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Influence of Fatty Acids on the Binding of Warfarin and Phenprocoumon to Human Serum Albumin with Relation to Anticoagulant Therapy

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Abstract

Warfarin and phenprocoumon binding to human serum albumin was studied by equilibrium dialysis.

The first stoichiometric binding constant was 1.89×10^5 M^{-1} for warfarin and 2.40×10^5 M^{-1} for phenprocoumon. The affinity of warfarin was markedly increased on addition of up to 3 mol mol^{-1} albumin of palmitic, stearic, oleic or linoleic acids with energetic couplings for co-binding of one molecule of each of the fatty acids and one molecule of warfarin of 0·9, 1·1, 0·7 and 0·6 kJ mol^{-1}, respectively. The affinity of phenprocoumon was only increased slightly on addition of palmitate with an energetic coupling of 0·3 kJ mol^{-1}.

Six consecutive serum samples were obtained from each of 14 patients undergoing surgery. The serum affinity of the drugs varied considerably corresponding to free drug concentrations between 0·7 and 2·7% for warfarin and between 0·8 and 4·9% for phenprocoumon. The affinity of warfarin but not of phenprocoumon was correlated to the increasing plasma fatty acid concentration. Anticoagulant therapy with phenprocoumon may thus be less sensitive than warfarin to changes in the fatty acid concentration of plasma.

The coumarin derivatives, warfarin and phenprocoumon, are widely used for oral anticoagulant therapy. Knowledge of the albumin binding is important for therapy since the anticoagulant effect seems to be related to the concentration of the free drug (Yacobi & Levy 1975) although other factors are important (Yacobi et al 1976). The dose requirement is critical and adverse effects have often been reported. Symptoms similar to those of overdosage have been described after giving phenylbutazone (Serlin & Breckenridge 1983) and other drugs to patients in anticoagulant therapy and have been ascribed to displacement of the coumarin derivative on binding of the second drug to serum albumin (MacLeod & Sellers 1976). Other factors, such as non-esterified fatty acids (NEFA) have also been shown to influence the binding (Havel et al 1963). The aim of the present study was to study the influence of long-chain fatty acids on the binding of warfarin and phenprocoumon in-vitro and to elucidate the magnitude of binding affinity variations in-vivo where varying fatty acid concentrations are seen in patients undergoing surgery.

Materials and Methods

Materials

Human serum albumin was obtained from AB Kabi Vitrum, Stockholm, Sweden. More than 95% of the total protein of this preparation was serum albumin. The serum was defatted with charcoal (Chen 1967) in solution acidified with sulphuric acid, lyophilized, and stored over silica gel at 4°C. The fatty acid content of the defatted albumin was less than 0·1 mol mol^{-1} albumin.

Racemic warfarin sodium salt was a gift from Nyco MED, Rødovre, Denmark. Racemic phenprocoumon was a gift from F. Hoffman-La Roche & Co Ltd., Basel, Switzerland. [^{14}C]Warfarin (spec. act. 56 Ci mol^{-1}) was obtained from Amersham International plc, Buckinghamshire, England. [^{3}H]Phenprocoumon (102 Ci mol^{-1}) was obtained as a gift from F. Hoffman-La Roche. These preparations were purified by thin-layer chromatography, using toluene : methanol (90 : 10) for warfarin and toluene : ethylacetate : ethanol (90 : 5 : 5) for phenprocoumon, on silica gel plates from Merck AG, Darmstadt (Germany), and were kept in ethanolic solutions at −20°C. The radiochemical purity was checked as previously described (Honoré 1987). In both preparations the content of loosely bound, dialysable radioactive impurities accounted for less than 0·5% of the total radioactivity. A volume of the solution, sufficient for one or a few days work, was evaporated in a stream of nitrogen and the fatty acid was dissolved in ethanol and added under stirring to the sample (buffered albumin solution or serum) to give an ethanol concentration less than 1%.

Palmitic, oleic, and linoleic acids were obtained from Fluka AG, Buchs, Switzerland. Stearic acid was from Merck. The purity of all acids was ~99% by gas-liquid chromatography. Oleic and linoleic acids were stored at −20°C in glass vials sealed under nitrogen.

