Formation and stability of dispersed particles composed of vitamin $\text{K}_1$, soybean oil and phosphatidylcholine

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Abstract The purpose of this study was to develop an intravenous formulation composed of vitamin $\text{K}_1$ (VK) for the treatment of blood coagulation with warfarin-induced hypoprothrombinaemia. VK was dispersed using sonication with soybean phosphatidylcholine (PC) and the dispersion mechanism was evaluated by characterizing the dispersed particles with dynamic light scattering, fluorescence spectroscopy and surface monolayer techniques. VK has an appreciable solubility in PC bilayers (approximately 20 mol%). Within the VK molar fraction of 0.1–0.9, the size of the dispersed particles increased at room temperature within 3 months. By addition of soybean oil (SO) to VK (molar ratio of VK:SO = 1:1), the solubility of the VK/SO mixtures in PC bilayers was decreased (approximately 5 mol%). The size of the aqueous dispersions at molar fractions of 0.1–0.7 was 50–70 nm and did not change for 3 months at room temperature. The solubility of the VK and VK/SO in PC bilayers is crucially important in the production of the stable aqueous dispersions of VK particles.

Key words Vitamin $\text{K}_1$ · Phosphatidylcholine · Soybean oil · Solubility · Stability

Introduction

Vitamin $\text{K}_1$ (VK) has been found in plants as a component of electron transport chains of chloroplasts participating in the photoreduction steps of the photosynthetic process [1]. VK is known to have “vitamin K activity” when fed to vitamin K deficient animals [2]. “Vitamin K activity” in animals is thought to be mainly the activation of a carboxylase which gives modification of prothrombin, blood clotting factor and other proteins in plasma and tissues [3].

It was reported that VK associated with liposomes, administered orally, enhanced the recovery of blood coagulation in rabbits with warfarin-induced hypoprothrombinaemia [4]. In addition, VK was solubilized by bile salts (sodium deoxycholate, sodium cholate and their corresponding glycine conjugates) as mixed micelles for oral administration [5]. However, very little attention has been given to VK as an intravenous formulation. In this study, we focused on the preparation of injectable formulation of VK for the treatment of the previously mentioned diseases. Owing to its polyenic structure, VK is virtually insoluble in water and is chemically labile, which makes its manipulation difficult. In order to overcome these problems, we suspended VK in soybean oil (SO) and dispersed it with soybean phosphatidylcholine (PC) using sonication. For the formation of the dispersion for parenteral use, characterization of the physicochemical properties of the dispersed particle, such as the particle size, the structure and the physical stability should be clarified. The size should be smaller than that of the sterilization filter (0.22 μm) and should not increase during storage for a long period.

In this study, in order to clarify the interaction between VK and PC, we prepared dispersed particles of VK and PC by sonication and investigated the dispersion mechanism using several physicochemical techniques. In addition, SO was added to the VK and PC...
mixture to improve the dispersibility of VK as in the case of fat emulsions for intravenous administration [6]. The size and structure of the VK/PC and VK/SO/PC particles was determined by dynamic light scattering, fluorescence quenching and analysis of the trapped aqueous volume inside the particles. The miscibility and solubility of VK/PC and VK/SO/PC were evaluated by surface monolayer techniques.

Experimental

Materials

VK and SO were purchased from Sigma Chemicals Co. (St Louis, Mo., USA). PC was purchased from Ajinomoto Co. (Tokyo, Japan). The fluorophore lipid 1-(5-dimethylaminonaphthylene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphethanolamine trimethylammonium salt (Dansyl-DHPE) was from Molecular Probes (Eugene, Ore., USA). Copper (II) sulfate pentahydrate (CuSO₄·5H₂O) and calcine (3,3′-bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Methods

Preparation of the dispersed particles

VK and PC or VK, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, water was added to give a final concentration of 5 mM of total lipids. Dispersed particles composed of VK and PC at various VK mole fractions (XVK = VK/ [VK + PC]) and particles composed of VK, SO and PC at various VK/SO mole fractions (XM = [VK + SO]/[VK + PC + SO]) were obtained by sonication for 30 min under a stream of nitrogen gas at 50 °C. A model UD-200 probe-type sonicator (Tomy Seiko Co., Tokyo, Japan) was used at a power setting of 100 W. After cooling to room temperature, 5 ml of the dispersion was dispensed into the glass ampoules filled with nitrogen gas and the ampoules were sealed. The ampoules were stored at room temperature for 3 months.

