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Dose-dependent Inhibition in Plasma Protein Binding of Valproic Acid During Continued Treatment in Guinea-pigs

HSIU-YING YU AND YU-ZEN SHEN*

*School of Pharmacy and *Department of Pediatrics, College of Medicine, National Taiwan University, Taipei 10018, Taiwan, ROC*

Abstract—Plasma protein binding of valproic acid over a wide range of steady-state plasma concentration (11·3 ± 2·6–1303·0 ± 122·9 μg mL^{-1}: s.e.m., n = 5) in guinea-pigs has been studied. Valproic acid was given by intravenous constant infusion. At steady-state the plasma protein binding of valproic acid was analysed. Nonlinear binding was observed. Unbound fraction (f_u) of valproic acid increased from 25 to 95% with the increase of steady-state plasma concentration (C_{ss}). The plasma protein-bound drug concentration (C_b) of valproic acid increased initially with C_{ss} but decreased after the C_{ss} exceeded 345·0 μg mL^{-1}, where the C_b was 152·5 ± 26·8 μg mL^{-1}. At a C_{ss} of 1303·3 ± 122·9 μg mL^{-1} the C_b was significantly ($P < 0·05$) decreased to 72·8 ± 20·2 μg mL^{-1}. Binding characteristics of valproic acid in-vitro were studied using drug-free guinea-pig plasma with added valproic acid (10–1000 μg mL^{-1}). The binding behaviour was also nonlinear in-vitro. The f_u increased from 14 to 79% with the increase of valproate concentrations. No decrease in C_b was observed throughout the range. The study demonstrated that binding characteristics of valproic acid in-vivo and in-vitro are not parallel. The results suggest that valproic acid may produce or induce plasma protein binding competitors; metabolites of valproic acid may be implicated.

Valproic acid is an antiepileptic drug effective in the treatment of intractable seizures (Wu et al 1984; Rall & Schleifer 1985). This branched chain fatty acid, like most fatty acids, is highly bound to plasma protein (Löscher 1978; Yu 1981; Yu et al 1985). Plasma protein binding of a drug is an important factor affecting pharmacokinetic parameters such as plasma concentration (Greenblatt et al 1982; Yu 1984), volume of distribution and clearance (Levy 1976; Tozer 1981). The saturability in plasma protein binding within therapeutic plasma concentration (Cramer & Mattson 1979), inhibition of plasma protein binding by other substances (Monks et al 1978; Cramer & Mattson 1979; Zimmerman et al 1981; Yu et al 1989) and variation in plasma protein binding of valproic acid (Marty et al 1982; Cramer et al 1984; Yu et al 1989) may result in an unpredictable dose–concentration–response relationship for valproic acid.

In general, detailed study of the characteristics of plasma protein binding of a drug is performed in-vitro using drug-free plasma or serum to which has been added serial concentrations of the investigated drug. However, metabolites of the parent drug or drug-induced biochemical changes in endogenous substances, which may interfere with plasma protein binding of the parent drug in-vivo, are not present in the medium in such in-vitro binding studies. Application of in-vitro plasma binding data for interpretation of in-vivo pharmacokinetics may therefore be misleading. It is, therefore, worthwhile to elucidate the correlation between in-vitro and in-vivo binding characteristics of valproic acid. Dosing to achieve a steady-state was designed for the in-vivo binding study because clinically valproic acid should be maintained at steady-state for the treatment of epilepsy. In order to avoid the effects of free fatty acids (Patel & Levy 1979; Zimmerman et al 1981) produced by in-vitro lipolysis of triglycerides in plasma during equilibrium dialysis, a method using ultrafiltration was used in the present study.

Correspondence: H.-Y. Yu, School of Pharmacy, College of Medicine, National Taiwan University, 1 Jen-Ai Road, Section 1, Taipei 10018, Taiwan, ROC.

Materials and Methods

Chemicals

Sodium valproate crystalline powder was a gift from Labaz, France. All other reagents were of analytical grade from E. Merck, Germany.

Animal experiments

Male guinea-pigs, 230–300 g, were obtained from the Experimental Animal Center, College of Medicine, National Taiwan University. The animals were given a bolus dose of sodium valproate solution followed by constant infusion through a jugular vein PE-50 catheter (Clay Adams) using a constant rate infusion pump (B. Braum Melsungen AG, Germany). The bolus doses were estimated as

$$\text{dose} = C_p \times Vd_{ss}$$

where C_p was the expected plasma level and Vd_{ss} was the volume of distribution at steady state for valproic acid previously determined (Yu et al 1987). The infusion doses ranged from 50 to 2000 μg min^{-1} kg^{-1}, and infusion times ranged from 5 to 8 h. At the steady-state assessed by a constant plasma concentration in three consecutive blood samples at 30 min time intervals, blood samples were collected from the carotid artery catheter (PE-50 tubing, Clay Adams) and analysed for total and unbound valproic acid immediately.

