Solubilization of Saponins of Bupleuri Radix with Ginseng Saponins: Effect of Malonyl-ginsenosides on Water Solubility of Saikosaponin-b₁

Xiao-hong ZHOU,^a Ryoji KASAI,^{*a} Masayuki YOSHIKAWA,^b Isao KITAGAWA^b and Osamu TANAKA^a

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,^a Kasumi, Minami-ku, Hiroshima 734, Japan and Faculty of Pharmaceutical Sciences, Osaka University,^b Yamada-oka, Suita, Osaka 565, Japan. Received December 6, 1990

Saikosaponins, the active principles of Bupleuri Radix, are generally sparingly soluble in water. The solubilizing effect of Ginseng saponins on saikosaponin-b₁ (Sb₁) which is formed from saikosaponin-a (Sa) by mild acid treatment, was investigated. It was revealed that the significant increase of the water-solubility of Sb₁ with Ginseng saponin mixture was due mainly to malonyl-ginsenosides. Some solubilizing effect was also observed with ginsenoside-Ro (Ro). No solubilizing effect was found with the neutral dammarane saponins, while the effects of malonyl-ginsenosides and Ro were remarkably potentiated in the presence of these neutral dammarane saponins. The water-solubility of Sa was also increased in the presence of malonyl-ginsenosides under cooperation with the neutral dammarane saponins.

Keywords Ginseng saponin; solubilizing effect; malonyl-ginsenoside; ginsenoside; saikosaponin; Bupleuri Radix; dammarane saponin; cooperative solubilization

In our serial studies on surface-active properties of oligo-glycosides, it has been reported that the water solubility of saikosaponin-a (Sa), one of the active principles of the Bupleuri Radix, is greatly increased in the presence of glucuronide saponins of Ginseng^{1,2)} and licorice³⁾, which are sometimes coprescribed with Bupleuri Radix in oriental

traditional medicines. Investigation of the structure-solubilization relationship revealed the important role of the carboxyl group of the glucuronide moiety for increasing the water solubility of Sa.

It has been known that the allyloxide moiety of Sa is very unstable and readily converted into the diene-saponin, saikosaponin-b₁ (Sb₁) on heating or by mild acid treatment.^{4,5)} Formation of some of Sb₁ from Sa was also observed during the decoction of Bupleuri Radix. Ogihara *et al.* reported on the pharmacological importance of diene-saponins of this type.⁶⁾ The present paper deals with the solubilization of Sb₁ with Ginseng saponins.

The results of the solubilizing effects are illustrated in Fig. 1. It was found that Sb₁ is much less soluble in water than Sa; saturated concentrations of Sa and Sb₁ in water at 37 °C are 0.1 and 0.02 mg/ml, respectively. An aqueous extract of Ginseng was subjected to chromatography on highly porous synthetic polymer resin with water, 40% methanol, and then methanol as eluates. An increase of the water solubility of Sb₁ was observed not with the water and 40% methanol

Chart 1

Chart 2

Fig. 1. Solubilizing Effects on Sb₁ (37°C, 24 h, in H₂O)

eluates but with the methanol eluate (ME). This eluate (ME) was separated by chromatography on silica gel, affording four fractions tentatively named frs. 1—4 according to an increasing order of polarity. Fractions 1—3 showed no solubilizing effect, while the solubility of Sb₁ is greatly increased in the presence of fr. 4; 1 ml of 0.1% solution of fr. 4 dissolved more than 1 mg of Sb₁ at 37°C. At thin layer chromatogram (TLC) of each fraction demonstrated that fr. 1 contained no saponin and fr. 2 consisted of neutral saponins of 20(*S*)-protopanaxatriol. Fraction 3 contained neutral saponins of both 20(*S*)-protopanaxatriol and 20(*S*)-protopanaxadiol, while fr. 4 was composed mainly of neutral saponins of 20(*S*)-protopanaxadiol and acidic saponins "Ro and malonyl-ginsenosides," the latter of which were recently isolated from Ginseng by Kitagawa *et al.*⁷⁾ This suggested that the acidic saponins play a major role in the solubilization of Sb₁.

