Effect of Glycyrrhizinate on Dissolution Behavior and Rectal Absorption of Amphotericin B in Rabbits

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The effects of dipotassium glycyrrhizinate (GLYK) on the dissolution behavior and bioavailability of amphotericin B (AMB) were investigated. The mixtures of AMB and GLYK were prepared at different molar ratios by lyophilization. Lyophilization resulted in amorphous AMB either alone or in the mixture. Dissolution rates of AMB of the mixtures were markedly faster than that of lyophilized AMB alone, which was followed by a decrease of dissolution. The initially-enhanced dissolution rate was likely to be due to the improvement of surface wettability of drug particles with GLYK rather than the amorphous state of AMB. A phase solubility study of AMB with GLYK indicated that the increasing solubility was caused by micellar solubilization. The *in vitro* release rate of AMB from suppositories containing the lyophilized mixtures was significantly accelerated by increasing the amount of GLYK.

The rectal absorption of AMB from suppositories containing either the drug alone, a physical mixture or a lyophilized mixture was studied using rabbits. The absorption of the mixture (AMB/GLYK = 1/9) was about 35 times greater in the area under the serum concentration–time curve (0—24 h) than that of lyophilized AMB alone. These results suggest that GLYK is useful for improving the dissolution property of AMB and the bioavailability of the drug incorporated in suppositories.

Keywords amphotericin B; dipotassium glycyrrhizinate; dissolution; rectal absorption; solubilization; drug release; suppository; bioavailability; rabbit

Amphotericin B (AMB) is one of the most effective antibiotics currently available for the treatment of systemic fungal infections in humans. The drug is usually administered in the form of an intravenous infusion which is supplied commercially as a combination of AMB and deoxycholate, a solubilizing agent for AMB. However, the infusion exhibits a variety of toxic side effects, the most serious being nephrotoxicity.[1)]

In recent years AMB has been administered orally for systemic fungal infections in order to avoid the severe side effects. However, the poor gastrointestinal absorption leads to very large doses of AMB in order to maintain an effective therapeutic blood level.[2)] Patients are given a daily aqueous suspension of the drug. Because of these disadvantages, the utilization of AMB in therapy is limited, and an appropriate new dosage from has been requested.

It had been reported that the poor bioavailability of AMB might be related to its inherent poor solubility in water.[3)] The poor water solubility is also one of the important factors limiting its application in the therapy of systemic fungal infections. To improve its water solubility, many attempts such as preparation of derivatives of AMB,[4)] complex formation with γ-cyclodextrin[5)] and solid dispersion in macrogol[6)] have been made, but none of these have yet led to practical use.

The present study was undertaken to investigate the modification of the dissolution characteristics of AMB by lyophilizing AMB with dipotassium glycyrrhizinate (GLYK), which may act as a solubilizing agent[7)] and an absorption promoter.[8)] Rectal absorption of AMB in the lyophilized mixtures was also investigated in rabbits.

Experimental

Materials AMB (886 μg/mg as potency) was obtained from Bristol-Myers Squibb Co., New Jersey, U.S.A. The amount of AMB is hereinafter expressed with its real potency. GLYK was purchased from Maruzen Pharmaceutical Co., Ltd., Hiroshima, Japan. Witepsol H-15 was used as a suppository base (Dynamit Nobel, Ltd.). All other chemicals were of reagent grade.

Lyophilized Mixture of AMB and GLYK Lyophilization of the mixture of AMB and GLYK at molar ratios of 1:1, 1:2, 1:4 and 1:9 was performed in accordance with the procedure reported by Bartner *et al.*[9)] A suitable amount of GLYK to AMB (1 g) was dissolved slowly in 60 ml of distilled water, and the pH of the solution was adjusted to 12.5 by adding 1 N NaOH in a beaker chilled in a refrigerant. AMB equivalent was dissolved in the solution with vigorous stirring under a nitrogen atomosphere. The pH of the solution was adjusted to 7.5 by the addition of 1 N H_3PO_4 in a steady stream. The solution was filtered through a 0.45 μm membrane filter. After the solution was frozen completely, the flask was fitted with a lyophilizing apparatus (model FD-1, Tokyo Rikakikai, Co.) and sublimation of water was initiated to obtain a solid AMB–GLYK mixture in a reduced pressure of 0.05 Torr. Approximately 10 h were required to dry 30 ml of the frozen solution. The fraction of powder passed through a 100 mesh screen was always used. AMB alone was also lyophilized in the same manner as above without adding GLYK. The physical mixture of AMB and GLYK was prepared by mixing each powder (< 100 mesh) in a beaker.

