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Differential Binding of Warfarin to Maternal, Foetal and Non-pregnant Sera and its Clinical Implications

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Abstract

The object of this study was to determine whether differential binding of sodium warfarin in paired maternal and cord sera accounts for its adverse effects on the foetus.

In-vitro binding of sodium warfarin to human serum albumin in maternal, foetal, and non-pregnant (control) subjects was determined by equilibrium dialysis at 37°C. Our data suggest that at therapeutic concentrations, sodium warfarin has a single high affinity binding site on human serum albumin with an association constant of $1{\cdot}65 \times 10^{-3}$ M. Serum albumin concentration in the control sera ($4{\cdot}42 \pm 0{\cdot}08$ g dL^{-1}) was comparable with that in the cord sera ($4{\cdot}54 \pm 0{\cdot}26$ g dL^{-1}) but was significantly higher ($P < 0{\cdot}01$) than the maternal levels ($4{\cdot}03 \pm 0{\cdot}21$ g dL^{-1}). Binding data indicate that the fraction of unbound warfarin in the foetal sera ($6{\cdot}8 \pm 1{\cdot}9$ g dL^{-1}) was significantly higher than in the maternal ($3{\cdot}60 \pm 1{\cdot}3$ g dL^{-1}; $P < 0{\cdot}01$) and non-pregnant sera ($1{\cdot}96 \pm 0{\cdot}6$ g dL^{-1}; $P < 0.001$). The maternal and foetal fractions of free warfarin were directly proportional to the concentrations of free fatty acids ($y = 1268 - 110x$; $r = 0{\cdot}93$; $P < 0{\cdot}001$), and bilirubin ($y = 8{\cdot}7 + 1{\cdot}4x$; $r = 0{\cdot}91$; $P < 0{\cdot}001$), respectively.

This study indicates that warfarin was more strongly bound in the maternal sera than in the foetal sera; this was probably because of the competitive and allosteric effect of free fatty acid and bilirubin in the maternal and foetal sera, respectively. The clinical significance of this observation is discussed in this paper.

Although the use of the oral anticoagulant drug sodium warfarin is beneficial for both prevention and treatment of thrombo-embolic disorders during pregnancy, its usage is restricted because of its adverse effects on the foetus (Ginsberg & Hirsh 1989), including chondrodysplasia punctata, stippled epiphysis, microcephaly, optic atrophy, and microphthalmia (Whitfield 1980; Kaplan et al 1982; Lamontagne et al 1984). The foetotoxic effects of sodium warfarin not only result in increased perinatal and infant mortality, but might be responsible for significant physical and mental handicap in the survivors (Hall et al 1980; Tamburrini et al 1987).

Although warfarin crosses the human placenta (Bajoria & Contractor 1993), its embryopathy is reported in only 4 to 33% of the foetuses exposed antenatally (Hall et al 1980; Vitali et al 1986). The mechanisms by which it causes structural and neurological foetal abnormalities is far from clear. One mechanism could be its albumin-binding function as inter-individual variation in albumin binding is shown to be associated with differences in its therapeutic efficacy (Yacobi et al 1976). Alteration in protein binding might, furthermore, alter the volume of distribution, clearance and elimination of a drug, thereby potentiating its therapeutic effect. Recent reports suggest that warfarin embryopathy occurs secondary to impairment in synthesis of vitamin K-dependent coagulation proteins or because of failure in carboxylation of bone proteins by sodium warfarin (Pauli et al 1987). As sodium warfarin is highly protein bound (O'Reilly & Kowitz 1967), we hypothesize that differences in albumin binding of sodium warfarin in the maternal–foetal unit might lead to relatively high levels of unbound sodium warfarin in the foetal circulation. Given the limited capacity of the foetal liver to metabolize sodium warfarin, the higher levels of the fraction of free sodium warfarin might prolong its therapeutic effect in the foetus and explain some of its foetal adversity.

To test this hypothesis and to enable us to understand the pharmacokinetic characteristics of sodium warfarin in maternal and foetal circulation in normal and pathological conditions we have compared the in-vitro albumin binding of sodium warfarin in maternal, foetal and non-pregnant sera.