Determination of particle size

The sizes of the VK/PC particles or VK/SO/PC particles stored at room temperature for 0, 1 and 3 months were measured with a DLS-7000DL submicron analyzer (Ohtsuka Electronics Co., Osaka, Japan) at 25 °C. The data were analyzed by the histogram method [7], and the weight-average particle sizes were evaluated.

Determination of the trapped volume inside the dispersed particles

In order to obtain information on the structural changes of VK/PC and VK/SO/PC particles as a function of XVK and XM, the volume trapped inside the particles was determined using the aqueous space marker calcine. Dried mixtures of VK and PC or VK, SO and PC (total 25 μM) were dispersed in a 5 ml of 70 mM calcine solution (pH 7.0) instead of water for the preparation of the dispersion. Untrapped calcine was removed by gel filtration (Sephadex G-50, buffer 5 mM trihydroxymethylaminoethane-HCl, 150 mM NaCl, pH 7.0). The volume of the calcine solution trapped in the dispersed particles was determined fluorometrically [8] after dissolution of the lipid particles after addition of 10% Triton X-100, and the aqueous volume trapped per mole of PC was evaluated. The PC in the dispersion was measured as described before [9].

Measurements of spreading pressure

In order to evaluate the miscibility of VK, SO and PC in the bulk phase, the spreading pressure of the lipid mixtures was measured. The VK, SO and PC were dissolved in chloroform and mixed. After evaporation of the solvent, the dried lipid mixtures were added on the surface of distilled water in a tensiometer (model CBVP-A3, Kyowa Kaimenkagaku Co., Tokyo, Japan). The spreading pressure of the lipid mixtures at the air/water interface was the steady-state value of surface pressure at 6–8 h after addition of the lipid or the lipid mixtures on the water surface at 25 °C. Details of the monolayer techniques were described elsewhere [10, 11].

Fluorescence quenching

The fluorescence quenching technique [12] was used to obtain information on structural changes [ratio of the number of PC molecules which exists in external and total (external plus internal) membrane] in the VK/SO/PC dispersed particles. CuSO₄ was used as a quencher for the Dansyl-DHPE fluorescence embedded in the lipid particles. The VK/SO/PC dispersed particles containing 1 mol% of Dansyl-DHPE were titrated with small aliquots of 1 M CuSO₄. The fluorescence intensity, I, at 515 nm (with excitation at 335 nm) was measured as a function of the Cu²⁺ concentration [Q]. Assuming that only the fluorescence of the Cu²⁺ accessible Dansyl-DHPE is quenched according to the Stern–Volmer equation [13], one can estimate the exposed fraction of Dansyl-DHPE, P, so

\[ I_0/Q = (1/P)Q + 1/KP, \]

where \( I_0 \) is fluorescence intensity in the absence of the quencher, \( I \) the intensity after quenching by Cu²⁺, \( [Q] \) the concentration of Cu²⁺ and \( K \) the Stern–Volmer constant.

Results and discussion

Size and stability of the dispersed particles

Figure 1 shows that the diameter of the dispersed particles was a function of XVK. (Fig. 1a, for VK/PC particles) and XM (Fig. 1b, for VK/SO/PC particles). Immediately after preparation, up to \( X_{VK} = 0.7 \), the particle size of VK/PC dispersions was almost 40 nm and at \( X_{VK} = 0.8 \) and 0.9, the size was increased to 80 and 150 nm, respectively. At \( X_{VK} = 0.3–0.9 \), the size was increased during storage at room temperature for 3 months. Up to \( X_M = 0.7 \), the size of the particles in the VK/SO/PC mixture varied between 50 and 70 nm. Separation of the dispersion into an air and a water phase or an increase in the size was not observed for VK/SO/PC at \( X_M < 0.7 \) within 3 months at room temperature; however, at \( X_M = 0.8 \), the particle diameter became considerably larger (160 nm), and phase separation was observed 72 h after preparation. At \( X_M = 0.9 \), the particle diameter was 220 nm and the separation was detected within 24 h after preparation.