[9,10-^{3}H]Palmitic acid (38 000 Ci mol^{-1}) was from New England Nuclear Research Products, NEN Chemicals GmbH, Dreieich, Germany. One millicurie of the tritiated palmitic acid was received from the manufacturer in 200 μL ethanolic solution. The solution was diluted with ethanol to 20 mL to minimize deterioration by self-irradiation and kept at −20°C.

Preparation of fatty acid/albumin solutions

Albumin was dissolved in water to a concentration of 300 μM and pH was brought to 9 with sodium hydroxide. The fatty acid was dissolved in ethanol, 90 mM for palmitic, oleic, and linoleic acids, 45 mM for stearic acid. One volume of the

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ethanolic solution was added to 50 vol defatted albumin solution under stirring. The solutions were lyophilized and the dried material stored over silica gel at 4°C until use when it was dissolved in 33 mM sodium phosphate buffer, pH 7·4. When dissolved, each of the fatty acid albumin preparations formed clear and stable solutions that were mixed with defatted albumin solutions in various proportions.

Equilibrium dialysis

Binding equilibria of warfarin and phenprocoumon to the albumin-fatty acid preparations were studied by equilibrium dialysis in 1-mL dialysis chambers (Honoré & Brodersen 1984). Cellophane membranes were cut from dialysis tubing (Union Carbide Corp.). A 33 mM sodium phosphate buffer, pH 7·4, with varying concentrations of warfarin or phenprocoumon (5–200 μM) was placed on both sides of the membrane. Albumin (30 μM), with or without fatty acid, was present only on the left side. The chambers were rotated in an air thermostat at 37°C. The fatty acids do not diffuse into the protein-free chamber under these circumstances. The time necessary for obtaining equilibrium was determined. Constant drug concentrations in the protein-free chamber were present for 3 h to more than 5 h and a dialysis time of 4·5 h was chosen. Binding of warfarin and phenprocoumon to the membranes and chamber walls could not be detected. Warfarin or phenprocoumon was measured in the protein-free solution by spectrophotometry at 307 nm. The concentration of unbound drug, c, and the molar ratio of bound drug to albumin, r, were calculated.

Dialysis exchange rate method

The binding affinity of warfarin and phenprocoumon to albumin-fatty acid preparations with added trace concentrations of the drugs was measured by determination of rate of dialysis exchange (Brodersen et al 1982). The binding affinity was expressed as the concentration of reserve albumin for binding of the drug. This is related to the free drug concentration as follows:

$$p = (1/K_{1,St})/(c_{bound}/c_{free}) \quad (1)$$

where p is the reserve albumin concentration, c_{bound} and c_{free} are bound and free trace equilibrium concentrations of the drug, and $K_{1,St}$ is the first stoichiometric binding constant for the drug to a standard albumin preparation. The defatted albumin was used as a standard.

Reserve albumin for binding of [^{14}C]warfarin and [^{3}H]phenprocoumon was measured in a buffered solution of defatted albumin (100 μM) with variable added amounts of each of the long-chain fatty acids. Reserve albumin concentration for binding of [^{3}H]palmitate was also measured in a buffered solution of defatted human serum albumin (30 μM) with variable, added amounts of warfarin or phenprocoumon.

The binding affinity of warfarin and phenprocoumon in serum samples with added trace concentrations of the drugs was measured by determination of rate of dialysis exchange and expressed as the concentration of reserve albumin for binding of warfarin or phenprocoumon.

Energetic coupling of binding of two ligands

The interaction between two ligands with a carrier can be quantitated by the energetic coupling, ΔG, as previously described by Weber (1975) and Brodersen et al (1982):

$$\Delta G_{D,FA} = RT \ln \frac{K_{(FA)D}}{K_D} \quad (2)$$

where $DG_{D,FA}$ is the energetic coupling between the first molecule bound of a drug and the first molecule of a fatty acid, K_D is the first stoichiometric binding constant for the drug to albumin and $K_{(FA)D}$ is the first stoichiometric binding constant for the drug to albumin complexed with one molecule of the fatty acid. Details of how to calculate the energetic coupling from data of reserve albumin measurements have previously been reported (Brodersen et al 1982). A zero value of ΔG

