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THE INTERACTION OF ENFLURANE, HALOTHANE AND THE HALOTHANE METABOLITE TRIFLUOROACETIC ACID WITH THE BINDING OF ACIDIC DRUGS TO HUMAN SERUM ALBUMIN

AN *IN VITRO* STUDY

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Abstract—The interaction of the volatile anaesthetics enflurane, halothane and the halothane metabolite trifluoroacetic acid with the binding of two highly bound acidic drugs (warfarin, phenytoin) to albumin has been studied *in vitro* by equilibrium dialysis. Trifluoroacetic acid (TFA) inhibited the binding of both drugs to human serum albumin (HSA). Halothane, on the other hand, increased the binding of warfarin to HSA, while enflurane inhibited only the binding of phenytoin. It seems that the binding of the acidic drugs warfarin and phenytoin to HSA is more sensitive to the structures of the gases than for the basic drug diazepam which was previously shown to be equally affected by both gases. Furthermore, it seems that drugs competing for the same binding site (warfarin, phenytoin) may respond differently to conformational changes of the site. It is suggested that drugs bound to the "diazepam site" are more easily affected by the volatile anaesthetics than drugs bound to the "warfarin site".

It has recently been shown [1, 2] that volatile anaesthetics are able to suppress the binding of xenobiotics to serum albumin by lowering the affinity constants without changing the number of binding sites. It has furthermore been shown that halothane, enflurane and the halothane metabolite trifluoroacetic acid (TFA) displace the basic drug diazepam from human serum proteins probably by inducing conformational changes in human serum albumin (HSA) [2, 3]. If the anaesthetics inhibit drug serum protein binding by conformational changes of the albumin molecule, the two different binding sites on HSA, the "warfarin and diazepam sites" [4] could theoretically be affected simultaneously.

The present study was undertaken in order to evaluate the interaction of halothane, enflurane and TFA with the binding of the acidic drugs warfarin and phenytoin to human serum proteins and to discuss differences between acidic and basic drugs in this respect.

MATERIALS AND METHODS

Determination of serum protein binding. The binding of warfarin and phenytoin was determined by equilibrium dialysis performed at 23 ± 1 using plexiglass cells with two compartments separated by a dialysis membrane as described previously [2]. Equilibrium was achieved within 8 and 5 hr for warfarin and phenytoin, respectively, and the binding was stable for at least 24 hr. [^{14}C]labelled drug dissolved in ethanol was added to the buffer compartment to achieve an initial concentration of 2.25 μmol/l in all experiments. The final concentration of the solvent ethanol was always less than 0.5‰. Thin layer chromatography (TLC) in two different solvent systems, ethylenedichloride:aceton (90:10) and toluene:dioxane (90:10), on silica gel 60 plates (Merck 5553, F.R.G.) demonstrated identity between [^{14}C]-warfarin and warfarin. The purity of [^{14}C]warfarin was more than 98%. Stability of the isotope during dialysis was demonstrated by TLC after extraction [5] from buffer and serum after terminated dialysis. Minor radioactive impurities behaved identically with the isotope itself. Gas chromatographic analysis of phenytoin [6] showed no significant impurities as well as identity between [^{14}C]phenytoin and unlabelled drug. No sign of degradation was seen in serum or buffer post dialysis.

The concentration of drug in the protein and buffer compartment after equilibrium dialysis was determined as described previously [2, 7]. Duplicate aliquots of 100 μl were drawn from each dialysis cell compartment. The counting efficiency of the Packard Tri Carb (3255) operated at 4° was 68% for both drugs, and their binding to serum proteins was calculated from the distribution of labelled compound, radioactive recovery (93–101%) and added amount of drug.