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Materials and Methods

Analytical grade sodium warfarin was a gift from Flockhart Laboratories, Middlesex, UK. Cibacron blue dye, epichlorohydrin, and other reagents were obtained from Sigma, Poole, Dorset. Sepharose 4B was from Pharmacia LKB Biotechnology, Sweden.

Isolation and purification of human serum albumin

Albumin was isolated from human plasma by affinity chromatography using a 50 mL cibacron blue Sepharose conjugate column equilibrated with tris buffer (0·05 M Tris HCl – 0·05 M NaCl; pH 8; Travis et al 1976). Out-of-date frozen human plasma (20 mL; transfusion laboratories, Charing Cross Hospital, London) was applied to the column. Albumin bound to the cibacron blue dye, was eluted as a single broad peak by thiocyanate buffer (0·05 M Tris HCl – 0·2 M NaSCN; pH 8), and collected as a single fraction. Albumin was concentrated to 10 mL by ultrafiltration using an Amicon UM-2 membrane and then dialysed against water for 48 h to remove any contaminating thiocyanate particles. The dialysed albumin solution was freeze-dried overnight at a vacuum of 0·1 torr (Speedivac Model 5PS, centrifugal freeze dryer; Edwards High Vacuum Ltd). The purity and quality of the albumin was determined by electro-

phoresis of the albumin preparation on a cellulose acetate membrane with the Beckman Microzone system. A single broad band corresponding to the R_F of albumin was obtained.

Selection of patients

Blood was collected from eighteen mothers (aged 18 to 35 years) who had uneventful pregnancies and normal vaginal deliveries of full-term infants. Patients who had complications during pregnancy such as pre-eclampsia, intrauterine growth retardation, antepartum haemorrhage, and diabetes mellitus or were taking any medication were excluded from the study, as were premature babies and those having congenital abnormalities. All mothers were on iron tablets during pregnancy and had either pethidine or epidural for pain relief during labour. Control sera were obtained from eighteen healthy non-pregnant women aged 20 to 35 years, who were not taking any medication. All the women gave informed verbal consent as approved by the hospital ethics committee.

Collection of blood

Blood (20 mL) was drawn by means of a disposable plastic syringe from both the mother and her new-born baby (cord blood) immediately after delivery. The blood was left to stand for 12 h at room temperature and was then centrifuged at 1500 g for 15 min. The serum was divided into two portions of 1 mL and 8 mL and stored at − 20°C until used for binding studies. Control sera were collected and stored in the same way as maternal or cord sera.

Equilibrium dialysis

Determination of sodium warfarin–albumin binding. Binding of sodium warfarin to human serum albumin and serum proteins was determined by equilibrium dialysis with phosphate buffer (0·067 M; pH 7·4) in dialysis bags at 37°C. Dialysis bags were made from visking cellophane casing of 10 or 15 inch diameter and washed thoroughly with distilled water and phosphate buffer before use. A series of experiments was performed to determine (a) the time taken by sodium warfarin to achieve equilibrium between bound and unbound forms under physiological conditions, and (b) whether binding of sodium warfarin was dependent on the concentration of albumin and sodium warfarin. Initially two types of experiment, using either varying concentrations of albumin (0·04–4 g dL^{-1}) and constant concentrations of sodium warfarin (15 $\mu g\ mL^{-1}$) or a fixed concentration of albumin (0·4 g dL^{-1}) and varying concentrations of warfarin (1–60 $\mu g\ mL^{-1}$), were undertaken to optimize the experimental conditions and to determine the number of binding sites.

A series of experiments was then undertaken to determine whether there was any difference in albumin-binding of warfarin in the maternal, foetal, and non-pregnant sera .

Experimental design. Experiments were conducted in duplicate and the maximum error for the duplicates was less than 3·5%. Albumin solution (2 mL; 0·4 g dL^{-1}) in phosphate buffer was added to the dialysis bags. Each dialysis bag was placed in a glass tube (15 mm × 100 mm). Freshly prepared sodium warfarin solution (3 mL; 15 $\mu g\ mL^{-1}$) in phosphate buffer was added to the compartment outside the dialysis bags. The tubes were covered with Parafilm to prevent fluid loss and placed on a horizontal shaker to achieve adequate mixing between the two compartments. Temperature was maintained at 37°C in a thermostatically-controlled water bath.