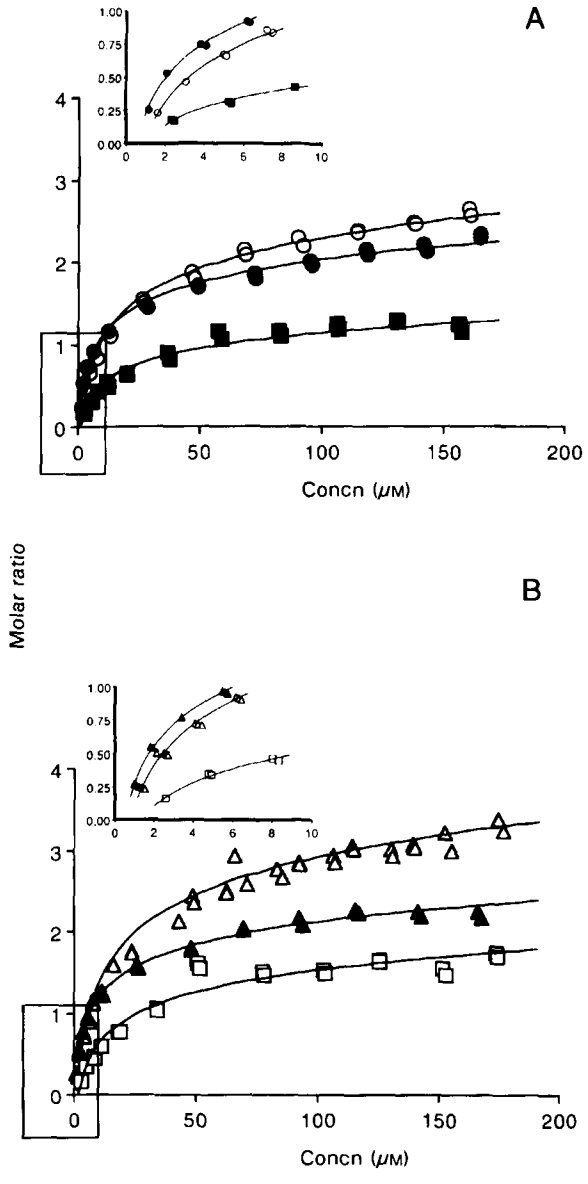

FIG. 1. Co-binding of warfarin and palmitate (A) and phenprocoumon and palmitate (B). The molar ratio of bound warfarin or phenprocoumon to albumin, r, was plotted against the concentration of free warfarin or free phenprocoumon, c. Binding isotherms for warfarin to albumin without palmitate (○), with 3 mol of palmitate mol^{-1} albumin (●), with 6 mol of palmitate mol^{-1} albumin (■). Binding isotherms for phenprocoumon to albumin without palmitate (△), with 3 mol of palmitate mol^{-1} albumin (▲), with 6 mol of palmitate mol^{-1} of albumin (□). Buffer was 33 mM sodium phosphate, pH 7·4, 37°C.

indicates that the two ligands are bound independently of each other whereas a positive value indicates that the binding of one ligand increases the binding of the other ligand. A negative value of ΔG signifies that one ligand inhibits the binding of the other ligand and a negative infinite value indicates that co-binding of the ligands at low concentrations can not be demonstrated.

Analytical methods

The concentration of non-esterified fatty acids (NEFA) in serum samples was determined by an enzymatic method, NEFAC, Wako chemicals GmbH, Neuss, Germany. The concentration of albumin in serum was measured according to Laurell (1966).

Statistical analyses

Linear regression analyses were performed by the method of least squared deviations. Probit analysis of the regressional residuals were in all cases normally distributed. The residuals were neither correlated to the NEFA concentration nor to that of albumin, indicating homogeneity of variance.

Patients and serum samples

Serum samples were obtained from 14 patients (9 females and 5 males, aged 52–79 years, mean 68 years) diagnosed as arthritic and hospitalized for knee-joint surgery. None of the patients received oral anticoagulants or other drugs capable of competing with warfarin and phenprocoumon.

Six venous blood samples were obtained from each patient, at 2000 h on the day before the operation, at 1500 h just after the operation, at 2100 h, and then at 0800 h on each of the following three days. The samples were left to clot and serum was separated and stored at −20°C.

