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Research Article

Nonlinear Binding of Valproic Acid (VPA) and E-Δ^2-Valproic Acid to Rat Plasma Proteins

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The binding characteristics of valproic acid (VPA) and its pharmacologically active monounsaturated metabolite, E-Δ^2-VPA, to rat plasma proteins were compared. The plasma free fraction was determined by a rapid equilibrium procedure, which minimizes the interfering effects of nonesterified fatty acids liberated by *in vitro* lipolysis. Nonlinear binding behavior was observed with both compounds over their respective pharmacologic concentration range. Multiple binding-site models were invoked to explain the binding isotherm. The 2-unsaturated compound has a much higher affinity for the rat plasma proteins (mainly albumin) than its saturated precursor. The equilibrium association constants for the high- and intermediate-affinity sites were more than an order of magnitude higher with E-Δ^2-VPA than with VPA (10^4–10^6 versus 10^3 M^{-1}). This difference in binding affinity was also reflected by a lower plasma free fraction for E-Δ^2-VPA compared with VPA ($<<10$ versus $>20\%$ at total concentrations of less than 100 μg/ml). A more pronounced dose- and concentration-dependent variation in the distribution and clearance kinetics is predicted for the 2-unsaturated analogue compared to VPA. Also, the structural dependency in plasma protein binding observed with these branched-chain fatty acids may provide insights into the mechanism of interaction between fatty acyl molecules and albumin.

KEY WORDS: rat plasma proteins; protein binding; valproic acid; E-Δ^2-valproic acid; branched-chain fatty acids.

INTRODUCTION

Valproic acid (VPA) is a branched-chain fatty acid used in the treatment of various types of generalized and partial seizures. In spite of broad-spectrum efficacy and absence of significant sedative side effects, the use of VPA has been restricted in young children and pregnant women due to concern over the potential of VPA to induce fulminant hepatotoxicity and teratogenicity (1,2).

The monounsaturated compound E-Δ^2-valproic acid (E-Δ^2-VPA) was one of the first metabolites of VPA to be identified as having significant anticonvulsant activity in experimental seizure models (3–5). Subsequent *in vivo* and *in vitro* studies in rodents indicate that E-Δ^2-VPA is relatively free of teratogenic effects, even at very high embryonic concentrations (6,7). There are also data suggesting that E-Δ^2-VPA is less hepatotoxic than VPA and some of the other valproate metabolites (8,9). This apparent lack of organ toxicities has stimulated efforts to develop E-Δ^2-VPA as an alternate to VPA.

As part of our current investigation into the pharmacokinetic and pharmacodynamic properties of the 2-unsaturated compound in rats with experimentally induced seizures, we compared the binding characteristics of E-Δ^2-VPA to its saturated precursor in rat plasma. Plasma protein binding is an important determinant of VPA pharmacokinetics in man and experimental animals (10–13). The most notable feature of valproic acid binding to plasma proteins is its saturability within the therapeutic plasma concentration range (50–150 μl/ml or 350–1000 μ*M*). The nonlinear behavior in plasma protein binding results in a dose and concentration dependency in the distribution volume and hepatic clearance of VPA.

Relatively little is known concerning the plasma protein binding of E-Δ^2-VPA. Studies in epileptic patients receiving chronic valproate therapy have shown that the 2-unsaturated metabolite is much more highly bound than the parent drug (>99% versus 86%) (14,15). Also, in a recent study of the dose-dependent tissue distribution of VPA and synthetic E-Δ^2-VPA in mice, Löscher and Nau (16) noted that the *ex vivo* free fraction of E-Δ^2-VPA in a 30-min plasma sample rose from <1 to 60% as the dose was increased from 0.1 to 1.0 mmol/kg. Hence, a detailed comparative study of the binding characteristics of E-Δ^2-VPA and VPA is warranted.

A major difficulty in the measurement of VPA plasma protein binding has been the interference by *in vitro* lipolysis. Nonesterified fatty acids (NEFA) released from hydrolysis of triglycerides by constitutive lipases in plasma are known to displace VPA from plasma protein binding sites (17–22). *In vitro* lipolysis, which occurs during handling and storage of plasma specimen, can be minimized by freezing the samples soon after collection and by storing them at or

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below −20°C (21,23,24). Lipolysis during binding measurement, especially in the case of equilibrium dialysis at 37°C, has proven difficult to control. Although inhibitors of plasma lipases can be used to reduce lipolysis, the inhibitors themselves may also displace drug from the plasma proteins. Riva *et al.* (25) have found that when dialysis time was limited to less than 120 min, no significant elevation in NEFA level was observed with human plasma samples. Therefore, a second objective of this study was to adapt the rapid equilibrium dialysis technique of Riva *et al.* (25) to the measurement of the free fraction of E-Δ^2-VPA and VPA in rat plasma.