As, in common with most ligands, sodium warfarin is adsorbed to some extent by the dialysis bags; calculation of the amount actually bound to albumin was corrected for bag binding. The amount bound to the dialysis bag was determined by performing the dialysis of sodium warfarin without adding albumin to the dialysate.

At the end of the experiment the fluid outside the dialysis bag was tested with 1% aceto-acetic acid for any leakage of albumin through the semi-permeable membrane. The pH of both compartments was measured at the beginning and end of the experiment and was found to vary between 7·38 and 7·45. Equilibrium was attained between bound and free sodium warfarin within 5 h of the start of the experiment.

Similarly, binding of sodium warfarin to maternal, foetal ($n = 18$) and control sera ($n = 18$) was studied by adding 15 $\mu g\ mL^{-1}$ of sodium warfarin to the serum. Each experiment was conducted in 18 matched pairs, maternal and foetal, for 5 h at 37°C. For each pair a correction was applied to compensate for binding of sodium warfarin to the dialysis bag.

Analytical methods

Sodium warfarin assay. The concentration of sodium warfarin was determined by the fluorimetric method of Corn & Berberich (1967). The sensitivity of the assay was 0·5 $\mu g\ mL^{-1}$ and coefficient of variation was < 12%.

Albumin assay. The concentration of serum albumin in maternal and foetal samples was determined by the spectrophotometric method of Doumas et al (1971). The total serum protein concentration was not determined in this study because the albumin concentration was more relevant to assessment of the protein binding capacity of sodium warfarin.

Bilirubin assay. The concentration of bilirubin was determined using a commercial assay kit (Sigma).

Free fatty acids assay. The concentration of free fatty acids was measured by the colorimetric method of Duncombe (1964).

Statistical analysis

All data are expressed as means ± s.d. unless otherwise indicated. The Student's *t*-test was used to compare data between the two groups. *P* values less than 0·05 were considered to be indicative of statistical significance. Correlation between two groups of data was determined by linear regression analysis using Spearman's correlation coefficient (r).

Calculation of binding

Two methods were used to determine the concentration of albumin-bound sodium warfarin. In the first method, albumin binding of sodium warfarin was determined by measuring its concentration both inside (total concentration) and outside (free concentration) the dialysis bag. The free and bound fractions of sodium warfarin were calculated as follows:

$$\text{Percent free sodium warfarin} = \frac{\text{Concn. of free}}{\text{Concn. of total}} \times 100 \quad (1)$$

Percent bound sodium warfarin

$$= \frac{\text{Concn. of total} - \text{Concn. of free}}{\text{Concn. of total}} \times 100 \quad (2)$$

In the second method, the concentration of free warfarin at equilibrium was determined by measuring its concentration in the compartment outside the dialysis bag. The amount of albumin-bound warfarin was calculated by subtracting the concentration of free and membrane-bound warfarin from the amount added initially. For all concentrations of sodium warfarin the difference between the two methods was only $0{\cdot}97 \pm 0{\cdot}23$.

The number of sodium warfarin binding sites on the albumin molecule (n) and the association constants (K) of the complexes were calculated by use of the Scatchard equation: (Scatchard 1949):

$$V/A = Kn - KV \quad (3)$$

where V is the molar ratio of bound sodium warfarin to albumin, A is the molar concentration of free sodium warfarin at equilibrium, K is the association constant for the binding at each site, and n is the number of binding sites on the albumin molecule.

Results

Five experiments were undertaken to determine the equilibration time for free and albumin-bound sodium warfarin. Fig. 1 shows that equilibrium was established within 5 h between free and bound sodium warfarin. When the dialysis bag containing sodium warfarin–albumin complex was placed in a test tube containing fresh phosphate buffer without warfarin, re-equilibration between free and bound warfarin was established within 10 min at a lower concentration of sodium warfarin, indicating that binding of sodium warfarin with human serum albumin was rapid and reversible in nature.

