J. Pharm. Pharmacol. 1987, 39: 416–420
Received October 14, 1986

A comparative study of the interaction of warfarin with human α_1-acid glycoprotein and human albumin

M. OTAGIRI,* T. MARUYAMA, T. IMAI, A. SUENAGA, Y. IMAMURA

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan

The interaction of warfarin with human α_1-acid glycoprotein (α_1-AGP) and human albumin (HA) has been investigated using fluorescence and circular dichroism techniques. The fluorescence of warfarin is greatly enhanced following binding to α_1-AGP or HA, the binding constant for a single site being estimated by the Scatchard method. The binding constants for the two serum proteins are similar, but the thermodynamic parameters differ. The binding constants increase as the pH is raised to 9·0. Various basic drugs, such as chlorpromazine, propranolol and imipramine, markedly inhibited the binding of warfarin to α_1-AGP. But, some acidic drugs, including phenylbutazone, effectively displaced warfarin bound to HA. The difference in CD spectra observed for α_1-AGP and HA indicated that the drug-binding sites of the two proteins might have different asymmetries. It thus appears that the mode of interaction of warfarin with the two proteins differs.

It is well-known that the human albumin (HA) and human α_1-acid glycoprotein (α_1-AGP) are major circulating serum proteins in drug binding. HA is generally considered to be the major binding component in human plasma for weakly acidic drugs (Kragh-Hausen 1981; Wilkinson 1983). On the other hand, α_1-AGP is the main serum protein for the binding of basic drugs (El-Gamal et al 1983; V beeck et al 1983). Urien et al (1982) have shown that some acidic drugs, including warfarin, bind to α_1-AGP with a high affinity. However, their investigations gave little insight into the nature of the interaction. The present study was undertaken to investigate the mechanism of binding of warfarin to α_1-AGP and HA using fluorescence and circular dichroism techniques.

MATERIALS AND METHODS

Materials

HA (lot No 84F-9399) and α_1-AGP (lot No 26F-9330) were obtained from Sigma Chemical Co. (St Louis, MO). The molecular weights of HA and α_1-AGP were taken as 66500 (Putnam 1975) and 44100 (Smith et al 1950), respectively. Potassium warfarin was supplied from Eisai Co. Ltd (Tokyo, Japan). All other materials were of reagent grade. All solutions were prepared in deionized and distilled water throughout. All protein and warfarin solutions were prepared in 1/15 M phosphate buffer. Protein solutions of $0{\cdot}2 - 10 \times 10^{-5}$ M were prepared, and a wide concentration range of $0{\cdot}5 - 15 \times 10^{-6}$ M of warfarin was used.

Apparatus and methods

Fluorescence measurements were made on a Hitachi 650-60 fluorescence spectrophotometer (Tokyo, Japan). Fluorometric titrations were carried out as follows: protein solutions of appropriate concentration were titrated by successive additions of warfarin solution (to give a final concentration of $0{\cdot}5 - 15 \times 10^{-6}$ M) and the fluorescence intensity was measured (excitation at 340 nm and emission at 389 nm) at 25 °C.

Circular dichroism (CD) measurements were made on a Jasco J-50A recording spectropolarimeter (Tokyo, Japan) using a 10 mm cell at 25 °C. All solutions were scanned from the wavelength at which no induced optical activity was observed. The induced ellipticity is defined as the ellipticity of the drug protein mixture minus the ellipticity of the protein alone at the same wavelength and is expressed in degrees.

Treatment of binding data

The fraction of warfarin bound, X, is usually determined by using the equation (Weber & Young 1964):

$$X = \frac{F_p - F_o}{F_b - F_o} \quad (1)$$

where F_p and F_o are the fluorescence intensities of a given concentration of warfarin in a solution of low

* Correspondence.

protein concentration and in a solution without protein, and F_b is the fluorescence of the same concentration of fully bound warfarin. After values for the fraction of bound warfarin had been found for all points along the titration curve, the results were plotted according to the Scatchard equation (1949):

$$r/C_f = nK - rK \qquad (2)$$

where r is the number of moles of warfarin bound per mole of protein, n is the number of binding sites, K is the binding constant and C_f is the concentration of free warfarin.

