Formation and Conformation of Baicalin–Berberine and Wogonoside–Berberine Complexes

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It is well-known that baicalin–berberine complex (1) precipitates in the water decoction of numerous Chinese Medicinal formulae containing Radix Scutellariae and Rhizoma Coptidis or Cortex Phellodendri. In the current study, ionic interaction between the carboxylate ion of baicalin and the quaternary ammonium ion of berberine was revealed to be responsible for the formation of 1 and wogonoside–berberine (2) by using FAB-MS and NMR titration experiments. In addition, nuclear Overhauser effect spectroscopy (NOESY) correlations observed in 1 and 2 suggested quite different conformation of the two complexes, which was further supported by the fact that the $[\alpha]_D$ of the canadine obtained by reduction of 1 is of an opposite sign to that obtained from 2. Partition coefficients (*n*-octanol/water) determination demonstrated 12–20 times larger partition coefficient of each complex (1, 2) than that of each single compound (baicalin, wogonoside, and berberine), indicating the significant role of the formation of the complex in the bioavailability enhancement of these pharmacologically active constituents.

Key words baicalin; berberine; wogonoside; baicalin–berberine complex; ionic interaction

It is well-known that notable amount of precipitate yield in the water decoction of Chinese Medicinal formulae containing Radix Scutellariae ("Huang-Qin") and Rhizoma Coptidis ("Huang-Lian") or Cortex Phellodendri ("Huang-Bo"). "Huang-Qin" and "Huang-Lian" is a well-known "medicine couple" whose combinative usage is believed to produce enhanced therapeutic effect, and therefore are very frequently co-prescribed in Chinese Medicinal formulae, such as "Shigao Tang," "Huanglian Jiedu Tang," "Huanglian Ejiao Tang" and "Gegen Qinlian Tang."[1)] Moreover, this "medicine couple" is also commonly used in "herbal tea" which is regularly consumed by the people in Southern China.[2)] Baicalin and berberine, the characteristic and representative chemical constituents of "Huang-Qin" and "Huang-Lian" respectively, were identified in the above mentioned precipitate, and formation of a baicalin–berberine complex has been proposed to be responsible for the precipitation.[3–5)] Precipitation in above decoction resulted in significantly decreased content of baicalin and berberine in the supernatant, suggesting that majority of these two pharmacologically-active compounds formed complex when they co-exist in the water decoction.[6)]

In our previous study, we have revealed that hydrophobicity of tannins can be increased by hydrophobic association with water-soluble compounds contained in the crude drugs used in Japanese and Chinese Traditional Medicine.[7)] Other studies also showed that inter-molecular association by hydrophobic or ionic interaction of herbal constituents may lead to alteration in their physico-chemical properties.[8)] Consequently, the apparent behavior such as bioactivity and bioavailability of the resultant complex may be quite different from that of the single compound alone. For example, berberine in the ion-pair compound of berberine–glycyrrhizin was found to be absorbed in gastro-intestinal tract faster than that in "Huang-Lian" water extract, and the maxim serum concentration (C_{max}) of berberine from the complex was also higher than that from water extract of "Huang-Lian."[9)] As for the complex of baicalin–berberine, previous studies have suggested that relevant precipitate exhibited bioactivities different from that of supernatant, *e.g.*, the acute toxicity of the precipitate in the decoction of "Huang-Qin" and "Huang-Lian" (1 : 0.5) was much lower (with an maxim tolerance dosage of 200 g/kg) than that of supernatant (with an oral LD_{50} of 98.29 g/kg), although 4 folds higher content of berberine, which is mainly responsible for the toxicity, were detected in the precipitate than in the supernatant.[10)] The anti-bacterial activity of the precipitate was shown to be lower than that of berberine, but was more potent than Scutellariae extract.[11)]

Since the precipitate recruit majority of baicalin and berberine of the decoction of Chinese Medicinal formulae containing "Huang-Qin" and "Huang-Lian," and the precipitate exhibited different bioactivities from that of single baicalin and berberine, studies on the nature of precipitate is of great significance for better understanding the pharmacological activities, therapeutic effects, and rational application of those traditional Chinese medicine (TCM) formulae. Several studies on the precipitate have been made to evaluate kinetic process of precipitation, and gastro-intestinal absorption of the formed precipitate.[11–13)] However, little attempt has been made to elucidate the chemical nature of baicalin–berberine and wogonoside–berberine complexes. Therefore, we herein investigate the chemical and biological properties of baicalin–berberine (**1**) and wogonoside–berberine (**2**) complexes.

