

***In Vivo* Assessment of Oral Administration of Probucol Nanoparticles in Rats**

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Pharmacokinetic profiles of probucol were evaluated after oral administration of the various nanosuspensions in rats. Probucol nanoparticles were prepared by co-grinding with various molecular weights of polyvinylpyrrolidone (PVP K12, PVP K17 and PVP K30) and sodium dodecyl sulfate (SDS). The average particle sizes of probucol after dispersing the ternary ground mixtures (GMs), probucol/PVP K12/SDS, probucol/PVP K17/SDS and probucol/PVP K30/SDS into water were 28, 75 and 89 nm respectively. The ternary GM suspensions with PVP K17/SDS and PVP K30/SDS were stable at 25 °C. However the particle size of probucol from the ternary GM with PVP K12/SDS gradually increased. Pharmacokinetic profiles of probucol indicated that variation in particle surface condition covered with PVP and SDS in addition to the particle size affected the improvement of *in vivo* absorption of probucol. The ternary GM with PVP K12/SDS exhibited a superior improvement of probucol absorption compared to the GMs with PVP K17/SDS and PVP K30/SDS. The binary GM with PVP or SDS and physical mixtures with PVP and/or SDS did not show significant differences in the area under the plasma concentration–time curve compared to the unprocessed probucol. In conclusion, preparation of probucol nanoparticles by co-grinding with PVP K12 and SDS could be a promising method for bioavailability enhancement.

Key words nanoparticles; grinding; probucol; polyvinylpyrrolidone; *in vivo* absorption

Probucol, 4,4'-{(1-methylethylidene)bis(thio)}-bis{2,6-bis(1,1-dimethylethyl)}phenol, is mainly used as a cholesterol-lowering agent. Various studies suggest that probucol lowers serum cholesterol by increasing the fractional rate of low density lipoprotein (LDL) catabolism in the final metabolic pathway for cholesterol elimination from the body.¹⁾ Probucol also inhibits the oxidation and tissue deposition of LDL cholesterol, thereby inhibiting atherogenesis.¹⁾ Probucol can not be injected intravenously due to the poor water solubility (5 ng/ml at 25 °C).^{1,2)} The bioavailability of probucol is very poor (up to 6%) through oral administration.³⁾ The tablet form of probucol is absorbed at different rates and in different amounts depending on the individual patients.¹⁾ When probucol was co-administered with arachis oil, the absorption enhancement of probucol in rats was observed.⁴⁾ Thus, the formulation seems to be important to improve the bioavailability.

Reducing the particle size is one of the ideas to improve the bioavailability of drugs with poor absorption. Several cutting-edge technologies such as high pressure homogenization, supercritical fluid processing, precipitation techniques and grinding are currently utilized to generate drug nanoparticles.^{5–8)} Co-grinding, where a drug is ground together with excipients, is a promising method for an effective reduction of particle size. A certain number of studies revealed that the co-ground mixture enhanced dissolution, resulting in the improvement of oral absorption and bioavailability.^{9–11)} Wet co-grinding method has been known as an effective method to produce stable nanosuspensions.^{12–15)} Liversidge and Cundy proposed a preparation method for crystalline nanoparticle by wet co-grinding of danazol with polyvinylpyrrolidone (PVP).¹²⁾ Oral absorption of poorly water-soluble drugs in nanocrystal form was remarkably improved.^{12,13)} However, the wet process may cause drug degradation by hydrolysis during the preparation. In addition, a

drying process should be required to prepare the solid dosage form. Compared with the wet co-grinding method, the dry co-grinding process has some advantages for solid pharmaceutical applications due to its simple preparation with free solvents. However, *in vivo* absorption experiments have been rarely reported.

Our previous studies demonstrated that drug nanoparticle was successfully produced by the dry co-grinding of a poorly water-soluble drug with polymer and surfactant.^{16–18)} The drug/PVP K17/sodium dodecyl sulfate (SDS) was profitably applied for the preparation of hydrophobic drug nanoparticles.^{16,18)} The nanosuspension prepared from the ground mixture (GM) dispersed into distilled water exhibited good stability as the particle agglomeration was effectively inhibited by the adsorption of both PVP and SDS on the surface of crystalline particles.¹⁸⁾ ¹³C-NMR studies revealed that the nanoparticle formation and stabilization were attributable to grinding-induced solid-state interactions among components of the drug/PVP/SDS ternary system.¹⁹⁾ We designed the present study to investigate the effects of particle size and PVP molecular weight on the oral absorption of probucol. Pharmacokinetic profiles of probucol nanoparticles were evaluated after oral administration in rats. Different sizes of probucol nanoparticles were produced by co-grinding with various molecular weights of PVP (*i.e.* PVP K12, PVP K17 or PVP K30) and SDS. The Probucol/PVP/SDS at the weight ratio of 1:3:0.5 was employed because it was one of the most appropriate ratios to reduce particle size with the least ratio of SDS.¹⁹⁾ Effects of the ternary composition of the GM on the improvement of absorption were also discussed.