Results

In-vitro studies

Bound and free equilibrium concentrations for warfarin and phenprocoumon to human serum albumin having 0, 3, and 6 mol palmitate attached per mol protein, are plotted in Fig. 1. The first stoichiometric binding constant to albumin without fatty acids is $1{\cdot}89 \times 10^5\ M^{-1}$ for warfarin and $2{\cdot}40 \times 10^5\ M^{-1}$ for phenprocoumon. The binding isotherms in

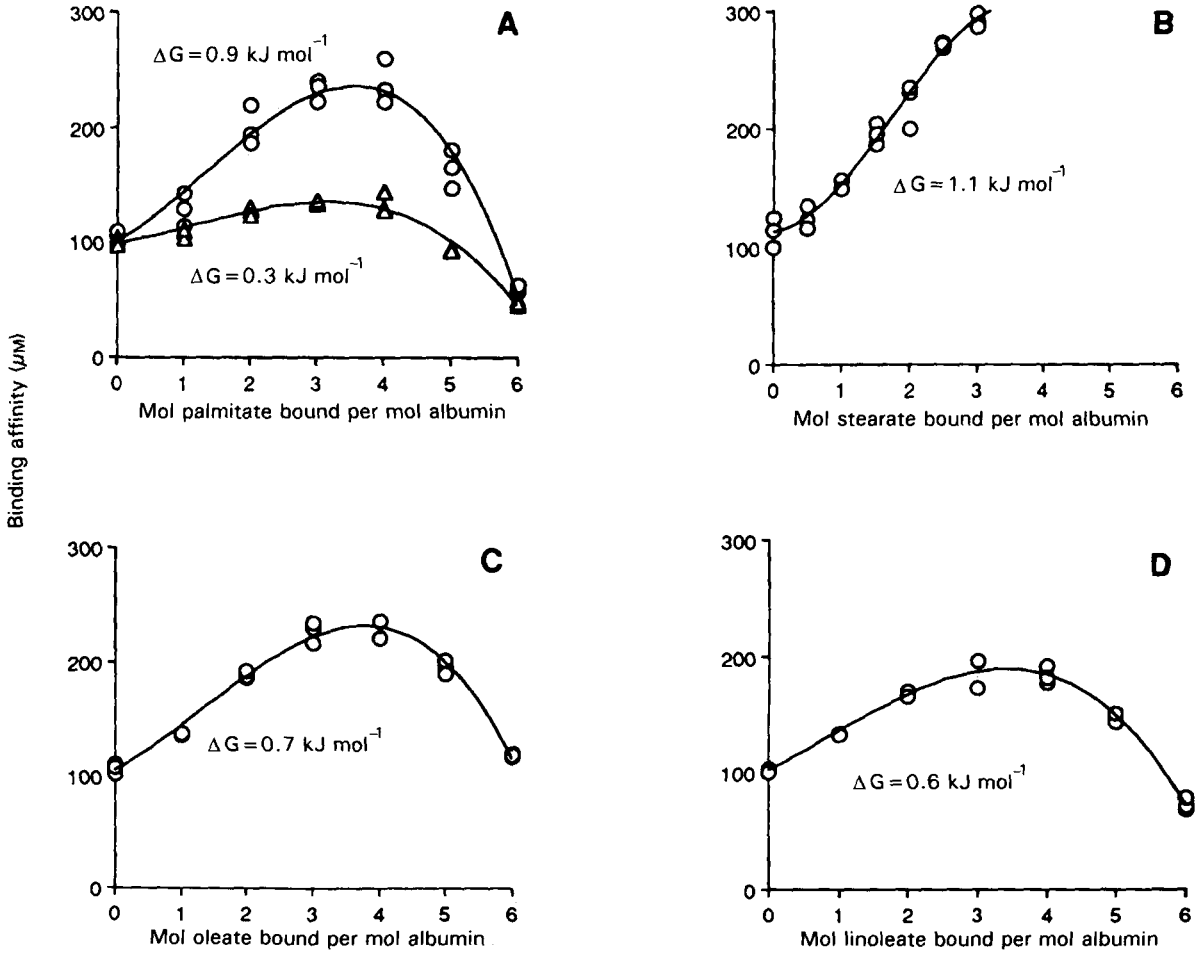

FIG. 2. Reserve albumin for binding of warfarin, $p_{warfarin}$, (○) and phenprocoumon, $p_{phenprocoumon}$ (△) as a function of palmitate/albumin ratio (A), stearate/albumin ratio (B), oleate/albumin ratio (C), and linoleate/albumin ratio (D). Serum albumin concentration was 100 μM, pH 7·4, 37°C. A 33-mM sodium phosphate buffer was used.