Powder X-Ray Diffraction A powder X-ray diffractometer (RAD-C; Rigaku Denki Co., Ltd., Japan) was operated under the following conditions: target Cu, filter Ni, voltage 30 kV, current 20 mA, scanning speed 4°/min.

Dissolution Studies A powder sample containing 10 mg of AMB equivalent in each mixture was transferred directly into the JP XI disintegration 2nd fluid (300 ml, pH 6.8) and was stirred with a magnetic stirrer bar at 400 rpm. The temperature was 37 ± 0.5 °C. An aliquot of the solution (2 ml) was pipetted at definite time intervals and filtered through a 0.45 μm membrane filter. One ml of the filtrate was diluted with ethanol to make exactly 5 ml. The concentration of the dissolved AMB was determined spectrophotometrically (UV-240, Shimadzu) at 407 nm. All studies were done in triplicate.

Solubility Studies Solubility measurements were carried out according to the method of Higuchi and Connors.[10)] An excess amount of AMB (100 mg) was placed in 20 ml of a 1/15 M phosphate buffer (pH 7.0) containing various amounts of GLYK. The solutions were shaken in a water bath at 37 ± 0.5 °C for 2 d. After equilibrium was attained, and aliquot was withdrawn using a 0.22 μm membrane filter. One ml of the filtrate was appropriately diluted with ethanol and analyzed spectrophotometrically at 407 nm.

Preparation of Suppositories AMB alone or a mixture of AMB and GLYK was suspended in Witepsol H-15 with occasional stirring after the base had been melted at 50 °C. The molten mass was then poured into a mold and allowed to solidify at room temperature. The content of AMB incorporated was adjusted according to the body weight of animals used. The dose was 10 mg of AMB equivalent/kg. All suppositories were stored in a refrigerator (5 °C) and used within 24 h after preparation.

Release of AMB from Suppositories The release test was performed according to the rotatory basket method using a JP XI dissolution test apparatus.[11] The suppository containing 10 mg of AMB equivalent was always used. The test solution was 900 ml of phosphate buffer (1/15 M, pH 7.5) kept at 37±0.5 °C. One suppository was put in each basket, which was then immersed in the solution. The baskets were rotated at 100 rpm. A 6 ml portion of the solution was removed at definite intervals and filtered through a 0.45 μm membrane filter. The concentration of AMB in the solution was determined by high performance liquid chromatography (HPLC).[12]

Animal Experiments White male rabbits, weighing from 2.6 to 3.2 kg, were fasted for a 24 h period before the experiments but they were allowed free access to water. The suppository (10 mg/kg) was manually inserted into the rectum. Retention of the suppository was ensured by fastening the anus with a clip after insertion. Blood samples of 1—2 ml were collected from the ear vein at appropriate intervals.

Assay of AMB in Serum AMB in serum was assayed by a modified Nilsson–Ehle *et al.* method.[12] To 0.2 ml of serum, 0.6 ml of methanol was added and mixed well. The mixture was allowed to stand at ambient temperature for 10 min, and then centrifuged at 3000 rpm for 5 min. The clear supernatant (50 μl) was injected into the chromatograph. An HPLC apparatus (LC-6A, Shimadzu) equipped with a UV-VIS detector (SPD-6AV, Shimadzu) and a data processor (C-R3A, Shimadzu) was used. The conditions for analysis were as follows: column Superspher RP-18 (125 mm × 4 mm i.d., Merck & Co., Inc.); mobile phase, 0.01 M solution of dipotassium salt of ethylenediaminetetra acetic acid (EDTA) (pH 4.2)–acetonitrile (61 : 39); flow rate, 0.8 ml/min; wavelength, 407 nm; sensitivity, 0.001 a.u.f.s.

Results and Discussion

Characterization of AMB in Lyophilized AMB–GLYK Mixture by X-Ray Diffraction The X-ray diffraction patterns of lyophilized and physical mixtures of AMB with GLYK are shown in Fig. 1. Since a sharp diffractional peak ($2\theta = 21.5°$) of AMB crystals did not overlap the peaks of GLYK, the peak was regarded as the characteristic peak of AMB crystals in the mixture. The peak of AMB was observed in the physical mixture, but the diffractional patterns of AMB in the lyophilized mixture generally displayed halo patterns which were characterized by a broad and weak peak with a high background. Lyophilized AMB alone also showed a halo pattern which was characteristic of an amorphous state. It was demonstrated that the original crystalline nature of AMB was mostly lost, whether it was lyophilized alone or in the mixture.