In-vitro study

Blood was drawn from a catheter in the carotid artery of drug-free guinea-pigs by a syringe previously treated with the anticoagulant sodium citrate, and pooled. To the plasma was

then added valproic acid to give the following final concentrations: 10, 25, 50, 100, 200, 300, 500 and 1000 μg mL^{-1}.

Determination of drug concentrations

The concentration of valproic acid was determined by GLC (Yu 1981). In brief, a 100 μL sample acidified with 50 μL of 3 M sulphuric acid was added to 100 μL of chloroform containing octanoic acid (1/10000) as an internal standard. After vortex mixing and centrifugation, 1 μL of the chloroform layer was analysed on a Hewlett-Packard GLC (HP 5840A), equipped with a flame ionization detector and a glass column packed with 10% FFAP on Chromosorb W AW-DMCS 80–100 mesh. The oven temperature was maintained at 190°C and nitrogen was used as the carrier gas. Plasma unbound drug was separated by a CF 25 Centriflo Membrane Cone (Amicon, Lexington, MA). After centrifugation, the ultrafiltrate was assayed for unbound valproic acid by GLC.

Determination of free fatty acid

Free fatty acid concentrations were determined by a spectrophotometric method using reagent kits (Wako Pure Chemical Industries Ltd, Japan). Since valproic acid is also a fatty acid, its presence would lead to an overestimation of endogenous free fatty acid. A calibration curve of spectrophotometric absorbance vs valproate concentration was used for the correction of free fatty acid concentration.

Parameter estimation

Scatchard plots (i.e. C_b/C_u vs C_b) (Scatchard 1949) were initially constructed to reveal multiplicity in the binding sites and to provide rough estimates of the binding parameters as the initial values for curve fitting. The plasma protein binding data were analysed according to the Langmuir model (eqns 1, 2) and a modified two-site binding model (eqn 3) (Ağabeyoğlu et al 1979; Semmes & Shen 1990).

$$C_b = N \times C_u/(K_d + C_u) \quad (1)$$

$$C_b = N_1 \times C_u/(K_{d1} + C_u) + N_2 \times C_u/(K_{d2} + C_u) \quad (2)$$

$$C_b = N_1 \times C_u/(K_{d1} + C_u) + (N_2/K_{d2}) \times C_u \quad (3)$$

where C_b is the concentration of bound drug obtained from subtracting unbound (C_u) from total (C_t) drug concentrations. K_d is the apparent dissociation constant for the binding site, N is the concentration of binding sites available on plasma protein. Subscripts 1 and 2 denote the first and the second class of sites, respectively. Akaike's information criterion (AIC) (Yamaoka et al 1978) was used as the criterion for choosing the best model.

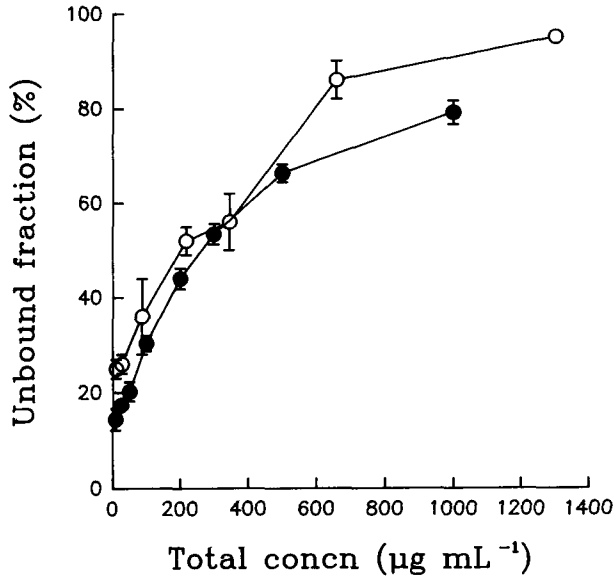

FIG. 1. Relationship between unbound fraction and total plasma concentration of valproic acid. Each point with vertical bar represents mean ± s.e.m. of five determinations. Key: ○, steady-state plasma samples; ●, plasma samples with added valproic acid.