METHODS

Dialysis Procedure

Equilibrium dialysis was performed in either 0.5 or 1.0-ml Plexiglas cells separated by a Spectrapor-2 high diffusion-capacity membrane (MW cutoff, 12,000–14,000; Spectrum Medical Industries, Los Angeles, CA). The cells were placed in a horizontal-shaker water bath preheated to 37°C. A 0.3-ml (VPA) or 0.8-ml (E-Δ^2-VPA) volume of spiked rat plasma was dialyzed against an equal volume of 0.067 *M*, pH 7.40, phosphate buffer. During the dialysis period, the positions of the cells were rotated to ensure thorough mixing. At the end of the dialysis, plasma and buffer samples were quickly removed from the Plexiglas cells, frozen in liquid nitrogen, and stored at −80°C pending analysis. The integrity of the dialysis membrane in each cell was verified by adding a 25-μl aliquot of postdialysis buffer to 0.5 ml of a 10% trichloroacetic acid solution and checked for turbidity. Samples were discarded if the turbidity test was positive, indicating leakage of plasma protein into the buffer phase.

Plasma Collection

Arterial blood was collected by abdominal aortic puncture from male Sprague–Dawley rats (250–350 g, from Charles River, Portage, MI) anesthetized with ether. The blood sample was quickly transferred to a Vacutainer tube containing ethylenediaminetetraacetic acid (EDTA) and centrifuged for 5 min at 2000*g*. Plasma was immediately harvested and used for studies within 30 min.

Time to Dialysis Equilibrium

Experiments to assess the rate of equilibration were performed with plasma collected from four rats. Experiments were performed on each individual rat plasma in order to evaluate the variability in the equilibration kinetics and the extent of plasma protein binding between animals. E-Δ^2-VPA at a final concentration of 130–160 μg/ml or VPA at a concentration of 250 μg/ml was added to the plasma compartment. In the case of VPA, a tracer quantity (0.18 μCi equivalent to 46.9 ng) of ^{3}H-VPA was also added. It has been shown that a more rapid equilibration can be achieved by adding drug to the plasma compartment as opposed to the buffer compartment (25,26). Distribution of E-Δ^2-VPA or VPA between the plasma and the buffer phases was determined after 20, 40, 50, 60, 80, 120, and 570 min of dialysis and expressed in terms of the buffer-to-plasma concentration ratio. The dialyzed plasma samples were processed within 10 min of collection for the assay of NEFA. Postdialysis plasma and buffer volumes were measured to check for fluid shifts. When the dialysis period was under 120 min, minimal volume shifts from buffer to plasma (≤5%) were observed. At 570 min, the plasma volume expanded by as much as 10%. For the dialysis of both E-Δ^2-VPA and VPA, material recovery, as determined by gas chromatographic and radioactivity assays of plasma and buffer phases, was always greater than 96%.

Binding Experiments and Parameter Estimation

To characterize the binding isotherm, equilibrium dialysis measurements were carried out at varying drug concentrations. Aliquots of fresh, pooled rat plasma were spiked with the sodium salt of E-Δ^2-VPA at concentrations of 15, 20, 25, 30, 45, 65, 130, 290, 430, and 730 μg/ml. Dialysis was carried out in quadruplicate for 80 min. Another set of pooled plasma was spiked with VPA at concentrations of 10, 30, 50, 100, 200, 400, 600, 800, 1000, and 1200 μg/ml and dialyzed for 60 min in duplicate. Again, each aliquot of plasma spiked with unlabeled VPA contained a tracer amount of tritiated VPA. The concentration range of each of the substrates was chosen to match that observed in our *in vivo* pharmacokinetic and pharmacodynamic experiments (unpublished data) as well as to ensure that most of the saturation curve was covered.

The plasma protein binding data were analyzed according to the classical Langmuir model, which assumes multiple classes of independent, noninteracting binding sites. The following general equation describes the binding equilibrium involving multiple binding sites:

$$C_b = \sum_{i=1}^{n} \frac{N_i \cdot K_i \cdot C_f}{1 + K_i \cdot C_f} \quad (1)$$

where C_b is the concentration of drug bound to plasma proteins, C_f is the concentration of free drug, N_i is the concentration of the i^{th} class of binding sites (maximum binding capacity), K_i is the equilibrium association constant for the i^{th} binding site, and n denotes the number of classes of sites.

