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# THE EFFECT OF CYCLODEXTRIN INCLUSION COMPLEXATION ON THE IN VITRO PROTEIN BINDING AND IN VIVO PROTHROMBIN TIME OF WARFARIN*

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## ABSTRACT

That the inclusion complexation occurred in an aqueous solution was proved by solubility determination and a membrane permeation study. The effects of inclusion complexation on the protein binding and prothrombin time of warfarin were studied by the technique of ultrafiltration and prothrombin time measurements respectively. The apparent stability constant of 1:1 complex was obtained from the initial portion of the straight line of phase solubility diagrams. The apparent stability constant of α or β-cyclodextrin complex is 10.29 $M^{-1}$ or 148.88 $M^{-1}$. The greater the stability constant of the inclusion

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** Correspondence

complex the less the permeability of the warfarin. The magnitude of the stability constant of the inclusion complex also determined the protein binding and the prothrombin time of warfarin. The greater the stability constant of the inclusion complex, the lesser the protein binding and the more prolonged the prothrombin time of warfarin. β-cyclodextrin showed a significantly different behavior from α-cyclodextrin in vitro protein binding and in vivo prothrombin time. The present results indicate a greater stability constant of the inclusion complex formed might determine the binding of warfarin to ablbumin and lead to an increased anticoagulant activity of warfarin.

## INTRODUCTION

Cyclodextrins are cyclic oligosaccharides possessing a doughnut-shaped hydrophobic cavity in which a number of drugs may be clathrated forming non-covalently bonded inclusion complexes (1). Once a guest drug molecule is included in the cavity, the original physicochemical and biochemical properties of the drug may be altered by complexation. Recently, this inclusion phenomenon has received considerable attention in pharmaceutical fields because of their ability to form inclusion complexes with many drugs either in the solid phase or in solution (2). Thus,

cyclodextrins have been extensively ultilized in pharmaceutical formulations to improve debittering, chemical stability, aqueous solubility, dissolution rate and bioavailability of many pharmaceuticals (3-7).

Warfarin is an extensively used rodenticide and is a clinically effective oral anticoagulant for treatment of thromboembolic occlusive vascular diseases (8). It is an indirect anticoagulant which has no action in vitro whereas heparin inhibits the coagulation of blood both in vivo and in vitro. Since the effect of warfarin appears about 12-24 hours after administration, the absorption and fate of warfarin in vivo is significantly influenced by other drugs (9,10). Many studies strongly show that there are significant differences in the bioavailability of warfarin from different dosage forms, and also show that absorption of warfarin in man is dissolution rate controlled (11,12). This may be attributed to the practically poor water solubility of warfarin and its easy interaction with various excipients and drugs in vitro and in vivo.

In the present study, the complex formation of warfarin with cyclodextrin in water was proved by solubility methods and membrane permeation studies. The effect of cyclodextrins on the protein binding of warfarin has been studied by use of ultrafiltration

techniques in vitro. Complexation of warfarin with cyclodextrin affecting the prothrombin time of warfarin in rats was also examined.

## MATERIALS AND METHODS

### Animals and Materials

Male Sprague-Dawley rats weighing between 250-275 grams served as the subjects. The animals were fasted, feed was withheld the night before and throughout the experiment, but water was allowed at all times during the experiments. The α-cyclodextrin(α-CD)[1], β-cyclodextrin(β-CD)[1], warfarin[2] and human albumin[2] were used.

### Solubility Determinations

Solubility determinations were carried out according to the method of Higuchi and Connors (13). Excess amounts of warfarin were added to glass stoppered conical flasks with had various concentrations of cyclodextrin aqueous solution contained in them. The flasks were set in a water bath and shaken continuously at 37 ± 0.5 °C. After equilibration was attained an aliquot was pipetted and filtrated through a membrane filter. A portion of the sample was adequately diluted with 0.1N NaOH solution and analyzed spectrophotometrically at 308 nm.

### Membrane Permeation Studies

The membrane permeation apparatus and method

described previously (3) were used for the measurements of the permeation behavior of warfarin. The cellophane membrane[3] was previously boiled in distilled water and washed with distilled water several times before being used. pH 7.0 Mcllvaine buffer solution was used as a solvent. One hundred milliliters of warfarin solution ($4.638 \times 10^{-4}$ M) in the absence and presence of α-CD or β-CD ($4.638 \times 10^{-4}$ M) was put into a donor compartment. while the same volume of Mcllvaine buffer solution was put into a receptor compartment. The studies were undertaken at 37±0.5°C. At prescribed intervals, a sample was removed from the receptor solution and assayed spectrophotometrically at 308 nm.[4]