Contents of the major saponins in ME determined by high performance liquid chromatography (HPLC)⁸⁾ were as follows: ginsenoside-Rg₁: 11.7%, -Rf: 12.2%, -Re: 3.6%, -Rd: 3.3%, -Rc: 3.4%, -Rb₂: 5.5% and Rb₁ (Rb₁): 8.2%, Ro: 1.9% and malonyl-ginsenosides-Rb₁ (MRb₁): 10.3%, -Rb₂: 2.8% and -Rc: 2.3%. Based on these analytical results, a mixture of neutral and acidic saponins in a ratio of contents in ME was prepared, being designated as Mix; 1.96 mg of Mix is equivalent to 3 mg of ME with respect to the composition of the major saponins. As shown in Fig. 1, a 0.196% aqueous solution of Mix exhibited almost the same solubilizing effect as that of 0.3% aqueous solution of ME. On the other hand, no solubilizing effect was observed with the neutral saponin mixture (Mix without Ro and malonyl-ginsenosides designated as Mix-MRo) at the concentration of 0.144% [=Mix 1.96 mg—acidic saponins 0.52 mg in 1 ml]. A 0.189% aqueous solution of a saponin mixture without Ro (Mix-Ro) [=Mix 1.96 mg—Ro 0.07 mg in 1 ml] showed a similar solubilizing effect to that of a 0.3% solution of ME. It was further noted that a 0.05% aqueous solution of MRb₁ which is approximately equivalent to the concentration of total

malonyl-ginsenosides in a 0.3% solution of ME, exhibited the same solubilizing effect as a 0.3% solution of ME. These results indicated that the solubilization of Sb₁ with ME (0.3% solution) is mainly due to the presence of malonyl-ginsenosides.

In our study on the solubilization of Sa with Ro, it was found that the solubilizing effect of Ro on Sa was significantly potentiated in the presence of the neutral dammarane saponins, which themselves do not increase the water solubility of Sa.²⁾ A similar cooperation of the neutral dammarane saponin was also observed in the present study. A 0.025% aqueous solution of MRb₁ did not show a solubilizing effect on Sb₁, while a 0.05% aqueous solution of a mixture of MRb₁ and Rb₁ (1:1) dissolved Sb₁ at the same concentration as that in a 0.05% solution of MRb₁ (*vide supra*).

It was reported that malonyl-ginsenosides are unstable and partly or completely decomposed to give the parent neutral ginsenosides⁷⁾ during the extraction or separation process under vigorous conditions. Decomposition of malonyl-ginsenosides is observed also during the process of the preparation of Red-Ginseng (treatment with hot steam). As shown in Fig. 1, some solubilizing effect was observed also with a 0.15% aqueous solution of a mixture of the neutral saponins and Ro (Mix-M) [=Mix 1.96 mg—(malonyl-ginsenosides) 0.46 mg/ml]. This evidence indicates that an increase in solubility of Sb₁ can be expected for Ginseng extract or saponin fractions prepared even under the vigorous conditions which result in decomposition of malonyl-ginsenosides. The potentiation of the solubilizing effect by concurring neutral dammarane saponins is operative also in case of the solubilization of Sb₁ with Ro. It was found that 1 ml of 0.1% aqueous solution of Ro dissolved 0.38 mg of Sb₁, while no solubilizing effect was observed with a 0.006% aqueous solution of Ro which approximately corresponds to a 0.3% aqueous solution of Mix-M with respect to the concentration of Ro. However, as mentioned above, addition of the neutral dammarane saponins to this dilute solution of Ro (0.15% solution of Mix-M) resulted in the regeneration of the solubilizing effect.

In our previous study on the solubilization of Sa,²⁾ a saponin mixture was obtained by the extraction of the aqueous suspension of an aqueous extract of Ginseng with 1-butanol. It was found that in the case of this butanolic participation, most malonyl-ginsenosides remain in an aqueous layer. Accordingly the saponin mixture used in the previous study²⁾ contained only a trace of malonyl-ginsenosides. In the present study, an increase in the water solubility of Sa with Ginseng saponins was reinvestigated using the fraction (ME) which contains malonyl-ginsenosides. The result revealed the important role of both Ro and malonyl-ginsenosides.¹⁰⁾ The saturated concentration of Sa in water at 37°C is 0.1 mg/ml. It was found that more than 5 mg of Sa were dissolved in 1.0 ml of 0.1 and 0.3% aqueous solutions of ME at 37°C. Aqueous solutions of Mix exhibited a similar solubilizing effect on Sa at the corresponding concentration, 0.065 and 0.196%, respectively. Solubilizing effects of Mix-MRo, Mix-Ro and Mix-M at the concentrations which correspond to 0.1 and 0.3% solutions of ME were determined. No solubilizing effect was observed with 0.048 and 0.144% solutions of

Mix – MRO. A 0.15% aqueous solution of Mix – M showed a similar effect to that of a 0.3% aqueous solution of ME, while a 0.05% aqueous solution of Mix – M dissolved Sa only at the concentration of 1.0 mg/ml, which was less than that with a 0.1% aqueous solution of ME. On the other hand, with regard to Mix – Ro, 1 ml of its 0.064% aqueous solution dissolved more than 5.0 mg of Sa but 1 ml of its 0.189% aqueous solution dissolved only 2.1 mg of Sa. This result suggested that the participation of the solubilizing effect of Ro is more significant in a relatively higher concentration, while that of malonyl-ginsenosides is more remarkable in relatively lower concentrations in the case of Sa.