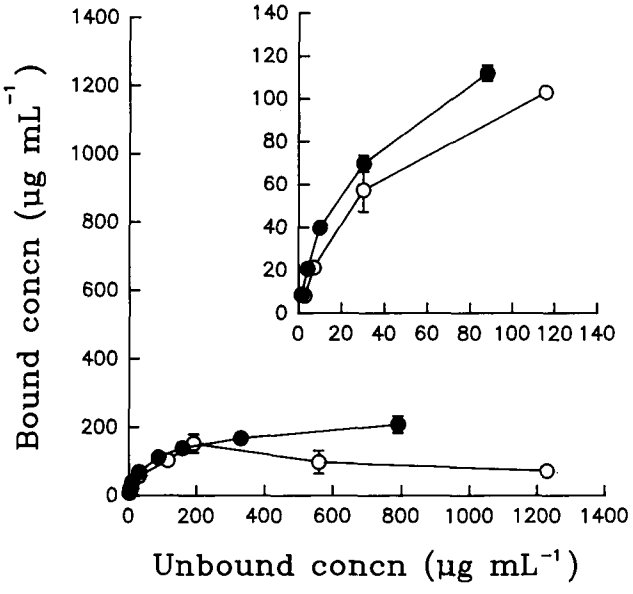

FIG. 2. Plasma bound concentration of valproic acid vs unbound plasma concentration. The insert shows an expanded plot of the data in the low concentration range. Each point with a vertical bar represents mean ± s.e.m. of five determinations. Key: ○, steady-state plasma samples; ●, plasma samples with added valproic acid.

Results

Fig. 1 shows a plot of the in-vitro and the in-vivo f_u as a function of total plasma concentration (C_t, which is the same as C_{ss} in the in-vivo study) of valproic acid. The concentration-dependent binding of valproic acid to guinea-pig plasma is presented in a plot of C_b as a function of C_u (Fig. 2). Plasma protein binding of valproic acid at various C_{ss} values is summarized in Table 1. The C_b was significantly decreased at a C_{ss} of 1303·3 μg mL^{-1} compared with that at a C_{ss} of 345·0 μg mL^{-1}.

Free fatty acids were approximately 0·3 mEq L^{-1} and were not significantly altered in the presence of valproic acid. The interference of valproic acid with the spectrophotometric method was negligible when valproate concentration was less than 200 μg mL^{-1}.

The Scatchard plots (Fig. 3) of plasma protein binding observed from the in-vitro experiment were nonlinear, which indicated that at least two binding sites were responsible for valproate binding. A steep initial descending portion of the

Table 1. Plasma protein binding of valproic acid at steady-state plasma concentration in guinea-pigs.

		A	B	C	D	E	F	G
Steady-state concentration ($\mu g\ mL^{-1}$)		11·3 2·6	28·6 3·8	87·5 7·3	218·6 15·8	345·0 52·6	659·1 51·8	1303·3 122·9
Unbound concentration ($\mu g\ mL^{-1}$)		2·9 0·7	7·4 1·5	30·1 5·0	115·7 14·4	192·5 40·3	560·7 30·9	1230·5 114·7
Unbound fraction (%)		25 2	26 2	36 8	52[a] 3	56[a] 6	86[a] 4	95[a] 1
Bound concentration ($\mu g\ mL^{-1}$)		8·1 2·2	21·2 2·3	57·4 10·2	103·0 2·3	152·5 26·8	98·4 33·6	72·8[b] 20·2
Free fatty acid ($mEq\ L^{-1}$)	0·32[c] 0·03	0·33 0·02	0·29 0·02	0·31 0·03	0·34 0·03	0·30 0·03	0·32 0·02	0·32 0·03

Data are mean ± s.e.m. of five determinations. [a] Significantly different ($P<0·05$) from group A by Student's t-test. [b] Significantly different ($P<0·05$) from group E by Student's t-test. [c] Pre-dose value.

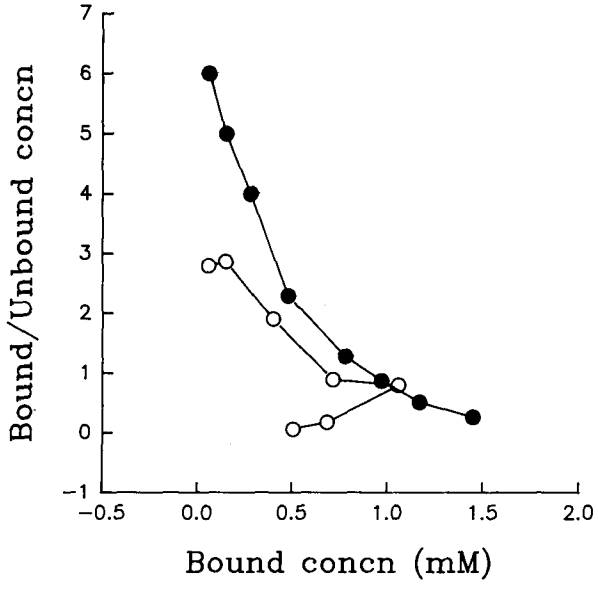

FIG. 3. Scatchard plots for binding of valproic acid to plasma of guinea-pigs. Each point represents mean of five determinations. Key: ○, steady-state plasma samples; ●, plasma samples with added valproic acid.