MATERIALS AND METHODS

Materials Probucol, 4,4'-{(1-methylethylidene)bis(thio)}-bis{2,6-bis(1,1-dimethylethyl)}phenol, was supplied by Dai-

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ichi Sankyo Co., Ltd. (Tokyo, Japan). Polyvinylpyrrolidone, PVP K12 (MW *ca.* 2500), was obtained from BASF Japan Ltd. (Tokyo, Japan). PVP K17 (MW *ca.* 10000) and PVP K30 (MW *ca.* 50000) were obtained from ISP Technologies, Inc. (Texas City, TX, U.S.A.). Sodium dodecyl sulfate (SDS) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Hydroxypropylcellulose was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). α -Tocopherol acetate, ethyl acetate, methanol, trifluoroacetic acid and acetonitrile were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). All other chemicals used were of reagent grades.

Preparation of Physical Mixture (PM) Probuco (0.67 g), PVP (2.00 g) and SDS (0.33 g) (weight ratio of 1:3:0.5) were physically mixed in a glass vial using a vortex mixer for 5 min. For the binary physical mixture, probu (0.75 g) and PVP (2.25 g) or probu (2.00 g) and SDS (1.00 g) were physically mixed following the same method as described above.

Preparation of Ground Mixture (GM) Probu (0.67 g), PVP (2.00 g) and SDS (0.33 g) (weight ratio of 1:3:0.5) were physically mixed and then ground in a vibrational rod mill (TI-500ET, CMT Co., Ltd., Fukushima, Japan) for 30 min. The grinding was performed under temperature control and monitoring at $10 \pm 5^\circ\text{C}$. The grinding cell and rod were made of stainless steel. For the binary ground mixture, probu (0.75 g) and PVP (2.25 g) or probu (2.00 g) and SDS (1.00 g) were ground respectively by the same method as described above.

Particle Size Analysis The GM was dispersed into distilled water, then sonicated for 2 min to make the suspension. The drug concentration in the suspension was 0.50 mg/ml. The Japanese Pharmacopoeia (JP XIV) first fluid (pH 1.2) and second fluid (pH 6.8) were also used as liquid media instead of distilled water. The particle size was determined by the dynamic light scattering method using Microtrac UPA, with a measurement range of 0.003–6 μm (Nikkiso Co., Ltd., Tokyo, Japan) and by the light scattering method using Microtrac FRA, with a measurement range of 0.1–700 μm (Nikkiso Co., Ltd., Tokyo, Japan).

Stability Study Stability studies of the GM suspensions were investigated by particle size analysis after storage at 25°C for 0 (initial), 1, 2, 3, 7, 10, 12, 24 and 48 h.

Quantitative Determination of Nanoparticles The GM suspensions were filtered through a 0.8 μm membrane filter (Millipore Corp., Tokyo, Japan) and then diluted with acetonitrile. The concentration in nanoparticle fractions was determined by HPLC (LC-6A, Shimadzu Corp., Kyoto, Japan). The mobile phase (acetonitrile/water=185/15, v/v) was delivered at a flow rate of 1.0 ml/min through a C-18 column (Inertsil ODS-2, 5 μm , 4.5 $\times$ 150 mm, GL Sciences Inc., Tokyo, Japan) at 40°C and the detection wavelength was 254 nm. The concentrations of probu in the samples were determined by the linear equation obtained from the standard curve (correlation coefficient=0.9999).

