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# Spectroscopic study of the interaction of coumarin anticoagulant drugs with polyvinylpyrrolidone

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**Abstract**: The interaction of coumarin anticoagulants with polyvinylpyrrolidone (PVP) was investigated using a fluorescence technique. The fluorescence intensities of warfarin and phenprocoumon were greatly enhanced following binding to PVP, while the fluorescence of 4-hydroxycoumarin was little enhanced in the presence of PVP. The enhanced fluorescence of warfarin and phenprocoumon bound to PVP can be explained by their incorporation into the hydrophobic environment in the PVP and by a decrease in the internal rotation of the α-substituted benzyl group in the drugs. The binding parameters of warfarin and phenprocoumon were estimated by the Klotz method; the binding constants for phenprocoumon and warfarin were found to be $2.6 \times 10^4$ and $2.2 \times 10^4$ $M^{-1}$, respectively. The $^{13}C$-NMR measurements suggest the lactone moiety in the 4-hydroxycoumarin and the substituted benzene ring play an important rôle in the binding to PVP.

**Keywords**: *Coumarin anticoagulants; polyvinylpyrrolidone; fluorescence; binding constant; interaction mode.*

## Introduction

Numerous investigations have been conducted on the binding of polyvinylpyrrolidone (PVP) with various pharmaceutical agents in aqueous solution [1–3]. Fluorescence spectrophotometry is used widely to study macromolecule–drug interactions including those of plasma proteins, because of its high sensitivity and convenience [4]. Hsiao *et al.* [5] have estimated the binding parameters of sulphonamides to PVP using a fluorescent probe technique. In general, the binding parameters were determined from the measurements of the fluorescence intensity of the probe in the presence of competitors. With this technique, probes with high specificity should be used for determining the binding parameters, because the displacement using probes having several affinity sites might complicate the interpretation of the data. In this respect, the determination of the binding parameters using the enhanced fluorescence of the drug–macromolecule mixture is a more accurate method than the competitive method. The present study was

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undertaken to investigate the interaction of PVP with coumarin anticoagulants using fluorescence spectroscopy.

## Experimental

*Materials*

PVP (K-30, Tokyo Kasei Co. Ltd., Tokyo) with an average mol. wt of 40,000 (10,000–80,000) was purified by passing an aqueous solution through a cationic and anionic ion-exchange resin, then dialysed against distilled water in dialysis tubing and finally lyophilized. Potassium warfarin (Eisai Co. Ltd., Tokyo), 4-hydroxycoumarin (Tokyo Kasei Co. Ltd., Tokyo) and phenprocoumon (Hoffman-LaRoche, Basel) were used without further purification. All other materials were reagent grade and all solutions were prepared in deionized and distilled water. All solutions were prepared in 0.1 M phosphate buffer of pH 7.4 at 25 ± 1°C. PVP concentrations of $2 \times 10^{-5}$–$1 \times 10^{-3}$ M were used. The pH values were checked at 25°C using a suitably standardized pH meter.

*Apparatus and methods*

Fluorescence measurements were made on a Hitachi MPF4 fluorescence spectrophotometer (Tokyo). The fluorimetric titrations were carried out as follows: 3.0 ml of the PVP solutions of appropriate concentrations in a 10 mm pathlength cell were titrated by the successive additions of 2.0 μl vol of drug solution to give a final drug concentration of $1 \times 10^{-5}$–$4 \times 10^{-5}$ M in the cell. The fluorescence intensity was measured at an appropriate wavelength of emission. The quantum yield of fluorescence was determined according to the method of Parker and Rees [6], quinine sulphate in 0.5 M sulphuric acid being used as the standard.