The binding experiments were performed, as previously described [2], in a gas-tight glass desiccator

* Abbreviations used: HSA, human serum albumin; K_D, dissociation constant; n, number of binding sites; n/K_D, binding capacity; B, molar concentration of bound drug; F, molar concentration of unbound drug; P, molar concentration of protein; MAC, minimum alveolar concentration; TLC, thin layer chromatography; TFA, trifluoroacetic acid.

in which the volatile anaesthetics were introduced. The concentration of the anaesthetics in the desiccator was determined before, during and at the end of equilibrium dialysis and were always within ±10% of the intended concentration. The concentrations were chosen to compare the gases at equimolar concentrations (5%) as well as equianaesthetic concentrations (2.5 and 5% correspond to 3 MAC* of halothane and enflurane, respectively). The analysis of gas concentrations was performed on a gas–liquid chromatograph (Carlo Erba 2350) equipped with a flame ionization detector. A 2 mm inside diameter glass column packed with 10% Carbowax 30 on 80/100 Chromosorb WAW was used. The analytical coefficients of variations were similar for both gases (within day: <2%; day to day: <3%; N = 6 for both). TFA was added to the buffer compartment to achieve initial concentrations of 4 and 10 mmol/l.

Calculation of binding. The dissociation constant, K_D, was determined from the reciprocal of the slope of the computerized straight line achieved by plotting B/P (ordinate) against B (abscissa) as described by Romer and Bickel [9] according to their modification of the Scatchard relationship [10]:

$$B/P = \frac{1}{K_D}(B_{max} - B) \quad (1)$$

where B_{max} is the maximum molar concentration of bound drug and P the molar concentration of the protein. The concentration of protein ranged from 25 to 700 μmol/l, while the mean concentrations of phenytoin and warfarin were 1.3 and 2.0 μmol/l, respectively, as discussed previously [7].

The binding capacity (n/K_D) of HSA for warfarin and phenytoin was calculated according to mass law expressions [11] shown in equation where (n/K_D) is the slope of the computerized straight line [7].

$$B/F = \left(\frac{n}{K_D}\right) \times P \quad (2)$$

The concentration of protein was determined as described by Lowry *et al.* [12] using bovine serum albumin (fraction V) as standard.

Chemicals. Human serum albumin (HSA) fraction V, (A 1653, two different batches, not free of fatty acids) and bovine serum albumin (fraction V) were purchased from Sigma, U.S.A. Warfarin and phenytoin were obtained from Nyegaard & Co. and the Norwegian Drug Monopoly, Norway, respectively. [^{14}C]phenytoin (47.05 mCi/mmol, purity >99%) and [^{14}C]warfarin (49 mCi/mmol, purity > 98%) were purchased from New England Nuclear, U.S.A., and Amersham, U.K. respectively. Halothane (CF_3–CHBrCl) containing 0.02% thymol as preservative and enflurane (CHFCl–CF_2–O–CHF_2) free of other agents were supplied by Hoechst and Abott, Norway, respectively. All chemicals were standard commercial products of best quality (pro analysis).

Statistics. All lines were drawn by a computer (Hewlett Packard 85). Linear least square regression analysis was used for all straight lines. The fitness of experimental coordinates to straight lines are given as r^2 values. The significance of the lines for the relationship between x and y is given as P values. Two-tailed two sample t-test was used in all group comparisons, and P-values less than 0.05 were considered significant.

* The alveolar concentration at 1 atm. which produces immobility in 50% of subjects exposed to a noxious stimuli [8].

RESULTS

The binding parameters for the interactions of warfarin and phenytoin with HSA are given in Table 1 and displayed in Figs. 1 and 2. Warfarin was more strongly associated with HSA than phenytoin with K_D values of 5.9 ± 1.1 and 125 ± 20 × 10^{-6} M, respectively. The number of binding sites (n), however, approached one for phenytoin (0.89 ± 0.17) while the corresponding value for warfarin was 0.51 ± 0.15. The capacity (n/K_D) of HSA to bind warfarin was more than 10 times that of phenytoin, with values of 8.7 ± 2.4 and 0.71 ± 0.08 × 10^4 M^{-1}, respectively.

The effect of the volatile anaesthetics (see also Figs. 1 and 2) and TFA on drug binding to HSA is displayed in Tables 2 and 3. Enflurane 5% did not affect the binding capacity of HSA for warfarin being in contrast to the decrease (62% of control) observed for the interaction between phenytoin and HSA. Halothane 2.5 and 5% increased the binding capacity of HSA for warfarin in a dose-dependent fashion with 15 (not statistically significant) and 29%, respectively, while the binding of phenytoin was unaffected.