The present results in the surface-active interaction between the constituents of the crude drugs must be noteworthy in view of the pharmaceutical superiority of extracts of the oriental traditional medicines, which are prepared by coprescription of several crude drugs.

Experimental

Materials All of the standard saponins in the present study were obtained in our serial studies.

Extraction and Separation of the Saponin Mixture from Ginseng Roots Commercial dried Ginseng roots without peeling (1 kg) were extracted with H₂O at room temperature. The water extract was subjected to chromatography on a highly porous synthetic polymer resin (Diaion HP-20), Mitsubishi Chemical Industries Co., Ltd., Japan) with H₂O, 40% MeOH and MeOH successively. The fraction eluted with MeOH was further chromatographed on silica gel with solvent: CHCl₃–MeOH–H₂O (6:4:1) to give four fractions, fr. 1 (yield from Ginseng 0.017%), fr. 2 (0.097%), fr. 3 (0.092%) and fr. 4 (2.77%).

Quantitative Analysis of Ginsenosides in ME by HPLC An HPLC 803D (Toyo Soda Co., Ltd., Japan), equipped with a variable wave length detector (UV 8000, Toyo Soda) and an Ultron N-18 column (Chromato Packings Center, Kyoto, Japan, particle size, 5 μ m; 4.6 $\times$ 150 mm) was used; flow rate: 1.0 ml/min, detection: absorption at 203 nm.

For HPLC analysis of Re and Rg₁, CH₃CN–0.5% H₃PO₄ (20:80 v/v) was used as a mobile phase. A methanolic solution of Rg₁ was subjected to HPLC analysis. The calibration plots for peak area ratio vs. concentration were found to be linear in the range of 5–10 μ g per injection of Rg₁, and the curves could be extrapolated through zero. 10 μ l of 1.0 mg/ml methanolic solution of Re was subjected to HPLC analysis under the same conditions as for Rg₁. The relative ratio of the peak area of Re to that of the same concentration of Rg₁ was obtained, being used for the quantitative analysis of these saponins.

For HPLC analysis of MRb₁, MRb₂, MRc, Rb₁, Rb₂, Rc, Rd, Rf and Ro, 50 mm KH₂PO₄ in 31% (v/v) CH₃CN was used as a mobile phase. The calibration plots for peak area ratio vs. concentration were found to be linear in the range of 5–15 μ g per injection of Rc, and the curve could be extrapolated through zero. 10 or 15 μ l of 1.0 mg/ml methanolic solutions

of the other saponins to that of the same concentration of Rc were obtained, respectively, being used for the quantitative analysis of these saponins.

For analysis of saponins in ME, a solution of ME was prepared to 10 mg/ml, and 10 or 15 μ l of this solution were subjected to HPLC analysis.

Determination of Solubilizing Effects A solution of an excess of Sb₁ in MeOH containing a Ginseng saponin (or a mixture), was concentrated to complete dryness. The residue was taken up in water, and after sonication for several minutes, the mixture was shaken at 37 °C for 24 h. The mixture was filtered to remove insoluble material. A measured volume of the Sb₁ content was determined by TLC-densitometry on a Shimadzu Dual wave length TLC-scanner, CS-930, equipped with a Shimadzu data-recorder DR-2 according to the methods reported in previous papers^{1,2,9}; TLC plate: Kieselgel 60F₂₅₄ (20 $\times$ 20 cm, Art. 11798, Merck), solvent: CHCl₃–MeOH–H₂O (30:10:1, homogeneous), s, 256 nm; r, 370 nm.

A solution of an excess of Sa in MeOH containing a Ginseng saponin (or mixture), was concentrated to complete dryness. The residue was taken up in water and after sonication for several minutes, the mixture was shaken at 37 °C for 24 h. The mixture was filtered to remove insoluble material. A measured volume of an aliquot of the filtrate was lyophilized, and the residue was treated with a mixture of 1.5 N HCl and MeOH (1:1) at 25 °C for 16 h. The reaction mixture was neutralized with 1.5 N aqueous NaOH and diluted with MeOH to a definite volume. The content of Sa was determined by TLC densitometry of the resulting diene-saponin Sb₁ under the same conditions as described above.

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References and Notes

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- 10) It should be noted that the exact comparison of the data on the solubilization in the present experiments with those of the previous study²⁾ is difficult, since the compositions of saponins in ME are somewhat different from those of FII in the previous study.