Table 2. Binding parameters of valproic acid in guinea-pig plasma estimated from in-vitro experiments.

One binding-site model	A	
	(mM)	($\mu g\ mL^{-1}$)
N	1·49 (0·08)	214·9
K_d	0·50 (0·10)	72·1
AIC	−24·05	

Two binding-site model	B		C	
	(mM)	($\mu g\ mL^{-1}$)	(mM)	($\mu g\ mL^{-1}$)
N_1	1·04 (0·18)	149·9	1·06 (0·06)	152·9
K_{d1}	0·23 (0·07)	33·2	0·24 (0·04)	34·6
N_2	4·95 (37·34)	713·8		
K_{d2}	53·17 (461·14)	7667·1		
N_2/K_{d2}			0·08 (0·01)	
AIC	−34·88		−36·39	

The standard error of nonlinear estimation is in parentheses.
A: $C_b = N \times C_u/(K_d + C_u)$.
B: $C_b = N_1 \times C_u/(K_{d1} + C_u) + N_2 \times C_u/(K_{d2} + C_u)$.
C: $C_b = N_1 \times C_u/(K_{d1} + C_u) + (N_2/K_{d2}) \times C_u$.

Scatchard plot which represented a high affinity with low capacity site appeared in in-vitro but not in in-vivo data. The in-vivo data revealed an unusual plot which indicated a pronounced decrease in binding following the increase of C_{ss} beyond 2·39 mM (345 $\mu g\ mL^{-1}$). The binding parameters estimated from in-vitro data by equations 1, 2 and 3 are presented in Table 2. The AIC test (Yamaoka et al 1978) indicated that the modified two binding-site model is more adequate than the one binding-site model for describing the plasma protein binding of valproic acid in-vitro. The in-vivo data, however, were not appropriate to be fitted by these equations. A more precise model including factors causing the decrease of C_b with increase of plasma concentration of valproic acid is required for accurate estimation of in-vivo binding parameters.

Discussion

The finding of two classes of valproic acid binding sites in guinea-pig plasma as observed from the present in-vitro study is in agreement with other studies on human or animal plasma (Barre et al 1985; Semmes & Shen 1990). The N_1 and N_2 values (Table 2B) are almost equivalent to those found in human serum, N_1 0·928 mM and N_2 4·188 mM, estimated by the same method (Barre et al 1985). The result with respect to the best fit model for the binding data is in agreement with that observed from an in-vitro study on rat plasma (Semmes & Shen 1990).

Plasma protein binding of valproic acid is saturable, and the unbound fraction of valproic acid is concentration-dependent. To discriminate between inhibition and simple saturation in the plasma protein binding of a drug by a C_u or f_u vs C_t plot is difficult (Fig. 1), but a plot of C_b vs C_u or C_t (Fig. 2) demonstrated the difference. According to the saturable binding theory, the C_b should remain constant after saturation regardless of the increase in C_t. Two possible mechanisms for the decreased binding were considered: decrease in binding ability owing to conformational modification of the protein induced by the very high valproate concentrations; and increase in binding competitors. Brodersen et al (1990) have studied the plasma protein binding of

valproic acid in the range 20 μM–50 mM (2·88–7210 μg mL^{-1}). They found that free valproate concentration above 10 mM (1442 μg mL^{-1}) may cause irreproducible results, and suggested that a slow irreversible change may take place in the albumin molecule above 10 mM free valproate concentration. In the present article the highest valproate concentration, either total or unbound, was far below their reported critical concentrations. Accordingly, the conformational modifications of the protein would not be expected at the valproate concentration levels in our study. Furthermore, a decrease in C_b was observed only in the in-vivo study but not in the in-vitro study with equivalent high valproate concentrations. This would appear to rule out an effect on the conformation of the binding protein. We suggest that there might be induction and production of binding competitors by valproic acid in-vivo. Free fatty acid could inhibit the plasma protein binding of valproic acid (Monks & Richens 1978; Bowdle et al 1982; Brodersen et al 1990). However, it is unlikely that an increase in valproate concentration without an increase in free fatty acids would potentiate the inhibitory effect of the free fatty acids on valproate protein binding. In general, binding competition between two highly bound drugs favours that of the higher concentration. The present study showed that valproate binding was decreased in spite of the increased valproate-to-free fatty acid concentration ratio at the very high valproate concentration in-vivo. The decreased valproate binding seems not due to free fatty acid.

