5.00, 5.37 (1H each, all d, J=7.6, 6.4, 7.7, 7.6 Hz, respectively, anomeric H × 4), 5.34 (1H, t, J=6.4 Hz, C24-H). ^{13}C-NMR (pyridine-d_5) δ: 105.1 (C-3 glc), 106.1 (C-2′ glc), 98.4 (C-20 glc), 105.0 (C-6‴ arap) (anomeric C × 4), 39.3 (C-1), 26.8 (C-2), 89.1 (C-3), 39.8 (C-4), 56.6 (C-5), 18.5 (C-6), 37.1 (C-7), 40.9 (C-8), 45.9 (C-9), 36.8 (C-10), 31.9 (C-11), 67.7 (C-12), 47.1 (C-13), 49.4 (C-14), 30.5 (C-15), 25.3 (C-16), 46.2 (C-17), 20.1 (C-18), 18.0 (C-19), 83.2 (C-20), 21.8 (C-21), 36.0 (C-22), 23.1 (C-23), 126.3 (C-24), 131.0 (C-25), 25.9 (C-26), 16.7 (C-27), 28.1 (C-28), 16.6 (C-29), 15.6 (C-30).

2) Treatment with $LiAlH_4$ II was refluxed for a predetermined time in THF with $LiAlH_4$. The reaction mixture was worked up as described above, and the residue was subjected to HPLC analysis.

3) Treatment with $NaBH_4$ II was refluxed for a definite time in various solvents with $NaBH_4$. Methanol was added to the reaction mixture, and refluxed for 30 min. A clear solution was neutralized by application of a Dowex 50 W × 8 (H^+ form), and evaporated to dryness *in vacuo*. The

residue was subjected to HPLC analysis.

4) Deuterium Labeling of Rb_2 with $NaBD_4$ II (50 mg) was refluxed for 15 h in 2-PrOH (5 ml) with $NaBD_4$ (240 mg). The reaction mixture was worked up as described above, and the residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield 12-D Rb_2 (2 mg) and 12-D-epi Rb_2 (17 mg). The identification of both D-labeled Rb_2 was performed by fast atom bombardment mass spectrometry (positive FAB-MS) data. 12-D Rb_2: FAB-MS *m/z*: 1080 $(M+H)^+$, 1102 $(M+Na)^+$. 12-D-epi Rb_2: FAB-MS *m/z*: 1102 $(M+Na)^+$.

5) Tritium Labeling of Rb_2 with ^3H-$NaBH_4$ II (20 mg) was refluxed for 24 h in 2-PrOH (10 ml) with $NaBH_4$ (100 mg) and ^3H-$NaBH_4$ (185 MBq in 500 μl of 0.01 N NaOH solution). The reaction mixture was worked up as described above, and the residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield ^3H-labeled Rb_2. The radioactivities of 12-^3H Rb_2 and 12-^3H-epi Rb_2 were 18.2 and 19.4 kBq/μmol, respectively.

Biological Experiment For intravenous injection, 0.4% solution of 12-D-epi Rb_2 dissolved in 0.9% saline was given *via* the femoral vein at a dose of 10 mg/kg to non-fasted rats. Urine sample was collected after 24 h by the use of a metabolic cage (KN-646, Natsume, Tokyo, Japan), and methanol was added to extract 12-D-epi Rb_2. After centrifugation at 3000 rpm for 15 min, the supernatant was evaporated *in vacuo*. The residue was treated with a SEP-PAK® C_{18} cartridge in the same manner as in our previous paper,[1] and 12-D-epi Rb_2 (1 mg) was obtained by preparative HPLC as described above. Identification was done by FAB-MS.

Results and Discussion

Ginseng saponins, isolated from the root of *Panax ginseng*, have been regarded as the principal components responsible for the pharmacological activities of the drug. Investigations, however, have still not completely elucidated the absorption, distribution, excretion and metabolism of ginseng saponins. The basic reason for this is that there is no satisfactory analytical method for biological samples; the usual analytical methods have problems such as sensitivity, specificity and other complications. The easiest way is to use radioisotopic ginseng saponins, which are labeled with ^{14}C and/or ^3H. However, the synthesis of a radioisotopic ginseng saponin has been thought to be very difficult owing to its natural product. In fact, there is no report on labeling of a specific position in a ginseng saponin using ^{14}C or ^3H, although there are reports describing their overall labeling.[5]