In Vitro Protein Binding Measurements

In vitro protein binding measurements were carried out according to the method of Solomon et al. (14) with slight modificition. pH 7.0 Mcllvaine buffer solution was also used as a solvent. In the group without cyclodextrins, the warfarin solution (15 mg/ml) was prepared with and without human albumin (9 mg/ml) as a stock solution. Ten milliliters of each stock solution were incubated at 37±0.5°C in a water bath for 30 minutes. Four milliliters of solution were pipetted into Centriflo membrane cone[5] and then centrifuged at 3000 rpm for one hour during which temperature was controlled at 37 ± 1 °C. After centrifugation, the

concentration of the unbound drug leached out from the membrane cone was analyzed spectrophotometrically. However in the presence of the cyclodextrins group, cyclodextrin powder (α: 160 mg; β : 186 mg) was directly added and dissolved into the warfarin solution before human albumin was added. Each mixture was also incubated at 37±0.5°C for 30 minutes. The following step was according to the above process.

In Vivo Determination of 24 hour Prothrombin Times

Rats were lightly anethetized with ether and warfarin powder administrated by gavage (10 mg/ 100 g BW). α-CD solution (32 mg/ 100 g BW), β-CD solution (36.8 mg/ 100 g BW) and the same amounts of warfarin-cyclodextrin solutions were also orally administrated by gavage. The control group received the distilled water orally.

Twenty-four hours after administration of each sample, the rats were etherized and blood was directly withdrawn from the heart. 0.9 ml of blood was immediately and carefully mixed with 0.1 ml of 3.8% sodium citrate solution in a conical test tube. This mixture was transfered to a clean centrifuge tube free of thrombin and detergents, then centrifuged for 10 minutes at 3000 rpm in a cold condition. 0.1 ml of supernatant of citrated plasma in test tube was incubated for one minute at 37±0.5°C. Then, 0.2 ml of

the prewarmed (37±0.5 °C) Thromborel solution[6] was added to this test tube and the 24 hour prothrombin time was determined with Behring Fibritimer[6] which was an automatic detector for the determination of clotting time.

## RESULTS

### Inclusion Complexation in Aqueous Solution

The phase solubility diagrams for warfarin with α-CD and β-CD in water at 37 °C are shown in Fig. 1. It is obvious that interaction between warfarin and cyclodextrin increased slightly in an upward curvature as a function of the α-CD concentration, and the resulting solubility curve can be classified as a typical $A_P$ type phase-solubility diagram although a plateau region appeared (13). But in the β-CD system, the solubility of warfarin rapidly increased as a straight line with increasing concentrations of β-CD, showing a typical $A_L$ type phase-solubility curve. The ascending part of the curve is a straight line or nearly straight line and may be ascribed to the formation of a 1:1 complex (2,13).

Another proof for an inclusion complexation in aqueous solution was discovered in membrane permeation studies. Fig. 2 shows the permeation profiles of warfarin from the donor solution through cellophane