Experimental

General The ^{1}H-NMR spectra were obtained using Varian Gemini 300 spectrometers operating at 300 MHz; chemical shifts (δ) are presented in ppm relative to Me_4Si as the internal standard. To determine the chemical shift changes caused by complex formation, the ^{1}H-NMR of baicalin, baicalin sodium salt, wogonoside, wogonoside sodium salt, berberine hydrochloride, as well as baicalin–berberine (**1**) and wogonoside–berberine (**2**) were measured in CD_3OD and DMSO-d_6 respectively. Assignments of the signals were made based on the nuclear Overhauser effect spectroscopy

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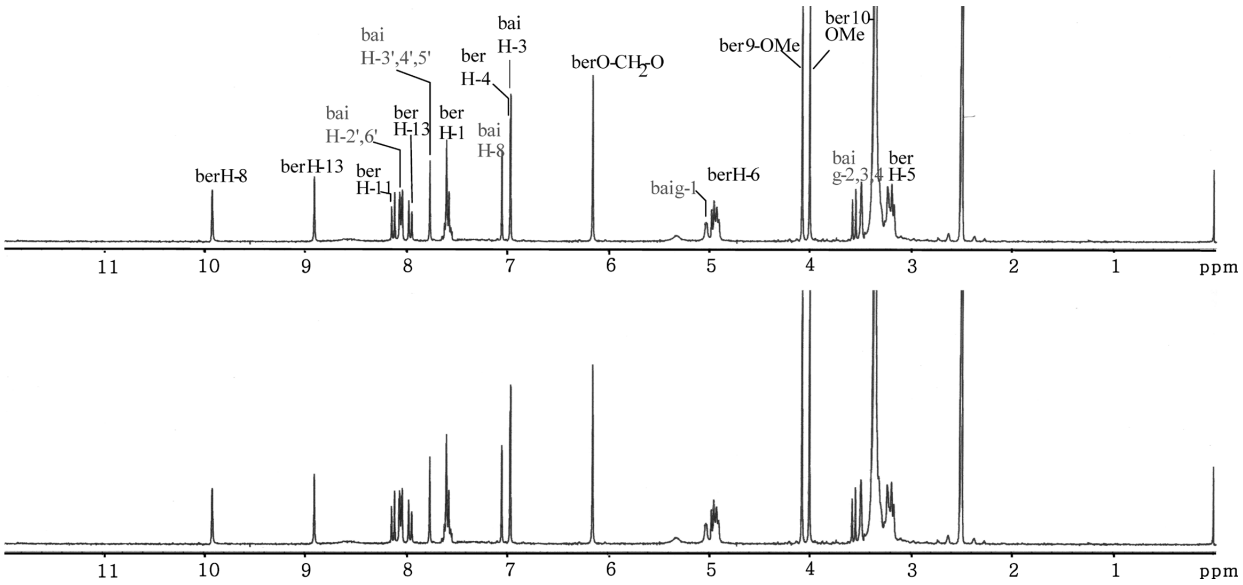

Fig. 1. ^{1}H-NMR Spectrum (300 MHz, DMSO-d_6) of Baicalin–Berberine Complex (**1**) (Upper) and Precipitate Obtained from the Water Decoction of Rhizoma Coptidis ("Huang-Lian") and Radix Scutellariae ("Huang-Qin") (Lower)

(NOESY) spectrum (500 MHz, in DMSO-d_6) of **1** and **2**. FAB-MS were recorded on a JEOL JMS DX-303 spectrometer (U.S.A.). Optical rotation was measured on a JASCO DIP-370 digital polarimeter.

Materials Baicalin, wogonoside, and berberine hydrochloride (berberine-HCl) were isolated from "Huang-Qin" and "Huang-Lian" in our lab. NaOH (1 N) was added to baicalin (500 mg) in 10 mL H_2O until it was completely dissolved. The solution was lyophilized to obtain powdered baicalin sodium salt (baicalin-Na). The same method was applied to wogonoside to obtain wogonoside sodium salt (wogonoside-Na).

Preparation of Precipitate Formed in the Decoction of "Huang-Qin" and "Huang-Lian" Twenty grams of Rhizoma Coptidis ("Huang-Lian") and 20 g of Root Scutellariae ("Huang-Qin") were decocted in 200 mL water for 10 min. The filtrate of decoction was left at room temperature for 3 h to yield yellow precipitate (330 mg).

