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Studies on the Pharmaceutical Quality Evaluation of Crude Drug Preparations used in Orient Medicine "Kampoo". II.[1]

Precipitation Reaction of Berberine and Glycyrrhizin in Aqueous Solution

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Precipitation reaction was found to occur when each 5×10^{-4} M aqueous solution of berberine (I) and glycyrrhizin (II) were blended. The molar ratio of I and II in the precipitate (III) was revealed to be 2: 1 by observation of UV absorption at 251 and 350 nm. By comparison of the IR absorption near at 1715 and 1620 cm^{-1} of II, III, soyasaponin III and glycyrrhetinic acid after treated at various pH, it was revealed that the quartery base moiety of I bound on the glucouronic acid moieties of II ionically in compound III. The solubility of III in aqueous solution was 0.09% at pH 3.5, while it increased up to 1.9% at pH 6.8. The solubility of III was slightly decreased by addition of II in aqueous solution.

Keywords——precipitation reaction of berberine and glycyrrhizin; berberine; glycyrrhizin; quality evaluation of crude drug preparations; crude drug; salt formation between berberine and glycyrrhizin; soyasaponin III; glycyrrhetinic acid; Amberlite XAD-2; glucuronic acid

In the previous paper,[1] it was shown that precipitation reaction occurred between berberine (I) in Coptidis Rhizoma (黄連) and glycyrrhizin (II) in Glycyrrhizae Radix (甘草) when they were boiled together in aqueous solution and that the supernatant solution thus obtained did not show any antibacterial activity which was derived from I.

This phenomenon may cause the lowering of recovery of both substances and also fall in pharmacological activity of the preparation which contain both crude drugs. However, it may also be regarded as a pharmaceutical pecuriarity of the orient medicine "Kampoo", in which the pharmacological activity of the particular drug is converted by the addition of many other kinds of crude drugs. Therefore, it is important to clarify the mechanism of this phenomenon.

The present study was undertaken to elucidate the precipitation mechanism through the investigation of the chemical structure and physico-chemical properties of the precipitate made from I and II in aqueous solution.

Experimental

Reagents——Berberine (I) was prepared by treatment of berberine chloride with moistened silver oxide as described in the literature[3] and evaporated *in vacuo*. Glycyrrhizin was once recrystallized from AcOH and twice from hot EtOH–water (8: 2).

Apparatus——Measurement of the specific conductance was carried out on Metrohm Konduktoskop E-365 B with Electrode EA-608. UV spectra were measured with Hitachi Double Wave Length Spectrophotometer Model 556. IR spectra were obtained with Hitachi Spectrophotometer Model G-2. NMR spectra were taken in D_2O solution at 60 MHz on a Varian A-60A Spectrometer. Optical rotation was measured with Hitachi Polarimeter EPI-181. Dissociation constants were determined with Beckman Zeromatic SS-3 pH Meter.

Measurement of the Specific Conductance of the Mixture of Berberine (I) and Glycyrrhizin (II) Solutions——Each 10^{-3}, 5×10^{-4} or 2×10^{-4} M of aqueous solution of I and II (ammonium salt, dipotassium salt and free

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[1] Part I: M. Noguchi, M. Kubo, T. Hayashi, and M. Ono, *Shoyaku.*, 32, 104 (1978).

[2] Location: *6, Hoenzaka-machi, Higashi-ku, Osaka.*

[3] B. Skinner, *J. Chem. Soc.*, **1950**, 823.

form) were mixed with different ratio and the specific conductance was measured 15 minutes later. Mixing ratio of both samples, the specific conductance and pH values are shown in Table I.

Preparation of the Precipitate (III) from I and II in Aqueous Solution——Each 100 ml of 5×10^{-4} M solution of I and II were mixed and centrifuged after 12 hr. The precipitate was dissolved in MeOH, filtered and the solvent was evaporated *in vacuo*. Amorphous powder, mp 228—235° (dec.), $[\alpha]_D^{21}$ +45.7° (c=0.707 MeOH), UV λ_{max} (log ε): 234 (4.78), 265 (4.73), 350 (4.63), 430 (4.01). NMR (in D_2O) ppm; 0.77, 0.88, 1.07, 1.16, 1.38 (s, 12H), 3.55 (m, 4H), 4.17 (d, 4H), 6.12 (s, 2H), 6.95 (s, 2H), 8.08 (s, 2H).