Trifluoroacetic acid, the metabolite of halothane,

Table 1. Binding parameters* for the interaction of warfarin and phenytoin with human serum albumin (HSA)

	$K_D \times 10^{-6}$ M	n	$n/K_D \times 10^4$ M^{-1}
Warfarin†	5.9 ± 1.1	0.51 ± 0.15	8.7 ± 2.4
Phenytoin†	125.0 ± 20.0	0.89 ± 0.17	0.71 ± 0.08

* Mean ± S.D. (n = 16).
† Initial concentration in the buffer compartment was 2.25 μmol/l.

Table 2. Effects of enflurane, halothane and trifluoroacetic acid (TFA) on the binding of warfarin* to human serum albumin (HSA)

	$n/K_D \times 10^4$ M^{-1}‡	
	Controls	Exposed
Enflurane 5%†	8.6 ± 0.6	8.3 ± 0.6
Halothane 5%	7.3 ± 0.6	9.4 ± 1.2§
Halothane 2.5%†	7.4 ± 0.6	8.5 ± 1.5
TFA 10 mM	11.6 ± 3.2	5.1 ± 0.5§
TFA 4 mM	11.6 ± 3.2	6.6 ± 0.8§

* Initial concentration in the buffer compartment was 2.25 μmol/l.
‡ Mean ± S.D., four separate determinations (each in duplicate).
† 3 MAC.
§ P < 0.05 (two-sample t-test).

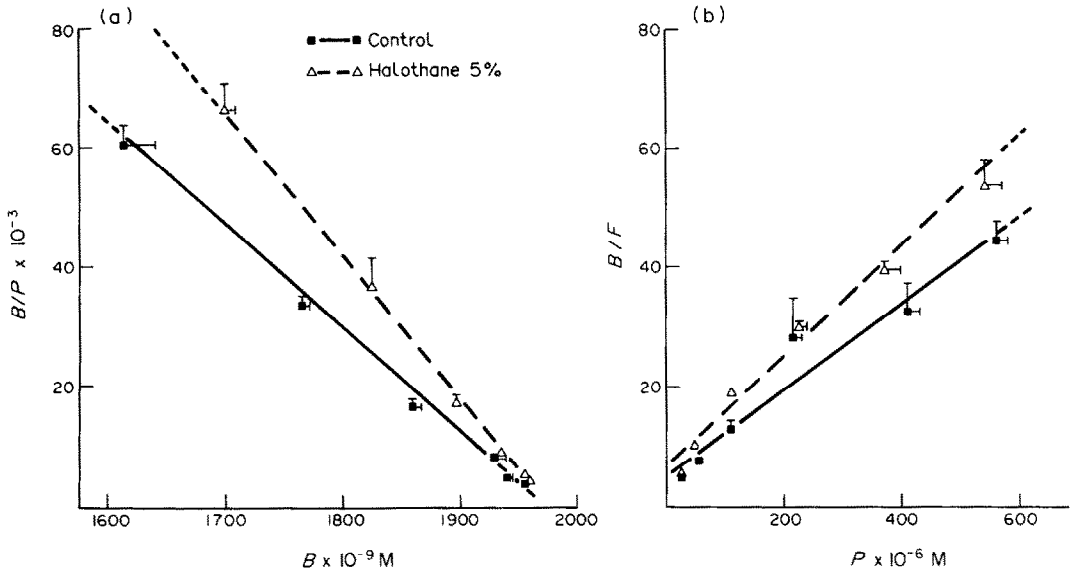

Fig. 1. The influence of 5% halothane on the binding of warfarin to human serum albumin (HSA). (a) Plotted according to Romer and Bickel [9] using the Scatchard relationship: $y = 344.755 - 0.175\,x$, $r^2 = 0.99$, $P < 0.01$ (control); $y = 470.612 - 0.238\,x$, $r^2 = 0.99$, $P < 0.01$ (halothane). (b) Plotted according to a modificiation of the law of mass action: $y = 4.905 + 0.073\,x$, $r^2 = 0.96$, $P < 0.01$ (control); $y = 6.375 + 0.094\,x$, $r^2 = 0.98$, $P < 0.01$ (halothane). B, F and P represent the molar concentrations of bound and unbound (free) drug, respectively. The concentration of albumin ranged from 25 to 700 μmol/l. Warfarin and phenytoin were added to the buffer compartment to an initial concentration of 2.25 μmol/l. Bars indicate standard deviations of mean values of four separate determinations (each in duplicate).

decreased the binding capacity of HSA for both drugs. At a TFA concentration of 4 mM the binding of warfarin and phenytoin was reduced to 56% and 85% of control, respectively, whereas the corresponding values at 10 mM of TFA were 44% and 77%.

