Studies on the Dissolution Behavior of Doxorubicin Hydrochloride Freeze-Dried Product

Eiji HAYAKAWA,* Kunitoshi FURUYA, Tokuyuki KURODA, Masuo MORIYAMA, and Akira KONDO

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan.
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Doxorubicin hydrochloride interacts with various metal salts in aqueous solution and is associated with the prolongation of dissolution time. When a doxorubicin hydrochloride freeze-dried product was dissolved with isotonic sodium chloride solution, several small jelly-like spheres appeared locally. The further addition of NaCl promoted the gel formation and the whole solution turned into a viscous liquid.

A diffusion experiment using filter paper and X-ray microanalyzer analysis indicated that the highly viscous structure, which was the cause of the prolongation of dissolution time, was formed on the surface of the small spheres by the interaction of doxorubicin with NaCl.

We found that methyl parahydroxybenzoate, L-phenyl alanine, urea, and citric acid, *etc.* had a shortening effect on the dissolution time of the doxorubicin hydrochloride freeze-dried product, and moreover, these additives reduced the viscosity of the aqueous solution of doxorubicin hydrochloride enhanced by the addition of NaCl.

Keywords doxorubicin hydrochloride; dissolution time; X-ray microanalyzer; ionic strength; gel formation; freeze drying

Introduction

Doxorubicin (Fig. 1) is a widely used antitumor agent. The formulation is a freeze-dried product of doxorubicin hydrochloride and its excipients. On reconstitution, water for injection, JP, or isotonic sodium chloride solution, JP, is used as a diluent.

When reconstituted with water for injection, it is generally satisfactory. But, it has a tendency for gel to appear locally when it is reconstituted with isotonic sodium chloride solution. Consequently, it leads to the prolongation of dissolution time.[1,2]

Murphy *et al.*[1] reported that the addition of hydroxybenzoate decreased the dissolution time. However, a detailed mechanism is not clear. We investigated the dissolution behavior of the doxorubicin hydrochloride freeze-dried product and the effect of additives on the dissolution behavior. We found that several compounds including methyl parahydroxybenzoate had a shortening effect on the dissolution time of the doxorubicin hydrochloride freeze-dried product. The shortening effect seems to be closely related to the reduction of the viscosity of the aqueous solution of doxorubicin hydrochloride enhanced by the addition of NaCl.

Experimental

Materials Doxorubicin hydrochloride was provided by Farmitalia Carlo Erba Co. All other reagents employed were commercial special-grade products.

Dissolution Time A freeze-dried formulation containing 10 mg of doxorubicin hydrochloride and 100 mg of lactose in a vial was prepared. When reconstituted with 5 ml of water for injection, the pH of the solution was in the range of 5.3—5.7.

Five milliliters of each reconstitution solvent was added to the vial within 10 s with a syringe with a No. 1/2 needle for subcutaneous use. The mixture was shaken at a rate of 5 times per second and was allowed to stand for gross observation. After that, it was shaken 5 times every 30 s and the time, t (s), required for the contents to dissolve completely from the start of the addition of the solvent was measured with a stopwatch.

Diffusion Distance into the Filter Paper The testing assembly, as illustrated in Fig. 2, consisted of a filter paper, a petori dish and a glass capillary tube which was 50 mm long and had a 5-mm inner diameter with both ends open.

Filter paper (Toyo Filter Paper Co., No. 5A) was put in a petori dish with a radius of 4.5 cm and immersed in water for injection (3 ml) or isotonic sodium chloride solution (3 ml).

The doxorubicin hydrochloride freeze-dried product described above was packed into the glass capillary tube in a layer of about 10 mm. Then the capillary tube was stood vertically in the center of the filter paper and radius of the circle, diffusion distance L(cm), which developed a red color, was measured with the passage of time.

X-Ray Microanalysis In order to examine their surface character, the gelatinous small spheres which appeared in the above described doxorubicin hydrochloride freeze-dried product when it was reconstituted with isotonic sodium chloride solution, were collected with dissecting forceps.

After drying, these spheres were cut with a knife and coated with carbon by a vacuum deposition apparatus (Nihondenshi JEOL JEE-3X), and observed under a scanning electron microscope (Nihondenshi JEOL JSM-820) at an acceleration voltage of 20 keV.

