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On the modulating effects of temperature, albumin, pH and calcium on the free fractions of phenobarbitone and phenytoin

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The effects of temperature, albumin, pH and Ca^{2+} on the binding of phenobarbitone and phenytoin to human serum albumin in buffer have been investigated using equilibrium dialysis. The free fractions of both anticonvulsants were much increased by raising the temperature. Lower free fractions were observed by increasing the albumin concentration from 5–8 g litre^{-1} and by raising pH from 6 to 9. No significant effect on the free fractions was observed by changing (at pH 7·4) the Ca^{2+} concentration from 0 to 5 mM. The observed differences in free fractions at 37 °C, as determined in phosphate, borate and Krebs-Ringer buffer at pH 7·4, indicate that great care is needed in the choice of dialysis fluid for dialysis of clinical samples.

In the management of seizure disorders with anticonvulsant drugs, clinical assessment and therapeutic drug monitoring are related to obtain maximal clinical efficacy and minimal side effects of these drugs. Although only the free fractions of the drugs are pharmacologically active, usually the schedules being evaluated are based on the total drug concentration in serum or plasma. However, changes in the protein binding of drugs can induce subtherapeutic or toxic effects, even when the total drug concentration is within the therapeutic range (Rowland 1980; Bogaert 1981; Belpaire 1981). In these cases, determination of free drug levels can be of value.

The protein binding of a drug can be affected by changes in the concentration of the binding protein(s). For example, the concentration of albumin—one of the major serum binding proteins for both acid and basic drugs —will be lower in cases of pregnancy (Krauer et al 1980; Dean et al 1980), as well as in diseases such as acute infections, alcoholic cirrhosis, rheumatoid arthritis, renal dysfunction and protein malnutrition (Rowland 1980; Belpaire 1981). Protein binding may also be affected by the presence of competing (or otherwise interfering) substances, for example as in uraemia (Reidenberg et al 1971; Shoeman & Azarnoff 1972), or as a result of polypharmacy (Fraser 1980). Also, changes in the concentration of electrolytes such as H^+, Ca^{2+} and Cl^- can influence albumin drug binding (Vallner et al 1979; Perrin & Juni 1982; Shaw et al 1982).

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Little is known about the effects of these and other factors on the free fractions of anticonvulsant drugs. We now present data on the effects of several of these factors on the albumin binding in-vitro of two widely used anticonvulsants, phenobarbitone and phenytoin, obtained by equilibrium dialysis.

MATERIALS AND METHODS

Materials

Phenobarbitone and phenytoin were obtained from Brocacef B.V., Maarssen, The Netherlands. Albumin (Human serum albumin, A-1886, essentially fatty acid-free, prepared from fraction V, lot 52F-9335) was obtained from Sigma Chemical Company, St Louis, USA. All other chemicals were of analytical grade, obtained from E. Merck A.G., Darmstadt, GFR, or J. T. Baker Chemicals, Deventer, The Netherlands. Sørensen phosphate buffer, pH 7·4 (modified) mM: NaH_2PO_4 13·5, Na_2HPO_4 53·3, NaCl 75·3. In the pH range 6 to 8, iso-osmotic phosphate buffers were prepared (Wade 1980). Borate buffer, pH 7·4: H_3BO_3 180·5 mmol litre^{-1}, $Na_2B_4O_7.10\,H_2O$ 5·0 mmol litre^{-1}, NaCl 46·2 mmol litre^{-1}. In the pH range 7 to 9, iso-osmotic borate buffers were prepared (Wade 1980). Krebs-Ringer bicarbonate buffer, pH 7·4 (modified) mM: NaCl 121, KCl 4·8, KH_2PO_4 1·2, $MgSO_4$ 1·2, $NaHCO_3$ 25·3, $CaCl_2$ 0–5. pH was adjusted by gassing with 5% CO_2 (v/v) in O_2.

Methods

Samples were prepared as follows: phenobarbitone or phenytoin was dissolved in ethanol (1 g litre^{-1}).

Aliquots were added to test tubes and evaporated to dryness before addition of an albumin/buffer mixture (albumin 40 g litre^{-1} unless otherwise stated). The final drug concentration in the samples was for phenobarbitone 25 mg litre^{-1} and for phenytoin 15 mg litre^{-1}, being concentrations in the middle of their respective therapeutic ranges.

Equilibrium dialysis was performed for 3½ h at 37 °C (unless otherwise stated), using a Dianorm equilibrium dialyser with Teflon dialysis cells (type Macro-1-S) (Diachema A.G., Rüschlikon, Zürich, Switzerland). Under these conditions, equilibrium was achieved between albumin and each of the drugs. Samples (1 ml aliquots of buffer with albumin and phenobarbitone or phenytoin) were dialysed against the same buffer (1 ml), using cellulose dialysis membranes (Diachema, type 10·14) with a declared molecular weight cut-off of 5000. The dialysis cells were rotated 8 times per minute, in a waterbath. In our experiments the albumin concentration in the buffer compartment after dialysis was found to be approximately 0·01% of that in the sample compartment, indicating that protein retention was effective.