Rosenthal plots (i.e., C_b/C_f versus C_b) were initially constructed to reveal multiplicity in the binding sites and to provide guess estimates of the binding parameters for each class of sites (27). Nonlinear least-squares regression analysis of the data was then performed using the program PAR in the BMDP statistical software package (28). A stepwise approach to the regression analysis was followed as recommended by Priore and Rosenthal (29). Initially, C_f was assigned as the independent variable and C_b was assigned as the dependent variable [as shown in Eq. (1)] to facilitate the identification of the best binding model (i.e., the number of independent binding sites and functional form of each binding site). Once an appropriate model was decided upon, the regression strategy was evaluated by comparing the fits with various algebraic permutations of the model equation involving different pairing of dependent and independent variables.

Several measures of goodness of fit were examined including the coefficient of variation in parameter estimates, distribution of points in the residual versus X variable plots,

and percentage deviation in each predicted Y variable from the observed Y variable. A reduction in the sums of squares and Akaike's information criterion (AIC) (30) was used as the criterion for choosing the best model and the appropriate regression strategy.

Drug Assays

E-Δ^2-VPA in plasma and buffer was assayed directly by a gas chromatographic procedure described by Semmes and Shen (31). The maximum interday coefficient of variation of the assay was 7.0%. The equilibrium bound concentration of C_b was calculated by subtracting the postdialysis buffer concentration (or C_f) from the total postdialysis plasma concentration (C_t). The plasma free fraction was calculated by dividing the buffer concentration by the total plasma concentration at equilibrium.

Tritium-labeled valproic acid (4,5-^{3}H-VPA), with a specific activity of 550.80 Ci/mol, was custom synthesized by Dupont, New England Nuclear Research Products (Boston, MA). Radiochemical purity was established by thin-layer chromatography in three solvent systems: (i) hexane:dioxane:acetic acid, 80:20:1, by volume; (ii) hexane:ethyl acetate, 1:1; and (iii) hexane:acetic acid, 100:1. Labeled VPA and unlabeled VPA were applied separately to 20 × 20-cm precoated silica gel 60 F254 TLC plates from EM Reagents (EM Science, Cherry Hill, NJ) and developed for a distance of 15 cm. Afterward, the unlabeled VPA was visualized by spraying the plate with a 0.1% thymol blue solution adjusted to pH 10 and exposing it to ammonia vapor. VPA appeared as a yellow spot on a blue chromatographic background. A radiochromatogram of the labeled VPA was constructed by cutting 1-cm sections of the silica gel along the entire 20-cm length of the TLC plate. The sections were placed in scintillation cocktail and counted for radioactivity. The ^{3}H-VPA peak was identified using the R_f estimated from the unlabeled VPA chromatogram. The purity of ^{3}H-VPA, calculated by comparing the tritium activity under the ^{3}H-VPA peak to the total radioactivity recovered from the TLC plate, was 93, 94, and 99.6% in the three respective solvent systems.

Tritium-labeled VPA in plasma and buffer samples was assayed by liquid scintillation counting. Two hundred-microliter aliquots of either plasma or buffer were added to 3 ml of Ready-Safe (Beckman Instruments, Fullerton, CA) scintillation cocktail. Samples were counted to a 95.5% confidence level on a Tri-Carb 2000CA liquid scintillation analyzer (Packard Instruments, Downers Grove, IL). External standard quench correction was applied.

The total concentration of VPA in postdialysis plasma was calculated using the specific activity method (32). The specific activity of ^{3}H-VPA (dpm/μg) was calculated by dividing the total dpm placed in the plasma compartment by the total amount (μg) of labeled and unlabeled VPA added. The total plasma concentration of VPA (C_t) at equilibrium was equal to the disintegrations per minute per milligram (dpm/ml) in the postdialysis plasma divided by the specific activity. The free fraction (f_p) was calculated by dividing dpm/ml in buffer by dpm/ml in plasma. The concentration of VPA in the buffer (C_f) was calculated by multiplying free fraction by C_t. This method was preferred over the usual mass balance method since no assumptions about recovery of drug from the cell or changes in plasma and buffer volumes were necessary.

NEFA Assay

Plasma nonesterified free fatty acids (NEFA) were assayed using a colorimetric assay reported by Bergman *et al.* (33). This method involved acidification of the plasma with 0.1 *M* glycine buffer (pH 2.7) followed by extraction with methoxyethanol:butyl ether (1:1, v:v). Spectrophotometric detection was provided by a color complex between a copper-fatty acid soap and 4-(2-thiazolylazo)-resorcinol. The extraction procedure was selective for long-chain fatty acids, which comprise more than 99% of plasma NEFA. Short- and medium-chain fatty acids did not interfere with the assay. The sensitivity limit was 40 μ*M* in a 0.25-ml sample. Blanks, calibration standards, and plasma samples were prepared in duplicate. The intrabatch coefficient of variation of the assay ranged from 6.6 to 11.7% over the range of 100 to 400 μ*M*.