$^{13}$C-NMR (100 MHz) spectra were recorded on a JNM-GX400 spectrometer (Tokyo) at 27 ± 0.5°C, using a 5 mm sample tube. Averages of 100,000 accumulations with 16,384 data points were made at 25,000 spectral widths (resolution 3.0 Hz). The concentration of drug and PVP in 0.1 M phosphate buffer (pH 7.4) were $1 \times 10^{-2}$ and $1 \times 10^{-3}$ M, respectively. The $^{13}$C-chemical shifts were recorded with an accuracy of 0.03 ppm using DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate) as the external reference.

Dialysis experiments were carried out using a Sanko plastic dialysis cell (Fukuoka). PVP solution (2 ml) was poured into one compartment and 2 ml of drug solution into the opposite compartment. Adsorption of drugs onto the membrane was negligible. After the 15 h dialysis period, the concentrations of the free drug were assayed by HPLC.

The viscosity of sucrose or PVP in phosphate buffer was determined using a viscometer (Low-Shear 30, Contraves, Zurich, measurable range $1.5 \times 10^{-3}$–$6.0 \times 10^{6}$cp, measurable minimum vol 0.5 ml). The assay was carried out at 30 ± 0.5°C.

## Results and Discussion

The fluorescence of warfarin in the presence and absence of PVP is illustrated in Fig. 1 which, for comparison, includes the effect of human serum albumin (HSA). The fluorescence intensity of warfarin in aqueous solution was very weak, whereas its fluorescence intensity was greatly enhanced by adding PVP. The relative increase in fluorescence intensity when PVP was added can be explained by the binding of warfarin to PVP, which is similar to the interaction of warfarin and HSA. Similarly, the

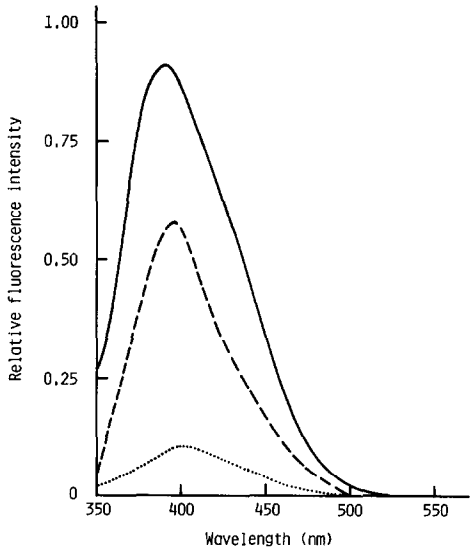



**Figure 1**
Fluorescence spectra of warfarin in the presence of PVP at pH 7.4 and 25°C. ····, Warfarin ($5 \times 10^{-6}$ M); ----, warfarin ($5 \times 10^{-6}$ M) + PVP ($5 \times 10^{-4}$ M); ———, warfarin ($5 \times 10^{-6}$ M) + HSA ($5 \times 10^{-5}$ M).

fluorescence of phenoprocoumon was markedly increased when PVP was added. However, fluorescence of 4-hydroxycoumarin was not induced in the presence of PVP, implying that the α-substituted benzyl group in coumarin anticoagulants is important in the binding process and/or the enhancement of fluorescence.

The enhanced fluorescence of warfarin bound to albumin has been considered to be due to the transfer of the warfarin molecule to a hydrophobic site in HSA [7] and to the rigid configuration of the warfarin molecule [8]. In fact, warfarin displays markedly high fluorescence in less polar solvents such as methanol, compared with that in aqueous buffers [9]. Moreover, as shown in Fig. 2, the fluorescence spectroscopic properties (quantum yield, $\phi$, and emission wavelength maximum, $\lambda_{max}$) of warfarin bound to PVP are similar to those in a viscous medium containing sucrose. This clearly suggests that the fluorescence behaviour of warfarin in the presence of PVP depends upon a decrease in