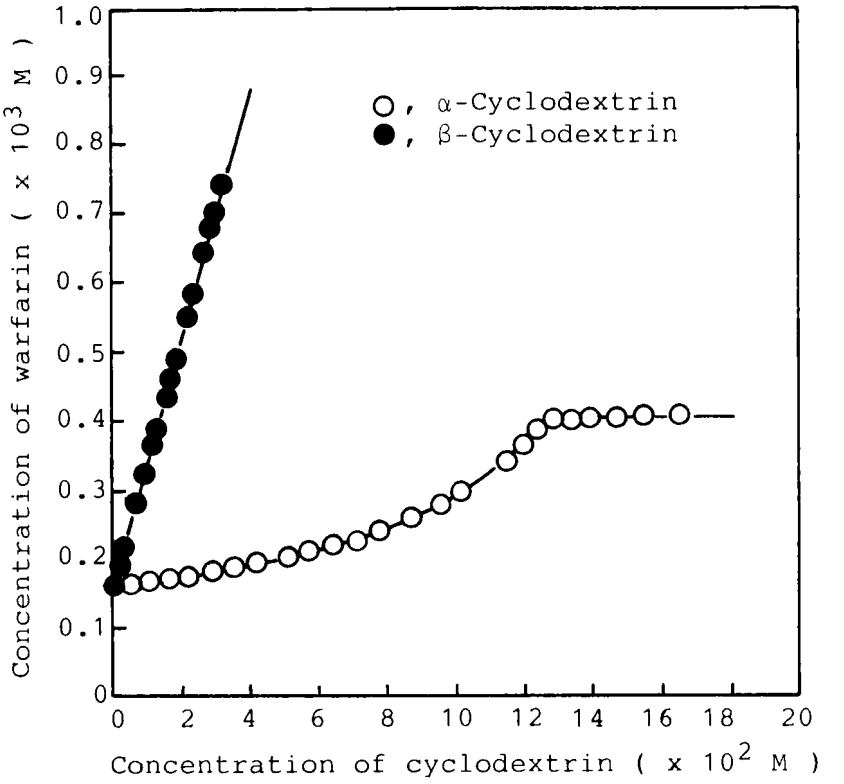


FIG. 1

Phase Solubility Diagrams of Warfarin-Cyclodextrins System in Water at 37°C

membrane in the absence and presence of α-CD or β-CD. It is evident that in the presence of α-CD or β-CD warfarin permeation was slower than in the warfarin alone group. Inclusion complexation was formed in the donor cell before permeation, this results in a lower concentration of warfarin in the receptor cell. There is no doubt that inclusion complexation occurred between warfarin and cyclodextrins in the aqueous solution.

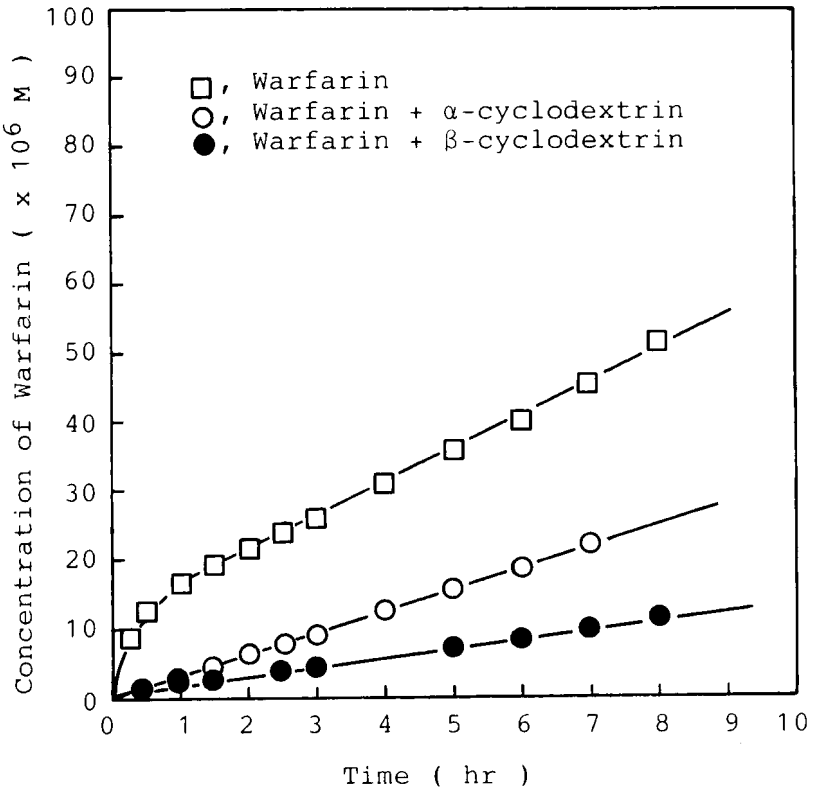


FIG. 2

Permeation Profiles of Warfarin in the Absence and Presence of Cyclodextrins in pH 7.0 McIlvaine Buffer Solution at 37°C

## In Vitro Protein Binding

Table I indicates that in the presence of human albumin all the percentages of unbound warfarin were significantly smaller than in the warfarin alone group ($p<<0.001$). Interaction of warfarin and human albumin inhibits the permeation from membrane cone even if ultrafiltration technique is used. Moreover, in the α-CD group, there was no significant difference ($p<0.2$)

TABLE I

Effect of α and β-Cyclodextrin on the Protein Binding of Warfarin In Vitro

| Groups | Test No. | % of Unbound Warfarin | P Value |
|---|---|---|---|
| Warfarin | 10 | 16.95±1.359 | |
| Warfarin + Albumin | 9 | 2.175±0.465 | p<<0.001* |
| Warfarin + α-cyclodextrin + Albumin | 8 | 2.914±0.482 | p<<0.001*, NS** (p<0.2) |
| Warfarin + β-cyclodextrin + Albumin | 10 | 4.142±0.546 | p<<0.001*, p<0.01** |

Note:
*: Significant difference when compared with warfarin group
**: Significant difference when compared with ( warfarin + albumin ) group

TABLE II

Effect of Different Excipients on the 24-hr Prothrombin Time of Warfarin in Rats Following Oral Administration

| Groups | Rat No. | Prothrombin Time (sec) (mean±S.E.M.) | P Value |
|---|---|---|---|
| Control | 12 | 10.25±0.366 | |
| Warfarin | 14 | 33.83±1.004 | p<<0.001* |
| α-Cyclodextrin | 12 | 9.146±0.246 | NS*(p<0.02) |
| β-Cyclodextrin | 12 | 9.392±0.338 | NS*(p<0.05) |
| Warfarin + α-cyclodextrin | 16 | 37.47±1.104 | p<<0.001*, NS**(p<0.05) |
| Warfarin + β-cyclodextrin | 16 | 41.85±1.171 | p<<0.001*, p<<0.001** |

Note:
*: Significant difference when compared with control group
**: Significant difference when compared with warfarin group

but significant difference was shown in the group which contained β-CD ($p<0.01$).