Then elemental analysis of the surface and central part of small spheres was conducted with an energy dispensing X-ray microanalyzer (Link Co. AN10000S QX200J).

Water Content The water content of the gelatinous small spheres was measured from the moisture loss after drying at 105 °C for 3 h.

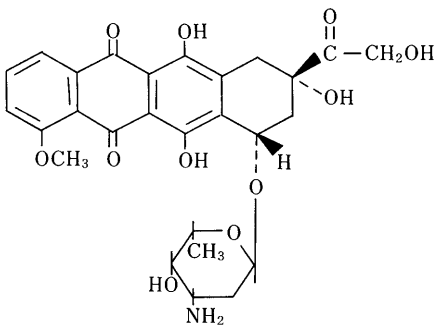

Fig. 1. Structure of Doxorubicin

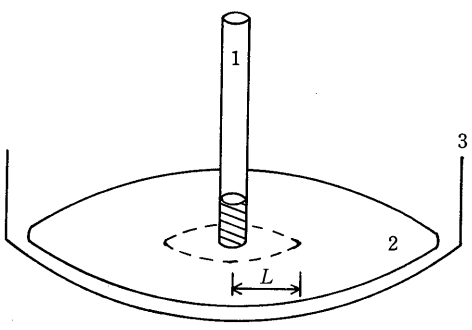

Fig. 2. Apparatus for Measuring Diffusion Distance

1, capillary tube; 2, filter paper; 3, petri dish; L, diffusion distance.

Effect of Additives on the Dissolution Time The dissolution times of the following three systems were compared. (1) Doxorubicin hydrochloride, lactose and each additive were mixed and ground to a powder and dispensed into a vial (physical mixture). After that, the mixture was reconsituted with an isotonic sodium chloride solution. (2) Doxorubicin hydrochloride, lactose and each additive were dissolved in water and dispensed into the vial. After that, the solution was freeze-dried and reconstituted with the isotonic sodium chloride solution. (3) Doxorubicin hydrochloride and lactose were dissolved in water and dispensed into the vial. After that, the solution was freeze-dried and reconstituted with the isotonic sodium chloride solution containing each additive.

Viscosity The measurement of the solution viscosity was made using an Ubbelohde-type viscometer at 25 °C. The kinematic viscosity, v (cSt), of the solution was obtained as a function of the concentration of various additives.

The solution was prepared as follows.

Each additive was mixed with an aqueous solution containing doxorubicin hydrochloride and NaCl, and then dissolved completely.

Thermal Analysis A Seiko SSC-580 II differential thermal analyzer (DTA) was used to study the influence of methyl parahydroxybenzoate on the thermal properties of doxorubicin and lactose in the freeze-dried product. *ca.* 3 mg of the sample was analyzed in an aluminum pan on the apparatus. Thermograms of the samples were run at a heating rate 10 °C/min from room temperature to 280 °C, at a sensitivity of 100 μV.

Results and Discussion

Dissolution Time of Doxorubicin Hydrochloride Freeze-Dried Product in Various Metal Salt Aqueous Solutions

When a doxorubicin hydrochloride freeze-dried product is reconstituted with isotonic sodium chloride solution, a gel is formed and the dissolution time is prolonged.[1,2] We investigated the effect of various metal chloride salts on the dissolution time in order to provide a basis for the understanding of the prolonged dissolution time.

After preparing a doxorubicin hydrochloride freeze-dried product by the method mentioned in the experimental method, it was reconstituted with various concentrations of NaCl or, 0.9% of various metal chloride aqueous solutions, and the dissolution time was measured (Fig. 3, Table I).

According to Fig. 3, as the concentration of NaCl increased, the dissolution time was prolonged. We found that when the doxorubicin hydrochloride freeze-dried product was dissolved not only with NaCl but also with all the metal chloride salt solutions, prolongation of the dissolution time occurred.