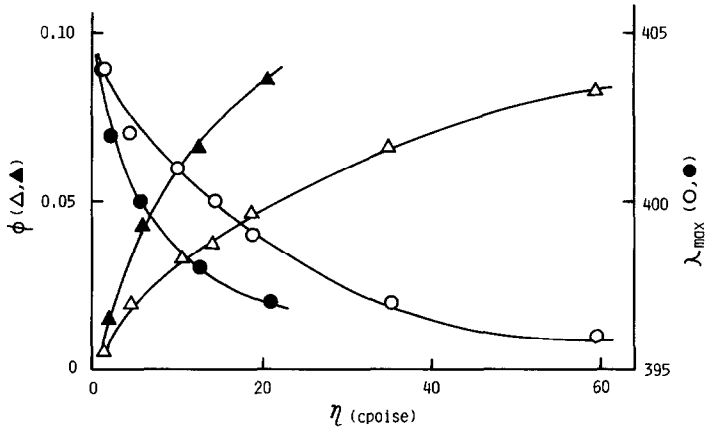



**Figure 2**
Effect of PVP and sucrose on the quantum yield of fluorescence and maximum wavelength of emission of warfarin: ●, ▲, in PVP solution; ○, △, in sucrose solution.

the internal rotation of the acetonylbenzyl group in the warfarin molecule. A similar effect on the fluorescence spectra of warfarin occurs on interaction with HSA [8]. However, the $\phi$ values and the hypsochromic shifts of the $\lambda_{max}$ values of warfarin in the warfarin–PVP system are larger than those in the presence of sucrose. Therefore, this difference may be ascribed mainly to the hydrophobic effect.

Figure 3 shows the fluorescence titration curves of warfarin with PVP. The fluorescence intensities for two titrations with high PVP concentrations ($5 \times 10^{-4}$ and $7 \times 10^{-4}$ M) were essentially identical [straight line (a)], indicating that the drug added was fully bound in both PVP solutions. At low PVP concentrations, the drug was only partially bound [curve (b)]. The observed fluorescence in the presence of PVP is a function of the amount bound at the individual sites and the associated fluorescent yield.

The fraction of the drug bound ($X$) is usually determined by using equation (1):

$$X = \frac{F_p - F_0}{F_b - F_0}, \tag{1}$$

where $F_p$ and $F_0$ are the fluorescence intensities of a given concentration of drug in a solution of low PVP concentration and in a solution without PVP, and $F_b$ is the fluorescence of the same concentration of fully bound drug. This treatment yields the true value of $X$, provided that the fluorescence intensity of the bound drug is a linear function of the concentration. However, the linearity of $F_b$ with the concentration of the drug–PVP complex applies only when the absorbance of the complex at the wavelength of excitation is low according to the fluorescence–absorbance relationship equation [10]:

$$F_b - F_0 = \phi I_0[2.3A - \frac{(2.3A)^2}{2!} + \frac{(2.3A)^3}{3!} - \frac{(2.3A)^4}{4!} + \text{---}], \tag{2}$$

where $\phi$ is the quantum yield of the emitting species, $I_0$ is the intensity of the exciting radiation, and $A$ is the absorbance. When the absorbance is low, the higher power terms in the equation become negligible. When the absorbance is high, the second and even the

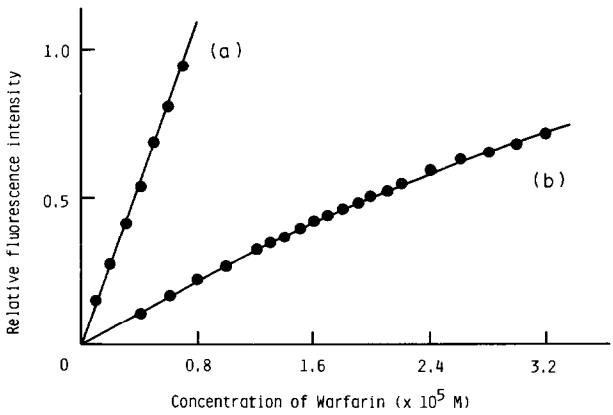



**Figure 3**
Plots of relative fluorescence intensities for the warfarin–PVP interaction as a function of the PVP concentration at pH 7.4. PVP concentration: a, $5 \times 10^{-4}$ and $7 \times 10^{-4}$ M; b, $2.2 \times 10^{-5}$ M.