RESULTS AND DISCUSSION

The fluorescence intensity of warfarin was greatly enhanced when bound to HA or α_1-AGP, and there was an accompanying slight blue shift of the emission maximum. The fluorometric titrations of warfarin with α_1-AGP are shown in Fig. 1. The fluorescence intensities for two titrations with high α_1-AGP concentrations (8–10 × 10^{-5} M) were identical (straight line a), suggesting that the warfarin added was fully bound. For this treatment to be valid, the fluorescence intensity of the bound warfarin must be a linear function of concentration. This is so only when the absorbance of the complex at the exciting wavelength is low. A correction for this absorption can be made by the method of Naik et al (1975), and was made for all the data when the absorbance at 340 nm was greater than 0·02. At low α_1-AGP concentrations, warfarin was only partially bound (curve b). Similar titration curves were also obtained for the HA system, as previously reported (Otagiri et al 1979).

To determine the maximum number of binding sites, Job's plots (1928) were constructed for the warfarin-protein systems by keeping the total concentration of warfarin and protein at 1·0 × 10^{-5} M. These plots are shown in Fig. 2. The inflection points for the two plots are near 0·5, the values expected for 1:1 complex formation. Therefore, it is reasonable to conclude that the maximum number of binding sites contributing to the fluorescence of warfarin-protein is one.

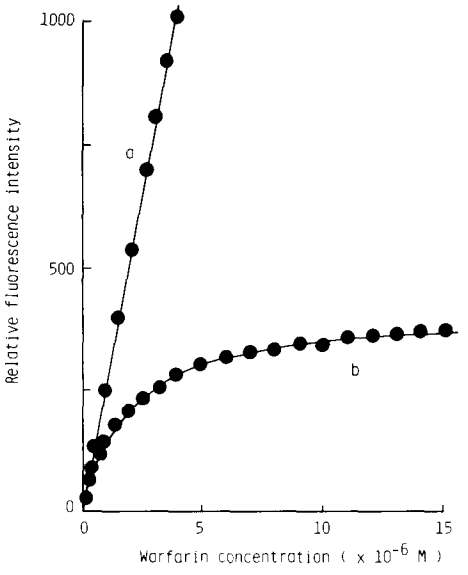

FIG. 1. Plots of relative fluorescence intensities as a function of warfarin concentration for warfarin-α_1-AGP interaction in 1/15M phosphate buffer (pH 7·4) at 25 °C. α_1-AGP concentration: a 8–10 × 10^{-5} M, b 2 × 10^{-6} M. Warfarin concentration: 0–1·5 × 10^{-5} M.

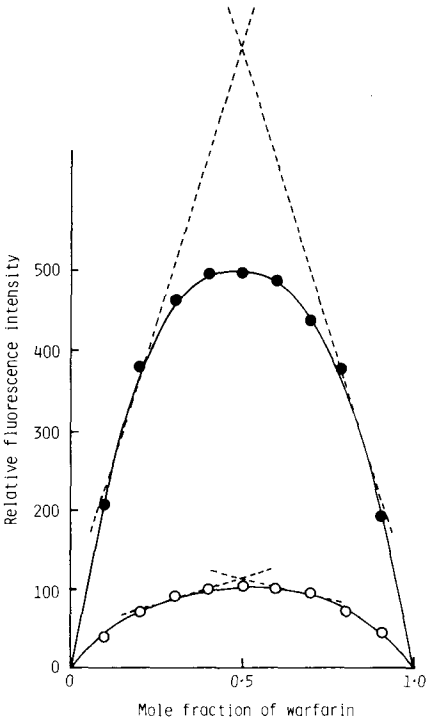

FIG. 2. Job's plots of relative fluorescence intensities for the warfarin-serum protein interaction. The final concentration is fixed at 10 μM. ●—● α_1-AGP, ○—○ HA.

Protein binding equilibria have traditionally been evaluated by means of Scatchard plots for the warfarin-α_1-AGP system, using data like that in Fig. 1. Fig. 3 shows the Scatchard plots for the warfarin-α_1-AGP system. The linearity of the Scatchard plots indicates that warfarin binds to one class of sites on

α_1-AGP. The binding constant of the complex of warfarin with α_1-AGP is almost the same as that obtained for HA, as shown in Table 1. This may indicate that the warfarin binding environment on α_1-AGP is similar to that on HA. Recently, El-Gamal et al (1982) and Sugiyama et al (1985) suggested that the high affinity site on α_1-AGP might also be located in a hydrophobic region of α_1-AGP, similar to some sites postulated for HA. Therefore, it seems that warfarin is bound to a hydrophobic area of α_1-AGP similar to that on HA. This hypothesis is also supported by the finding that the warfarin molecule quenched the intrinsic fluorescence of α_1-AGP that might arise from tryptophan and/or tyrosine residues (not shown).

Table 1. Binding parameters of warfarin-serum protein interaction at pH 7·4 and 25 °C.