We attempted to obtain ^3H labeled Rb_2 as shown in Chart 1. Rb_2 was partially acetylated to I by acetic anhydride and pyridine, that is, the C-12 hydroxyl group of I was not acetylated. This selective acetylation was previously reported by Tanaka *et al.*[6] Then, the C-12 hydroxyl group of I was oxidized by chromic acid and pyridine to yield II. The formation of ^3H labeled Rb_2 was expected when Rb_2 was reproduced by reduction of II with ^3H labeled reductants. Though C-12 epimerization occurs in this reduction, the method for yielding 12β-OH (*i.e.*, Rb_2) predominantly by the reduction with sodium and 2-PrOH was reported by Tanaka *et al.*[6] On the other hand, ^3H-$NaBH_4$ is widely used because of its high relative radioactivity, and ^3H-$LiAlH_4$ is also occasionally used as a ^3H labeled raductant.

Therefore, we began the study by selecting the reductant in this reduction. First, II was refluxed for 24 h in THF with $LiAlH_4$ or $NaBH_4$, and the reaction products were determined by HPLC. As shown in Fig. 1, the major product was 12-epi Rb_2 in this reduction with both reductants, but the yield of Rb_2 was better with $NaBH_4$ than with $LiAlH_4$.

Then, the suitability of using a protic solvent for the reduction of II with $NaBH_4$ was examined. II was refluxed for 24 h with $NaBH_4$ in methanol, ethanol, 1-PrOH, 2-PrOH, 1-butanol and 2-butanol, respectively, and the reaction products in each case were determined by HPLC. Figure 2 shows that the most suitable solvent in this reduction was 2-PrOH.

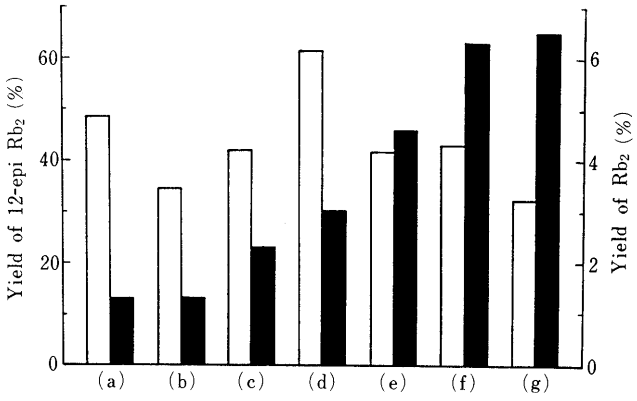

Fig. 1. Reduction of II with $LiAlH_4$ or $NaBH_4$ in THF for 24 h

□, 12-epi Rb_2; ■, Rb_2; (a), $LiAlH_4$ (57 mol ratio to II); (b), $LiAlH_4$ (117 mol ratio); (c), $LiAlH_4$ (154 mol ratio); (d), $NaBH_4$ (53 mol ratio); (e), $NaBH_4$ (132 mol ratio); (f), $NaBH_4$ (216 mol ratio); (g), $NaBH_4$ (305 mol ratio).

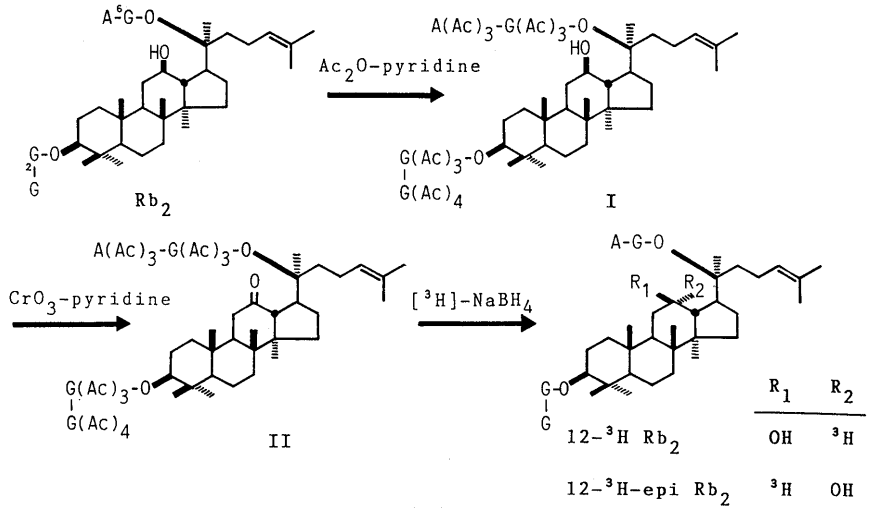

Chart 1. Synthetic Route of ^3H–Rb_2

A, α-L-arabinopyranosyl; G, β-D-glucopyranosyl; Ac, acetyl.