In Vivo Absorption Studies Probu formulations were administered by oral gavages to male Sprague-Dawley rats. Twenty-seven adult rats with a weight of 270–330 g were obtained from Charles River Laboratories Inc. (Wilmington, MA, U.S.A.). All rats were made to fast overnight prior to the dose administration and remained fasting until

6 h after the dose administration. The sample suspensions were prepared with sonication by dispersing the probu formulations into distilled water to obtain a probu concentration of 25 mg/ml, then administered to the rats at 8 ml/kg by oral gavage. In the case of unprocessed probu, due to its low wettability, 0.5% hydroxypropylcellulose solution was used instead of distilled water to facilitate the dispersion. Blood samples (*ca.* 0.3 ml) were collected into vacutainer tubes containing EDTA, 0, 1, 2, 3, 4, 7, 10, 24 and 48 h after drug administration. After this collection, the blood samples were centrifuged at approximately 2800 rpm (*ca.* 1000 $\times g$) at $2-8^\circ\text{C}$ for about 15 min. Each plasma specimen was collected, and stored at *ca.* -80°C until analysis. No adverse reaction was observed following dose administration during the study.

Determination of Probu in Rat Plasma by HPLC.
Preparation of Plasma Samples Probu in the plasma samples were determined by HPLC. α -Tocopherol acetate was used as internal standard. A solution of internal standard was prepared with ethyl acetate at 10 $\mu\text{g/ml}$. Samples were prepared as follows: 50 μl of plasma were extracted with 1 ml of internal standard solution in polypropylene tubes. Samples were then vortexed and centrifuged at 12000 rpm for 10 min. Supernatants were transferred into glass test tubes. A blank (50 μl of blank plasma extracted with internal standard) and double blank (50 μl of blank plasma extracted with blank ethyl acetate) were also prepared. Samples were dried under nitrogen, reconstituted with 100 μl of methanol, and transferred into a glass insert in an autosampler vial.

Preparation of Standard Samples Standards were prepared in the same manner as samples (using blank plasma) and reconstituted with methanol containing probu at known concentrations. Eight calibration standards were prepared for probu concentration in plasma, between 0.02 and 8.75 $\mu\text{g/ml}$ probu in plasma. The limit of quantitation was described by the lowest concentration of the standard (0.02 $\mu\text{g/ml}$).

HPLC Conditions The probu concentration in plasma samples was determined by HPLC (HP 1100, Agilent Technologies Inc., Santa Clara, CA, U.S.A.). The mobile phase was delivered at a flow rate of 1.5 ml/min through a reverse-phase column (Zorbax 300SB-C8, 5 μm , 4.6 $\times$ 150 mm, Agilent Technologies Inc., Santa Clara, CA, U.S.A.) at 40°C and the detection wavelength was 220 nm. The injection volume was 20 μl . The gradient was the change in the proportion of phase A solvent (0.1% trifluoroacetic acid in water) and phase B solvent (0.1% trifluoroacetic acid in acetonitrile) that make up the mobile phase. The steps of gradient shown were: phase B solvent concentration in the mobile phase was 75% (at 0 min) and changed from 75 to 90% (after 10 min) and returned from 90 to 75% (after 15 min).

HPLC Data Analysis Analysis of the plasma samples was carried out using Agilent Chem Station software (V. A. 09. 01, Agilent Technologies Inc., Santa Clara, CA, U.S.A.). The concentrations of probu in the samples were determined by the linear equation obtained from the standard curve (correlation coefficient=0.9987).

Statistic Analysis Data are expressed by the mean $\pm$ S.E. The area under the plasma concentration–time curve (AUC) value (0–48 h) of the plasma profiles was calculated using the logarithmic and linear trapezoidal rules. *p*-values were

calculated using *F*-test and *T*-test in Microsoft Excel 2003 and less than 0.05 considered significant.

RESULTS AND DISCUSSION

Stability Study of Probucol/PVP/SDS Ternary GM Suspension We employed the mixing ratio of probucol/PVP/SDS at the weight ratio of 1 : 3 : 0.5 for ternary ground mixtures (GMs) and ternary physical mixtures (PMs) in this study, in order to reduce the amount of excipients and the administration volume though our previous experiments confirmed that the best suitable ratio was 1 : 3 : 1.¹⁹⁾

Variations in particle size of probucol obtained from probucol/PVP/SDS ternary GMs dispersed into distilled water and physiological fluids, JP XIV first fluid (pH 1.2) and JP XIV second fluid (pH 6.8), are shown in Fig. 1. After dispersion into distilled water, the initial particle sizes of probucol nanoparticles obtained from probucol/PVP K12/SDS, probucol/PVP K17/SDS and probucol/PVP K30/SDS ternary GMs were 28, 75 and 89 nm, respectively. During the storage period, the size of probucol nanoparticles obtained from the ternary GM prepared with PVP K12 increased steadily, while those of the ternary GMs prepared with PVP K17 and PVP K30 showed slight increases only in the initial stage then became constant. Finally, the particle size of the ternary GM with PVP K12 was higher than that of the ternary GMs prepared with PVP K17 or PVP K30.

