Clove Oil Prevents Glycyrrhizin Gel Formation in Aqueous Solution

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One persistent problem with using therapeutic concentrations of glycyrrhizin (GZ) is that, at these high concentrations, it forms a gel in an aqueous solution. We previously solved this problem by dissolving GZ in a highly concentrated phosphate buffer. Unfortunately, the resulting GZ solution has a hyperosmotic pressure that renders it unsuitable for use in patients. The aim of this study was to prepare a highly concentrated GZ solution having an osmotic pressure ratio of 1 and a pH of 7.4. By adding small amounts of oil and using a 100 mM phosphate buffer, we achieved an emulsified GZ solution that is stable at room temperature and has a physiological osmotic pressure and pH. When clove oil was used as an emulsifier, the gel formation temperature of GZ solution decreased appreciably compared to that of GZ solution without clove oil. Using scanning electron microscopy (SEM), we examined the detailed characteristics of GZ gels prepared from solutions with or without clove oil. SEM of cross sections of GZ gels revealed an irregular structure in gels prepared with clove oil, indicating that clove oil prevented the formation of the intermolecular GZ networks typically characterized by gels derived from pure GZ solutions.

Key words glycyrrhizin; gel; clove oil; fennel oil; mentha oil; scanning electron microscopy (SEM)

Glycyrrhizin (GZ), a major component of licorice (*Glycyrrhiza glabra* L.), is used as a remedy for chronic hepatitis, allergies, and other maladies. Because GZ is poorly absorbed by the intestinal tract and readily forms gels, the only way it can be therapeutically delivered is by parenteral, low-dose injection. Indeed, prescription grade GZ is currently available in only a 2 mg/ml solution. To obtain a sufficient therapeutic dose to treat chronic hepatitis, however, a patient must receive 100 mg GZ per day or 50 ml of a 2 mg/ml solution per day.[1,2] We previously showed that GZ aqueous solutions prepared in ≥300 mM phosphate buffer (pH 7—8) did not form gels at room temperature.[3] In fact, with this procedure, we achieved a 200 mg/ml solution of GZ. However, this came at a high cost. The phosphate salt concentration required to prepare such a highly concentrated GZ solution causes it to have a high osmotic pressure, making it difficult to deliver *via* injection. The purpose of this study was to prepare a 100 mg/ml GZ solution that can be used under physiological conditions. Thus, we aimed to prepare a GZ solution having an osmotic pressure ratio of 1 and a pH of 7.4.

Experimental

Chemicals GZ (purity >98%) and Glycyrrhetic acid were gifts from Koki Co. (Tokyo, Japan) and Maruzen Pharmaceutical Co. (Hiroshima, Japan), respectively. Soybean oil, olive oil, fennel oil, and clove oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mentha oil was purchased from Hoei Pharmaceutical Co. (Osaka, Japan). All other materials were reagent grade.

Osmolarity Measurement The osmolarity of phosphate buffered solutions (50—300 mM) and GZ solutions (1—100 mg/ml) was measured using a Freezing point osmometer (model OM-802, VOGEL GmbH, Germany).

Preparation of GZ Solution After 1.0 g GZ was added to a screw vial containing 5 ml of phosphate buffer and 0—150 μl of oil, the samples were stirred for 16 h at 100 rpm at 60 °C on a Hotplate stirrer (model DP-1L, Asone Co., Osaka, Japan). Any oil drops that separated from the GZ aqueous solution were carefully removed using a micropipette.

Measurement of Gel Formation Temperature After preparing the GZ solution, we placed all samples into a 60 °C water bath equipped with a Cool-pipe (model 80LF, Taitec, Tokyo, Japan), and gradually decreased the water bath temperature at a rate of 1 °C every 10 min (range: 60 °C—3 °C). We determined the starting point of gel formation in each sample by tilting the vial and noting the temperature at which a thin layer of gel formed on the boundary surface of the tube.

HPLC Assay of GZ We determined GZ concentration according to an HPLC method previously described.[4] Briefly, a Nucleosil 5C18 column (inner diameter: 4.6 mm; length: 250 mm) was used and maintained at a temperature of 40 °C during separation. Detection was performed at a UV wavelength of 254 nm. The solvent used for the mobile phase was comprised of methanol, distilled water, 25% ammonia solution, and 60% perchloric acid (53 : 47 : 0.5 : 0.5, v/v); flow rate was 0.8 ml/min. The correlation coefficients of the calibration curves were 0.999 or better; the detection limit was 0.2 μg/ml.