Fig. 2 shows a Scatchard plot of the ratio of the molar concentration of bound to free sodium warfarin against the ratio of the molar concentration of bound sodium warfarin to albumin. A curvilinear plot was obtained, indicating the presence of at least two independent albumin binding sites (n_1 and n_2), for sodium warfarin. The association constants of sodium warfarin for both binding sites were calculated from a

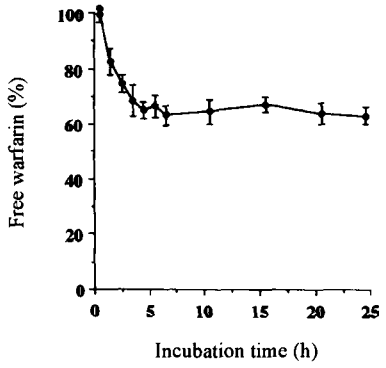

FIG. 1. Concentration of sodium warfarin outside the dialysis bag with increasing time of equilibrium dialysis experiments. In these experiments the concentrations of sodium warfarin and albumin were 35 μg mL^{-1} and 0·04 g dL^{-1}, respectively. All values are expressed as mean ± s.d. (n = 5).

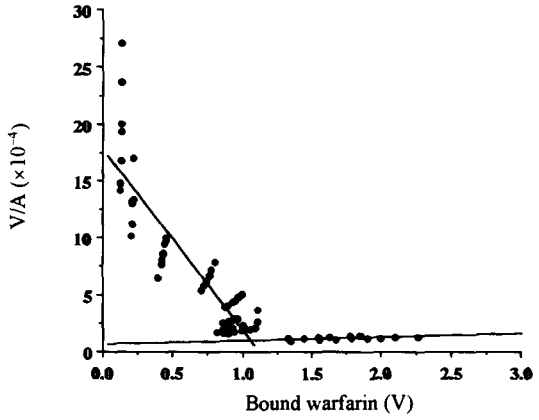

FIG. 2. Graphical determination of number of binding sites and association constants for interaction of sodium warfarin with human serum albumin. V represents moles of sodium warfarin bound per mole of albumin and A is the concentration of unbound drug. The line was obtained by means of the Scatchard equation where values of V/A were plotted as a function of V. K was the association constant and n was the number of binding sites of sodium warfarin per molecule of protein. These data, obtained from five equilibrium dialysis studies, consisted of 80 points.

regression line by applying the method of least squares analysis. The association constant (K_1) for first binding site (n_1) was $1{\cdot}65 \times 10^{-3}$ M at 37°C with K_1n_1 of 16·81 and n_1 of 1·02. The association constant calculated for the possible second binding site gave a non-significant apparent association constant (K_2) of $9{\cdot}49 \times 10^{-3}$ M with K_2n_2 of 2·47 and n_2 of 2·60.

Fig. 3. compares the albumin binding of warfarin in maternal, foetal and control sera. Albumin levels in foetal sera ($4{\cdot}54 \pm 0{\cdot}26$ g dL^{-1}) were significantly higher ($P < 0{\cdot}001$) than in maternal sera ($4{\cdot}03 \pm 0{\cdot}20$ g dL^{-1}) but were comparable with those of the control sera ($4{\cdot}42 \pm 0{\cdot}08$ g dL^{-1}; Fig. 3A); the latter levels were significantly higher ($P < 0{\cdot}01$) than those in the maternal sera ($4{\cdot}03 \pm 0{\cdot}21$ g dL^{-1}).