Blank rat plasma was spiked with either VPA or E-Δ^2-VPA at the respective concentrations of 50 μg/ml (347 μ*M*) and 100 μg/ml (694 μ*M*) and carried through the assay procedure. No interference from these compounds was observed.

Statistical Comparisons

Comparison between two means was performed using either the paired or the unpaired Student t test. When means from more than two groups were compared, a one-way analysis of variance was employed to test for a significant difference between groups. When variations occurred among groups, pairs of means were compared by Scheffe's test (34). Statistical significance was defined as $P \leq 0.05$.

RESULTS

E-Δ^2-Valproic Acid

Time to Dialysis Equilibrium

Figure 1A shows a plot of the mean buffer-to-plasma concentration ratio (×100) of E-Δ^2-VPA versus dialysis time for the four individual rat plasma samples. The percentage (%) buffer/plasma rose steadily over the first 60 min and appeared to level off between 60 and 80 min at about 12–13%. A further increase in % buffer/plasma was observed beyond 80 min, reaching 17% at 570 min (9.5 hr). At 60 min, the between-rat coefficient of variation in % buffer/plasma was 18% (12.0 ± 2.2%); by 80 min, the %CV decreased to 6% (13 ± 0.77%). At time points beyond 80 min, the %CV in % buffer/plasma ranged from 3.8 to 8.8%. From these data, the optimal equilibration time was determined to be 80 min. Only the mean % buffer/plasma at 570 min was found to be significantly different (ANOVA, $P < 0.05$) from the mean at the equilibrium time point of 80 min.

Time Course of NEFA

To investigate the cause of the rise in % buffer/plasma beyond 80 min, NEFA concentrations in the postdialysis plasma were measured. Figure 1B shows the mean postdialysis plasma NEFA levels at each dialysis time point. The

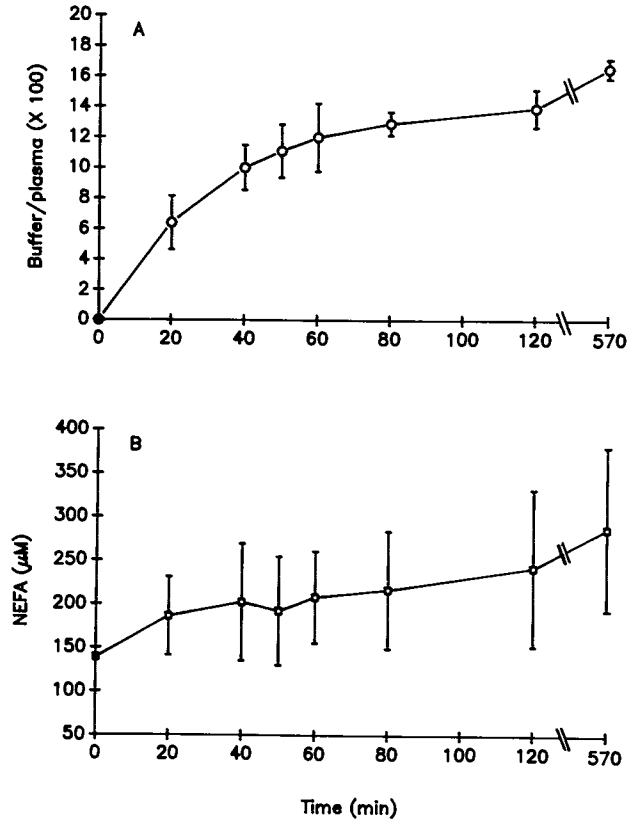

Fig. 1. (A) Time course of equilibration of E-Δ^2-VPA between dialysis cell compartments as expressed by the mean buffer-to-plasma concentration ratios (×100). (B) Corresponding time course of plasma NEFA concentrations (μM). Each data point represents the mean ± standard deviation of results from four individual rat plasma.

basal NEFA concentration at time 0 represents the result from a separate set of measurements on fresh, undialyzed rat plasma, which were assayed within 10 min of blood withdrawal. There was a large variability in individual rat plasma NEFA concentration (ca. twofold). Although the mean NEFA levels appear to increase slightly over the initial 40-min period, there were no statistically significant differences between any of the time points prior to equilibration (i.e., 80 min). A more discernible rise in NEFA level was observed beyond 80 min. By 570 min, the mean NEFA level had increased to approximately twice that of baseline. However, the increase did not reach statistical significance (ANOVA, $P < 0.05$). Nonetheless, the time course of elevation in dialyzed plasma NEFA appeared to parallel the increase in % buffer/plasma during the postdistribution equilibrium phase (i.e., after 80 min).