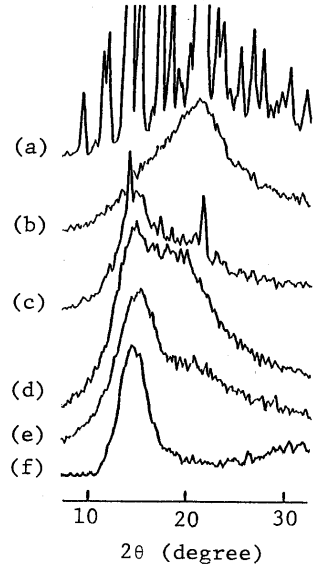

Fig. 1. Powder X-Ray Diffraction Patterns of AMB-GLYK Systems

(a) AMB crystalline bulk powder; (b) lyophilized AMB; (c) AMB: GLYK = 1 : 9 physical mixture; AMB–GLYK lyophilized mixture: (d) 1 : 4; (e) 1 : 9; (f) GLYK.

Dissolution Behavior of AMB Figure 2 shows the dissolution behavior of AMB alone, physical mixture and lyophilized mixtures of AMB and GLYK in a JP XI 2nd disintegration fluid (pH 6.8). The lyophilized mixtures exhibited markedly higher dissolution rates of AMB than that of either the lyophilized or crystalline drug alone, followed by a decreasing tendency of dissolution with time. The maximal AMB concentration was dependent on the molar ratio of the mixtures: the more GLYK, the higher the AMB concentration. AMB–GLYK with a 1 : 9 molar ratio yielded several hundred times higher concentration of AMB than that of the crystalline form at the concentration peak, and still held 35 times higher concentration, even at 30 min. The dissolution behavior of lyophilized AMB alone was slightly higher that that of the crystalline form. The results, however, indicate that the amorphous state of AMB itself contributed little to the enhanced dissolution, but the concomitant GLYK possibly improves the surface wettability of lyophilized AMB, resulting in efficient exposure of the lyophilized particles to water and dispersion.[13] By contrast, the dissolution of crystalline AMB in the physical mixture (1 : 9) was little enhanced by GLYK. This result suggests that there is a great difference in surface properties between

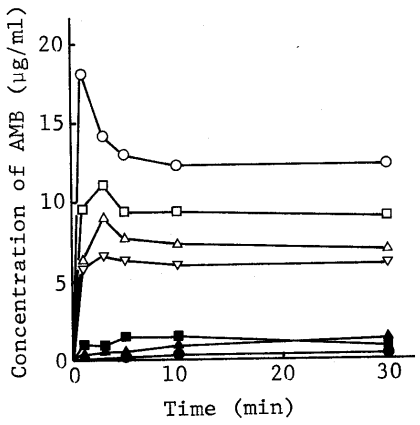

Fig. 2. Dissolution Profiles of AMB from the AMB–GLYK Systems in JP XI 2nd Fluid at 37 °C

●, AMB crystalline bulk powder; ▲, lyophilized AMB; ■, AMB : GLYK = 1 : 9 physical mixture; AMB–GLYK lyophilized mixture: ▽, 1 : 1; △, 1 : 2; □, 1 : 4; ○, 1 : 9.

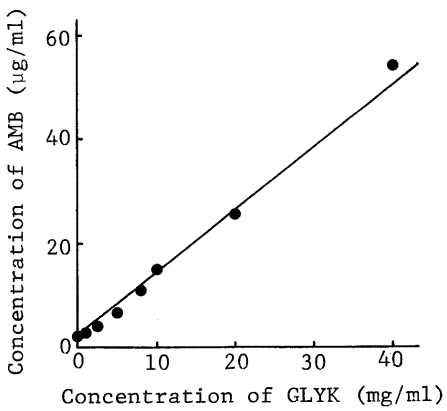

Fig. 3. Effect of GLYK on Solubility of AMB in pH 7.0, 1/15 M Phosphate Buffer at 37 °C

crystalline and amorphous AMB in terms of its interaction with GLYK. In addition, crystalline AMB has a disadvantage in that it needs to cross over a barrier of potential energy to break the bonds between adjacent molecules, compared with the amorphous form.