Although it is not clear for an univalent metal ion, it is reported that bivalent and trivalent metal ions coordinate to the functional group of C_{11}-OH and $C_{12}=0$ of doxorubicine and form a six-member chelate.[3] It is also reported that Cl^-, which is a counterion, has the effect of shielding the charge of $-NH_3^+$ ($pK_a=7.4$) in the sugar moiety.[4] When the concentration of Cl^- is increased under a fixed pH condition, the electrostatic repulsion force of the positively charged doxorubicin molecules is inhibited, and the self-association of doxorubicin is promoted.[4]

Therefore, it is considered that the addition of various metal chloride salts promotes the self-association of

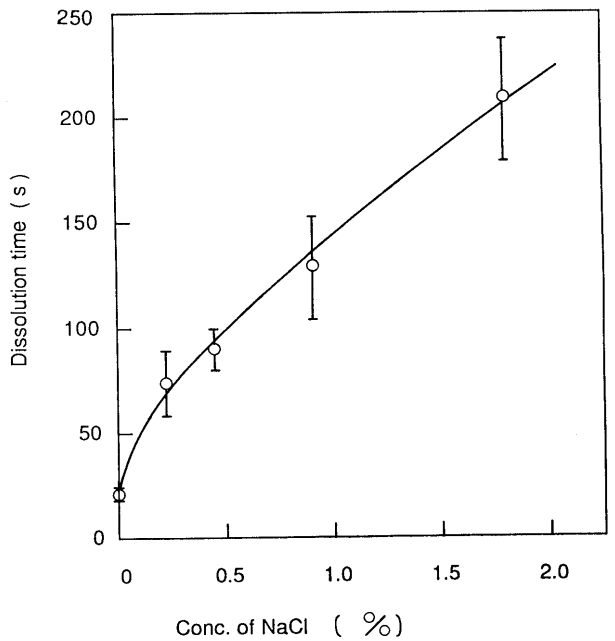

Fig. 3. Dissolution Time of the Freeze-Dried Product of Doxorubicin Hydrochloride (10 mg) and Lactose (100 mg) on Reconstitution with Various Conc. of NaCl Aq. Soln. at 25 °C

The data are expressed as mean ± S.D. ($n=5$).

TABLE I. Dissolution Time of Freeze-Dried Mixture of Doxorubicin Hydrochloride (10 mg) and Lactose (100 mg) on Reconstitution with Water for Injection, Isotonic Sodium Chloride Solution, and Various Metal Chloride Solutions at 25 °C

Solvent	pH		Dissolution time (s) mean ± S.D. ($n=5$)
	Before dissolution	After dissolution	
Water for injection	6.2	5.5	21 ± 2
Isotonic sodium chloride solution	6.0	5.5	130 ± 25
0.9% KCl	5.6	5.2	130 ± 16
0.9% $CaCl_2$	8.5	6.7	125 ± 20
0.9% $MgCl_2$	6.9	5.7	105 ± 31
0.9% $BaCl_2$	5.8	5.3	100 ± 36
0.9% $MnCl_2$	5.7	5.3	95 ± 36
0.9% $NiCl_2$	5.8	4.8	90 ± 18
0.9% $CuCl_2$	4.0	2.8	80 ± 36
0.9% $FeCl_3$	2.1	2.0	65 ± 25
0.9% $AlCl_3$	3.4	3.2	80 ± 27

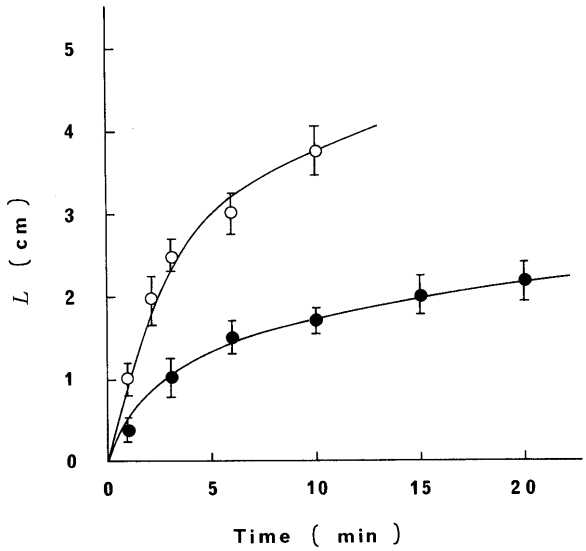

Fig. 4. Diffusion Curves of Doxorubicin Hydrochloride into Filter Paper at 25 °C

○, water for injection; ●, isotonic sodium chloride solution. The data are expressed as mean ± S.D. ($n=5$).

doxorubicin, which leads to gel formation and the prolongation of the dissolution time.