Binding Isotherm

Figure 2 shows a plot of the plasma protein binding data of E-Δ^2-VPA in the form C_b versus C_f. An initial plateau in C_b appeared at free concentrations around 0.30 μg/ml (see inset, Fig. 2). Beyond 0.5 μg/ml, C_b rose again until a second plateau was approached in the C_f range of about 100 μg/ml. Figure 3 shows a corresponding Rosenthal plot of the data (i.e., C_b/C_f versus C_b). A curvilinear relationship was clearly evident. Both types of plots indicated that at least two distinct binding sites existed for E-Δ^2-VPA. The initial steep descending portion of the Rosenthal plot represented a high-affinity, low-capacity site, and the shallow terminal portion represented a low-affinity, but high-capacity site. The need to invoke multiple sites for the binding of E-Δ^2-VPA was confirmed by the failure to fit the C_b versus C_f data to a one-site model equation using nonlinear least-squares regression analysis. Although a two-site model visually improved the fit at low free concentrations (≤5.0 μg/ml) accompanied by a small reduction in the residual sum of squares of 18% and a reduction in AIC test (30) from 438 for one site to 433, a poor fit was still observed at high C_f levels, where C_b was consistently underestimated. Instead of reaching a plateau, C_b continued to increase steadily with C_f, when C_f exceeded 100 μg/ml. This suggested the existence of a third class of binding sites. Since saturation of this last binding site could not be achieved within the pharmacological concentration range, linear binding was assumed. Addition of the linear component of a third binding site resulted in an excellent fit as indicated by visual inspection and a substantial reduction in the AIC value to 272. The final equation for the three-site binding model was

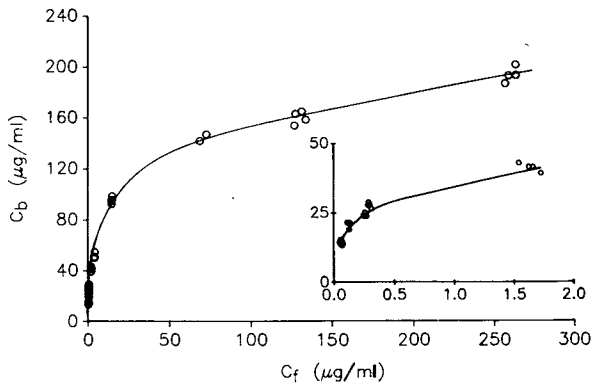

Fig. 2. Plasma bound concentration (C_b) of E-Δ^2-VPA plotted against free plasma concentration (C_f). The solid line represents the nonlinear least-squares regression fit to the three-site binding model. The inset shows an expanded plot of the data in the low concentration range. The appearance of filled circles is due to the overlap of several data points.

$$C_b = \frac{N_1 \cdot K_1 \cdot C_f}{1 + K_1 \cdot C_f} + \frac{N_2 \cdot K_2 \cdot C_f}{1 + K_2 \cdot C_f} + NK_3 \cdot C_f \qquad (2)$$

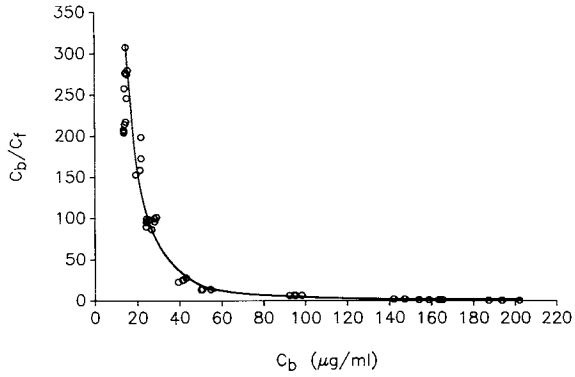

Fig. 3. Rosenthal plot of the plasma protein binding data for E-Δ^2-VPA.

where N_1 is the maximum binding capacity for the high-affinity site, K_1 is the apparent association-rate constant for high-affinity binding, N_2 is the maximum binding capacity for the intermediate-affinity site, K_2 is the apparent association-rate constant for intermediate-affinity binding, and NK_3 is the product of the binding capacity and association constant for the low-affinity site (the limiting linear binding constant).

The parameter estimates from the regression analysis are presented in Table I. A plot of the residual (observed C_b minus predicted C_b) versus C_f was evenly distributed around zero and lay roughly between two bands parallel to the C_f axis. The percentage deviation ranged from 0.069 to 15.0%, with a median of 3.7%.