Reduction of Berberine Baicalin-Na (63 mg) and berberine hydrochloride (5 mg) were dissolved in ice water (200 mL); then 1 mL water solution containing 5 mg $NaBH_4$ was added. After 1 h, the reaction mixture was extracted with EtOAc. The resulting EtOAc extract was dried with Na_2SO_4 and applied to silica gel chromatography eluted with an increasing gradient of hexane–EtOAc (4 : 1–3 : 1–2 : 1, v/v) to afford canadine (3.5 mg). Wogonoside-Na (130 mg) and berberine hydrochloride (10 mg) were dissolved in ice water and reduced in a manner similar to that described for baicalin–berberine to yield 8.4 mg of canadine.

Determination of Partition Coefficient Baicalin-Na (0.97 mg), wogonoside-Na (0.77 mg), berberine hydrochloride (0.77 mg), **1** (1.79 mg) and **2** (1.77 mg) were dissolved in water (1 mL) respectively and partitioned with *n*-octanol (1 mL) at room temperature. The upper and lower phases were analyzed by HPLC performed on a TSK gel ODS-80TM column (8×150 mm) [mobile phase, 0.5 mM H_3PO_4 (A) and acetonitrile (B); 60% of B for analyzing baicalin, wogonoside, baicalin–berberine and wogonoside–berberine, 40% of B for analyzing berberine; flow rate: 1.0 mL/min; detection wavelength: 270 nm]. The partition coefficient was calculated based on the peak area ratio (peak area of the compound in the organic layer/peak area of the compound in the aqueous layer).

Results and Discussion

Formation of Baicalin–Berberine Complex in the Water Decoction of "Huang-Lian" and "Huang-Qin" To confirm the formation of the baicalin–berberine complex in the decoction of two herbs, *i.e.*, "Huang-Qin" and "Huang-Lian," we firstly prepared baicalin–berberine complex (**1**) by collecting precipitate formed in an aqueous solution of pure baicalin sodium and berberine hydrochloride. The obtained precipitate gave a peak corresponding to 1 : 1 baicalin–berberine complex (*m/z* 780, [baicalin+berberine−H]$^-$) in the negative FAB-MS. ^{1}H-NMR experiments of **1** and the precipitate produced in the decoction of mixed herbs of "Huang-Qin" and "Huang-Lian" were then carried out. As can be seen from Fig. 1, a set of signals assignable to berberine and a set of signals due to baicalin can be apparently observed in the ^{1}H-NMR spectrum of **1** (Fig. 1). Similar signals can be observed in the ^{1}H-NMR spectrum of the precipitate from the herbs (Fig. 1), suggesting that the major component in the precipitate is baicalin–berberine complex (**1**). The ratio of the integral value of the proton signals arising from berberine to that of the signals deriving from baicalin is approximately one, indicating that the stoichiometric ratio of baicalin and berberine in **1** is 1 : 1, which is in agreement with the result of the FAB-MS experiment.

Interactions between Baicalin and Berberine, and between Wogonoside and Berberine To achieve a comprehensive understanding of the nature of this kind of complex, we examined the interaction between berberine and baicalin in **1**, as well as that between berberine and wogonoside in the wogonoside–berberine complex (**2**), in which wogonoside is another representative flavone glucuronide in the herb "Huang-Qin." Firstly, the changes in the ^{1}H-NMR chemical shifts of **1** compared to that of baicalin sodium salt and berberine hydrochloride were examined. As presented in Table 1 and Fig. 2, a significant upfield shift of the signal due to H-8