The initial probucol size of the ternary GM with PVP K12 increased from 28 nm in distilled water to 48 nm in JP first fluid and 56 nm in JP second fluid. In contrast, the particle size obtained from the ternary GMs with PVP K17 and PVP K30 slightly decreased from 75 and 89 nm in water to 69 and 816 nm in JP first fluid, and 75 and 85 nm in JP second fluid, respectively. Changes in the particle size with time, for all ternary GMs, exhibited a comparable trend regardless of the dispersion media. The results demonstrated that the probucol particle size was effectively reduced at the initial stage when the low molecular weight PVP was employed. In pH-adjusted conditions, the particle size of probucol prepared with PVP K12 became large, while the other two PVPs showed little influence on the particle size. We assumed that the surface conditions of nanoparticles obtained from PVP K12/SDS might be different from those of PVP K17/SDS and PVP K30/SDS. It was clarified that SDS formed micelles together with the PVP polymer as a micelle-like aggregate in PVP/SDS solution.^{20–22)} For the ternary GMs with PVP K17 and PVP K30, the surfaces of probucol nanoparticles were probably covered by PVP–SDS micelle complex as a layered structure, so that ternary GMs with PVP K17 and PVP K30 were able to become stable in the suspensions. For the ternary GM with PVP K12, PVP K12 and SDS might not make a PVP–SDS complex with micelle-like structure, due to the shortest length of the chain (the molecular weight was 1/4 of PVP K17, 1/20 of PVP K30). The PVP K12 and SDS might cover insufficiently the surface of probucol nanoparticles in the suspension. Therefore ternary GM with PVP K12 induced a relatively rapid agglomeration of probucol nanoparticles.

Drug Absorption from the Ternary GM *in Vivo* Pharmacokinetics of different probucol formulations administered by oral gavages to male Sprague-Dawley rats was evaluated. The plasma concentration–time profiles of probucol

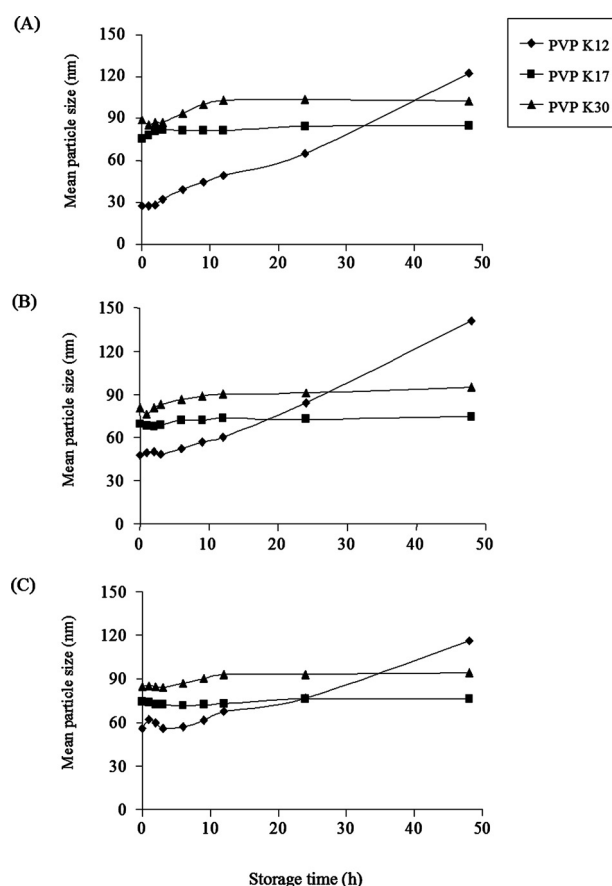


Fig. 1. Variation in the Particle Size of Probucol Nanoparticles Obtained from Ternary Ground Mixtures of Probucol/PVP/SDS at the Weight Ratio of 1 : 3 : 0.5 after Dispersion in (A) Distilled Water, (B) JP 1st Fluid and (C) JP 2nd Fluid, at Various Time Intervals and at a Temperature of 25 °C.

after oral administration in rats are shown in Fig. 2A. The *in vivo* absorptions were remarkably improved by probucol/PVP/SDS ternary GMs compared to those of the ternary PM and unprocessed probucol. After the administration of ternary GMs, the plasma concentrations increased rapidly and peaks were observed after 3–4 h.