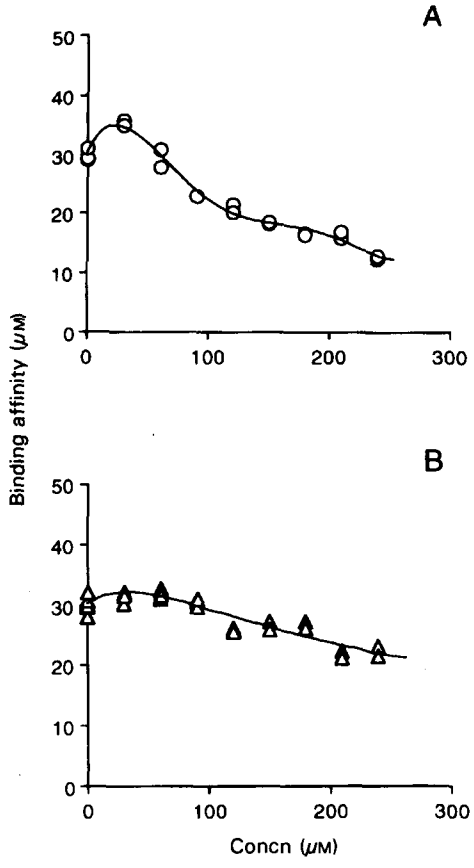

FIG. 3. Reserve albumin concentration for binding of palmitate as a function of warfarin (A) and phenprocoumon (B) added in varying concentrations. Serum albumin concentration was 30 μM, pH 7·4, 37°C. A 33-mM sodium phosphate buffer was used.

Fig. 1 indicate that the binding affinity of the first molecule of warfarin is markedly increased to albumin carrying an average of 3 mol palmitate, while albumin carrying 6 mol palmitate per mol albumin possesses lower affinity than albumin without fatty acids. This is more clearly demonstrated in the inserted figure where the binding isotherm for warfarin to albumin containing 3 mol palmitate per mol albumin is placed above the isotherm for defatted albumin. The isotherm representing the binding of warfarin to albumin having 6 mol palmitate attached per mol albumin is placed below that of albumin without palmitate. The affinity of the first molecule of phenprocoumon is slightly increased to albumin containing 3 mol palmitate per mol albumin. The effect of palmitate on phenprocoumon binding is apparently weaker than on warfarin binding. The binding isotherm for phenprocoumon to albumin containing 6 mol palmitate per mol albumin is placed below the other isotherms throughout the whole concentration range.

Fig. 2 demonstrates the effect of different long-chain fatty acids on the binding of each of the anticoagulant drugs measured as the reserve albumin concentration for binding of warfarin, $p_{warfarin}$, or phenprocoumon, $p_{phenprocoumon}$. The reserve albumin increased on addition of palmitate up to about 3 mol mol^{-1} albumin whereas further addition of palmitate decreased the concentration. In agreement with the binding isotherms in Fig. 1 the effect of palmitate on phenprocoumon binding was considerably weaker than on warfarin binding. The results of $p_{warfarin}$ with stearate, oleate and linoleate all show increased binding of warfarin up to 3 mol mol^{-1} albumin. The findings in Fig. 2 may also be expressed in quantitative terms by the energetic coupling, ΔG, calculated from equation 2 by using the $K_{(FA)D}/K_D$ values for warfarin or phenprocoumon (Brodersen et al 1982). The ΔG-values for co-binding of warfarin with palmitate, stearate, oleate and linoleate were 0·9, 1·1, 0·7 and 0·6 kJ mol^{-1}, respectively; the value for co-binding of phenprocoumon with palmitate was 0·3 kJ mol^{-1}. We found that all the ΔG-values are positive, indicating that the drugs and the fatty acids bind with heterotropic cooperativity. These findings show increased binding of warfarin and to a lesser extent phenprocoumon upon binding of long-chain fatty acids to albumin. The reverse interaction, the effect of warfarin on palmitate binding is shown in Fig. 3. As expected, $p_{palmitate}$ increases at low drug concentrations, most pronounced in the case of binding of warfarin and to a lesser extent in the case of binding of phenprocoumon. A decrease in reserve albumin concentration is found on addition of higher concentrations of warfarin or phenprocoumon, indicating that the drugs at these concentrations interact negatively with binding of palmitate.