Determination of I and II in III by UV Absorption Method——Each 9 mg of III, 2.5 and 5.0 mg of I or II were dissolved in 50% MeOH and UV absorption was measured. The content of I in III was obtained by the comparison of the absorbance of I and III at 350 nm. The content of II in III was obtained by the comparison of the absorbance of II and III at 251 nm after subtracting the absorbance owing to I.

Chromatography of II on Amberlite XAD-2 Column——20 mg of II was dissolved in 10 ml of buffer solution of different pH values (pH: 3.0, 3.6, 4.0, 4.5, 5.0, 6.0, 7.0) prepared by mixing 0.1 M KH_2-citrate and 0.1 N NaOH (or 0.1 N HCl) and charged on the column of 15 g of Amberlite XAD-2 which was previously swollen in water.[4] The column was washed with 100 ml of water and then eluted with 100 ml of 66% EtOH. The eluate was filtered and evaporated *in vacuo*.

Measurement of the Ionization Constant——2×10^{-3} mol of sample was dissolved in 45 ml of distilled water and pH change was observed by each addition of 0.4 ml of 0.01 N NaOH. The pH value in which a half of the sample was neutralized was determined from the titration curve.[5]

Preparation of Soyasaponin III (V)——150 mg of prosapogenol II of soyasaponin I was dissolved in 5 ml of MeOH and added 2 ml of 10% KOH. The solution was refluxed for 4 hr and evaporated *in vacuo* to half volume. After 12 hr the precipitate was filtered and recrystallized from EtOH–water (95:5).[6]

Determination of Solubility of III——10 ml of the buffer solution (pH: 3.52, 5.0) or glycyrrhizin solution (0.3, 0.4 M) was added to 20—200 mg of (III) and stirred for 2 hr with an ultrasonic mixer and the absorbance at 350 nm was compared with that of the standard solution of known concentration.

Results and Discussion

In order to elucidate the interaction of berberine (I) with glycyrrhizin (II) in aqueous solution, the specific conductance was measured on the mixture of 10^{-3}—10^{-4} M solution of I and II by different ratio. As the result, distinct bending was found at molar ratio of 2:1 for I and II, demonstrating the existence of ionic interaction between I and II in the solution (Fig. 1).

Precipitation occurred immediately after addition of both solutions by 10^{-3} M and one hour later by 5×10^{-4} M, however, precipitation did not occur by 2×10^{-4} M solution even

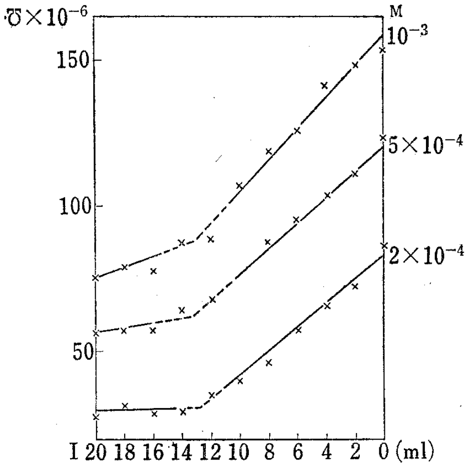

Fig. 1. Specific Conductance of the Mixture of Berberine (I) and Glycyrrhizin (II) Solutions

Table I. Specific Conductance of the Mixture of Berberine and Glycyrrhizin Solutions

No.	5×10^{-4} mol solution (ml)		$\mho\times10^{-6}$	pH
	Berberine	Glycyrrhizin		
1	20	0	55.8	7.25
2	18	2	57.0	6.38
3	16	4	57.2	5.65
4	14	6	61.8	5.00
5	12	8	67.5	4.60
6	10	10	77.3	4.40
7	8	12	85.7	4.25
8	6	14	94.2	4.15
9	4	16	102.5	4.15
10	2	18	110.0	4.08
11	0	20	123.0	4.05