third term must be considered. The straight line in Fig. 3 was obtained, after correcting the observed fluorescence intensities for the absorbance effect (the second term in the correction equation was considered). A similar titration curve was also obtained for protein, as previously reported [11, 12]. However, the plateau in the titration curve obtained for the protein–coumarin system was not observed for the PVP–coumarin interaction. This suggests that the PVP binding sites are not saturated with the ligand. Thus, PVP, unlike serum protein [13], appears to have multiple binding sites.

The binding parameters of the drug–PVP interaction can be estimated by equation (3) of the Langmuir isotherm:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nKD_f}, \tag{3}$$

where $r$ is the number of molecules of drug bound/mol of PVP, $n$ is the number of binding sites, $K$ is the binding constant and $D_f$ is the concentration of the free drug. Figure 4 shows the results of the double reciprocal plot for the warfarin–PVP interaction, using the data in Fig. 3. Table 1 lists the binding constants of the drug–PVP interaction, together with the free fractions of the coumarin compounds in the presence of PVP, obtained from dialysis experiments. Although the binding constant for 4-hydroxycoumarin was not determined by fluorimetry, the dialysis results clearly indicate that the 4-hydroxycoumarin is bound to PVP. However, the free fraction of 4-hydroxycoumarin was considerably larger than those of the other two drugs. The $K$ values and the free fraction values for warfarin and phenprocoumon were almost the same. Therefore, these results suggest that the α-substituted benzyl group in the coumarin drugs plays an important rôle in the binding to PVP.

$^{13}$C-NMR spectrometry was employed to gain further information on the mode of binding in the coumarin drug–PVP interaction in aqueous solution. The assignments of chemical shifts for warfarin were confirmed by off-resonance experiments and the additivity rule of the substituent chemical shifts [14]. Table 2 summarizes the effect of PVP on the $^{13}$C-chemical shifts of 4-hydroxycoumarin and warfarin. As a result of the binding of PVP, all the carbon signals on the lactone moiety of the coumarin ring were shifted significantly upfield or downfield, while all the carbon signals except C8 on the

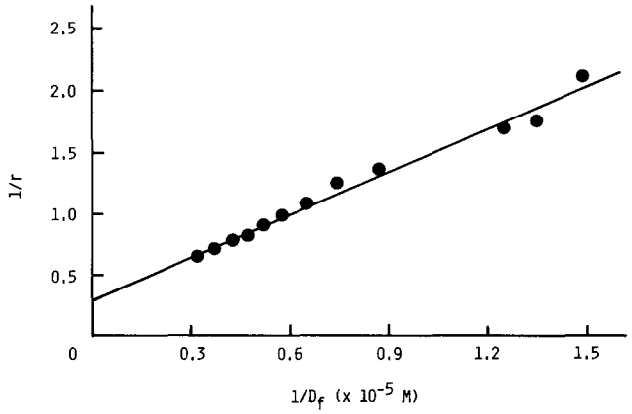
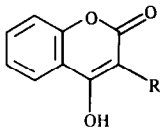
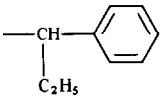
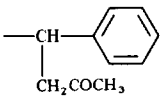