Figure 4 shows the change in free fraction (×100) or $\%f_p$ as a function of total plasma concentration (C_t). It is worth noting that $\%f_p$ increased continuously throughout the entire plasma concentration range (C_t). A steep rise in $\%f_p$ from 0.3 to 6.7% was observed as C_t was increased from 10 to 60 μg.ml. A more moderate increase in $\%f_p$ occurred between 6.7 and 33% as C_t increased from 60 to 220 μg/ml. Above 220 μg/ml, the increase in $\%f_p$ was gradual and appeared to level off beyond 450 μg/ml.

Valproic Acid

Time to Equilibrium

The data from the dialysis equilibration experiment are presented in Fig. 5. The mean % buffer/plasma for four individual rat plasma showed a steady rise over an initial period of 50 min. Distribution equilibrium appeared to be reached between 50 and 60 min at a % buffer/plasma of 35%. Again, as in the case with E-Δ^2-VPA, the % buffer/plasma resumed its increase beyond 60 min, reaching 43% at 570 min (9.5 hr). ANOVA and paired comparison test showed that the mean %buffer/plasma at 80, 120, and 570 min were all statistically different ($P < 0.05$) from the mean value at 60 min. The coefficient of variation in % buffer/plasma was approximately 12% during the early period of dialysis. The %CV declined to 6.5% at 60 min and remained in the range of 5–6% thereafter. Thus, 60 min was chosen as the optimal equilibration time for dialysis measurements with VPA.

Table I. Estimates of Model Parameters for the Binding of E-Δ^2-Valproic Acid to Rat Plasma Proteins

Parameter	Estimate	%CV
N_1	27.65 ± 1.84 μg/ml (1.947×10^{-4} M)[a]	6.65
K_1	18.26 ± 3.818 ml/μg (2.593×10^{6} M^{-1})	20.9
N_2	119.6 ± 3.661 μg/ml (8.422×10^{-4} M)	3.06
K_2	0.0779 ± 0.0079 ml/μg (1.052×10^{4} M^{-1})	10.1
NK_3	0.1993 ± 0.0161	8.08

[a] Mean ± standard deviation from the nonlinear regression analysis. The numbers in parentheses are the mean estimates expressed as molarity.

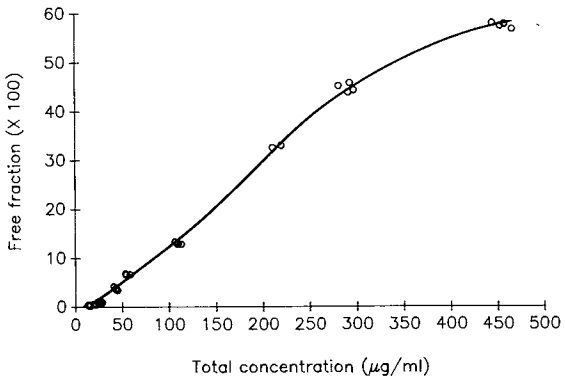

Fig. 4. Relationship between equilibrium free fraction (×100) of E-Δ^2-VPA and total plasma concentration. A smooth line was drawn to connect the data points.

Binding Isotherm

Figure 6 shows the concentration-dependent binding of VPA to rat plasma protein presented in a plot of C_b versus C_f. Except with the appearance of an inflection between 20 and 150 μg/ml, the bound concentration of VPA increased steadily throughout the entire range of free concentrations. Figure 7 shows a Rosenthal plot of the data: a biphasic concave curve, consisting of an initial descending portion up to a C_b of 120 μg/ml, beyond which an apparent plateau was reached. The steep descending portion suggests the presence of a high-affinity saturable binding site, whereas the flat portion suggests the presence of a linear binding site or a second saturable site with a very low equilibrium association constant. Based on these observations, the following modified two-site binding model was proposed.

$$C_b = \frac{N_1 \cdot K_1 \cdot C_f}{1 + K_1 \cdot C_f} + NK_2 \cdot C_f \qquad (3)$$

where N_1 is the maximum binding capacity for the high-affinity site, K_1 is the apparent association rate constant for the high-affinity binding site, and NK_2 is the product of the binding capacity and association constant for the lower-affinity site (the linear component of a second site). The binding data were fit successfully to the model equation us-

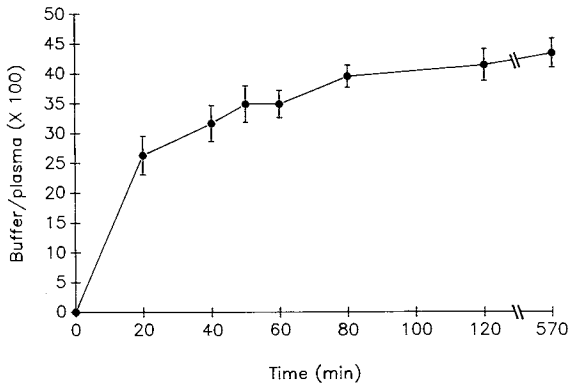

Fig. 5. Time course of the equilibration of VPA between dialysis cell compartments as expressed by mean buffer-to-plasma concentration ratios (×100). Each data point represents the mean ± standard deviation of four experiments.