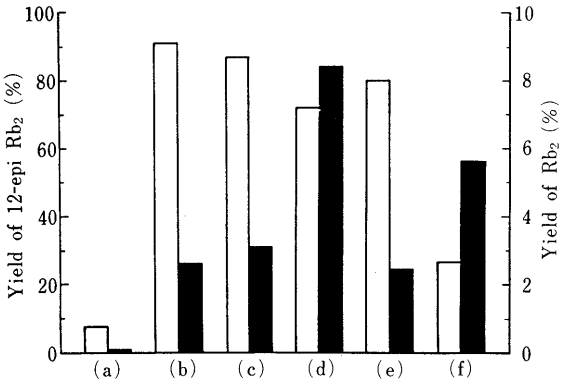

Fig. 2. Reduction of II with $NaBH_4$ in Several Solvents for 24 h

200 mol ratio of $NaBH_4$ to II was used in each reaction. □, 12-epi Rb_2; ■, Rb_2; (a), methanol; (b), ethanol; (c), 1-propanol; (d), 2-propanol; (e), 1-butanol; (f), 2-butanol.

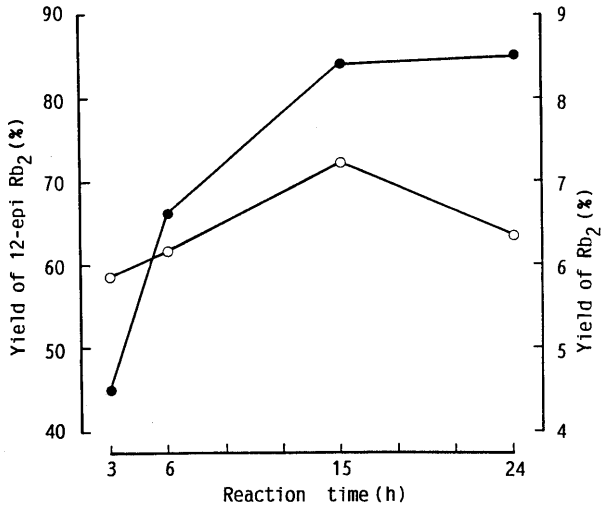

Fig. 3. Time Course of Reduction of II with $NaBH_4$ in 2-Propanol

200 mol ratio of $NaBH_4$ to II was used. ○, 12-epi Rb_2; ●, Rb_2.

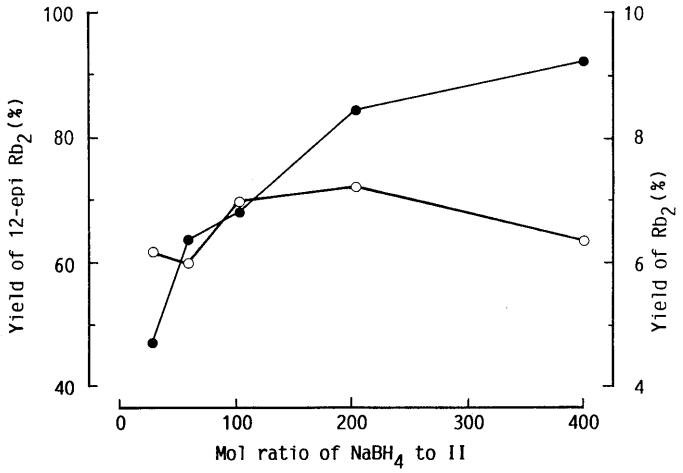

Fig. 4. Reduction of II with $NaBH_4$ in 2-Propanol for 24 h

○, 12-epi Rb_2; ●, Rb_2.

Next, to examine the time-course of this reduction, II was refluxed for 3, 6, 15 and 24 h, respectively. From the result in Fig. 3, we judged that the reaction time of 15 h might be enough to get Rb_2 with good yield.