**Figure 4**
Double reciprocal plots of the warfarin–PVP interaction.

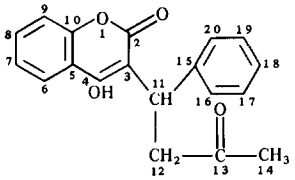
**Table 1**
Binding parameters of complexes and free fractions of drugs in PVP solution at 25°C



| Compound | R | $n$ | $K$ ($\times 10^4 M^{-1}$) | $nK^*$ ($\times 10^4 M^{-1}$) | Free fraction† (%) |
|---|---|---|---|---|---|
| 4-Hydroxycoumarin | H | — | — | 7.9 | 89.6 |
| Phenoprocoumon |  | 4.1 ± 0.5 | 2.6 ± 0.7 | 100.0 | 51.7 |
| Warfarin |  | 3.6 ± 0.3 | 2.2 ± 0.5 | 50.0 | 54.1 |

*See ref. 18.
†PVP ($2 \times 10^{-4}$M) and drugs ($1 \times 10^{-4}$M) were used throughout these experiments.

**Table 2**
Effect of PVP on the $^{13}$C-chemical shifts of 4-hydroxycoumarin and warfarin



| Carbon | 4-Hydroxycoumarin Without PVP $\delta_0$ | 4-Hydroxycoumarin $\Delta\delta$ | Warfarin Without PVP $\delta_0$ | Warfarin $\Delta\delta$ |
|---|---|---|---|---|
| 2 | 181.51 | −0.58 | 177.93 | −0.85 |
| 3 | 123.83 | 0.36 | 124.26 | 0.61 |
| 4 | 172.64 | −0.70 | 170.22 | −0.97 |
| 5 | 119.40 | 0.06 | 105.37 | 0.45 |
| 6 | 134.82 | 0.00 | 134.01 | −0.16 |
| 7 | 126.56 | 0.06 | 126.20 | −0.06 |
| 8 | 126.56 | 0.24 | 127.08 | 0.31 |
| 9 | 119.40 | 0.06 | 118.88 | 0.00 |
| 10 | 156.65 | 0.00 | 155.77 | 0.06 |
| 11 | | | 37.95 | — |
| 12 | | | 48.30 | — |
| 13 | | | 218.70 | −0.64 |
| 14 | | | 32.06 | 0.09 |
| 15 | | | 146.81 | 0.64 |
| 16, 20 | | | 129.81 | 0.36 |
| 17, 21 | | | 131.03 | −0.12 |
| 18 | | | 128.63 | −0.03 |

$\Delta\delta = \delta_{\text{with PVP}} - \delta_0$. The negative sign shows the upfield displacement.

phenyl moiety of coumarin ring showed no appreciable change, suggesting that the lactone moiety may be involved in the interaction with PVP. The chemical shifts of warfarin are significantly greater than those for 4-hydroxycoumarin, reflecting the magnitude of the binding constants. The carbon signals of the α-substituted acetonyl-benzyl group of warfarin were also shifted upfield or downfield. However, the C11 and C12 signals of the alkyl group could not be analysed due to overlapping of the PVP signals. It is noteworthy that the C15, C16, C20 of the α-substituted benzyl group displays significant downfield shifts compared with other carbons (C17, C18, C19). This phenomenon can be explained by the restriction of free rotation of the benzene ring due to the binding to PVP [15], in agreement with the fluorescence data. Thus, the $^{13}$C-NMR data indicate that the lactone moiety and the α-substituted benzyl group of the coumarin anticoagulants participate in the binding with PVP.

The binding constants obtained here are greater than that of dicoumarol–PVP complex reported by Cho *et al.* [16], and the number of binding sites is much smaller. These differences can be due to differences in chemical structure, buffer composition, as well as differences arising from the different experimental techniques employed. In particular, the large differences of the *n* values are due to the fact that the fluorescence technique may detect only those binding sites that enhance fluorescence. It should be noted that the binding affinities (*nK*) of PVP are one-fifth to one-tenth of that of serum albumin, comparable to the observation of Klotz and Shikama [17] who found that the binding affinity of Methyl Orange with PVP was one-third of that with serum albumin.

In conclusion, the data presented here suggest that the α-substituted benzyl group may play an important rôle in the binding to macromolecules.

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