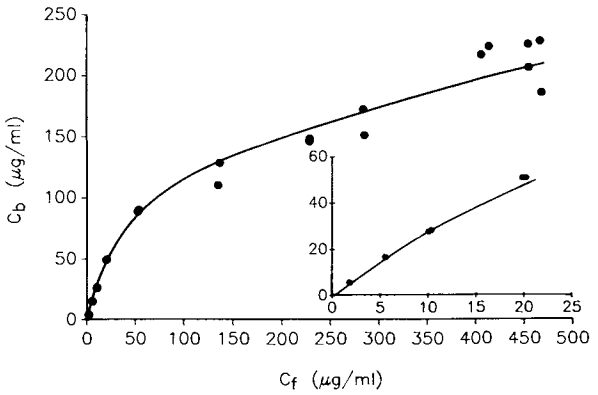

Fig. 6. Plasma bound concentration (C_b) of VPA plotted against free plasma concentration (C_f). The solid line represents nonlinear least-squares regression fit to the two-site binding model. The inset shows an expanded plot of the data in the low concentration range.

ing the nonlinear least-squares regression program. The parameter estimates from the regression analysis are shown in Table II.

A weighting function of $1/y^2$ was found significantly to improve the fit at low free concentrations and resulted in a dramatic reduction in the residual sum of squares. A plot of the weighted residual versus C_f showed that it was evenly scattered around 0 and formed two bands parallel to the C_f, thus indicating that $1/y^2$ was an appropriate weighting variable. The median percentage deviation was 2.8% and ranged from 0.17 to 4.6%.

A plot of valproic acid equilibrium free fraction (×100) versus total plasma concentration (C_t) is presented in Fig. 8. Unlike E-Δ^2-VPA, the VPA free fraction increased only slightly, from 22 to 28%, when a total plasma concentration was increased from 7.5 to 70 μg/ml. The $\%f_p$ gradually increased from 25 to 60% as C_t was increased from 70 to 400 μg/ml. From 400 to 700 μg/ml, only a small increase in $\%f_p$, from 60 to 70%, was noted. Hence, concentration-dependent binding was most apparent between valproate plasma concentrations of 100 and 400 μg/ml.

DISCUSSION

A rapid equilibrium dialysis procedure, similar to one previously developed by Riva *et al.* (24) for the study of VPA binding to human plasma proteins, was successfully applied

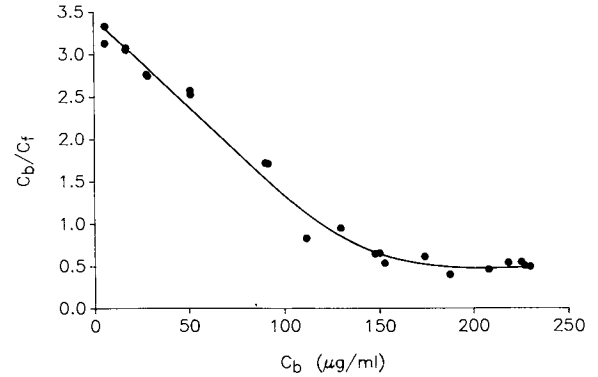

Fig. 7. Rosenthal plot of the plasma protein binding data for VPA.

Table II. Estimates of Model Parameters for the Binding of Valproic Acid Plasma Proteins

Parameter	Estimate	%CV
N_1	141.0 ± 12.85 μg/ml (9.794×10^{-4} M)[a]	9.11
K_1	0.0231 ± 0.0023 ml/μg (3.323×10^3 M^{-1})	9.76
NK_2	0.1715 ± 0.0367	21.4

[a] Mean ± standard deviation from the nonlinear regression analysis. The numbers in parentheses are the mean estimates expressed as molarity.