Diffusion Distance of Doxorubicin into Filter Paper In order to evaluate the interaction between doxorubicin and NaCl, the diffusion distance of the doxorubicin hydrochloride freeze-dried product packed into the glass capillary tube was examined.

Figure 4 shows the diffusion distance of doxorubicin into the filter paper with the passage of time. It was observed that the diffusion of doxorubicin into isotonic sodium chloride solution was slow compared with that of water for injection. The slow diffusion of doxorubicin suggests an interaction with NaCl at the front of the red circle which developed.

Analysis of Gelatinous Small Spheres When the doxorubicin hydrochloride freeze-dried product was reconstituted with isotonic sodium chloride solution, most of the doxorubicin dissolved comparatively rapidly (within about 30 s), however, a few transparent small jelly-like spheres with a diameter of about 2 mm were partially produced.

The elemental analysis of the surface and central part of the small spheres was conducted with an X-ray microanalyzer.[5] As shown in Fig. 5, the characteristic X-ray of the Na element (1.041 keV) was detected on the surface of the small spheres, but it was not detected in the central part. Since doxorubicin is hydrochloride salt, the characteristic X-ray of the Cl element (2.622 keV) was detected both on the surface and in the central part of the small spheres.

The water content of the small spheres was 83.0%, which was less than that of the bulk solution (91.2%).

From the above, it is speculated that NaCl interacts with doxorubicin hydrochloride on the surface of the small spheres and forms a gelatinous highly viscous structure, which prevents water from penetrating into the spheres and prolongs the dissolution time of the doxorubicin hydro-

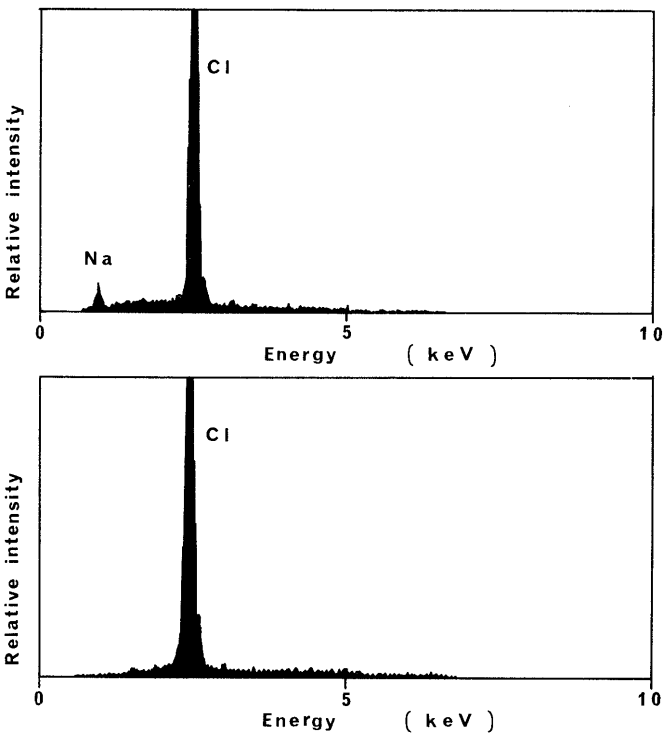

Fig. 5. Energy Dispensing X-Ray Spectrum of Gelatinous Small Spheres in the Doxorubicin Aqueous Solution Reconstituted with Isotonic Sodium Chloride Solution

Surface of gelatinous small sphere (top plot) and center of gelatinous small sphere (bottom plot).