In-vivo studies

The affinities were found to vary from one patient to another; $p_{warfarin}$ in the interval from 194 μM to 796 μM and $p_{phenprocoumon}$ between 81 μM and 531 μM. This corresponds to a variation in the percentage of free drug between 0·7 and 2·7% for warfarin and between 0·8 and 4·9% for phenprocoumon using the binding constants and the reserve albumin concentrations. Theoretically, this variation may result from differences in albumin concentration, albumin affinity or presence of other ligands that interfere with the binding of warfarin or phenprocoumon. By picturing values of the reserve albumin concentration vs the albumin concentration (Fig. 4), we find – as expected – that $p_{warfarin}$ as well as $p_{phenprocoumon}$ increases with the increasing albumin concentration. It is noted, however, that the regression line for $p_{warfarin}$ closely follows the albumin concentration, although we measured a NEFA/albumin ratio between 0·73 and 4·3, i.e. in an interval where albumin should have an increased affinity for warfarin (Fig. 2). For phenprocoumon we find that the regression line is localized more than 200 mM below the albumin concentration. In almost all patients the concentration of serum NEFA increased temporarily as a result of surgery. The reserve albumin concentrations for warfarin and phenprocoumon are plotted vs the serum NEFA concentration in Fig. 4. $p_{warfarin}$ increased significantly with the elevated serum NEFA concentration while there was no significant correlation between $p_{phenprocoumon}$ and NEFA. The same pattern was seen when the measured reserve albumin concentrations were corrected for differences in albumin concentration by plotting p divided by the albumin concentration vs the serum NEFA concentration.

Discussion

The main purpose of the present study was to assess the interaction between long-chain fatty acids and warfarin or phenprocoumon on binding to human serum albumin in-vitro as well as in-vivo.

