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PLASMA PROTEIN BINDING INTERACTION BETWEEN PHENYTOIN AND VALPROIC ACID *IN VITRO*

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1 Valproic acid or phenytoin were added to fresh human serum in varying concentrations and their binding characteristics determined by the method of Scatchard (1949).

2 Changes in serum albumin binding were investigated for phenytoin in the presence of 280, 560, 1050 and 2100 μmol l^{-1} valproic acid, and for valproic acid in the presence of 40, 120, 280 and 480 μmol l^{-1} phenytoin.

3 Phenytoin appeared to bind to a single site on the albumin molecule and could be competitively displaced from this site by concentrations of valproic acid above 280 μmol l^{-1}.

4 At high concentrations of valproic acid, the affinity of phenytoin for albumin was greatly decreased but the number of available binding sites was increased from one to four.

5 Valproic acid was bound to two high affinity and five low affinity binding sites but the latter were not detectable at valproic acid concentrations below 2100 μmol l^{-1}.

6 Phenytoin displaced valproic acid from its high affinity binding sites, although this was statistically significant only at a concentration of 480 μmol l^{-1} phenytoin.

Introduction

Most epileptic patients are treated with more than one antiepileptic drug and interactions frequently occur (Richens, 1977). Some of the commonly used antiepileptic drugs are highly bound to plasma proteins and displacement from binding sites represents one possible mechanism of interaction.

At therapeutic concentrations, phenytoin is approximately 90% bound to plasma proteins, mainly the albumin fraction (Glazko & Chang, 1972; Odar Cederlöf & Borgå, 1976) although a small amount of phenytoin binds to α-globulins (Lightfoot & Christian, 1966) and β-lipoproteins (Kramer & Richens, 1972). Drugs such as salicylate, sulfafurazole, phenylbutazone, acetazolamide and diazoxide have been shown *in vitro* to displace phenytoin from its binding sites, increasing the unbound concentration by as much as threefold (Andreason, 1973; Lunde, Rane, Yaffe, Lund & Sjöqvist, 1970; Roe, Podosin & Blaskovics, 1975). Phenytoin has been reported to displace certain of the tricyclic antidepressants from their binding sites, doubling the unbound levels (Borga, Azarnoff, Forshell & Sjöqvist, 1969).

Valproic acid is an antiepileptic drug with a simple two chain fatty acid structure which is approximately 90% bound to plasma proteins at a total valproic acid concentration of 350 μmol l^{-1}. Jordan, Shillingford & Steed (1976) found only negligible binding of the drug to either α or γ globulins. When valproic acid was added to the drug regime of patients taking phenytoin, a significant, although transient, fall in the total plasma phenytoin level was observed (Vakil, Critchley, Philips, Haydock, Cocks & Dyer, 1975; Lascelles, 1976).

Valproic acid has been reported to cause a significant *in vitro* displacement of phenobarbitone which did not increase with increasing valproic acid concentrations (Jordan *et al.*, 1976). These authors also found a small, but statistically non-significant, displacement of phenytoin by valproic acid. Patsalos & Lascelles (1977) found a significant displacement of phenytoin by valproic acid both *in vitro* using human plasma and *in vivo* using plasma/brain drug concentrations in rats.

The present investigation was undertaken to clarify the effect of valproic acid upon the binding of phenytoin and also to examine possible displacement of valproic acid by phenytoin.

Methods

Blood was drawn from the antecubital vein of normal healthy volunteers, allowed to clot for several hours

and spun to obtain serum. The serum was pooled and stored at −20°C until required.

To investigate plasma protein binding, aliquots of serum were incubated at room temperature (21–23°C) for 45 min with varying concentrations of either phenytoin (Parke-Davis) or valproic acid (Reckitt and Colman), spiked with [^{14}C]-phenytoin (N.E.N.) or [^{14}C]-valproic acid (supplied by Dr Harvey Kupferberg, N.I.H., Maryland) respectively to yield counts of approximately 50,000 disintegrations/min/ml (d/min) of serum. Following this incubation, the serum was divided into five smaller aliquots and incubated for 45 min at room temperature in the presence of varying concentrations of the displacing drug. Duplicate 2 ml samples were taken from each tube and subjected to ultrafiltration through visking tubing (Scientific Supplies) by centrifuging at 1000 *g* for 10 min, then 45 min (Lunde *et al.*, 1970). The 10 min ultrafiltrate was discarded and duplicate 100 µl samples of the 45 min ultrafiltrate were added to plastic vials containing 10 ml of Packard Instagel scintillant. Radioactivity was measured by counting the vials for 10 min in a Packard liquid scintillation spectrometer. The % of free drug was determined by expressing the d/min from the ultrafiltrate as a % of the total d/min in 100 µl of serum prior to ultrafiltration.