DISCUSSION

The method for the determination of binding constants varying the concentration of protein rather than drug concentration was chosen for several reasons. It provides accurate experimental data at

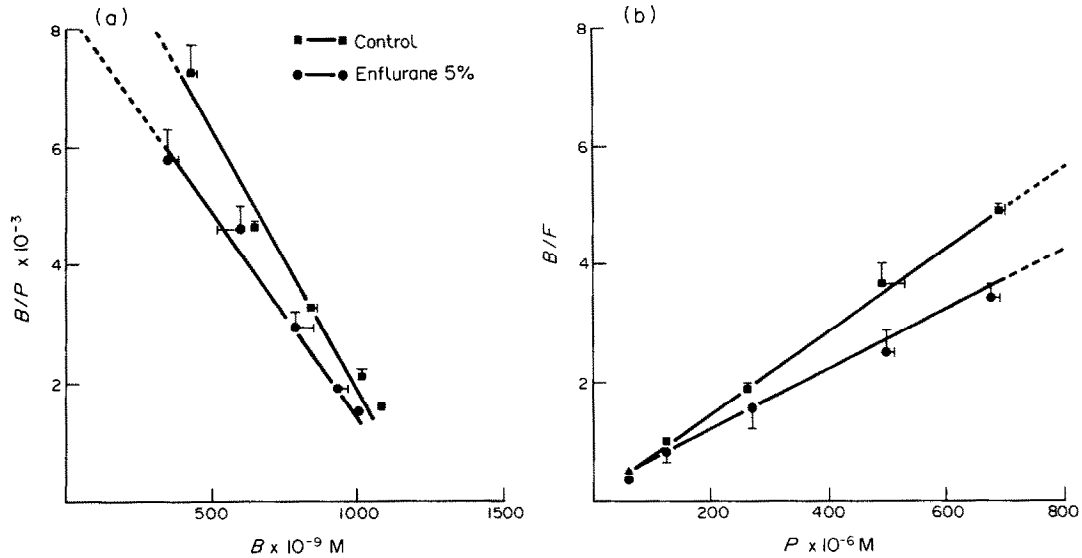

Fig. 2. The influence of 5% enflurane on the binding of phenytoin to human serum albumin (HSA). Conditions and designations are identical to those presented for warfarin in Fig. 1. (a) $y = 10.869 - 0.009\,x$, $r^2 = 0.99$, $P < 0.01$ (control); $y = 8.416 - 0.007\,x$, $r^2 = 0.98$, $P < 0.01$ (enflurane). (b) $y = 0.082 + 0.007\,x$, $r^2 = 0.99$, $P < 0.01$ (control); $y = 0.203 + 0.005\,x$, $r^2 = 0.99$, $P < 0.01$ (enflurane).

Table 3. Effects of enflurane, halothane and trifluoroacetic acid (TFA) on the binding of phenytoin* to human serum albumin (HSA)

	$n/K_D \times 10^4\,M^{-1}$‡	
	Control	Exposed
Enflurane 5%	0.82 ± 0.10	0.51 ± 0.02§
Halothane 5%	0.70 ± 0.08	0.68 ± 0.05
Halothane 2.5%	0.70 ± 0.02	0.70 ± 0.08
TFA 10 mM	0.65 ± 0.06	0.50 ± 0.01§
TFA 4 mM	0.65 ± 0.06	0.55 ± 0.06

Conditions and designations as described for Table 2.

low drug-to-protein concentration ratios [9]. Furthermore, under such conditions binding constants for one class of binding sites, the high affinity is usually determined [9, 13] making a discussion of binding interaction patterns possible [2, 14]. Finally, the method is favourable when the drug has a low aqueous solubility.