Protein	n	K (L M^{-1})
HA	1·02	$2·0 \times 10^5$
α_1-AGP	0·82	$2·2 \times 10^5$

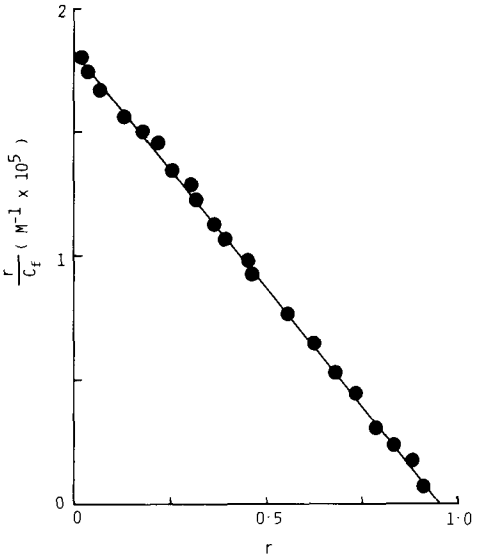

FIG. 3. Scatchard plots of warfarin-α_1-AGP interaction, using data of Fig. 1 (see text).

The effects of pH and temperature on the binding of warfarin to α_1-AGP and HA were also examined. The binding constants of the complexes of the drug with the two proteins increase with pH (Fig. 4). It is well-known that within the pH range of 6–9, HA exists in two conformational forms, the so-called N-form and B-form (Leonard et al 1963; Harmsen et al 1971). Recently, Friedman et al (1985) demonstrated that the microenvironmental changes in α_1-AGP occur around physiological pH. In fact, the intrinsic fluorescence of the two proteins increases with pH (Fig. 4), although the fluorescence of warfarin alone is not significantly changed on raising the pH from 6·5 to 9 (not shown). Therefore, the above dependence of the binding constants on pH can be explained by the conformational changes of the proteins rather than the changes in the degree of ionization of the warfarin molecule, as the pK_a of warfarin is 5·10 (Otagiri et al 1978).

The thermodynamic parameters were determined from a van't Hoff plot and the results are summarized in Table 2. With HA, the enthalpy variation (ΔH) was negative, signifying that the binding process was exothermic. The entropy variation (ΔS) was also negative, a finding also reported for the albumin binding of salicylate (Zaroslinski et al 1974) and cephalosporins (Briand et al 1982). O'Reilly (1971) previously indicated that ΔS is positive for the warfarin-HA interaction. The difference may be due to variations in albumin as well as buffer composition. In sharp contrast with the α_1-AGP system, both ΔH and ΔS are positive. From Table 2, it can be seen that the negative enthalpy makes a large contribution to the free energy term for HA. On the other hand, the positive entropy is the main source of free energy variation (ΔG) for the α_1-AGP system. For drug-ligand interaction, a positive entropy is frequently taken as evidence of hydrophobic interaction, but it has been pointed out that the positive

Table 2. Thermodynamic parameters of warfarin-serum protein at pH 7·4.

Temperature (°C)	HA			α_1-AGP		
	ΔG^*	ΔH^*	$\Delta S^\dagger$	ΔG^*	ΔH^*	$\Delta S^\dagger$
10	−30·2					
15				−28·2		
25	−29·6	−40·7	−37·1	−29·9	26·4	189
40	−29·1			−32·9		

* kJ mol^{-1}, † J K^{-1} mol.

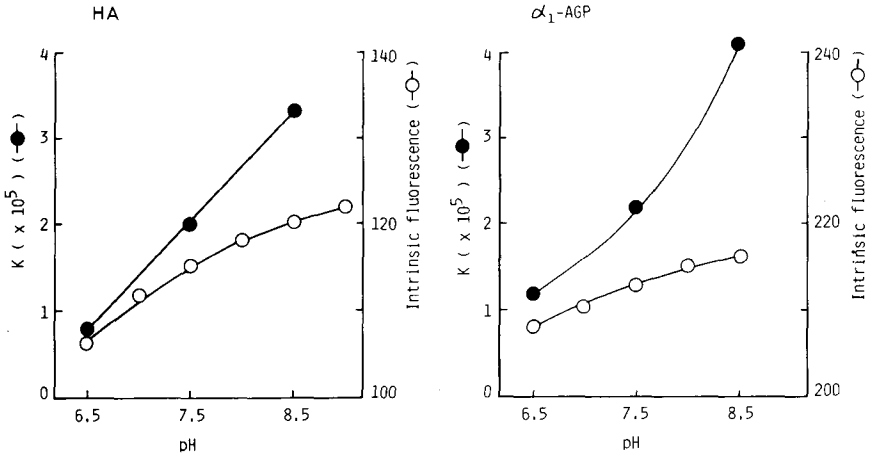

FIG. 4. pH profiles of binding constants for interaction of warfarin with serum protein and intrinsic fluorescence of serum protein. The fluorescence of 10 μM serum protein was monitored. The serum protein and warfarin concentration as in Fig. 1 were used for the estimation of binding constants. ●—● binding constant, ○—○ intrinsic fluorescence.