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[4] Y. Hashimoto (ed.) "Syoyaku Bunseki," Hirokawa Shoten, Tokyo, 1977, p. 98.
[5] S. Matsuura (ed.), "Ionization Constants" Maruzen, Tokyo, 1963, p. 118.
[6] I. Kitagawa, M. Yoshikawa, and I. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **24**, 121 (1976).

after 48 hr. The pH of the solution in which precipitation occurred were nearly 5.0, where both I and II were soluble in water (Table I). The precipitate (III) dissolved in acetone: water (1:1) gave a single spot which had different *Rf* value (0.57) from I or II by TLC on Wakogel B-5 using $CHCl_3$–MeOH–H_2O (65:35:10) as the developing solvent (Fig. 2).

By determination of the content of I and II in III using the absorbance at 251, 350 nm on UV spectra as shown in Fig. 3, it is revealed that the molar ratio of I and II in III is also 2:1 (Table II).

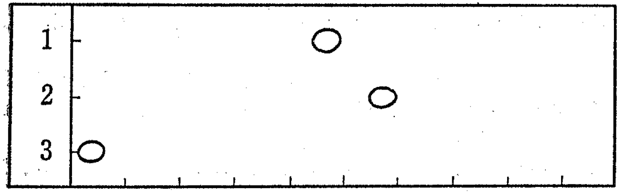


Fig. 2. Thin-Layer Chromatogram of Berberine-Glycyrrhizinate and Related Compounds

1) berberine-Glycyrrhizinate, 2) berberine, 3) glycyrrhizin.
Wakogel B-5FM, $CHCl_3$: MeOH: H_2O (65: 35: 10), Pan UV Lamp.

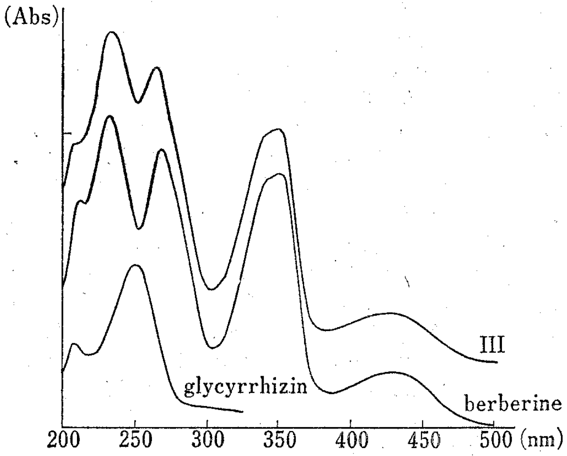


Fig. 3. UV Spectra of Berberine Glycyrrhizinate (III) and Related Compounds

TABLE II. Berberine Content (%) of Berberine Glycyrrhizinate precipitated from Aqueous Solution

	Molar ratio		Cont. (%)
Calculated	Ber-OH[a) : Glycyrrh.[c)	2 : 1	47.3
	Ber-OH : Glycyrrh.	1 : 1	31.0
Observed	Ber-Cl[b) : Glycyrrh.	2 : 1	48.1
	Ber-OH : Glycyrrh.	2 : 1	51.9

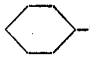
a) berberine hydroxide,
b) berberine chloride,
c) glycyrrhizin.

As II is a tribasic carboxylic acid which has two glucuronic acid (IV) moieties and one carboxylic acid moiety attached to the triterpene skeleton in II, two of them may bind with I ionically in compound III. So, an experiment was done to determine the binding sites between I and II by investigation of the nature of residual carboxylic acid in II which did not take part in bond formation.

As shown in Table III, ionization constant (pK_a) of the carboxylic acid which have a hydroxyl group at α-position lies in 3—4 and nearly 5 when it is attached to a saturated carbon ring system.[5) The pK_a values of IV and dipotassium salt of II obtained by titration with 1/100 N NaOH were 3.65 and 3.4, respectively (Table III). Two potassium ions, therefore, seem to bind on the IV moieties in II and the carboxylic acid attached to ring system remains free. However, one mol of III consumed three mol of NaOH and the pK_a value of free acid in III could not be determined.