Concentrations used to investigate displacement of valproic acid by phenytoin were 40, 120, 280 and 480 µmol l^{-1} phenytoin, and concentrations of valproic acid for investigation of phenytoin displacement were 280, 560, 1050 and 2100 µmol l^{-1}. As phenytoin is poorly soluble in water it was dissolved in methanol to give a standard solution of 80 µmol l^{-1}. Since methanol can displace drugs (Lund *et al.*, 1970) the volume of methanol was kept constant (6 µl/ml serum). Valproic acid standard was dissolved in distilled water to give a concentration of 140 µmol l^{-1}, and the maximum amount of this solution added to serum was 15 µl/ml.

Total concentrations of phenytoin for the construction of the Scatchard plots were measured by gas liquid chromatography. The excretion procedure was as follows: to 1 ml serum was added 250 µl of 0.66 M HCl, 100 µl internal standard (5-(4-methylphenyl)-5 phenylhydantoin, 56 µmol l^{-1}) and 10 ml chloroform. The mixture was shaken for 5 min and centrifuged to separate the aqueous and organic layers. The organic phase was removed and blown down to dryness under a stream of air in a water bath at 60°C. The dry residue was taken up in 10 µl of the methylating agent, 0.1 M trimethyl-phenyl-ammonium-hydroxide (Eastman Kodak) and 1 µl of the sample injected into a Perkin Elmer F30 gas chromatograph fitted with a nitrogen specific rubidium bead detector. Drug concentrations were measured from the ratio of the peak heights of phenytoin and the internal marker.

Total serum concentrations of valproic acid were measured on a Perkin Elmer F11 gas chromatograph fitted with a flame ionization detector using the method of Schultz & Toseland (1977).

The results were analysed by the method of Scatchard (1949) in which the number of moles of drug bound per mole of albumin, $\bar{V}$, are calculated and plotted as the abscissa against $\bar{V}/A$ as the ordinate, where A is the number of unbound moles of drug.

From this data n and K were calculated from a least squares fit by computer using the model $\bar{V} = nKA - \bar{V}KA$ for the straight line fit and

$$\bar{V} = \frac{n_1 K_1 A}{1 + K_1 A} + \frac{n_2 K_2 A}{1 + K_2 A}$$

for a curved relationship, where n is the number of drug binding sites available on the albumin molecule and K is the apparent association constant of the drug for these sites (the subscripts 1 and 2 for n and K refer to the primary and secondary affinity binding sites where applicable).

Upper and lower 95% confidence limits were calculated for n and K because n is dependent on two variables and the upper and lower limits were not symmetrical. Using Fieller's Theorem (Armitage, 1974) the equation for calculation of the non-symmetrical limits was

$$\frac{n - \frac{gz}{y} \pm \frac{t}{b}\left(x - 2nz + n^2 y - g\left(x - \frac{z^2}{y}\right)\right)^{\frac{1}{2}}}{1 - g}$$

Where n = number of binding sites
t = t statistic for 95% confidence limits for number of observations − 2
b = plus slope
x = standard error of the intercept^2
y = standard error of the slope^2
z = -mean (x) xy
$g = \frac{t^2 y}{b^2}$

Results

Figure 1 shows the Scatchard plots for phenytoin in the presence of 0, 280, 560, 1050 and 2100 µmol l^{-1} valproic acid. The values of n and K for phenytoin binding together with the upper and lower 95% confidence limits are given in Table 1. As none of the regression lines for phenytoin touch at any point, there appeared to be significant displacement of phenytoin by valproic acid at each of the four concentrations used.