to the present study of E-Δ^2-VPA and VPA binding to rat plasma proteins. By using a high-diffusion cellophane membrane and by spiking the drug in the plasma compartment, the equilibration time can be shortened to a period of 1 to 1.5 hr. This is in contrast to a typical period of 4 to 24 hr required for conventional equilibrium dialysis procedures. The slightly longer equilibration time with E-Δ^2-VPA (60 vs 80 min) may reflect the higher affinity of the unsaturated compound to plasma protein, compared to VPA. At equilibrium, the between-sample reproducibility in free fraction ranged from 3 to 5% for both VPA and E-Δ^2-VPA. This was comparable to the findings of Riva *et al.* (24), who reported a 7% coefficient of variation with replicate determinations in human plasma. Similar to the experience of Riva *et al.* (24), the free fraction of both VPA and E-Δ^2-VPA began to rise beyond 80 min, probably in part due to the accumulation of NEFA from continual lipolysis. This was suggested by a statistically significant correlation between free fraction of E-Δ^2-VPA and plasma NEFA (r = 0.552, P = 0.05) during the postequilibrium period.

Two distinct saturable binding sites plus a third linear binding site were suggested by our data analysis over a total plasma concentration of 10–500 μg/ml for E-Δ^2-VPA. Over a comparable plasma concentration range for VPA, only one saturable site plus a linear binding site was discernible. Most significant is the fact that E-Δ^2-VPA was more extensively bound to plasma proteins than was VPA. A comparison of Figs. 4 and 8 shows that the free fraction of E-Δ^2-VPA is far lower as compared to VPA, at total plasma concentrations

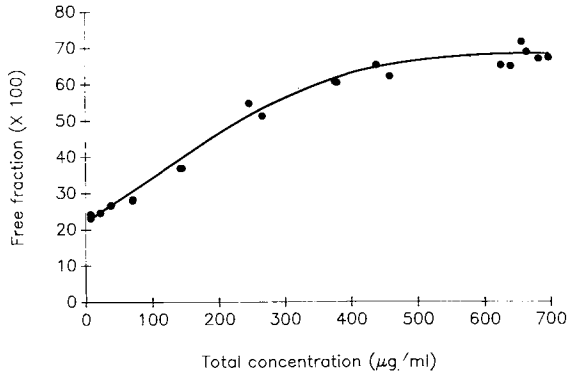

Fig. 8. Relationship between equilibrium free fraction (×100) of VPA and total plasma concentration. A smooth line was drawn to connect the data points.

less than 100 μg/ml. However, the free fraction of E-Δ^2-VPA began to approach that of VPA at concentrations around 200 μg/ml or higher. VPA as well as E-Δ^2-VPA is thought to bind primarily to albumin, since both saturated and unsaturated long- and medium-chain fatty acids have selective affinity for plasma albumin (35–37). The fact that endogenous free fatty acids, including palmitate, stearate, oleate, and linoleate, can displace VPA from its albumin binding site further suggests that VPA shares a common binding site with all of these fatty acids.

The high-affinity equilibrium association constant for E-Δ^2-VPA ($K_1 = 2.59 \times 10^6\ M^{-1}$) is similar to association constants reported for the binding of medium straight-chain fatty acids to human serum albumin. For example, the constants reported for nonanoate (C9) and decanoate (C10) were reported to be 2.5×10^6 and $1.0 \times 10^7\ M^{-1}$, respectively (38). The association constants for the intermediate-affinity site on human serum albumin for long-chain fatty acids, such as palmitate, stearate, oleate, and linoleate, are also in the range of 8.0×10^5 to $4.0 \times 10^6\ M^{-1}$ (39).

It is interesting to note that both the high- and the intermediate-affinity equilibrium association constants for E-Δ^2-VPA (10^4–$10^6\ M^{-1}$) are orders of magnitude greater than the observed high-affinity constant for VPA (ca. $10^3\ M^{-1}$). Assuming that E-Δ^2-VPA binds to the same site on rat albumin as does VPA, the interaction between the fatty acyl molecule and the protein binding site is seemingly enhanced by the introduction of a double bond between C2 and C3 that restricts rotational freedom of the carboxyl group and the propyl side chain. We are not aware of a comparable structure–binding relationship with long-chain saturated and unsaturated fatty acids. This phenomenon may be unique to medium-chain acids. Further investigation into this phenomenon may reveal important information on the binding mechanism of fatty acids to albumin.

Nonlinear plasma protein binding of VPA is known to influence significantly the systematic disposition kinetics of VPA, although its influence on the pharmacodynamic action of VPA is less clear. The very pronounced nonlinear plasma protein binding characteristics of E-Δ^2-VPA over the relevant pharmacological concentration range will most likely have a significant impact on the extravascular distribution, the hepatic clearance, and perhaps the anticonvulsant action of this unsaturated analog of VPA.

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