Particle Size Assay We measured the average particle size of GZ micelles in GZ solutions prepared in the absence or presence of clove oil using a Laser particle analyzer (model DLS-900, Otsuka Electronics Co., Osaka, Japan). Prior to analysis, the samples were filtered with a Millipore membrane filter (0.45 μm pore size). Accumulation times were 100, the incidence angle of the laser beam was set to 30°, and the average particle diameter was evaluated as a Z-average using a monomodal method (a cumulant analysis)[5,6] at 25 °C.

Scanning Electron Microscopy (SEM) in GZ Gel Sections GZ solutions (100 mg/ml) were prepared in the absence and presence of 2% clove oil, then incubated for 16 h at 60 °C. After incubation, excess clove oil drops were removed. The solutions were transferred into chloroethylene tubes (2 mm inside diameter), which were briefly immersed in liquid nitrogen to quickly freeze the samples. The samples were subsequently freeze-dried at 20 °C and 26 Pa by touching them to cooled metal to avoid disrupting gel formation. The gel specimens were sectioned and prepared for SEM analysis.

Results

Osmolarity The osmolarity of the phosphate buffered solution (pH 7.4) increased linearly with increasing concentrations of phosphate buffer (Fig. 1A). To determine the osmolarity of GZ (1—100 mg/ml), we used GZ solutions prepared in 200 mM phosphate buffer, because a 100 mg/ml GZ solution immediately forms a gel when dissolved in 100 mM phosphate buffer. The osmolarity increased exponentially with increasing concentrations of GZ (Fig. 1B).

Temperature of GZ Gel Formation We measured the

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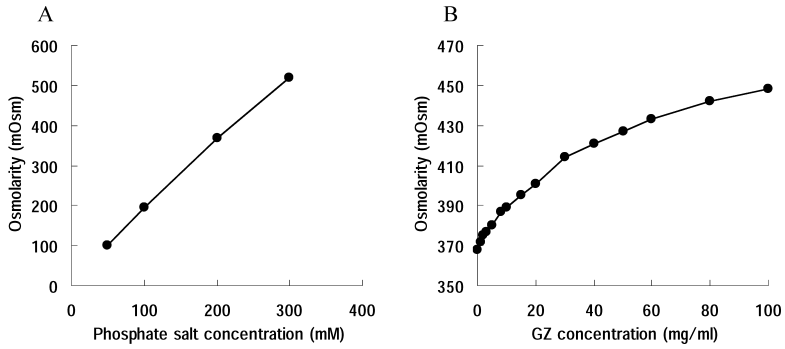

Fig. 1. Relationship between (A) Phosphate Salt Concentration and (B) GZ Concentration and Osmolarity

GZ was dissolved in 200 mM phosphate buffered solution (pH 7.4). Data represent the mean of three experiments. Standard deviation bars were too small to indicate in the graphs.

Table 1. Gel Formation Temperature of GZ Solution[a)] in the Absence or Presence of Various Types of Oil

Oil (%, v/v)	Temperature (°C)
no oil	32
1.0% olive oil	32
2.0% olive oil	32
1.0% soybean oil	32
2.0% soybean oil	32
1.0% mentha oil	30
2.0% mentha oil	24
3.0% mentha oil	24
0.1% fennel oil	31
0.5% fennel oil	30
1.0% fennel oil	25
2.0% fennel oil	24
3.0% fennel oil	23
0.1% clove oil	32
0.5% clove oil	27
1.0% clove oil	24
2.0% clove oil	4
3.0% clove oil	<3

a) Concentration of GZ in all samples was 100 mg/ml.

gel formation temperature of GZ solutions containing different kinds of oil (Table 1). Unadulterated GZ solution (control) formed a gel at 32 °C. GZ solutions containing either olive oil or soybean oil formed gels at the same temperature as that of the control solution. On the other hand, the gel formation temperature of GZ solutions containing mentha oil, fennel oil, or clove oil decreased with increasing oil content. The decrease in gel formation temperature related to the addition of clove oil was characteristic of several different essential oils. In fact, the gel formation temperatures of GZ solutions containing 2% and 3% clove oil were significantly lower (4 °C and <3 °C, respectively) than the control. Interestingly, GZ solutions containing clove oil turned a light dark-brown, even though very little separated oil remained.