Values for n_1 and n_2 and K_1 and K_2 derived by adding valproic acid to human serum in concentrations of up to 7000 µmol l^{-1} are shown in Table 2. Figure 2 shows the corresponding Scatchard

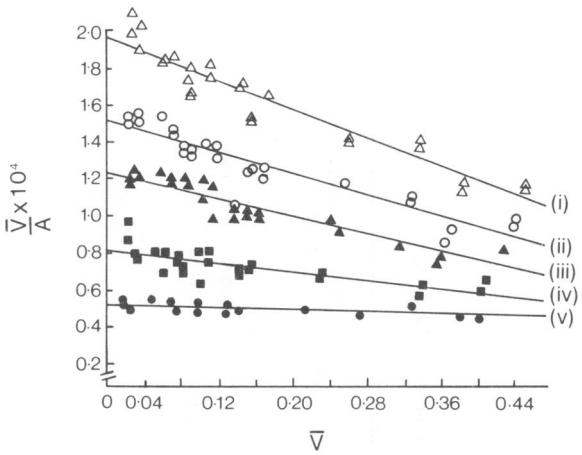

Figure 1 Scatchard plots for binding of phenytoin in human serum, and the effect of 0 (△); 280 (○); 560 (▲); 1050 (■); 2100 (●) μmol l^{-1} valproic acid. $\bar{V}$ molar ratio of bound ligand to albumin; A molar concentration of unbound ligand.

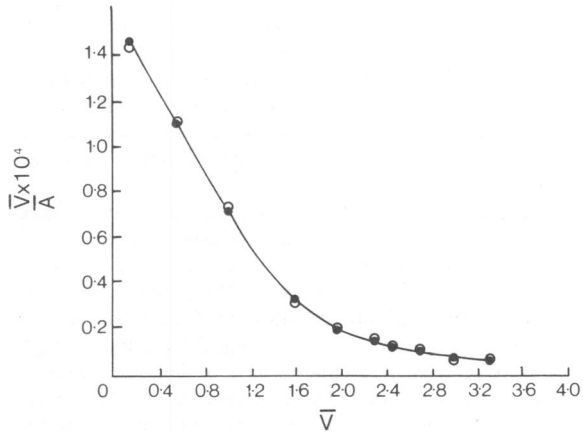

Figure 2 Scatchard plot for binding of valproic acid in human serum. $\bar{V}$ molar ratio of bound ligand to albumin; A molar concentration of unbound ligand. ○ experimental data; ● computer fitted data.

plot. Graphical representation of the effect of 0, 40, 120, 280 and 480 μmol l^{-1} phenytoin on the binding of valproic acid (below 2100 μmol l^{-1}) is shown in Figure 3 and the values for n and K in Table 3. The slopes and intercepts of the lines in Figure 3 were compared by Student's *t*-test and a significant difference was found between both slopes and intercepts of plot (i) (valproic acid alone) and plot (v) (valproic acid + 480 μmol l^{-1} phenytoin), and plot (ii) (valproic acid + 40 μmol l^{-1} phenytoin) and plot (v).

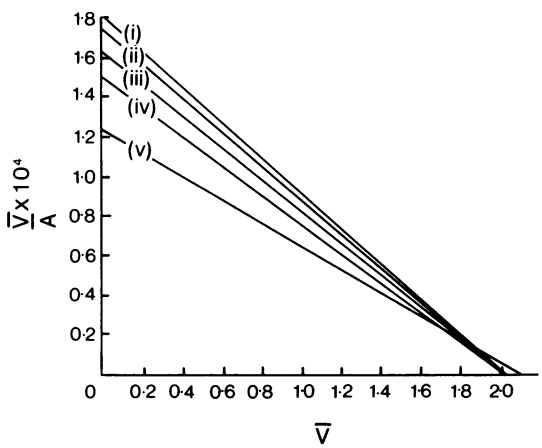

Figure 3 Scatchard plots for binding of valproic acid in human serum, and the effect of 0 (i); 40 (ii); 120 (iii); 280 (iv); 480 (v) μmol l^{-1} phenytoin. $\bar{V}$ molar ratio of bound ligand to albumin; A molar concentration of unbound ligand.

Discussion

The linearity of the Scatchard plot indicates that within the concentration range studied phenytoin bound to only one site on the albumin molecule with an apparent association constant (K) of 1.86×10^4 l/mol. Phenytoin was displaced *in vitro* from its binding site on serum albumin by concentrations of valproic acid above 280 μmol l^{-1} (therapeutic range 300–700 μmol l^{-1}), and this

Table 1 Number of binding sites, n, and apparent association constant K (l/mol) for binding of phenytoin in serum containing varying concentrations of valproic acid

Valproic acid (μmol l^{-1})	*n*	*95% confidence limits*		$K \times 10^4$	*95% confidence limits*	
		Upper	*Lower*		*Upper*	*Lower*
0	1.04	1.22	0.90	1.86	2.09	1.63
280	1.07	1.31	0.90	1.42	1.65	1.19
560	1.10	1.34	0.92	1.11	1.28	0.94
1050	1.44	2.18	1.12	0.57	0.76	0.38
2100	4.81	24.26	2.62	0.11	0.19	0.03

displacement of phenytoin appeared to increase proportionately with the concentration of valproic acid. The decrease in the value of K with increasing valproic acid concentration is indicative of competitive displacement of phenytoin. In the presence of 2100 μmol l^{-1} of valproic acid the number of phenytoin binding sites on the albumin molecule increased to four.