Consequently, another experiment was done for the classification of three carboxylic acid moieties in II by determining the difference in salt forming reaction in buffer solutions.

Table III. pK_a Values of Organic Acids

Compound	pK_a	
CH_3COOH	4.76	Literature[5]
-COOH	4.90	Literature
$HO\text{-}CH_2COOH$	3.83	Literature
$MeO\text{-}CH_2COOH$	3.53	Literature
Glucuronic acid	3.65	Observed
Glycyrrhizin-K_2	5.40	Observed

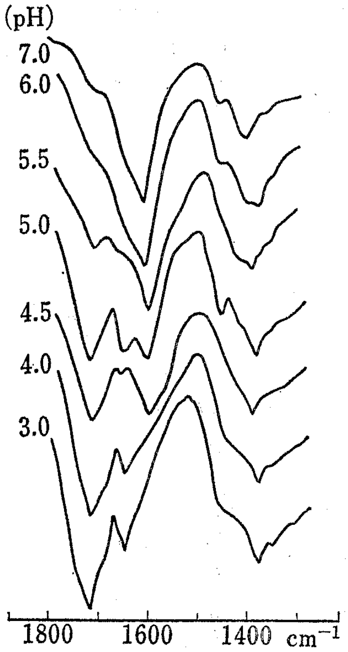


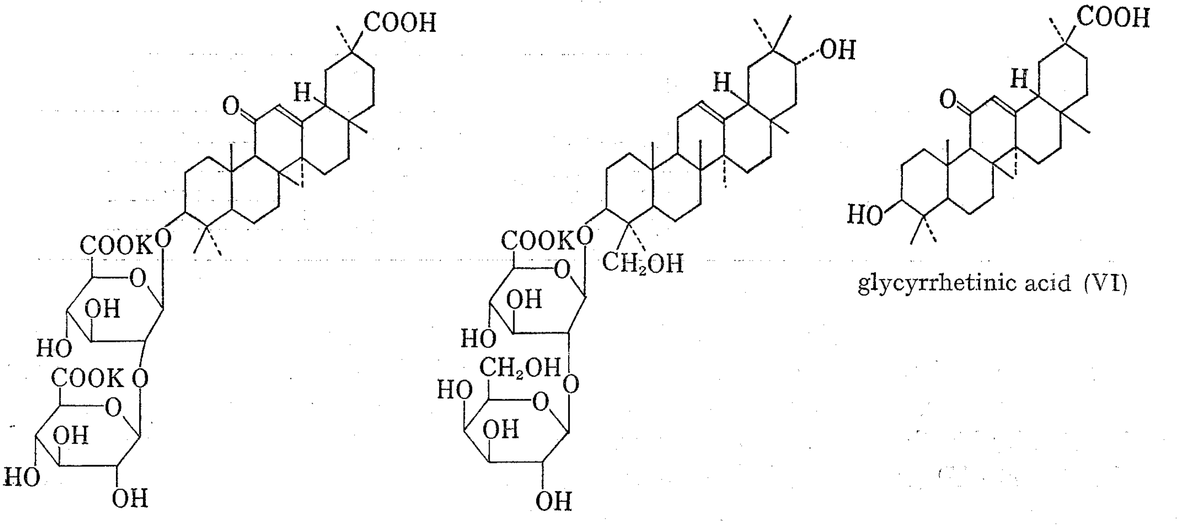
Fig. 4. IR Spectra of Glycyrrhizinic Acid (II) prepared under Various pH Conditions

II previously dissolved in each buffer solution of pH 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0 was adsorbed on the Amberlite XAD-2 column and eluted with 66% EtOH after washing and desalting the column with water. IR spectra were measured on the residue evaporated *in vacuo* by KBr tablet method. By observation of the IR absorption near at 1715 and 1620 cm^{-1} which are originated from the absorption of C=O of nonionic and ionic carboxylic acid, respectively, it is revealed that three carboxylic acid moieties in II exist in nonionic form below pH 3.0, in both ionic and nonionic forms between pH 4.5 and 5.5 and in ionic form over pH 6.0 (Fig. 4).