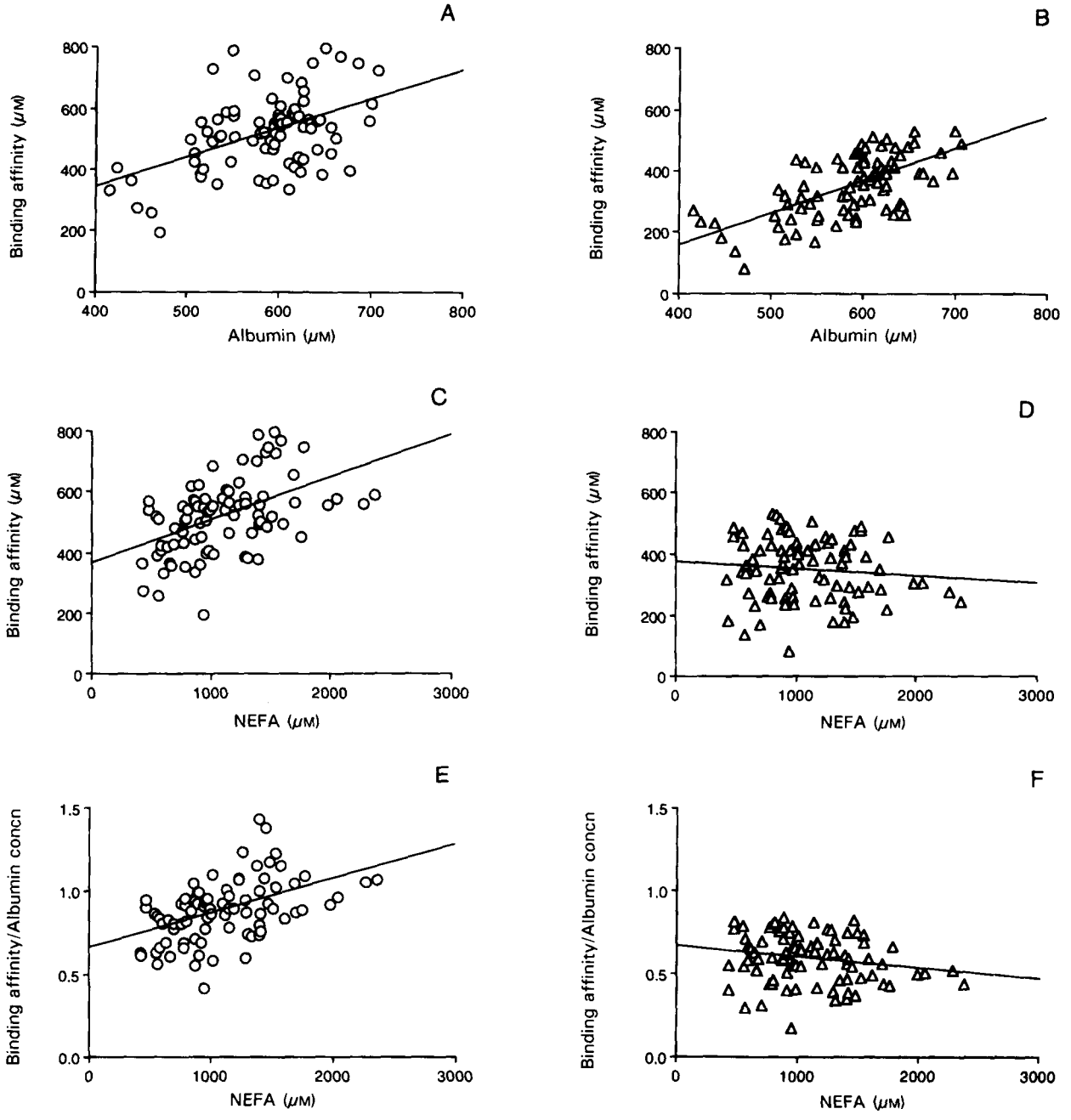

FIG. 4. A. Reserve albumin for binding of warfarin, $p_{warfarin}$ (○) as a function of the albumin concentration ($r^2 = 0{\cdot}24$, $P = 0{\cdot}0001$). B. Reserve albumin for binding of phenprocoumon, $p_{phenprocoumon}$ (△) as a function of the albumin concentration ($r^2 = 0{\cdot}41$, $P = 0{\cdot}0001$). C. $p_{warfarin}$ vs the concentration of non-esterified fatty acid, NEFA ($r^2 = 0{\cdot}24$, $P = 0{\cdot}0001$). D. $p_{phenprocoumon}$ vs the NEFA concentration ($r^2 = 0{\cdot}01$, $P = 0{\cdot}36$). E. $p_{warfarin}$ divided by the albumin concentration as a function of NEFA ($r^2 = 0{\cdot}22$, $P = 0{\cdot}0001$). F. $p_{phenprocoumon}$ divided by the albumin concentration as a function of NEFA ($r^2 = 0{\cdot}03$, $P = 0{\cdot}09$). All parameters were measured in fourteen patients undergoing surgery.

We found a considerable variation in the binding affinity of the anticoagulant drugs warfarin and phenprocoumon in serum. The variation can partly be explained by variations in albumin concentration and, for warfarin, by variations in serum NEFA. Anticoagulant therapy with phenprocoumon should thus be less sensitive to changes in the plasma level of free fatty acids, for example as seen during major surgery. However, we found that other factors also influence the binding, especially for phenprocoumon where the reserve albumin concentration was below the albumin concentration. This could be explained by the presence of another ligand in serum competing for binding to albumin. Thus even with a known concentration of albumin and NEFA other factors are relevant for the binding affinity in serum. A patient with a very low reserve albumin (giving a high free drug concentration) will be more prone to overdosage than a patient with a very high reserve albumin (giving a low free drug concentration). Currently, dose requirements are adjusted on a trial-and-error basis until the desired effect on

blood coagulation has been achieved. An initial measurement of $p_{warfarin}$ or $p_{phenprocoumon}$ in an individual patient could give an estimate of the dose required for proper therapy.

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