In Vivo 24 hour Prothrombin Time

Although fasting might enhance the prothrombin time of warfarin (9), this phenomenon can be omitted since all the present experiments occurred in the same conditions. Table II shows that when α-CD or β-CD was used there was no significant difference from the control group (α: $p<0.02$; β: $p<0.05$). A significant elevation of the 24 hour prothrombin time for warfarin containing groups was observed ($p<<0.001$). Furthermore, warfarin-β-CD group significantly increased the 24 hour prothrombin time ($p<<0.001$) whereas warfarin-α-CD group showed no significant difference compared to the warfarin alone group ($p<0.05$). There was also a significant difference between warfarin-α-CD group and warfarin-β-CD group ($p<0.01$). This suggests that a stability constant might act as an important parameter in biological effect.

## Discussion

The apparent stability constant, Kc, was calculated from the initial straight line portion of the phase-solubility diagrams according to the following equation (13).

$$Kc = \frac{\text{slope}}{\text{intercept} \; ( \; 1\text{-slope} \; )}$$

The Kc value of α-CD and β-CD is 10.29 $M^{-1}$ and 148.88 $M^{-1}$, respectively. The Kc value of β-CD is considerably larger than that of α-CD, suggesting that in aqueous media the larger the cavity size of β-CD the more favorable the fit of warfarin molecules. Moreover, it can also be predicted that the cavity of β-CD mainly entrapped the phenolic group of warfarin (15).

From the permeation study, it is obvious that the greater the stability constant of the complex the lesser the permeation of the drug. This may be due to the fact that the warfarin was located in an asymmetric cavity of β-CD leading to the higher molecular weight of a poor permeable complex with a much lower permeation rate constant since the mechanism of permeation through a membrane was mainly controlled by the pore-size (16).

Warfarin is bound to albumin in man about 97 percent, this is consistent with our data (17). The capacity and affinity are the main characteristics of the protein binding between the drug and the protein. Table I indicates that β-CD might partially inhibit the binding of warfarin to human albumin. Since interaction between warfarin and β-CD had previously occurred, when the human albumin is added, the stable inclusion complex might prevent protein binding leading

to the larger amount of unbound warfarin. However, the stability constant of α-CD was considerably smaller than that of β-CD leading to the higher binding capacity of albumin. In the absence of albumin, warfarin-α-CD or warfarin-β-CD easily leaches out from the Centriflo membrane cone just like warfarin alone since the inclusion complexation has a smaller molecular weight than the albumin-drug complex. In the presence of albumin, sterical conformation of the inclusion complex possibly hinders the interaction of warfarin with albumin although warfarin easily interacted with albumin.

Cyclodextrin has been studied to reduce prothrombine time of menadione owing to inclusion complex formation (18). In the present study, the results of the prothrombin time measurements also correspond with the in vitro protein binding determinations. The higher the stability constant the more prolonged the prothrombin time. There was no significant difference in prothrombin time for powder form of warfarin and solution form of warfarin-α-CD after oral administration, this might be due to the smaller stability constant of warfarin-α-CD. Moreover, the stability constant of warfarin-β-CD was larger than warfarin-α-CD (148.88 $M^{-1}$ >> 10.29 $M^{-1}$), thus warfarin could slowly dissociate from the cavity of inclusion

complex conformation and be continously absorbed by the GI tract leading to the prolongation of the prothrombin time. Since the cyclodextrin inclusion complex has acted as a "molecular capsule", a function similar to microcapsules or nanocapsules, controlled release of the drug from an inclusion complex to maintain a constant drug activity might be expected (19,20). Further detailed investigations are being undertaken.

## Footnotes

1. Nippon shokuhin Kako Ltd. Tokyo, Japan
2. Signa Chemical Co., St. Louis, USA
3. 3787-F45, 32mm, Thomas Dialyzer Tubing, A. H. Thomas Co., Philadelphia, USA
4. UVIKON-810, Kontron Co., Switzerland
5. Amicon CF-50A, Amicon Co., USA
6. Behring, West Germany

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