Pharmacokinetic parameters following oral administration of the probucol/PVP/SDS ternary GM suspensions are presented in Table 1. Interestingly, the suspension obtained from the ternary GM prepared with PVP K12 remarkably increased *AUC* and the maximum drug concentration ($C_{\max}$) values of probucol, 41.3-fold ($p < 0.005$) and 17.5-fold ($p < 0.005$) compared to the respective values of unprocessed probucol. For ternary GMs with PVP K17 and PVP K30 cases, the *AUC* and $C_{\max}$ values increased 10.7-fold ($p < 0.05$) and 7.9-fold ($p < 0.02$), 5.6-fold ($p < 0.05$) and 3.3-fold ($p < 0.02$) respectively. There were no significant differences in *AUC* and $C_{\max}$ values between the ternary PM and the unprocessed probucol. These results suggested that additives used have no effect on *in vivo* absorption of the unprocessed probucol.

The particle size reduction could lead to the enhancement of the *in vivo* absorption of probucol. But stability results shown in Fig. 1 demonstrated that the probucol particle obtained from ternary GM with PVP K12 has different characteristics from those of ternary GMs with PVP K17 and PVP K30. Not only the size of probucol nanoparticles but also the

Table 1. Pharmacokinetic Parameters of Probucol Following Oral Administration of Probucol/PVP/SDS Ternary, Probucol/PVP and Probucol/SDS Binary Suspensions

Suspension	$C_{\max}$ ($\mu\text{g/ml}$)	$T_{\max}$ (h)	AUC ($\mu\text{g h/ml}$)	Enhancement ratio ^{a)}
Unprocessed probucol	0.068 ± 0.009	3.7 ± 0.3	0.31 ± 0.02	1.0
Probucol/PVP K12/SDS GM	$1.188 \pm 0.062^{***}$	4.0 ± 0.0	$12.81 \pm 0.77^{***}$	41.3
Probucol/PVP K17/SDS GM	$0.539 \pm 0.062^{**}$	3.0 ± 0.6	$3.33 \pm 0.49^*$	10.7
Probucol/PVP K30/SDS GM	$0.227 \pm 0.028^{**}$	3.3 ± 0.3	$1.74 \pm 0.22^*$	5.6
Probucol/PVP K12/SDS PM	0.053 ± 0.003	3.7 ± 0.3	0.54 ± 0.15	1.7
Probucol/PVP K12 PM	0.059 ± 0.003	3.3 ± 0.7	0.36 ± 0.06	1.2
Probucol/PVP K12 GM	0.050 ± 0.004	2.7 ± 0.3	0.53 ± 0.16	1.7
Probucol/SDS PM	0.049 ± 0.003	3.7 ± 0.3	0.49 ± 0.05	1.6
Probucol/SDS GM	$0.170 \pm 0.008^*$	3.0 ± 0.0	1.09 ± 0.21	3.5

$C_{\max}$ =maximum drug concentration, $T_{\max}$ =time of maximum concentration, AUC =area under the plasma concentration–time curve. Results are expressed as mean $\pm$ S.E. ($n=3$). a) Enhancement ratio was calculated according to the following equation: the ratio=the corresponding AUC/AUC of unprocessed probucol. *** $p<0.005$; ** $p<0.02$; * $p<0.05$, compared to the corresponding parameters of the unprocessed probucol suspension.

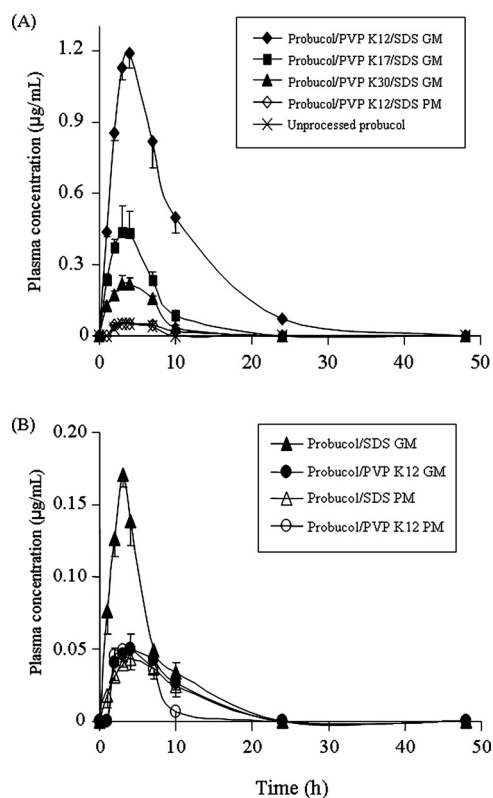


Fig. 2. Plasma Concentration of Probucol Following Oral Administration of (A) Probucol/PVP/SDS Ternary and (B) Probucol/PVP or Probucol/SDS Binary Mixture Suspensions