Valproic acid bound to both high and low affinity binding sites on the albumin molecule, although the 5 low affinity sites were only detectable at concentrations of valproic acid above 2100 μmol l^{-1}. Valproic acid had two high affinity sites, but from the data used to investigate low affinity binding, the number of high affinity sites was calculated as 1.6. This discrepancy may be due to the different ranges of concentration used. It is, however, more likely to have been due to the use of serum from non-fasting volunteers in the latter case. A higher level of endogenous fatty acids may have caused displacement of valproic acid thus reducing the apparent number of binding sites. This is substantiated by our unpublished data showing that palmitic acid competes strongly with, and will readily displace, valproic acid from its primary binding sites. Phenytoin tended to decrease K for valproic acid binding to the high affinity sites, but this decrease was significant only when the phenytoin concentration was greater than 480 μmol l^{-1}. Since n did not alter, the interaction between phenytoin and valproic acid appeared to be competitive and as K was decreased for both drugs they may be competing for the same binding site on the albumin molecule.

It is possible, therefore, that in an equimolar solution of both drugs, phenytoin, which has a greater affinity for the albumin molecule, will more readily displace valproic acid from their common binding site. However, as valproic acid has a second available primary binding site, the overall effect may not be as marked as the displacement of phenytoin (which has only a single binding site).

Rudman, Bixler & del Rio (1970) examined the effect of oleic and palmitic acid on the protein binding of several drugs including phenytoin. They found that 3.5 moles of fatty acid/mol of albumin caused a displacement of phenytoin which was measured as a reduction in the n_1K_1 value from a Scatchard plot. They believed this shift could have been caused by competitive displacement. However, a disproprotionately large change in the n_1K_1 value caused by addition of 7 moles of fatty acid per mole of albumin was most probably caused by a change in the conformation of the phenytoin binding site.

Valproic acid has the structure of a simple fatty acid and at a concentration of 2100 μmol l^{-1} (3.6 moles acid/mole albumin) caused an increase in the number of phenytoin primary binding sites on the albumin molecule from one to four. The association constant was reduced so that the strength with which

Table 2 Number of binding sites, n_2, n_2 and apparent association constants K_2 and K_2 (l/mol) for valproic acid binding in human serum in concentrations up to 7000 μmol l^{-1}

n_1	95% confidence limits		n_2	95% confidence limits		$K_1 \times 10^4$	95% confidence limits		$K_2 \times 10^4$	95% confidence limits	
	Upper	*Lower*		*Upper*	*Lower*		*Upper*	*Lower*		*Upper*	*Lower*
1.600	1.627	1.573	4.620	4.832	4.408	0.941	0.968	0.914	0.012	0.014	0.011

Table 3 Number of binding sites, n and apparent association constants K (l/mol) for valproic acid binding in serum containing various concentrations of phenytoin

Phenytoin ($\mu mol\ l^{-1}$)	*n*	*95% confidence limits*		$K \times 10^4$	*95% confidence limits*	
		Upper	*Lower*		*Upper*	*Lower*
0	2.00	2.35	1.79	0.90	0.97	0.83
40	2.00	2.43	1.79	0.87	0.95	0.79
120	2.02	2.30	1.74	0.81	0.88	0.74
280	2.01	2.51	1.65	0.75	0.82	0.67
480	2.10	2.86	1.40	0.58	0.63	0.53

phenytoin bound to this increased number of sites was not increased. However, as suggested above, valproic acid may have caused a conformational change in the albumin molecule resulting in more sites becoming available to which phenytoin could bind.

The displacement of phenytoin by valproic acid *in vitro* is in accordance with the slight transient fall in total serum phenytoin levels observed when sodium valproate is added to phenytoin therapy (Vakil *et al.*, 1976; Lascelles, 1976). Assuming that displacement of phenytoin occurs *in vitro*, the result would be an increase in the amount of unbound drug available for both pharmacological action and metabolism. An increase in hepatic clearance would lead to a fall in the total serum concentration. Thus, the displacement of phenytoin from its binding site would be compensated for by a reduction in the total drug concentration which promotes a new equilibrium between free and bound drug.