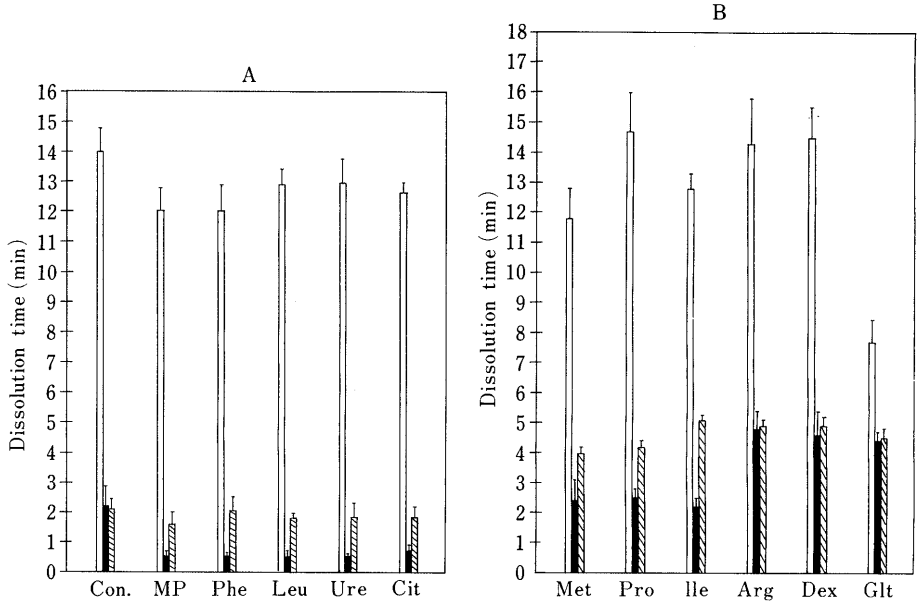

Fig. 6. Effect of Additives on the Dissolution Time of Doxorubicin Hydrochloride at 25 °C

The data are expressed as mean ± S.D. ($n=5$).

□: physical mixture of doxorubicin hydrochloride (10 mg), lactose (100 mg) and additives dissolved with isotonic sodium chloride solution (5 ml).

■: freeze-dried product of doxorubicin hydrochloride (10 mg), lactose (100 mg) and additives reconstituted with isotonic sodium chloride solution (5 ml).

▨: freeze-dried product of doxorubicin hydrochloride (10 mg) and lactose (100 mg) reconstituted with isotonic sodium chloride solution (5 ml) containing additives. additives, amount added (mg/vial), pH of the reconstituted solution

(A) Con: non, 0, pH 5.5; MP: methyl parahydroxybenzoate, 1, pH 5.5; Phe: L-phenylalanine, 10, pH 5.5; Leu: L-leucine, 10, pH 5.2; Ure: urea, 25, pH 5.3; Cit: citric acid, 15, pH 2.6.

(B) Met: L-methionine, 10, pH 5.3; Pro: L-proline, 10, pH 5.2; Ile: L-isoleucine, 10, pH 5.3; Arg: L-arginine hydrochloride, 10, pH 5.5; Dex: dextran 40, 10, pH 5.3; Glt: L-glutathione, 10, pH 3.0.