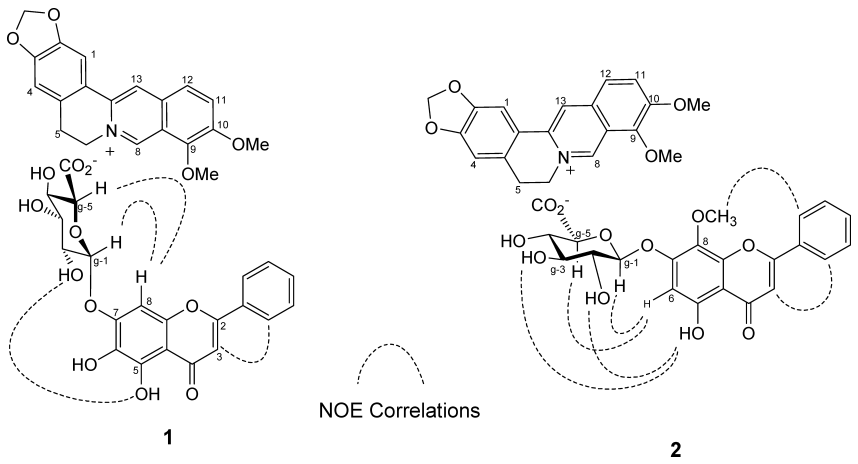

Fig. 2. NOESY Correlations Observed in Baicalin–Berberine (**1**) and Wogonoside–Berberine (**2**)

($\Delta\delta_H$=−0.131) of baicalin was observed, while smaller upfield shifts were observed for the signals arising from C ring (H-3) and B ring (H-2′,6′, H-3′,4′,5′) of baicalin, showing a reverse relationship between chemical shift change and the distance from the sugar moiety. These chemical shift changes were attributable to the anisotropic effect resulting from Pi-system of berberine, indicating an association of baicalin and berberine. Such association was further supported by the large upfield shifts of H-11, H-12, H-13 and 10-OMe of berberine moiety caused by the anisotropic effects of the Pi-system of baicalin (Table 2, Fig. 2), and smaller upfield shifts of H-1, H-4 and OCH_2O of berberine that resulting from the shielding effect of B ring and de-shielding effect of A ring of baicalin. This region-selective upfield shift of baicalin indicated that the association of berberine might occur at sugar moiety of baicalin, as additionally evidenced by the slight upfield shift of g-1 and g-2,3,4 signals of the glucuronic acid moiety of baicalin which just located in an area where the shielding effect and de-shielding effect from different aromatic ring of berberine was partially offset. However, a slight downfield shift ($\Delta\delta_H$=+0.022 ppm, Table 1) was observed for the signal of g-5 in baicalin moiety. As a proton attached to α-carbon of carboxylic acid group, a significant upfield shift of signal of g-5 ($\Delta\delta_H$=−0.275 ppm, Table 3, $\delta_3-\delta_2$) was observed during the formation of baicalin sodium salt (Table 3), which resulted from the decrease in the electronegativity of the carboxylic group when it was ionized to form a carboxylate anion.[14] The g-5 signal in **1** was almost superimposable to that observed in baicalin sodium, indicating the ionization of the carboxylic group of baicalin by berberine in the complex.

To further explore the nature of the interaction between baicalin and berberine in the complex, an NMR titration experiment was carried out by adding trifluoroacetic acid (TFA) into complex (**1**) in an NMR tube. Significant decreases in upfield shift of the signals arising from baicalin (Table 3) [for example, $\Delta\delta_{H\text{-}8}$=−0.131 (complex)→$\Delta\delta_{H\text{-}8}$=−0.044 (after addition of TFA)] were observed after the addition of TFA. This phenomenon indicated that baicalin was partially dissociated from **1**, which is the consequence of the formation of the berberine-TFA salt. This result confirmed that the interaction between berberine and baicalin is of an ionic mode.

^{1}H-NMR measurement of **2** for the purpose of revealing the interaction between wogonoside and berberine followed a similar pattern to that of baicalin, indicating an association of berberine with the sugar moiety of wogonoside (Table 4). However, the decrements of the chemical shifts of corresponding signals in **2** were smaller than that observed in **1**, implying that the aromatic ring of berberine was not so "close" to the B-ring and C-ring of wogonoside in space due to the stereohindrance effect caused by the 8-OMe group in wogonoside. This was also confirmed by less decrement in the chemical shifts of the aromatic signals of berberine (Table 5). Likewise,

Table 1. ^{1}H-NMR Data of Baicalin Moiety of Baicalin Sodium Salt (Baicalin-Na) and Baicalin–Berberine Complex (**1**)[a] (in CD_3OD, 300 MHz)

Position	Baicalin-Na (δ_1)	**1** (δ_2)	$\Delta\delta$ ($\delta_2-\delta_1$)
H-2′,6′	8.039	7.988	−0.051
H-3′,4′,5′	7.565	7.549	−0.016
H-8	7.109	6.978	−0.131
H-3	6.789	6.719	−0.070
Glucuronic acid moiety			
g-1	5.082	5.060	−0.022
g-2,3,4	3.585	3.580	−0.005
g-5	3.880	3.902	0.022

a) Signals were assigned on the basis of the NOESY spectrum of baicalin–berberine complex (**1**).