In equimolar concentrations it is likely that phenytoin and valproic acid will displace each other from a common binding site on the albumin molecule. However, as the therapeutic range of serum concentrations of valproic acid is about seven times higher than phenytoin on a molar basis, it is unlikely that phenytoin will cause an important displacement of valproic acid in the clinical situation. Conversely it is probable that valproic acid would produce a clinically important displacement of phenytoin. This possibility needs to be borne in mind when sodium valproate is added to existing phenytoin therapy.

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References

ANDREASEN, F. (1973). Protein binding of drugs in plasma from patients with acute renal failure. *Acta Pharmac. Tox.*, **32**, 417–429.

ARMITAGE, P. (1974). *Statistical Methods in Medical Research*, p 447. Oxford: Blackwell Scientific Publications.

BORGÅ, O., AZARNOFF, L., FORSHELL, G.P. & SJÖQVIST, F. (1969). Plasma protein binding of tricyclic antidepressants in man. *Biochem. Pharmac.*, **18**, 2135–2143.

GLAZKO, A.J. & CHANG, T. (1972). Diphenylhydantoin: absorption, distribution and excretion. In *Antiepileptic Drugs*. Eds: D.M. Woodbury, J.K. Penry & R.D. Schmidt, pp 127–136. New York: Raven Press.

JORDAN, B., SHILLINGFORD, J.S. & STEED, K.P. (1976). Preliminary observations on the protein-binding and enzyme-inducing properties of sodium valproate (Epilim). In *Clinical and Pharmacological Aspects of Sodium Valproate (Epilim) in the Treatment of Epilepsy*. Ed: Legg, N.J. pp 112–116. Tunbridge Wells: MCS Consultants.

KRAMER, R.L. & RICHENS, A. (1972). Two dimensional immunoelectrophoresis of human serum proteins for the investigation of protein binding of drugs. *Br. J. Pharmac.*, **45**, 184P.

LASCELLES, P.T. (1976). Plasma concentration of anticonvulsant drugs and the management of epilepsy. In *Twelfth Symposium on Advanced Medicine*, Ed: Peters, D.K. pp 108–117. London: Pitman Medical Publishers Ltd.

LIGHTFOOT, R.W. & CHRISTIAN, C.L. (1966). Serum protein binding of thyroxine and diphenylhydantoin. *J. clin. Endocrinol. Metab.*, **26**, 305–308.

LUNDE, P.K., RANE, A., YAFFE, S.J., LUND, L. & SJÖQVIST, F. (1970). Plasma protein binding of diphenylhydantoin in man: interaction with other drugs and the effect of temperature on plasma dilution. *Clin. Pharmac. Ther.*, **11**, 836–855.

ODAR-CEDERLÖF, I. & BORGÅ, O. (1976). Impaired plasma protein binding of phenytoin in uremia and displacement effect of salicyclic acid. *Clin. Pharmac. Ther.*, **20**, 36–47.

PATSALOS, P.N. & LASCELLES, P.T. (1977). Valproate may lower serum phenytoin. *Lancet*, **i**, 50.

RICHENS, A. (1977). Interactions with antiepileptic drugs. *Drugs*, **13**, 266–275.

ROE, T.F., PODOSIN, R.L. & BLASKOVICS, M.E. (1975). Drug interaction: diazoxide and diphenylhydantoin. *J. Pediatr.*, **87**, 480–484.

RUDMAN, D., BIXLER, T.J. & del RIO, A.E. (1971). Effect of

free fatty acids on binding of drugs by bovine serum albumin, by human serum albumin, and by rabbit serum. *J. Pharmac. exp. Ther.*, **176**, 261–262.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SCHULTZ, F.U. & TOSELAND, P.A. (1977). Determination of the anticonvulsant drug sodium di-n-propylacetate in human plasma by gas chromatography. *Ann. clin. Biochem.* (in press).

VAKIL, S.D., CRITCHLEY, E.M.R., PHILIPS, J.C., HAYDOCK, C., COCKS, A. & DYER, T. (1976). The effect of sodium valproate (Epilim) on phenytoin and phenobarbitone blood levels. In *Clinical Pharmacological Aspects of Sodium Valproate (Epilim) in the Treatment of Epilepsy*. Ed: Legg, N.J. pp 75–77. Tunbridge Wells: MCS Consultants.

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