To explain this phenomenon, it is necessary to determine the ion forming activity of both glucuronic acid and carboxylic acid attached to ring system separately. So, soyasaponin III[6] (V) which has only one IV moiety and glycyrrhetinic acid (VI) which has only one carboxylic acid attached to triterpene ring are chosen as the model compounds (Chart 1).

Potassium salt of V or VI dissolved in a small amount of MeOH were stirred with buffer solution of different pH for 3 hr at 40°. The precipitates thus formed were centrifuged and examined by IR method as described above.

As the result, it is revealed that carboxylic acid in V exists in ionic form over pH 6.0 and in nonionic form below pH 3.0 (Fig. 5), while the carboxylic acid in VI exists in ionic form



glycyrrhizinic acid (II) soyasaponin III (V) glycyrrhetinic acid (VI)

Chart 1

over pH 6.0 and in nonionic form below pH 6.0 (Fig. 6). Compound III also behaved similarly with V by treatment with buffer solution of different pH (Fig. 7). These results are summarized and shown in Table IV.

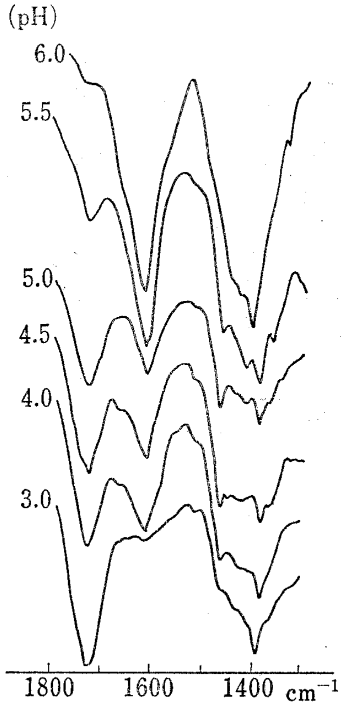


Fig. 5. IR Spectra of Soyasaponin III (V) prepared under Various pH Conditions

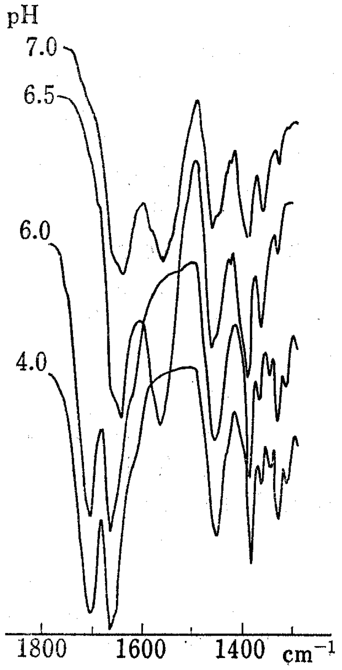


Fig. 6. IR Spectra of Glycyrrhetinic Acid (VI) prepared under Various pH Conditions

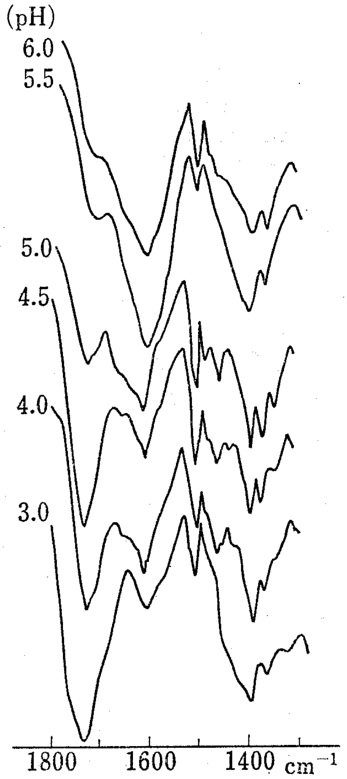


Fig. 7. IR Spectra of Berberine Glycyrrhizinate (III) prepared under Various pH Conditions

Table IV. IR Absorption of Carboxylic Acid in Diberberine Monoglycyrrhizinate and Related Compounds prepared under Various pH Conditions