Warfarin was more strongly bound in the control sera ($96{\cdot}23 \pm 0{\cdot}96\%$) than in maternal ($92{\cdot}54 \pm 1{\cdot}99\%$; $P < 0{\cdot}001$) and foetal ($88{\cdot}01 \pm 3{\cdot}79\%$; $P < 0{\cdot}001$) sera (Fig. 3B). Binding of warfarin in the maternal sera was, furthermore, significantly higher than in the cord sera ($88{\cdot}06 \pm 3{\cdot}79\%$; $P < 0{\cdot}001$). Similarly, the fraction of free warfarin in control sera ($1{\cdot}96 \pm 0{\cdot}59\%$) was significantly lower ($P < 0{\cdot}001$) than in maternal ($3{\cdot}61 \pm 1{\cdot}31\%$) and foetal sera ($6{\cdot}84 \pm 1{\cdot}92\%$) (Fig. 3C). The concentration of unbound warfarin in foetal sera ($6{\cdot}84 \pm 1{\cdot}92\%$) was significantly higher ($P < 0{\cdot}001$) than maternal levels ($3{\cdot}61 \pm 1{\cdot}31\%$). Foetal bilirubin levels ($18{\cdot}04 \pm 2{\cdot}88$ mg dL^{-1}) were significantly higher ($P < 0{\cdot}001$) than maternal levels ($1{\cdot}43 \pm 0{\cdot}85$ mg dL^{-1}) and those in control sera ($1{\cdot}44 \pm 0{\cdot}63$ mg dL^{-1}) (Fig. 3D). Bilirubin levels in maternal and control sera were comparable. Maternal levels of free fatty acids ($875{\cdot}5 \pm 154{\cdot}8$ μmol L^{-1}) were significantly higher ($P < 0{\cdot}001$) than foetal plasma levels ($195{\cdot}3 \pm 68{\cdot}8$ μmol L^{-1}) and those in control sera ($284{\cdot}3 \pm 89{\cdot}4$ μmol L^{-1}). The concentrations of free fatty acids in foetal and control sera were comparable (Fig. 3E).

Fig. 4A indicates that there was significant correlation between fraction of free warfarin and the albumin concentration in the control sera ($y = 5{\cdot}28 - 0{\cdot}44x$; $r = 0{\cdot}93$; $P < 0{\cdot}001$; $n = 18$). No such relationship was found between the concentration of albumin and the fraction of free warfarin in