The most probable binding competitors are metabolites of valproic acid. A metabolite, 2-dehydrovalproic acid, showed a much higher affinity for the rat plasma proteins than its saturated precursor (Nau et al 1984; Semmes & Shen 1990), and elimination of the metabolite was slower than that of valproic acid (Rettenmeier et al 1986; Nau & Löscher 1985). The concentration ratio of metabolite to valproic acid in mouse, rat and human plasma after a single dose of valproic acid was 3 to 16% (Löscher 1981a; Nau & Löscher 1982, 1984; Löscher & Nau 1983), and that in dog plasma after continued treatment is 56 to 81% (Löscher 1981b). Although the concentration ratio in guinea-pig plasma has not been reported, it is reasonable to assume it to be in the same range. Since the plasma elimination of this metabolite is slower than that of its parent drug (Rettenmeier et al 1986; Nau & Löscher 1985), the plasma concentration ratio in guinea-pig after constant intravenous infusion of valproic acid for several hours would be much higher than that observed after a single dose, and probably as high as that found in dog plasma after continued treatment, and hence might significantly interfere with the plasma protein binding of valproic acid. The characteristics of plasma protein binding of valproic acid in-vivo after high doses cannot, therefore, be properly described by the conventional binding models (Ağabeyoğlu et al 1979; Semmes & Shen 1990). A binding model involving dose-dependent concentrations of binding metabolites is required. The binding parameters estimated from the lower C_{ss} (up to 345·0 μg mL^{-1}, the deflection point of C_b) in the in-vivo study by the one binding site model (Table 3) are comparable with those from the in-vitro study (Table 1). This result suggests that at a therapeutic plasma level the concentration of metabolites might be too low to exert significant displacement on plasma protein binding of valproic acid. In order to reveal significant plasma binding displacement, a binding displacer must have not only strong affinity to the same binding sites but also a concentration approaching the molar concentration of protein to saturate the majority of the binding sites (Rowland 1980). The application of the in-vitro binding parameters for the interpretation of in-vivo kinetics of valproic acid is therefore, restricted only to the therapeutic concentration range and attention should be paid to the accumulation of binding competitors during long term therapy with valproate.

Table 3. Binding parameters of valproic acid estimated from the in-vivo data of steady-state plasma concentrations not exceeding 345 μg mL^{-1} in guinea-pigs.

One-binding site model		
	(mM)	(μg mL^{-1})
N	1·36 (0·21)	196·1 (30·3)
K_d	0·57 (0·21)	82·2 (30·3)
AIC	−18·11	
Two-binding site model		
	(mM)	(μg mL^{-1})
N_1	0·46 (207·9)	66·6 (29979·2)
K_{d1}	0·76 (135·9)	109·6 (19596·8)
N_2	0·85 (211·1)	122·6 (30440·6)
K_{d2}	0·41 (27·5)	59·1 (3965·5)
AIC	−14·11	

Data in parentheses are the standard errors of nonlinear estimation.

Dose-dependent pharmacokinetics of valproic acid in guinea-pigs has been reported previously (Yu et al 1987). The decrease in plasma protein binding with increase in doses may contribute in part to the nonlinear kinetics of valproic acid. Since the elimination of valproic acid is predominantly by hepatic metabolism (Gugler et al 1977; Gugler & Von Unruh 1980), and is limited (Löscher 1978), total body clearance (CL_{tot}) of valproic acid is therefore a function of its unbound fraction (f_u). A pronounced increase in f_u at toxic plasma concentrations could markedly decrease CL_{tot} subsequent to metabolic saturation.

The dose-dependent inhibition of plasma protein binding of valproic acid by its metabolites might also play a role in causing variation of plasma protein binding of valproic acid in epileptic patients (Bowdle et al 1980; Marty et al 1982; Patel et al 1982; Yu 1984) because of inter-subject variations in production and elimination of binding competitor. The presence of metabolic inducers which accelerate the production of binding competitive metabolites should also be considered.

In conclusion, the behaviour of plasma protein binding of valproic acid after continuous drug administration is not parallel to that observed from plasma with added valproic acid. The plasma protein binding of valproic acid is inhibited by its metabolites or by induced endogenous substances in-vivo and is dose-dependent. Further studies are necessary for the identification of these binding displacers.

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