It has been reported that GLYK carries a solubilizing ability for poorly soluble drugs since it forms micelles,[14] and the critical micelle concentration (cmc) was estimated to be about 0.3 mM (0.27 mg/ml) in the neutral pH region.[15]

In order to examine the possible interaction of AMB and GLYK in water, the solubility of AMB (crystalline) was measured as a function of GLYK. Figure 3 shows the phase solubility diagram of AMB with GLYK up to 40 mg/ml. The linear dependency of the solubility indicates that AMB was well solubilized by GLYK micelles, as the concentration of GLYK was examined up to far above the cmc. However, this result also indicates that the enhanced dissolution of lyophilized mixtures observed in Fig. 2, for example the 1:9 mixture, was not due to the micellar solubilization, because even this mixture of AMB and GLYK produced as little as 0.3 mg/ml of GLYK under the conditions where a 100 mg of the mixture was placed in 300 ml of water.

A method of expressing micellar solubilization data gives the following equation[16]:

$$D_t/D_f = 1 + K[S] \quad (1)$$

where D_t and D_f are the total solute concentration and the solute concentration in the aqueous phase, respectively. [S] is the surfactant concentration. K is a measure of the "binding" or "association" capacity of the surfactant and could be obtained from the slope of plots of D_t/D_f against [S]. Applying Eq. 1 to the AMB solubilization by GLYK (Fig. 3), the K value was calculated to be 4.7×10^2 (M^{-1}).

Thus, the potential role of GLYK for the dissolution of AMB in water includes: (1) the improvement of surface wettability and deflocculation of lyophilized AMB particles when the surfactant concentration is lower than the cmc, and (2) when GLYK concentration is greater than the cmc, the solubility of AMB increases due to micellar solubilization.

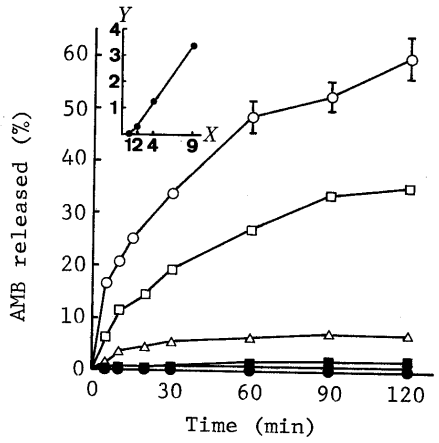

Fig. 4. Release Profiles of AMB from Various Suppositories in pH 7.5, 1/15 M Phosphate Buffer at 37 °C

●, AMB crystalline bulk powder; ▲, lyophilized AMB; ■, AMB:GLYK=1:9 physical mixture; AMB–GLYK lyophilized mixture: ▽, 1:1; △, 1:2; □, 1:4; ○, 1:9. Each suppository contains 10 mg of AMB equivalent. Inset: X, molar ratio of GLYK to AMB; Y, initial release rate (×10, mg/min).

Release of AMB from Suppository Figure 4 shows the release profiles of AMB from suppositories in which various forms of the drug are incorporated. The release rate of AMB from the lyophilized mixture was significantly enhanced, except for the 1:1 mixture, compared with that of the drug along and the physical mixture (1:9). The order of the release rates nearly corresponds to the results obtained in the dissolution testing, although even the 1:1 mixture produced an improved dissolution rate of AMB while the physical mixture produced no improvement in the release testing. At higher incorporations of GLYK, the initial release rate was proportional to the surfactant content (inset in Fig. 4).

When the suppository containing mixtures of AMB and GLYK is placed in a large amount of water assuming a perfect sink, as set up in this study, it is very likely that in the first place, GLYK dissolves easily into the medium as water penetrates into the vehicle, leaving the AMB behind.[17] It would therefore be reasonable that the less the GLYK was incorporated, the less remaining GLYK would be available for the the dissolution of AMB in the vehicle. A typical example could be seen in the 1:1 mixture: AMB was well dispersed and allowed to have effective contact with water in the dissolution testing, while in the release testing, most of the GLYK was washed away to the bulk solution before building up a sufficient concentration of the surfactant around the particles of the AMB. As a result, the particles, which had little freedom of migration in the vehicle, were left behind.