To better understand the structural features of GZ gel formation, we examined whether glycyrrhetic acid, a hydrolytic metabolite of GZ, forms a gel in aqueous solution. Glycyrrhetic acid (100 mg/ml) failed to form a gel.

GZ Stability We were interested in determining whether the GZ-clove oil solution was a true emulsion. To rule out the possibility that GZ and clove oil chemically react with each other in aqueous solution, we measured the concentration of GZ in a 100 mg/ml GZ solution containing 3% clove oil. Measurements were determined using an HPLC. The GZ concentration was 99.9±2.4 mg/ml, suggesting that the GZ-clove oil solution was an emulsion.

GZ Micelle Particle Size To verify that GZ and clove oil form an emulsion, we used a Laser particle analyzer to measure the micelle particle size of control and GZ solutions containing clove oil. In control solutions (without clove oil), we were unable to measure the average diameter of GZ micelles, suggesting that their diameters were less than 3 nm, the detection threshold of the DLS-900. On the other hand, the average particle diameter of GZ micelles in solutions containing 3% clove oil was 44.1 nm.

SEM of GZ Gel To clarify why clove oil prevented GZ gel formation in aqueous solution, we examined with SEM cross sections of freeze-dried GZ solutions prepared with or without clove oil. The structure of specimens of GZ prepared without clove oil had lamellar cavities (Figs. 2A, B), while those of GZ solutions with 2% clove oil did not have these cavities (Figs. 2C, D). This suggests that clove oil alters the structure of GZ solutions, lowering the temperature at which GZ gels form.

Discussion

Highly concentrated GZ rapidly forms gels in aqueous solution. To our knowledge, no reports exist that describe the formation of GZ gel in aqueous solution. GZ is a triterpenoid saponin with two glucuronic acids. Interestingly, glycyrrhetic acid, a GZ-derived compound lacking two glucuronic acids, fails to form gels in aqueous solution. Thus, the presence of the two glucuronic acids may be an important structural characteristic of GZ that underlies its ability to form gels in aqueous solution. Indeed, recent findings indicate that sugars may promote gelation of certain compounds in aqueous solutions.[7,8)] Generally, most gelatinizing agents are polymers, and very few low molecular weight gelatinizers exist. However, recently developed low molecular weight organic compounds containing sugar chains have proven to be effective gelatinizers,[7,8)] suggesting that one or more sugar chains are important for gel formation in aqueous solutions. These findings motivated us to find a way to inhibit GZ gel formation by altering the osmolarity of GZ solutions, with the aim of developing a concentrated GZ solution for therapeutic use.

First, to prepare a highly concentrated GZ solution, we systematically varied the diluent phosphate buffer concentration until we could obtain a 100 mg/ml GZ solution. To

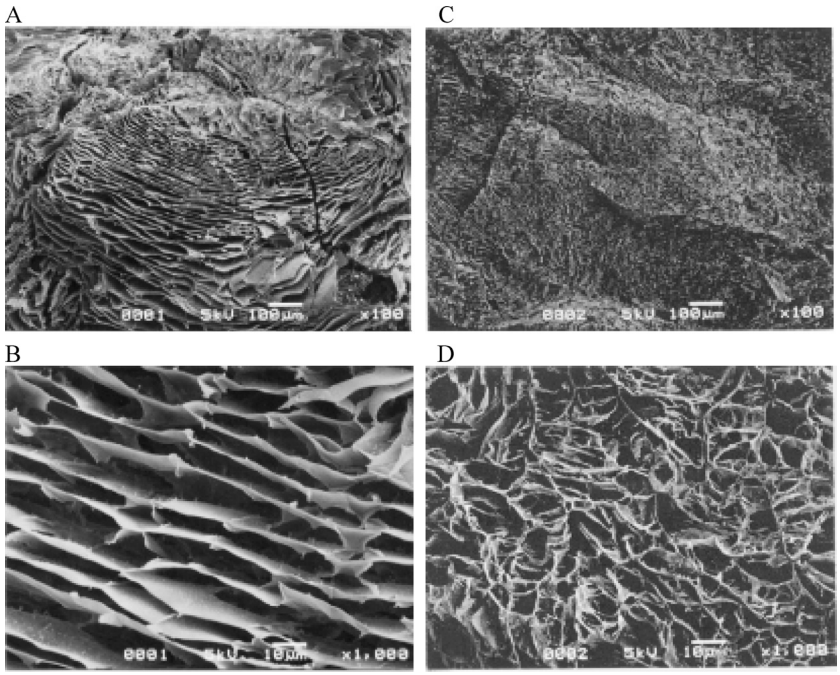

Fig. 2. SEM of Freeze-Dried Gel Sections Derived from GZ Solutions (A, B) without and (C, D) with Clove Oil
The concentration of GZ was 100 mg/ml in both samples; the concentration of clove oil was 2%.