Table 2. ^{1}H-NMR Data of Berberine Moiety of Berberine Hydrochloride (Berberine-HCl) and Baicalin–Berberine Complex (**1**)[a] (in CD_3OD, 300 MHz)

Position	Berberine-HCl (δ_1)	**1** (δ_2)	$\Delta\delta$ ($\delta_2-\delta_1$)
H-8	9.771	9.738	−0.033
H-13	8.713	8.641	−0.072
H-11	8.123	8.022	−0.101
H-12	8.000	7.935	−0.065
H-1	7.667	7.611	−0.056
H-4	6.964	6.896	−0.068
O–CH_2–O	6.108	6.081	−0.027
H-6	4.919	4.898	−0.021
9-OMe	4.206	4.181	−0.025
10-OMe	4.111	4.026	−0.085
H-5	3.252	3.233	−0.019

a) Signals were assigned on the basis of the NOESY spectrum of baicalin–berberine complex (**1**).

Table 3. Changes of the Chemical Shifts of Baicalin Moiety Due to the Addition of Trifluoroacetic Acid (TFA) to Baicalin–Berberine Complex (**1**) (in CD_3OD, 300 MHz)

Position	**1**+TFA (δ_1)	Baicalin (δ_2)	Baicalin-Na (δ_3)	$\Delta\delta$ ($\delta_1-\delta_2$)	$\Delta\delta$ ($\delta_1-\delta_3$)
H-2′,6′	7.970	8.010	8.039	−0.040	−0.069
H-3′,4′,5′	7.560	7.561	7.565	−0.001	−0.005
H-8	6.958	7.002	7.109	−0.044	−0.151
H-3	6.758	6.781	6.789	−0.023	−0.031
Glucuronic acid moiety					
g-1	5.180	5.190	5.082	−0.010	0.098
g-2,3,4	3.619	3.618	3.585	0.001	0.034
g-5	4.170	4.155	3.880	0.015	0.290

Table 4. ^{1}H-NMR Chemical Shifts of the Wogonoside Moiety of Wogonoside Sodium Salt (Wogonoside-Na) and Wogonoside–Berberine Complex (**2**)[a] (CD_3OD, 300 MHz)

Position	Wogonoside-Na (δ_1)	**2** (δ_2)	$\Delta\delta$ ($\delta_2-\delta_1$)
H-2′,6′	8.063	8.022	−0.041
H-3′,4′,5′	7.615	7.590	−0.025
H-6	6.853	6.793	−0.060
H-3	6.720	6.628	−0.092
OMe	4.001	3.946	−0.055
Glucuronic acid moiety			
g-1	5.156	5.120	−0.036
g-5	3.844	3.830	−0.014
g-2,3,4	3.600	3.580	−0.020

a) Signals were assigned on the basis of the NOESY spectrum of wogonoside–berberine complex (**2**).

Table 5. ^{1}H-NMR Chemical Shifts of the Berberine Moiety of Berberine Hydrochloride (Berberine-HCl) and Wogonoside–Berberine Complex (**2**)[a] (CD_3OD, 300 MHz)

Position	Berberine-HCl (δ_1)	**2** (δ_2)	$\Delta\delta$ ($\delta_2-\delta_1$)
H-8	9.758	9.735	−0.023
H-13	8.695	8.639	−0.056
H-11	8.125	8.076	−0.049
H-12	8.000	7.960	−0.040
H-1	7.660	7.608	−0.052
H-4	6.971	6.937	−0.034
O–CH_2–O	6.112	6.090	−0.022
H-6	4.932	4.926	−0.006
9-OMe	4.207	4.186	−0.021
10-OMe	4.117	4.091	−0.026
H-5	3.260	3.242	−0.018

a) Signals were assigned on the basis of the NOESY spectrum of wogonoside–berberine complex (**2**).