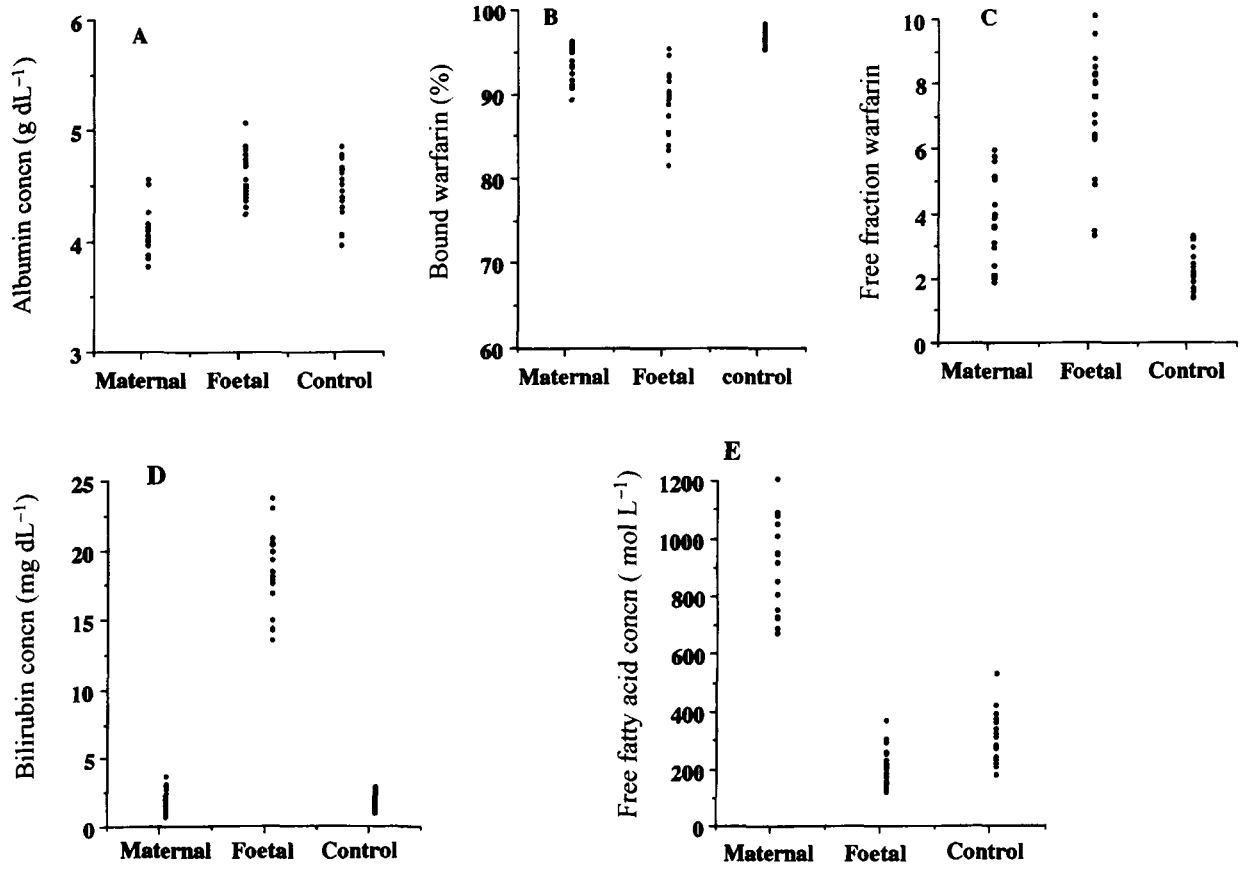

FIG. 3. Comparison between control, maternal and foetal sera (A) albumin concentration, (B) albumin binding of warfarin, (C) free fraction, (D) concentration of bilirubin, and (E) concentration of free fatty acids. The values were obtained from eighteen paired maternal and foetal samples.

maternal and foetal sera. These observations suggested that factors other than albumin concentration might account for different binding of warfarin in maternal and foetal blood.

A significant negative correlation was found between bilirubin levels and free warfarin in foetal blood ($y = 9{\cdot}7 + 1{\cdot}4x$; $r = 0{\cdot}91$ $n = 18$; $P < 0{\cdot}001$) (Fig. 4B), suggesting that bilirubin competes with warfarin for its binding site on the albumin. In maternal and control sera there was no significant correlation between the fractions of free warfarin and bilirubin levels.

A significant correlation was found between free fatty acid levels and the fraction of free sodium warfarin in the maternal sera ($y = 1268 - 110x$; $r = -0{\cdot}93$; $P < 0{\cdot}001$; $n = 18$) (Fig. 4C). In control and foetal sera there was no relationship between the fraction of free warfarin and the levels of free fatty acids.

Discussion

The results of our study showed that sodium warfarin binds more strongly to maternal than to foetal sera. Our data also indicate that binding of sodium warfarin is significantly higher in non-pregnant sera than the maternal sera. These findings have important therapeutic implications as protein binding function could substantially influence the pharmacokinetic and pharmacodynamic characteristics of a drug (Rowland 1980; Gibaldi & Koup 1981). As the unbound fraction of a drug is the primary factor which influences its rate of placental transfer and foetal exposure, understanding of maternal and foetal albumin binding might, furthermore, enable prediction of foetal adversity of warfarin in certain pathological conditions.

In this study we found albumin to have a single binding site for warfarin, which contradicts the findings of other investigators who showed the existence of at least two binding sites (O'Reilly & Kowitz 1967; Mais et al 1974; Wosilait 1975). The reason for this discrepancy is unclear. One explanation could be that as we used a low concentration of sodium warfarin, we were unable to demonstrate the second low-affinity binding site, which probably is activated only in the presence of the high concentrations of warfarin used by other investigators (O'Reilly & Kowitz 1967; Mais et al 1974). We did not investigate the second albumin-binding site because interaction of warfarin with this site occurs only at concentrations seldom found in clinical practice. Binding of warfarin to the second site accounts, furthermore, for less than 5% of its total binding.