obtain an isoosmotic 100 mg/ml GZ solution, we calculated that a phosphate concentration of 100 mM was necessary. After preparing a 100 mg/ml GZ solution in 100 mM phosphate buffer, we determined the osmolarity of this concentrated GZ solution to be 275 mOsm, with an osmotic pressure ratio of approximately 1 (physiological osmotic pressure=285 mOsm). This confirmed that a 100 mM phosphate buffered solution was needed to prepare an isoosmotic 100 mg/ml GZ solution.

Second, we sought to inhibit gelation of this 100 mg/ml GZ solution by systematically adding small amounts of various oils to emulsify the GZ solution. The ability of these oils to prevent gelation of concentrated GZ was assessed by examining the temperature at which the GZ-oil solutions form gels. We found that clove oil remarkably decreased the gel formation temperature of GZ. Because about one-third of the initial quantity of clove oil separated from the GZ solution after 16 h of incubation at 60 °C, it is possible that the remaining clove oil may have formed an emulsion with GZ or may have chemically reacted with GZ to form a water soluble reaction product. The former scenario seems reasonable because GZ has been demonstrated to be surface active and water soluble.[9] Because of these characteristics, GZ has been used to increase the solubility of drugs that have poor water solubility.[10,11]

If clove oil inhibits GZ gelation by forming a water soluble GZ-clove oil reaction product, then one would expect the GZ content in the aqueous solution to decrease in the presence of clove oil. HPLC analysis of GZ solutions with and without 3% clove oil revealed no differences in the GZ concentration of these two solutions, suggesting that clove oil does not react with GZ in the aqueous solution to form a water soluble reaction product. To verify that clove oil and GZ formed an emulsion by means of the surface activity of GZ, we measured the mean particle size of GZ-oil micelles. Although laser particle size analysis failed to measure particles in GZ solutions lacking clove oil, this analysis positively identified the presence of particles in the GZ-clove oil emulsion, confirming that clove oil was indeed emulsified in the GZ solution.

As part of our analyses, we also sought to determine whether the absence or presence of clove oil affected the crystalline structure of GZ gels. This was accomplished by examining freeze-dried GZ sections by SEM. It is difficult to accurately assess the molecular structure of gels derived from aqueous solutions because of the ice crystals that form during the freeze-drying process can obscure the true structure of the gel. To avoid this pitfall, ice crystal inhibitors such as glycerol are usually added to the solution. However, we avoided the addition of ice crystal inhibitors to the GZ sample, because their addition may affect GZ gel structure. For our SEM analyses, therefore, we postulated that the formation of ice crystals may be closely related to the intermolecular network that underlies GZ gel formation.

SEM examination of GZ gels revealed that GZ solutions form gels comprised of a network of regularly spaced GZ molecules arranged as lamellar cavities. In contrast, GZ gels derived from GZ-clove oil solutions formed gels comprised of a network of irregularly spaced GZ molecules; lamellar cavities were absent. These observations strongly indicate that the addition of clove oil to the GZ aqueous solution blocked the formation of the intermolecular networks of lamellar cavities that generally occurs during the normal gel-forming process. Although we could not definitively decipher the mechanism by which clove oil alters these networks, we proposed the following sequence of events. First, clove oil is either dissolved or dispersed in GZ solution by means of the surface active action of GZ. Second, clove oil suppresses GZ gelation by forming hydrogen bonds with the glucuronic acids of GZ.

Because individual clove oil droplets were too small to resolve with SEM, it was not possible to show that clove oil exists as an emulsion with GZ and that it inhibits GZ gelation. However, when taken together, our data lead us to conclude that clove oil inhibits GZ gel formation by collapsing the intermolecular network of GZ.

Conclusions

We have developed a new procedure for preparing concentrated GZ solutions that can be used under physiological conditions. These solutions have an osmotic pressure ratio of 1 and a pH of 7.4. Addition of clove oil to concentrated GZ aqueous solutions effectively inhibits the formation of GZ gels by preventing the expansion of intermolecular networks of GZ molecules. The prevention of GZ gelation by clove oil appears to be mediated by an emulsifying effect between clove oil and GZ.

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