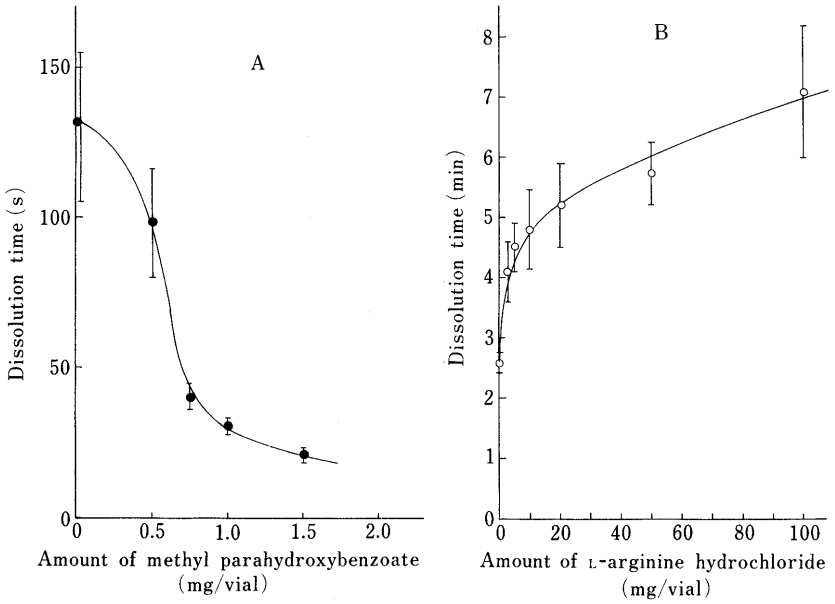

Fig. 7. (A) Effect of Methyl Parahydroxybenzoate on the Dissolution Time of Frecze-Dried Product of Doxorubicin Hydrochloride (10 mg) and Lactose (100 mg) and Methyl Parahydroxybenzoate (0—1.5 mg) on Reconstitution with Isotonic Sodium Chloride Solution (5 ml) at 25 °C, at pH 5.5, (B) Effect of L-Arginine Hydrochloride on the Dissolution Time of Freeze-Dried Product of Doxorubicin Hydrochloride (10 mg) and Lactose (100 mg) and L-Arginine Hydrochloride (0—100 mg) on Reconstitution with Isotonic Sodium Chloride Solution (5 ml) at 25 °C, at pH 5.5

The data are expressed as mean ± S.D. ($n=5$).

chloride freeze-dried product.

Effect of Additives on the Dissolution Time The dissolution times of the three systems described in the experimental method were compared. Many types of additives were screened from the viewpoint of their shortening effects on the dissolution time of the doxorubicin hydrochloride freeze-dried product.

As a result, aromatic compounds (methyl parahydroxy benzoate, *para*-amino benzoic acid, *n*-propyl gallate, L-phenylalanine, L-tryptophan, *etc.*), L-leucine, urea and organic acids (citric acid, thiomalic acid, *N*-acetylglutamic acid, *etc.*) were found to be additives which had a strong shortening effect on the dissolution time. The aromatic compounds, L-leucine and urea were especially effective at pH 5.3—5.5 which is close to the physiological pH and favorable in the preparation of the injection. The organic acids were effective around pH 2.5, but when the pH was elevated to around 5.5 with NaOH aqueous solution, the dissolution time was prolonged. Inorganic acids such as HCl or H_2SO_4 shortened the dissolution time around pH 2.0—2.5, however their effects were weaker compared with those of organic acids.

The dissolution time decreased as the amount of additives added was increased. Figure 6A shows the results concerning methyl parahydroxybenzoate, L-phenylalanine, L-leucine, urea and citric acid at the added amount required to effectively shorten the dissolution time of the freeze-dried product.

From Fig. 6A, the following points are found. (1) Although the additive was added to doxorubicin hydrochloride as a physical mixture, the shortening effect on the dissolution time was very slight. The additive was also added to the isotonic sodium chloride solution, and the effect was barely evident when compared with a control case (no additive). (2) In order for the additive to exert a shortening effect on the dissolution time, it was necessary to freeze-dry the additive together with doxorubicin hydrochloride and lactose.

Figure 6B shows the results concerning additives which had similar dissolution time or prolonged dissolution time compared with a control case, when the doxorubicin hydrochloride freeze-dried product containing an additive was dissolved with the isotonic sodium chloride solution.

According to Figs. 6A, 6B, the dissolution times of physical mixtures were much longer than those of freeze-dried products. The difference might be explained in terms of the concentration effect of doxorubicin on the self-association. Physical mixtures have higher concentrated regions of doxorubicin locally compared with freeze-dried products, and it is known that the self-association of doxorubicin increases by increasing the concentration.[3]

Figure 7A shows the relation between the added amount of methyl parahydroxybenzoate and the dissolution time in the isotonic sodium chloride solution. Methyl parahydroxybenzoate was freeze-dried together with doxorubicin hydrochloride and lactose.

It was observed that methyl parahydroxybenzoate possessed a sufficient shortening effect on the dissolution time when an amount of 1 mg was added per 1 vial.

Figure 7B shows the result concerning L-arginine hydrochloride. Contrary to methyl parahydroxybenzoate, an increase in the added amount of L-arginine hydrochloride prolonged the dissolution time.

Effect of Additives on the Viscosity of Doxorubicin Hydrochloride Under the conditions of Figs. 6, 7, the viscosity of all solutions was almost the same as that of water (about 1 cSt) after the dissolution of doxorubicin and the effect of additives on the viscosity was not observed.