entropy may also be a manifestation of electrostatic forces. However, in such a case, the enthalpy change is expected to be near zero for a purely electrostatic interaction (Klotz 1973). It is also possible that the changes in behaviour of the solvent (Nemethy & Scheraga 1962), water, after the binding process, account for a large fraction of thermodynamic parameters.

Most in-vitro investigations of the interaction of drugs and albumin (Tanford 1973) have indicated that the hydrophobic interaction plays an important role in the binding. With warfarin, a significant portion of the binding energy was derived from non-ionic sources (Chignell 1970; O'Reilly 1971). On the other hand, the binding process of basic drugs with α_1-AGP may be explained by the contribution

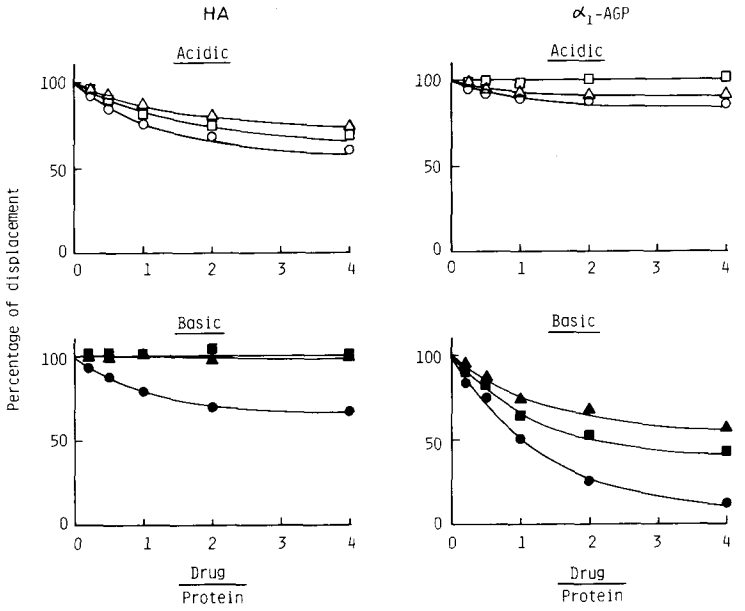

FIG. 5. Effect of various drugs on the fluorescence intensity of warfarin in the presence of serum protein warfarin concentration: 2×10^{-6} M; serum protein concentration: 1×10^{-5} M. The ordinate represents decrease in fluorescence as a percentage of original fluorescence. ○ Salicylate. △ Sulphadimethoxine. ■ Propranolol. □ Phenylbutazone. ● Chlorpromazine. ▲ Imipramine.

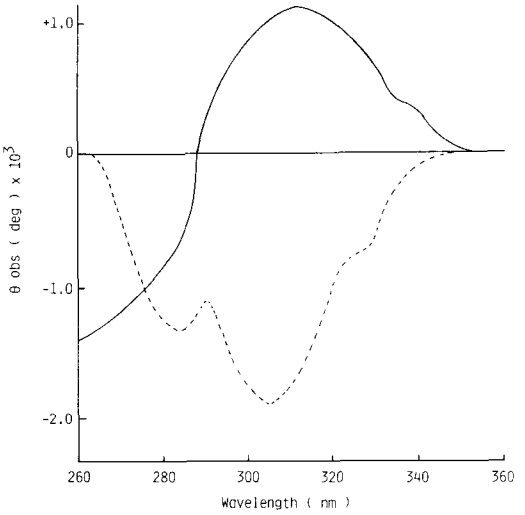

FIG. 6. Induced circular dichroism of warfarin binding to serum protein. Warfarin concentration 5×10^{-5} M; serum protein concentration 1×10^{-5} M. - - - - α_1-AGP, —— HA.

of electrostatic interaction (Kirley et al 1982). However, El-Gamal et al (1982) have demonstrated the involvement of a hydrophobic interaction in the binding of drugs to α_1-AGP. Therefore, the limited data in Table 2 suggest that the interaction mode of warfarin with α_1-AGP is different from that with HA, although the detailed mechanism requires further investigation.