Results are expressed as mean $\pm$ S.E. ($n=3$).

different surface states covered by PVP and SDS seemed to influence the *in vivo* absorption of probucol. Despite the fact that the particle size obtained from the ternary GM with PVP K17 was somewhat similar to that from the ternary GM with PVP K30, ternary GM with PVP K17 enhanced the absorption better than the ternary GM with PVP K30. It was considered that the long chain length of high molecular weight PVP (PVP K30) contributed to the thick layer structure of the PVP/SDS complex on the surface of nanoparticles. The thick layer structure might be attributed to the difficulty of probucol molecules to be dissolved and absorbed. On the contrary, the smallest nanoparticles without the layer structure, which were generated by ternary GM with PVP K12, might facilitate dissolution and absorption, though the stabil-

Table 2. Mean Particle Size of Probucol Obtained from the Binary and Ternary Physical Mixtures and Ground Mixtures in Distilled Water, Measured by FRA (0.1–700 μm)

Suspension	Mean particle size (μm)
Unprocessed probucol	27.7 ± 0.6
Probucol/PVP K12 PM	42.3 ± 0.4
Probucol/PVP K12 GM	17.3 ± 0.4
Probucol/SDS PM	46.0 ± 0.3
Probucol/SDS GM	58.4 ± 2.4
Probucol/PVP K12/SDS PM	47.1 ± 0.1
Probucol/PVP K12/SDS GM	$0.028 \pm 0.001^a)$

Results are expressed as mean $\pm$ S.D. ($n=3$). a) Particle size was measured by UPA (0.003–6 μm).

ity in water is not so good as shown in Fig. 1.

Interpretation of Drug Absorption from Binary GM with SDS The effects of probucol/PVP K12 and probucol/SDS binary GMs on plasma concentration of probucol through oral administration of the suspension are shown in Fig. 2B. The probucol/PVP K12 binary GM demonstrated a similar concentration–time profile to that of the binary PMs and unprocessed probucol. On the other hand, the probucol/SDS binary GM showed minor absorption improvement compared to all other binary mixtures and the unprocessed probucol. Table 1 also presents pharmacokinetic parameters of binary systems. The AUC and $C_{\max}$ values of the probucol/SDS GM increased respectively, 3.5-fold (not statistically significant) and 2.5-fold ($p<0.005$) compared to the unprocessed probucol.

Mean particle sizes of probucol obtained from the binary systems and ternary PM are shown in Table 2. The size of the unprocessed probucol was 27.7 μm . Probucol/PVP K12 and probucol/SDS GMs showed no significant particle size reduction compared to the unprocessed probucol even though absorption improvement in probucol/SDS GM was observed. However, there still remains the possibility that the particle size in the range of the submicron region might not be detected due to the detection limit of the FRA particle size analyzer. A filtrate sample of the probucol/SDS GM suspension was prepared with a 0.8 μm membrane filter to investigate whether the micronized particles exist or not. HPLC quantitative analysis revealed that approximately 17% of the probucol was recovered as nanometer-sized particles in the suspension of probucol/SDS binary GM. The average particle size

was around 287 nm. These results supported the reason that there was minor absorption improvement derived from the probucol/SDS binary GM. Because probucol nanoparticles from the probucol/SDS binary GM were unstable in JP first fluid,¹⁸⁾ their structure was different from that of ternary GMs, which was covered with PVP and SDS.

CONCLUSIONS

It was demonstrated that the ternary ground mixtures of probucol/PVP/SDS significantly enhanced the bioavailability of probucol. The ternary GM with PVP K12 showed a superior absorption improvement compared to the GMs with PVP K17 and PVP K30. The results suggested that not only reduced particle size of probucol but also different particle surface conditions covered with PVP and SDS seem to influence the *in vivo* absorption of probucol. On the contrary, the ternary PM and all binary GMs did not show significant differences in the *AUC* value compared to the unprocessed probucol. Preparation of probucol nanoparticles by co-grinding with PVP K12 and SDS was confirmed as the promising method for the bioavailability enhancement.

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