The latter is relevant where a large scale variation of drug concentrations is not feasible. However, by dissolving phenytoin in 2% ethanol, Kober *et al.* [15] calculated from scattered data the binding constants $K_D = 59 \times 10^{-6}$ M and $n = 1$ giving a n/K_D ratio of $1.69 \times 10^4\,M^{-1}$. As a consequence of the low solubility of phenytoin Bowmer and Lindup [16] reported only the binding capacity (n/K_D) of the phenytoin–HSA interaction ranging from 0.39 to $0.47 \times 10^4\,M^{-1}$. Although representing a wide range, these data are comparable with the results obtained in this study, especially those reported by Bowmer and Lindup [16].

Warfarin is usually considered to have one class of high affinity binding sites ($n = 1$) on HSA. It is, however, well known that fatty acids may interfere with drug binding to HSA [17] by altering both the number of binding sites as well as drug-protein affinities. The preparation of HSA used in this study was not free of fatty acids and this is probably the reason for the low number of binding sites. The apparent binding constants calculated in this study, however, compare favourably with those derived earlier for the high affinity class of warfarin in human serum ($K_D = 3.2 \times 10^{-6}$ M, $n = 0.30$) [14] giving a n/K_D ratio or binding capacity of 9.4×10^{-4} M.

The present study confirmed previous findings [1, 2] that volatile anaesthetics and the halothane metabolite TFA interact with the binding of xenobiotics to albumin. In this study, however, binding capacity (n/K_D) was preferred as parameter for the effect of interacting agents since by including both n and K_D it reflects the total binding differences. The effect of halothane and enflurane on the binding of the acidic drugs warfarin and phenytoin to HSA was smaller than and different from those earlier reported for the basic drug diazepam [2]. The main reason for these differences is probably that warfarin and phenytoin on one side and diazepam on the other side are bound to different binding sites on HSA, usually referred to as the "warfarin site" and the "diazepam site", respectively [4].

The two anaesthetics, halothane and enflurane, have previously been shown to affect to about the same degree the K_D value of the interaction between diazepam and HSA [2]. This indicates that different chemical structures may exert similar effect on the interaction between diazepam and HSA binding. The binding of warfarin to HSA, however, was more dependent on the structure of the interacting gases, since halothane increased the binding while enflurane did not influence the binding at all, indicating that warfarin or the "warfarin site" are more complex in nature than diazepam or the "diazepam site". Increased binding of warfarin to HSA due to interactions with fatty acids and other drugs has been shown by others and attributed to ligand-induced conformational changes of HSA [4, 14, 19]. However, most drugs displace warfarin from its binding site [4].

Although warfarin and phenytoin are shown to compete for the same binding site on HSA [4], the binding of the two drugs was affected differently by halothane and enflurane. This indicates that drugs competing for the same site do not necessarily respond similarly to allosteric changes of the site [18].

The binding of warfarin and phenytoin to HSA was much less affected by halothane and enflurane than the binding of diazepam to HSA [2]. Since warfarin binds with a lower and phenytoin with a higher dissociation constant than diazepam [2] (K_D values of 5.9 and 125×10^{-6} M, respectively, compared with 13×10^{-6} M for diazepam) [2] it seems unlikely that these differences are explained by the association "power" of the drugs. It seems more likely to assume that the investigated anaesthetics exert a more profound effect on the conformation of the "diazepam site" as compared to the "warfarin site". From this one may speculate whether the binding of drugs to the "diazepam site" in general will be more easily affected by volatile gases than those bound to the "warfarin site".

Trifluoroacetic acid decreased the binding of both drugs to HSA as also demonstrated for diazepam [2]. Compared to the gases it seems that TFA decreases drug binding to HSA in a more general fashion without preference for any of the binding sites. This indicates that TFA probably induces a more profound effect on the conformation than do the gases.

In conclusion, it has been demonstrated that enflurane, halothane and TFA are able to alter the binding of the acidic drugs warfarin and phenytoin to HSA, but to a lesser extent than reported earlier for the basic drug diazepam. Furthermore, it was shown that warfarin and phenytoin, bound to the same binding site, respond differently to halothane and enflurane. It is suggested that drugs bound to the "warfarin site" will be less affected by volatile anesthetics than those bound to the "diazepam site".

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