As can be seen in Fig. 5, warfarin, on the basis of decreased fluorescence, may be inferred to displace salicylate, phenylbutazone and sulphadimethoxine from HA binding sites, but has little effect on basic drugs, except chlorpromazine. On the other hand, warfarin is markedly displaced from α_1-AGP by chlorpromazine, propranolol and imipramine while the extent of displacement of warfarin by the weakly acid drugs was small. These results also indicate that the binding character for the two proteins may be different, as expected from the data in Table 2.

In an attempt to elucidate further the mechanism of interaction of warfarin with HA and α_1-AGP, the binding of warfarin to the two proteins was examined by CD spectroscopy. The binding of warfarin to HA generated a monophasic positive extrinsic Cotton effect around 310 nm and a positive shoulder peak around 340 nm, as previously reported by Brown et al (1977). In sharp contrast to the HA system, two negative ellipticity bands around 280 and 305 nm and a negative shoulder peak around 335 nm were observed following the binding to α_1-AGP (Fig. 6). The sign and magnitude of the extrinsic Cotton effect may depend upon the spatial arrangement and the rigidity of the drug-protein complex when comparisons are made between the interaction of the drug molecule with similar macromolecules. Therefore, the different spectra in the two systems may indicate that the complex is held rigidly in the region near the chromophore responsible for positive or negative contributions to the Cotton effect (Chignell 1969).

In conclusion, the data show that the interaction of warfarin with α_1-AGP differs from that with HA.

REFERENCES

Briand, C., Sarrazin, M., Peyrot, V., Gilli, R., Bourdeaux, M., Sari, J. C. (1982) Mol. Pharmacol. 21: 92–99

Brown, N. A., Jahnchen, E., Müller, W. E., Wollert, U. (1977) Ibid. 13: 70–79

Chignell, C. F. (1969) Ibid. 5: 455–462

Chignell, C. F. (1970) Ibid. 6: 1–12

El-Gamal, S., Wollert, U., Müller, W. E. (1982) J. Pharm. Pharmacol. 34: 152–157

El-Gamal, S., Wollert, U., Müller, W. (1983) J. Pharm. Sci. 72: 202–204

Friedman, M. L., Schlueter, K. T., Kirley, T. L., Halsall, H. B. (1985) Biochem. J. 232: 863–867

Harmsen, B. J. M., DeBruin, S. H., Janssen, L. H. M., Rodrigues de Miranda, J. F., Van Os, A. J. (1971) Biochemistry 10: 3217–3221

Job, P. (1928) Ann. Chim. 9: 113–203

Kirley, T. L., Sprague, E. D., Halsall, H. B. (1982) Biophys. Chem. 15: 209–216

Klotz, I. M. (1973) Ann. N.Y. Acad. Sci. 226: 18–35

Kragh-Hausen (1981) Pharmacol. Rev. 33: 17–53

Leonard, W. J. Vijai, K. K. Foster, J. F. (1963) J. Biol. Chem. 238: 1984–1988

Naik, D. V., Paul, W. L., Threatte, R. M., Schulman, S. G. (1975) Anal. Chem. 67: 267–270

Nemethy, G., Scheraga, H. A. (1962) J. Chem. Phys. 36: 3401–3417

O'Reilly, R. A. (1971) Mol. Pharmacol. 7: 209–218

Otagiri, M., Fokkens, J. G., Hardee, G. E., Perrin, J. H. (1978) Pharm. Acta Helv. 53: 241–247

Otagiri, M., Otagiri, Y., Perrin, J. H. (1979) Int. J. Pharm. 2: 283–294

Putnam, F. W. (1975) in: Putnam, F. W. (ed.) The Plasma Proteins, Academic Press, N.Y. pp 135–161

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51: 660–672

Smith, E. L., Brown, D. M., Weiner, H. E., Winzler, R. J. (1950) J. Biol. Chem. 185: 569–575

Sugiyama, Y., Suzuki, Y., Sawada, Y., Kawasaki, S., Beppu, T., Iga T., Hanano, M. (1985) Biochem. Pharmacol. 34: 821–829

Tanford, C. (1973) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley & Sons, N.Y., London, Sydney, Toronto

Urien, S., Albengres, E., Zini, R., Tillement, J. P. (1982) Biochem. Pharmacol. 31: 3687–3689

Verbeeck, R. K., Cardinal, J. A., Hill, A. G., Midka, K. K. (1983) Ibid. 32: 2565–2570

Weber, G., Young, L. B. (1964) J. Biol. Chem. 239: 1415–1423

Wilkinson (1983) Drug Metab. Rev. 14: 427–466

Zaroslinski, J. F., Keresztes-Nagy, S., Mais, R. F., Oester, Y. T. (1974) Biochem. Pharmacol. 23: 1767–1776