	$\nu_{\max}^{\text{KBr}}$ (cm^{-1})					
	(pH)	3	4	5	6	7
Glycyrrhizinic acid		—1710—→|	|←——1710, 1610—→|		|←——1610——	
Glycyrrhetinic acid		———1710———————→|				|←——1570—
Soyasaponin III		—1725—→|	|←——1725, 1610—→|		|←——1610——	
Berberine glycyrrhizinate		—1710—→|	|←——1710, 1610—→|		|←——1610——	

III precipitates from aqueous solution at nearly pH 5.0 and the IR spectrum shows that it has two types of carboxylic acid, ionic and nonionic, in its structure (Fig. 8). Under this pH region, however, only IV moieties exist in ionic form. So, it may be concluded that III precipitates by binding of two mol of I with two IV moieties of II ionically (Chart 2).

Otsuka *et al.*[7] have already shown that II dissolves the slightly soluble substances in water by its surface active reaction. However, the solubility of III was not incerased by ad-

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[7] A. Otsuka, Y. Yonezawa, K. Iba, T. Tatsumi, and K. Sunada, *Yakugaku Zasshi*, **96**, 203 (1976).

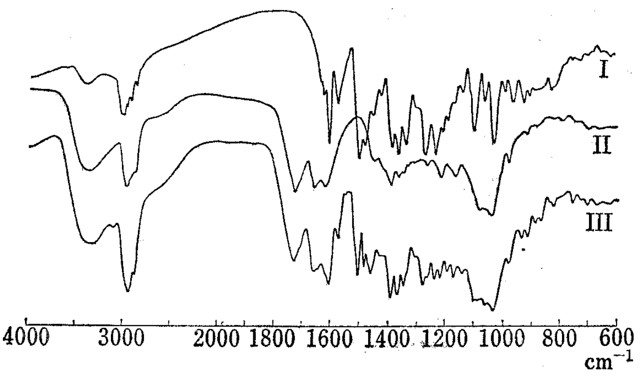

Fig. 8. IR Spectra of Berberine (I), Glycyrrhizin (II) and Berberine Glycyrrhizinate (III)

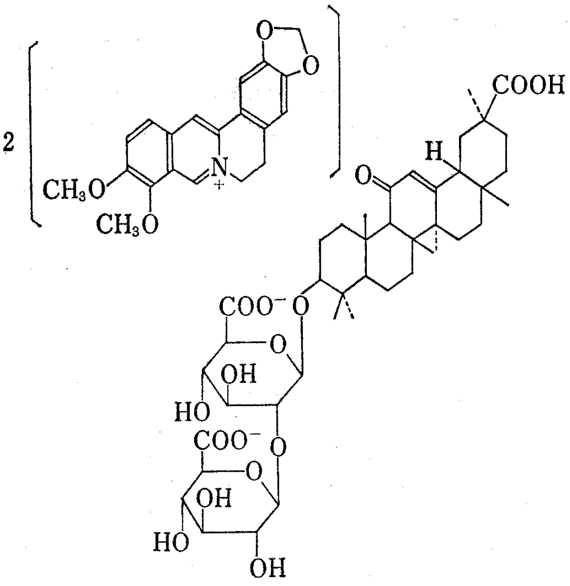

Chart 2. Chemical Structure of Diberberine Monoglycyrrhizinate (III)

Table V. Solubility of Diberberine Monoglycyrrhizinate in Aqueous Solution

Solvent	Solubility (mg) in 100 ml
pH 3.52 Buffer Soln.	89.55
pH 5.0 Buffer Soln.	211.95
pH 6.86 Buffer Soln.	1910.3
0.3 M Glycyrrhizin K_2 Soln.	38.21
0.4 M Glycyrrhizin K_2 Soln.	36.72

dition of II in aqueous solution. As shown in Table V the solubility of III in aqueous solution depends only upon the pH of the solution. It can therefore presumed from this result that the transformation of the carboxylic acid moiety attached to the triterpene ring in III from nonionic into ionic in higher pH region is responsible for the increase of the solubility of III.

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