Table 6. Changes of the Chemical Shifts of the Wogonoside Moiety Due to the Addition of TFA to Wogonoside–Berberine Complex (**2**) (in CD_3OD, 300 MHz)

Position	**2**+TFA (δ_1)	Wogonoside (δ_2)	Wogonoside-Na (δ_3)	$\Delta\delta$ ($\delta_1-\delta_2$)	$\Delta\delta$ ($\delta_2-\delta_3$)
H-2′,6′	8.044	8.050	8.063	−0.006	−0.019
H-3′,4′,5′	7.609	7.607	7.615	0.002	−0.006
H-6	6.829	6.851	6.853	−0.022	−0.024
H-3	6.627	6.654	6.720	−0.027	−0.093
OMe	3.976	3.992	4.001	−0.016	−0.025
Glucuronic acid moiety					
g-1	5.203	5.222	5.156	−0.019	0.047
g-5	4.095	4.097	3.844	−0.002	0.251
g-2,3,4	3.630	3.643	3.600	−0.013	0.030

the similar chemical shift of g-5 in **2** to that in wogonoside sodium salt indicated an ionic bond linking wogonoside and berberine in **2**. After the addition of TFA, these chemical shift changes caused by the anisotropic effect of berberine fell off rapidly as a consequence of the dissociation of berberine (Table 6), supporting the ionization interaction between wogonoside and berberine in **2**.

Conformation of Baicalin–Berberine (1) and Wogonoside–Berberine (2) Complexes Conformation of the complexes baicalin–berberine (**1**) and wogonoside–berberine (**2**) were further studied in detail. The NOESY of baicalin–berberine (**1**) and wogonoside–berberine (**2**) complexes was measured. The NOE correlations observed in each complex were observed and are shown in Fig. 2, suggesting large differences in the conformation of the two complexes. This was further supported by the fact that the $[\alpha]_D$ of the canadine which was obtained by reduction of baicalin–berberine (**1**) with $NaBH_4$ was of an opposite sign to that obtained from wogonoside–berberine (**2**) (+17.2° from **1**, −25.2° from **2**) (Fig. 3).

Partition Coefficient of Baicalin–Berberine and Wogonoside–Berberine Complexes Absorption of ionic drugs as alkaloids and carboxylic acids through the gastrointestinal tract is known to be enhanced by ion-pair formation, because it increases their solubility in fat.[15,16] Therefore, the partition coefficients (*n*-octanol/water) of baicalin, wogonoside, berberine, baicalin–berberine complex (**1**) and wogonoside–berberine complex (**2**) were determined by using HPLC analysis (ODS column). As shown in Fig. 4, The value of the partition

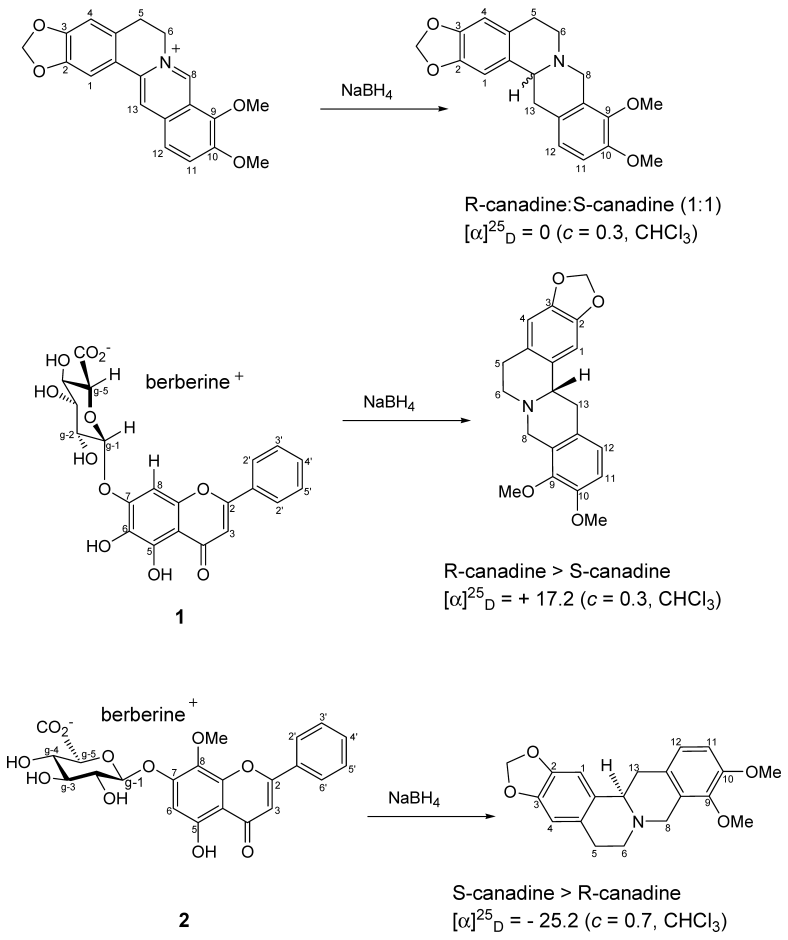

Fig. 3. Reduction of Berberine in Baicalin–Berberine (**1**) and Wogonoside–Berberine (**2**)