Our data confirmed the previously published report that foetal serum albumin concentrations were significantly higher than those in their mothers (Krauer et al 1984). Despite low maternal albumin concentrations, however, our data suggest that sodium warfarin is more strongly bound in maternal than in foetal sera. This behaviour is strikingly different from that of other highly protein-bound drugs such as salicylate, dexamethasone, phenytoin or diazepam (Hamar & Levy 1980a, b; Kuhnz & Nau 1983; Kuhnz et al 1983). Although pH has been proposed as the single most important factor determining the protein binding capacity of a drug, protein binding both of basic

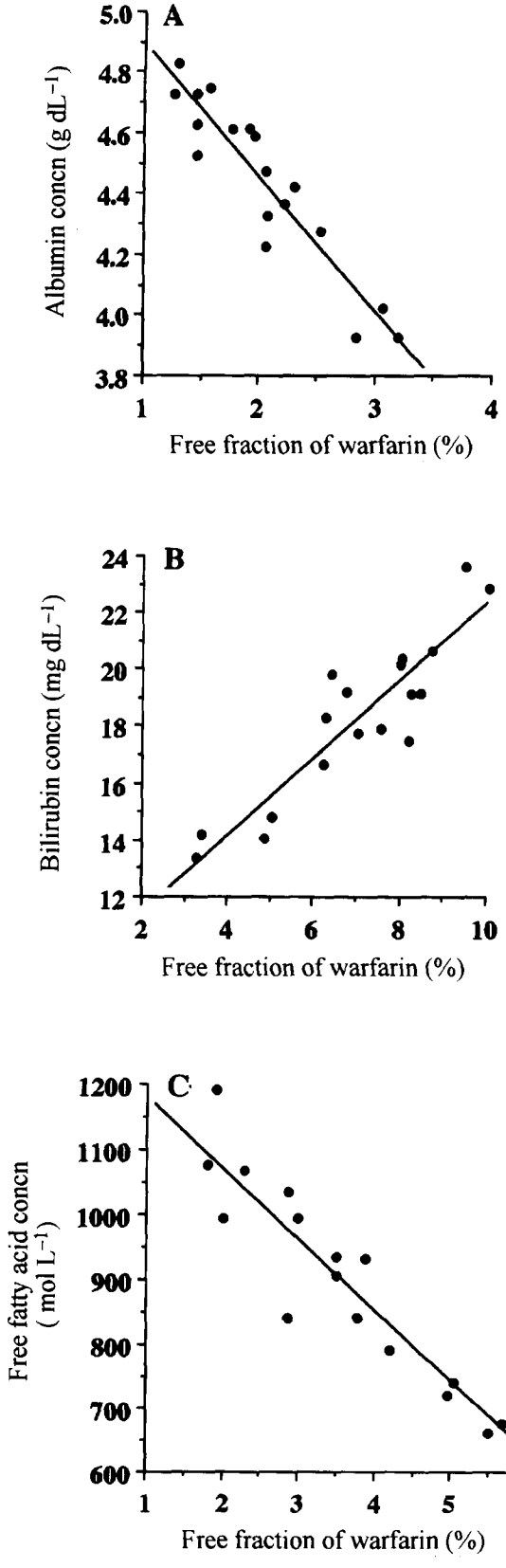

FIG. 4. The correlation between fraction of free sodium warfarin (as percentage) and albumin concentration in control subjects ($y = -5{\cdot}28 - 0{\cdot}44x$; $r = 0{\cdot}93$; $P < 0{\cdot}001$; $n = 18$), free fatty acid concentration in maternal blood ($y = -1268 - 110x$; $r = 0{\cdot}88$ $P < 0{\cdot}001$; $n = 18$) and bilirubin concentration in foetal blood ($y = 8{\cdot}7 + 1{\cdot}4x$; $r = 0{\cdot}91$; $P < 0{\cdot}001$; $n = 18$).

drugs (dexamethasone, diazepam) and weakly acidic drugs (phenytoin, sulphisoxazole) were higher in the foetus than in the mother (Hamar & Levy 1980b). Thus it seems likely that different binding of warfarin in maternal and foetal sera is governed by factors other than pH.