As the incorporated GLYK increased, higher concentra-

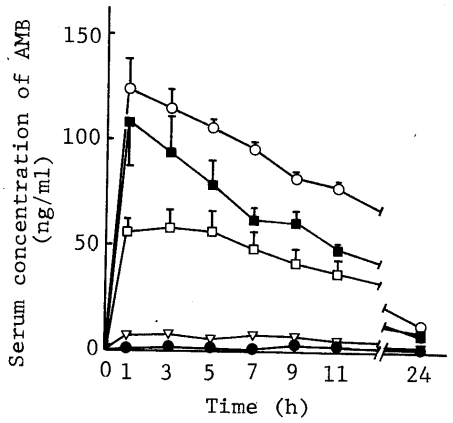

Fig. 5. Serum Concentration of AMB after Rectal Administration of Various Suppositories

●, AMB crystalline bulk powder; ■, AMB:GLYK=1:9 physical mixture; AMB–GLYK lyophilized mixture: ▽, 1:1; □, 1:4; ○, 1:9. Each value represents the mean±S.E. (n=3).

TABLE I. Bioavailability Parameters after Rectal Administration of Suppositories in Rabbits

	C_{max} (ng/ml)	T_{max} (h)	AUC_{0-24h} (h·ng/ml)
AMB[a]	4.6± 1.2	8.3±1.4	46.5± 8.7
1:1 LM[b]	8.3± 1.0	2.3±0.5	112.3± 26.1
1:4 LM	60.0± 8.2	3.0±0.9	819.7±136.5
1:9 LM	127.2±11.7	1.7±0.5	1638.2± 51.7
1:9 PM[c]	107.5±23.1	1.0±0	1152.5±116.7

Each value represents the mean±S.E. (n=3). *a*) Crystalline bulk powder. *b*) AMB: GLYK lyophilized mixture. *c*) AMB: GLYK physical mixture.

tions of the drug could be dissolved in the vehicle if the fraction of the surfactant to be washed away was almost equivalent. Furthermore, the spaces which were first occupied by GLYK in the vehicle could be occupied by water after the surfactant dissolves away into the bulk solution. This would provide easier access or channels for the solubilized AMB in the local region to the bulk solution.

Bioavailability Figure 5 and Table I show the results of the rectal absorption study of AMB from various suppositories in rabbits. While absorption of the AMB alone was extremely poor, the lyophilized mixtures of AMB with GLYK showed significant increases of absorption, particularly mixed at molar ratios of 1:4 and 1:9. The serum peak level (C_{max}) of those mixtures was significantly higher, and the time required to reach a maximum peak (T_{max}) was shorter compared to the AMB alone. The AMB absorption from the 1:9 lyophilized mixture was 35 times greater in the area under the serum concentration–time curve (AUC) than that of the AMB alone. The improved rectal absorption of AMB from suppositories is consistent with the results of the *in vitro* dissolution and release of lyophilized mixtures. On the other hand, the physical mixture also demonstrated considerably enhanced rectal absorption. However, there was a significant difference ($p<0.05$) of the AUC value between the 1:9 lyophilized mixture and the physical mixture. The absorption of AMB of the physical mixture was unexpectedly high in reference to the results of the dissolution and drug release tests.

However, there is apparently a reason leading to the enhanced rectal absorption of AMB from the physical mixture. Since there should be only a limited quantity of local fluids available for dissolution in the rectum, GLYK could maintain high concentrations in the area. This probably helps AMB be better solubilized, even in the crystalline form, and to be effectively conveyed to an absorptive membrane surface. Thus, the rectal dissolution behavior of AMB with the aid of GLYK is different from that in the *in vitro* studies, mainly due to the difference in aqueous phase available.

It is of interest that the initial absorption rates of the lyophilized and physical mixtures with the 1:9 molar ratio were comparable. This result suggests that the amount of GLYK incorporated is a determining factor for absorption improvement rather than whether AMB is either amorphous (lyophilized) or crystalline. However, it should be noticed that the 1:9 lyophilized mixture could hold higher blood concentrations than the corresponding physical mixture, resulting in an AUC about 40% larger.

It has been recently reported that some types of saponins have acted as drug absorption promotors,[18] probably facilitating the membrane crossing of a solute. Mishima *et al.* reported that nasal absorption of insulin was markedly enhanced by glycyrrhizin derivatives which have a chemical structure similar to saponins.[8] Hence, the increased absorption of AMB may be in part attributable to the promoting effect by GLYK. GLYK has been reported not to cause acute irritation to the mucosal membrane.[8] It was suggested from these results that GLYK may be a practically useful absorption enhancer of AMB.

The present study showed that the poor dissolution and absorption of AMB was significantly improved by lyophilization with GLYK. While there are some inevitable disadvantages associated with the intravenous and oral administration of AMB, rectal administration with suppositories of lyophilized AMB-GLYK may provide a promising means for controlling appropriate blood levels, making much safer use of this drug possible.

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