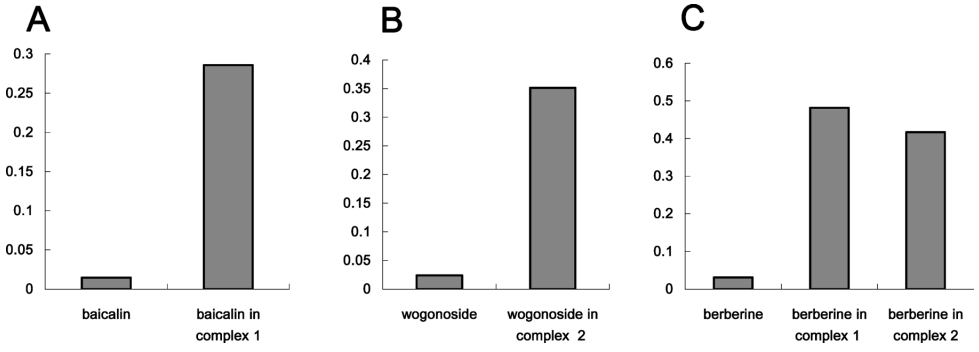

Fig. 4. Comparison of the Partition Coefficients of Baicalin (A), Wogonoside (B), and Berberine (C)

coefficient of each complex (**1**, **2**) is 12–20 times larger than that of the single compound, indicating more efficient transport of the complex through the lipid bilayer, which may in turn lead to greater bioavailability. This result supported the previous study conducted by Yang *et al*., who found that the C_{max} of baicalin after oral administration of precipitate obtained from decoction of "Huang-Qin" and "Huang-Lian" was higher than orally taken Radix Scutellariae extract.[9)]

Conclusions

In the current study, the precipitate formed in the water decoction of "Huang-Lian" and "Huang-Qin" was confirmed to be mainly a complex containing baicalin and berberine with a 1 : 1 ratio. Little wogonoside–berberine could be found in the precipitates. Both baicalin and wogonoside are flavone glucuronide which possess a carboxyl group, while berberine is a quaternary alkaloid with strong alkaline properties. Interactions between baicalin and berberine, and between wogonoside and berberine in the complexes were established to be of an ionic mode in this study, which occurred between the carboxylate ion of baicalin or wogonoside and the quaternary ammonium ion of berberine. Such association keeps the hydrophilic carboxyl group and quaternary ammonium ion inside the complex, and leaves the hydrophobic part of baicalin and berberine outside facing the water phase, thus resulting in the production of precipitate. It is worthwhile noting that the conformation of the wogonoside–berberine complex is significantly different from that of the baicalin–berberine complex

due to the stereo-hindrance effect caused by 8-OMe, which results in quite different properties in the binding to macromolecules.

After the formation of complexes, the partition coefficients (*n*-octanol/water) of baicalin, wogonoside and berberine increased markedly in comparison to the single compound. The increased lipophilicity of the complex is expected to contribute positively to the bioavailability of these pharmacologically active constituents.[11] On the other hand, efflux transporters are involved in the cell transport of berberine and baicalin,[17,18] it is speculated that the baicalin–berberine and wogonoside–berberine complexes, differing greatly in their hydrophobicity and confirmation, may exhibit quite different affinities for the relevant transporters, and consequently result in distinct pharmacokinetic behavior from that of single compounds. Moreover, binding to DNA, in which process the quaternary ammonium cation and planar structure of berberine play a critical role, is well-known to be closely associated with its biological activities and anti-cancer mechanisms.[19–21] Recent studies suggest that binding with polyadenylic acid may be a potential basis for the therapeutic action of isoquinoline alkaloids.[22] Therefore, the properties of berberine in terms of binding to the above described macrobiomolecules are expected to be altered significantly and hence lead to different pharmacological activities in the formation of complexes.

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