Finally, to determine the suitable quantity of $NaBH_4$, the reduction was done with various mole ratios of $NaBH_4$ to II. The result is shown in Fig. 4. From the results, we decided that the suitable mole ratio of $NaBH_4$ to II might be *ca.* 200. The yield of Rb_2 and 12-epi Rb_2 under the best conditions was 8.4% and 72.0%, respectively, and the ratio of Rb_2 to 12-epi Rb_2 was 8.6.

Thus, we concluded that the most suitable reduction conditions for II to obtain Rb_2 in good yield was 15 h for refluxing, using 2-PrOH as the solvent and 200-fold $NaBH_4$ to II in mole ratio.

Under these conditions, we synthesized 12-D labeled Rb_2 with $NaBD_4$. The yield was 8.8% for 12-D Rb_2 and 56.1% for 12-D-epi Rb_2. The ratio of Rb_2 to 12-epi Rb_2 was 6.4, almost the same as in the case of $NaBH_4$. We therefore went on the synthesize 12-^{3}H labeled Rb_2 using our best reduction condition. The yield of 12-^{3}H Rb_2 and 12-^{3}H-epi Rb_2 was 3.5% and 70.5%, respectively. The ratio of Rb_2 to 12-epi Rb_2 was 20.1. The specific activities of 12-^{3}H Rb_2 and 12-^{3}H-epi Rb_2 were 18.2 and 19.4 kBq/μmol, respectively. Total reduction percentage of II by ^{3}H-$NaBH_4$ was the same as that by $NaBH_4$, but the ratio of Rb_2 to 12-epi Rb_2 was poorer than that of $NaBH_4$. The cause of this is not clear, but may be due to "an isotopic effect," The alkali solution used to dissolve ^{3}H-$NaBH_4$ did not affect the reduction, and this was confirmed by $NaBH_4$. In practice, however, this is not a large problem; we are able to obtain highly radioactive ^{3}H-$NaBH_4$ commercially and so can get enough 12-^{3}H Rb_2 for animal experiments. We are also able to use 12-^{3}H-epi Rb_2, if the behavior of both 12-^{3}H Rb_2 and 12-^{3}H-epi Rb_2 in the animal body is proved effective in future.

We also examined the possibility of H–D isotope exchange in the rat body after intravenous injection of 12-D-epi Rb_2. A comparison of FAB-MS data for 12-D-epi Rb_2 isolated from rat urine with that of 12-D-epi Rb_2 showed that H–D exchange did not occur. Therefore, the pharmacokinetics such as absorption, distribution and excretion of Rb_2 in rats may be clarified using ^{3}H labeled Rb_2 in the near future. This method of specific position labeling of Rb_2 may also be applicable to other ginsenosides. ^{3}H labeling of Rb_2 to obtain high radioactivity is now under way.

Acknowledgements The authors are grateful to the Korea Ginseng and Tobacco Research Institute and the Japan–Korea Red Ginseng Co., Ltd., for the supply of pure Rb_2. They also thank Dr. M. Uchida, of the Central Analytical Laboratory of their university, for measurement of FAB-MS spectra. This work was supported in part by a grant from the Medical Society for Red Ginseng Research.

References

1) M. Karikura, T. Miyase, H. Tanizawa, T. Taniyama, and Y. Takino, *Chem. Pharm. Bull.*, **39**, 2357 (1991).
2) *a*) Y. Takino, T. Odani, H. Tanizawa, and T. Hayashi, *Chem. Pharm. Bull.*, **30**, 2196 (1982); *b*) T. Odani, H. Tanizawa, and Y. Takino, *ibid.*, **31**, 292 (1983); *c*) *Idem, ibid.*, **31**, 1059 (1983); *d*) *Idem, ibid.*, **31**, 3691 (1983).
3) U. Sankawa, "Panax Ginseng," ed. by A. Kumagai, H. Oura, and H. Okuda, Kyoritsu Shuppan, Tokyo, 1985, pp. 58—70.
4) S. Yahara, O. Tanaka, and I. Nishioka, *Chem. Pharm. Bull.*, **26**, 3010 (1978).
5) *a*) B. H. Han, E. B. Lee, M. C. Yun, and L. N. Woo, *Korean Biochem. J.*, **9**, 21 (1976); *b*) B. H. Han and I. M. Chang, *Korean J. Ginseng Sci.*, **2**, 17 (1977).
6) O. Tanaka, M. Nagai, and S. Shibata, *Chem. Pharm. Bull.*, **14**, 1150 (1966).