The dialysis membranes were rinsed with double-distilled water and were incubated overnight with dialysis buffer, before they were used. Sørensen phosphate buffer was used as dialysis fluid and for the preparation of the samples, unless otherwise stated. Both compartments were sampled at the end of each dialysis run, and the drug concentrations were measured. The percentage unbound drug ('free fraction') was calculated with reference to the *final* sample concentration at equilibrium.

Measuring drug concentrations: 0·5 ml aliquots of samples, dialysates or raffinates were shaken (1 min, whirl-mixer) with one drop of HCl (4 M) and 1 ml dichloromethane containing phenytoin or phenobarbitone as internal standard. The drug concentration was determined by HPLC, using a Partisil 5 column, 150 × 4·6 mm (28800, Chrompack Nederland B.V., Middelburg, The Netherlands). A Waters model Wisp 710B automatic injection module, a model 600A solvent delivery system and a model 440 adsorbance detector were used (Waters Associates Inc., Milford, Massachusetts, USA). Detection was at 254 nm. The eluent used was 94·5% dichloromethane, 5% tetrahydrofuran, 0·5% methanol and acetic acid 1 µl litre^{-1}. The flow rate was 2·0 ml min^{-1}.

The albumin concentration in the samples before and after dialysis were measured with GPC, using a LiChrosorb diol column sized 300 × 3·9 mm, where the mean particle size was 10 µm (E. Merck A.G., Darmstadt, GFR). UV detection was at 254 nm. The eluent used was Sørensen phosphate buffer pH 7·4, and the flow rate was 0·4 ml min^{-1}.

RESULTS

The effect of temperature on the free fractions of phenobarbitone and phenytoin in phosphate buffer pH 7·4, containing albumin 40 g litre^{-1}

By increasing the temperature from 23 to 39 °C, the free fraction of phenytoin was almost doubled, while the free fraction of phenobarbitone was increased, but to a lesser extent (see Fig. 1A).

The effect of albumin on the free fractions in phosphate buffer pH 7·4 at 37 °C

At an albumin concentration of 40 g litre^{-1}, the free fractions of phenobarbitone and phenytoin were 59 ± 1·6% and 19 ± 1·3% (n = 10), respectively. Varying the albumin concentration between 5 and 80 g litre^{-1} had a marked influence on the free fractions (see Fig. 1B).

The osmotic activity of the albumin molecules causes a concentration-dependent watershift across the dialysis membranes, resulting in a lower albumin concentration in the sample compartment at equilibrium. In Fig. 1B the free fractions are plotted versus the resulting albumin concentrations after dialysis.

The effect of pH on the free fraction in phosphate (pH 6–8) and borate (pH 7–9) buffers, containing albumin 40 g litre^{-1}

By increasing the pH from 6 to 9, the free fractions of both phenobarbitone and phenytoin decreased markedly (see Fig. 1C). In the pH range 7 to 8, free fractions were determined in both buffers, because significant differences between phosphate and borate buffers were observed at pH 7·4.

The effect of calcium on the free fractions in Krebs-Ringer bicarbonate buffer pH 7·4, containing albumin 40 g litre^{-1}, at 37 °C

Hardly any effect was seen on the free fractions of either drug, when the Ca^{2+} concentration was varied between 0 and 5 mM (see Fig. 1D). In the serum, the total Ca^{2+} concentration ranges between 2·15 and 2·5 mM. A Krebs-Ringer bicarbonate buffer was used in this experiment, because addition of $CaCl_2$ to the phosphate (or borate) buffer used in the other experiments, resulted in precipitation. During dialysis, no rise in pH occurred.