Doxorubicin interacts with NaCl in an aqueous solution and the viscosity of the solution increases quickly with the concentration of doxorubicin and NaCl.[6,7] The effect of various additives on the viscosity of doxorubicin in a NaCl

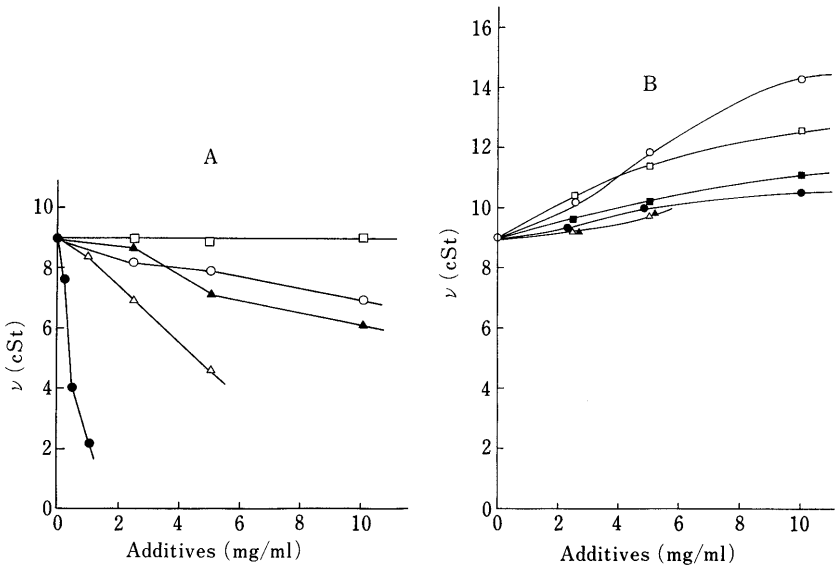

Fig. 8. Effect of Additives on the Viscosity of Doxorubicin Hydrochloride Aqueous Solution at 25 °C

[doxorubicin hydrochloride] = 5 mg/ml, [NaCl] = 15 mg/ml. The pH of the doxorubicin hydrochloride aqueous solution in the absence of additives was about 5.5. The pH of each solution in the presence of additives was not adjusted and it is described in the parenthesis.
(A) ○, citric acid (pH 2.5); ●, methyl parahydroxybenzoate (pH 5.5); △, L-phenylalanine (pH 5.5); ▲, urea (pH 5.5); □, L-leucine (pH 5.2).
(B) ○, L-arginine hydrochloride (pH 5.5); ●, L-isoleucine (pH 5.5); △, L-glutathione (pH 3.0); ▲, L-methionine (pH 5.5); □, dextran 40 (pH 5.5); ■, L-proline (pH 5.5).

aqueous solution was examined.

The kinematic viscosity, v, of the doxorubicin aqueous solution is shown in Fig. 8 as a function of the concentration of various additives. In the absence of additives, the doxorubicin aqueous solution containing NaCl was a viscous solution with a viscosity of about 9 cSt. Under the condition of Fig. 8, the concentration of doxorubicin is 2.5 times higher than that of Figs. 6, 7.

Figure 8A shows the results concerning additives which had a shortening effect on the dissolution time (described in Fig. 6A). On the other hand, Fig. 8B shows the results concerning additives which did not have a shortening or prolonging effect on the dissolution time of the freeze-dried product (described in Fig. 6B). The decrease in the viscosity caused by the addition of additives was observed for all except L-leucine in Fig. 8A. The effect depended upon the kind of additives and the concentration of additives.

The effect of methyl parahydroxybenzoate on the decrease in viscosity was the strongest of the additives. L-Phenylalanine, urea and citric acid also had fairly strong effects. But, L-leucine had no effect on the viscosity change. Unlike the other additives, the addition of citric acid caused a decrease in the pH of the solution.

An increase in the viscosity caused by the addition of additives was observed for all in Fig. 8B.

The results were fairly consistent with the results of the dissolution time of the freeze-dried product containing each additive described in Fig. 6.