It is possible that differences in albumin binding of sodium warfarin could be a result of competitive and allosteric effects of endogenous substances on its binding site. It has been shown that high concentrations of free fatty acids displace most drugs from their albumin binding sites, thereby increasing their free concentration (Tsutsumi et al 1975; Sager et al 1979), but as our data indicate a significant negative correlation between maternal levels of free fatty acids and the fraction of unbound warfarin, it is likely that free fatty acids enhance the binding of warfarin in maternal sera. This is in agreement with the results of other investigators who have also found that free fatty acids induce conjugation of sodium warfarin with albumin (Wosilait & Ryan 1979). As we did not find any correlation between the free warfarin and free fatty acids in the foetal sera, it is, furthermore, likely that differences in free fatty acids levels might be responsible at least in part for differences between foetal and maternal protein binding.

The difference between albumin–sodium warfarin binding in maternal and foetal sera could also be attributed to the relatively high foetal bilirubin concentration as both bilirubin and sodium warfarin compete for the same binding site, i.e., site 1 (sodium warfarin site) on the albumin molecules (Sjoholm et al 1979). As foetal albumin has a greater affinity for bilirubin than warfarin (K of $5{\cdot}2 \times 10^{-8}$ M in comparison with $1{\cdot}65 \times 10^{-3}$ M; Irollo et al 1987) it is, furthermore, plausible that bilirubin displaces warfarin from its binding site in foetal blood. Our data confirm this hypothesis; a significant positive correlation was found between the concentration of bilirubin and that of free sodium warfarin in the foetal blood. Protein binding of diphenylhydantoin in hyperbilirubinaemic infants has been reported to be significantly less than that in normal foetuses (Rane et al 1971). Foetal albumin might have greater affinity for bilirubin than the adult albumin (K value of $5{\cdot}2 \times 10^{-8}$ M compared with $2{\cdot}4 \times 10^{-7}$ M (Krasner et al 1973), which could explain why we failed to demonstrate any correlation between maternal bilirubin and sodium warfarin levels. Our data suggest, therefore, that competitive and allosteric effects of endogenous substances found in maternal and foetal sera might explain the different albumin binding in maternal and the foetal sera.

Another factor which could also explain lower drug binding to foetal proteins is the intrinsic qualitative difference in drug affinity between the adult and the foetal plasma proteins (Pruitt & Dayton 1971). Serum electrophoresis failed, however, to confirm any significant qualitative difference between mothers and their foetuses (Ganapathy & Cohen 1975).

The findings of our study might explain why antenatal administration of sodium warfarin causes structural and neurological abnormalities in 4 to 30% of foetuses only. We speculate that as sodium warfarin is more strongly bound in maternal sera, this minimizes foetal exposure to warfarin by reducing its net transplacental transfer. Despite relatively low foetal levels of total warfarin, it is likely that the fraction of unbound warfarin could be higher because it is less strongly bound to foetal albumin. This in turn can produce an exaggerated pharmacological response in the foetus because

there is strong correlation between the serum concentration of free warfarin and its anticoagulant response (Mungall et al 1984). The anticoagulant response is further potentiated because metabolism of warfarin by foetal liver is impaired because of the immaturity of the foetal hepatic enzyme system responsible for its biotransformation. Although available evidence indicates that human foetal hepatic microsomal monooxygenase enzyme activity is detectable from 6–7 weeks of gestation and increases throughout the pregnancy, the glucuronide conjugation pathway is poorly developed or absent (Pelkonen 1980). This glucuronide conjugation is important for elimination of hydrophilic metabolites of sodium warfarin by the kidney. Thus, the overall regulation of foetal exposure to sodium warfarin appears to depend on two factors: the albumin binding function and the foetal hepatic enzyme activity. As the functional activity of the drug–albumin interaction and the metabolic pathway depends on a number of factors, it is possible that foetal bilirubin concentration and genetic and environmental make-up will probably determine which foetuses are susceptible to the adverse effects of warfarin. Maternal factors such as hypoproteinaemia and drug interaction might, however, also potentiate foetal vulnerability to warfarin by increasing the free fraction of warfarin in the maternal circulation and hence its transplacental transfer. Further studies are necessary to determine the effect of albumin binding on transplacental transfer of warfarin.

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