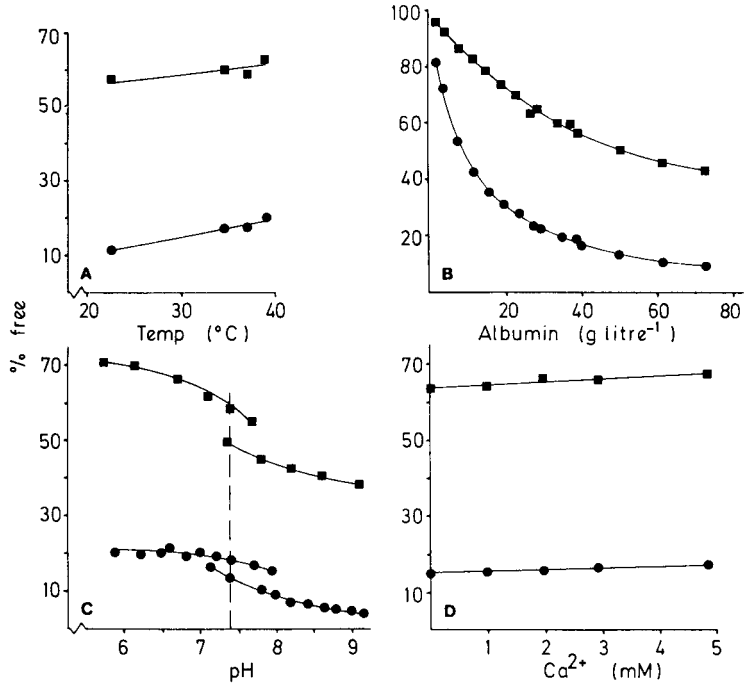

FIG. 1. The free fractions of phenobarbitone 25 mg litre^{-1} (—■—■—) and phenytoin 15 mg litre^{-1} (—●—●—) in buffer, containing albumin, as a function of: A, temperature (in phosphate buffer pH 7·4, containing albumin 40 g litre^{-1}); B, albumin concentration (in phosphate buffer pH 7·4, at 37 °C); C, pH (in phosphate buffer pH 6–8, and in borate buffer pH 7–9, containing albumin 40 g litre^{-1}, at 37 °C); D, Ca^{2+} concentration (in Krebs-Ringer buffer pH 7·4, containing albumin 40 g litre^{-1}, at 37 °C). The free fractions were determined by equilibrium dialysis. Data represent the means of 2–10 determinations.

DISCUSSION

In-vivo, several (patho)physiological factors can induce changes in the protein binding of a drug, which may lead to marked intra- and inter-individual differences in the free fraction of a drug. Because of the complexity of the situation in-vivo, we investigated the effects of several biochemical parameters separately in-vitro.

The effect of temperature

As can be seen from our data, in the range of temperature fluctuations that can occur under (patho)physiological conditions, the effect of temperature on the free fractions will only be small. Under in-vitro conditions, for several drugs e.g. disopyramide (David et al 1983), theophylline (Shaw et al 1982; Brørs et al 1983) and phenytoin (Lunde et al 1970), lower free fractions at lower temperature were reported. Our results agree with these data.

The effect of albumin

Under physiological conditions, the albumin concentration ranges between 35 and 50 g litre^{-1}. However, albumin concentration will be lower under several conditions (see Introduction). Our results show the marked influence of lowering the albumin concentration on the free fractions of phenobarbitone and phenytoin, and are in agreement with data found for phenytoin in literature (Lunde et al 1970; Lecomte et al 1979; Argyle et al 1984).

The effect of pH

Several investigators reported lower free fractions in-vitro at increasing pH values for some drugs, e.g. lignocaine (lidocaine) (Burney et al 1978), theophylline (Vallner et al 1979; Shaw et al 1982; Brørs et al 1983) and warfarin (Wilting et al 1980a). Our experiments show a similar decrease of free fractions for phenobarbitone and phenytoin. The pH dependence of the binding to albumin, as seen in Fig. 1C, is probably caused by the neutral to base transition of albumin over the pH range 6 to 9. Warfarin and diazapam were found to have a higher affinity for the basic conformation of albumin, resulting in a lower free fraction at higher pH (Wilting et al 1980a, b). Since warfarin binds to site I on the albumin molecule, and diazapam to site II, both binding sites are believed to be influenced by this conformation

change (Van der Giesen 1982). Like warfarin, phenytoin binds to site I (Perucca et al 1981). It seems likely that the albumin-drug binding is also influenced by the change in the ionization of the drugs over the pH range used (Vallner et al 1979). The pK_a values of phenobarbitone and phenytoin are 7·4 and 8·3, respectively (Wade 1980).

The effect of Ca^{2+}

Wilting et al (1980a) reported for warfarin that, over the pH range 6 to 9, Ca^{2+} 2·5 mM favoured the neutral to base transition of albumin at a concentration of 4 g litre^{-1}. This resulted in a lower free fraction of this drug. However, when serum (albumin concentration about 40 g litre^{-1}) was used at physiological pH, no effect of Ca^{2+} was observed on the free fraction of warfarin (Van der Giesen 1982; Van der Giesen & Wilting 1982). These data are in agreement with our results.

The effect of buffer composition

When we investigated the influence of pH on the free fractions, we used (at physiological pH values) phosphate as well as borate buffers. Significantly different free fractions were found with these buffers (see Fig. 1C). In contrast to these findings, no such differences were reported in a study about the binding of warfarin to albumin, in which phosphate and borate buffers were used (Wilting et al 1980a). In agreement with our data, comparable differences between free fractions of imipramine (Bruun Kristersen & Gram 1982) and theophylline (Shaw et al 1982) were reported, when phosphate buffer and Krebs-Ringer buffer were used as dialysis fluid. It is likely, that the reported differences are caused by the various electrolyte compositions of the buffers. These data emphasize the role of buffer composition on the validity of absolute values of the free fractions of drugs. Though most investigators use a phosphate buffer, the composition of a dialysis buffer should be as physiological as possible. For samples in therapeutic drug monitoring, the use of protein-free ultrafiltrate of pooled human serum as dialysis fluid, could overcome some of the above problems (Bruun Kristersen & Gram 1982; Van der Giesen 1982).

Our data clearly indicate that (patho)physiologically induced changes in pH and serum albumin can significantly alter the free fractions of phenobarbitone and phenytoin, and consequently the therapeutic effect of these drugs. In our opinion, for patients whose (total) serum drug concentrations are in disaccordance with the observed clinical effects, interpretation of free drug levels should be made with reference to the biochemical parameters.

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