Self-association of anthracycline compounds in solution has been studied using the UV/VIS absorption spectrum,[8] circular dichroism (CD)[9] and proton nuclear magnetic resonance (^{1}H-NMR).[10]

The mechanism of self-association is ascribed to the interaction between the planar aromatic rings. This process is called stacking and is based on the interaction between π-electron systems.[9]

Nishijo *et al.*[11] reported interaction between different molecules owing to π-π stacking. We also found interaction

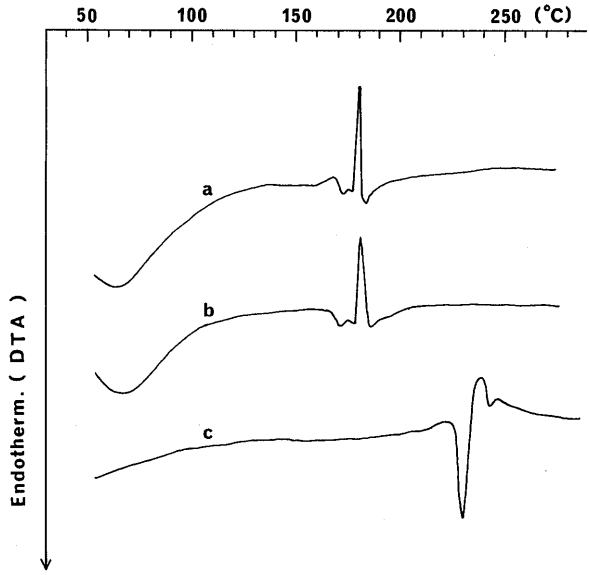

Fig. 9. Effects of the Addition of Methyl Parahydroxybenzoate on the Thermal Properties of Doxorubicin Hydrochloride Freeze-Dried Product

a, freeze-dried product of doxorubicin hydrochloride (10 mg) and lactose (100 mg); b, freeze-dried product of doxorubicin hydrochloride (10 mg) and lactose (100 mg) and methyl parahydroxybenzoate (1 mg); c, doxorubicin hydrochloride raw material.

between doxorubicin and methyl parahydroxybenzoate owing to π-π stacking using ^{1}H-NMR.[7]

The thermal properties of the doxorubicin hydrochloride freeze-dried product were examined by DTA (Fig. 9). The exothermic peak (about 180 °C) of amorphous lactose obtained by freeze-drying was observed.[12] The doxorubicin hydrochloride raw material is crystalline and the melting point is about 230 °C. It was found that both doxorubicin hydrochloride and lactose existed in an amorphous state in the freeze-dried product, and the addition of methyl parahydroxybenzoate had no affect on the amorphous state.

The self-association of doxorubicin increases with an increase in the concentration.[3] It is reported that the

molecules of doxorubicin are concentrated into the concentrated region during the freezing process.[6] Therefore, it is speculated that the concentrated region in the freeze-dried product has the tendency of self-association.

From the results mentioned above, it is thought that aromatic compounds such as methyl parahydroxybenzoate and L-phenylalanine inhibit the self-association of doxorubicin by interacting with the molecules of doxorubicin owing to π-π stacking and reduce the viscosity of the solution, exerting a dissolution promoting effect.

It is reported that urea interacts strongly with water molecules and acts as a water structure breaker.[13] Since urea reduces the viscosity of doxorubicin aqueous solution and shortens the dissolution time of the freeze-dried product, this strongly suggests the involvement of water molecules besides the self-association of doxorubicin as the mechanism of gel formation. Therefore, it is thought that urea inhibits gel formation by influencing the interaction between doxorubicin and water.

In the case of organic acid, it appears that by decreasing the pH of the solution, the electrostatic repulsion force between the positively charged doxorubicin molecules is elevated and it inhibits the self-association of doxorubicin. But, the difference between organic acids and inorganic acids is unclear now.

According to the result of the viscosity experiment, unlike other additives, L-leucine appears to have no influence on the self-association of doxorubicin, but, it had a strong effect on the dissolution time. L-Leucine is a hydrophobic amino acid. But, other hydrophobic amino acids (L-proline, L-methionine, L-isoleucine) did not have such a strong effect. Therefore, the effect of L-leucine on the dissolution time appears to be characteristic for L-leucine, but the mechanism is unclear.

According to Figs. 6, 7, 8, additives which had a shortening effect on the dissolution time reduced the viscosity of doxorubicin hydrochloride aqueous solution enhanced by the addition of NaCl as a whole. On the other hand, additives which did not have a shortening effect or prolonged the dissolution time enhanced the viscosity of the solution. On the basis of the results, it is considered that the shortening effect of additives on the dissolution time is closely related to the reduction of the viscosity of doxorubicin hydrochloride aqueous solution.

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