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Binding of Drugs to Human Serum Albumin

XIV. The Theoretical Basis for the Interaction between Phenytoin and Valproate[1]

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SUMMARY

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The binding of valproate and phenytoin to human serum proteins has been studied qualitatively and quantitatively by equilibrium dialysis and with albumin immobilized in microparticles. Albumin was shown to be the only protein significantly binding valproate in serum. Valproate is bound to two sites on the albumin molecule, the diazepam and warfarin sites, with the association constants, K_a, 3.1×10^4 M^{-1}. Phenytoin is bound primarily only to one site, ($K_a = 1.7 \times 10^4$ M^{-1}), which is identical to the second valproate site, i.e., the warfarin one. A theoretical evaluation of the possible interaction between valproate and phenytoin *in vivo* was made. It was shown that valproate, with its high therapeutic plasma concentration, would increase the free fraction of phenytoin by 100%. On the other hand, phenytoin at therapeutic concentrations would not significantly displace valproate. These theoretical calculations were confirmed by the experimental results obtained with sera from epileptic patients treated with phenytoin. The binding of phenytoin was markedly decreased when valproate was added to the sera *in vitro*, while the binding of valproate was not significantly impaired.

INTRODUCTION

Valproate has been introduced in the last years as an antiepileptic drug (1, 2). It is a short-chain branched fatty acid and is used in the form of the sodium salt. It is protein bound to about 90-94% in human plasma (3-6).

Many epileptic patients are treated with combinations of several drugs, some of which, as phenytoin and valproate, are strongly bound to plasma proteins. Some cases with adverse reactions involving valproate have recently been noticed, which may be due partly to interactions between the different drugs on the protein binding level.[2] Several papers have also reported that valproate decreases the binding of phenytoin both *in vivo* and *in vitro* (6-10). However, relatively high concentrations of phenytoin were needed to significantly displace valproate *in vitro* (6). This apparent contradiction has been studied in the present paper, and the theoretical basis for the binding interaction between valproate and phenytoin has been investigated. Thereby the binding sites on the albumin molecule have been identified and the binding characteristics (i.e., K_a and n) have been evaluated. The binding constants have been used to simulate the free fraction, f_u, of the drugs at different drug concentrations. The capacity of some other anticonvulsant drugs to displace phenytoin and valproate has also been studied.

MATERIALS AND METHODS

Drugs. [^{14}C]Valproate (1.55 mCi/mmol) was obtained from Abbott Laboratories, North Chicago, Ill. [^{14}C]-Phenytoin (59.8 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, England. The radiochemical purity, as found by thin-layer chromatography, was in all cases better than 98%. Other drugs were obtained from the respective manufacturers or their representatives in Sweden.

Human serum albumin (HSA) was obtained from AB KABI, Stockholm, and was freed from fatty acid by charcoal treatment or extraction with toluene-chloroform.

Preparation of microparticles with human serum albumin. Microparticles[3] with immobilized HSA were pre-

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[1] For part XIII, see Proc. IX Eur. Congr. Rheumatol., Wiesbaden, 1979.

[2] A. Rane, personal communication (1978).

[3] American patent 4,061,466; British patent 1,533,579.

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pared as described by Ekman and Sjöholm (11). In short, HSA (100 mg/ml) was dissolved with acrylamide and *N,N'*-methylenebisacrylamide in sodium phosphate buffer. After the addition of the catalyst, the solution was poured into a mixture of toluene and chloroform with a detergent. The mixture was homogenized to produce a water-in-oil emulsion. An accelerator was added to the emulsion, and polymerization started after a few minutes. During the whole procedure, oxygen was excluded from the system by bubbling nitrogen gas through the solution. After 20 min the phases were allowed to separate and the microparticles were washed repeatedly with buffer and centrifugated until no protein could be detected in the supernatant. The HSA content in the microparticles was determined from the capacity of the microparticles to bind [^{14}C]salicylic acid. Leakage of HSA from the particles was routinely checked by spectrophotometry. Normally, no leakage could be detected in 3–4 weeks.

Binding of drugs to HSA in microparticles. This was studied as described by Kober *et al.* (12). The experiments were carried out at room temperature (25°C). Microparticles containing HSA were suspended in 0.005 M sodium phosphate buffer, pH 7.4, with 0.1 M KCl. Aliquots of 0.5 ml were pipetted into plastic centrifuge tubes. Different amounts of the drug studied, also containing the ^{14}C-labeled compound, were dissolved in the same buffer and added to the tubes containing the microparticle suspension. Earlier studies (13) showed that equilibrium is obtained rapidly; the samples were generally centrifugated within 5–15 min for 30 min at about 3000*g*. After centrifugation, the concentration of the free drug in the system was determined by measuring the radioactivity in 0.1-ml aliquots taken in duplicate from the supernatant. The method yields results which coincide with those obtained by equilibrium dialysis with charcoal-treated HSA (12).

In the interaction studies, which followed the principles outlined by Sjöholm *et al.* (14), the displacing drug, which was not radioactively labeled, was added to the microparticle suspension to give drug:HSA molar ratios of 1:1, 5:1, and 10:1. Buffer-insoluble drugs were dissolved in a minimum amount of ethanol, not exceeding 1.5% in the final solution. After mixing and centrifugation, the radioactivity in the supernatant of the samples was determined.

Patients. Four epileptic children, aged 6 to 16 years, participated in the study. They had been treated with phenytoin for at least 2 years and the seizures were under control. Phenytoin was the only drug administered during this study. The children showed no signs of liver disorder. The sera were obtained from Dr. Anders Eriksson, Pediatric Department, Hospital of Boden, Sweden. The serum concentrations of phenytoin were 24–43 μM (or 6.1–10.8 μg/ml).

Equilibrium dialysis. This was performed as described by Sjöholm *et al.* (15). The binding to isolated human serum proteins and to human serum, respectively, was determined by equilibrium dialysis at room temperature (25°C) against isotonic phosphate buffer, pH 7.4, using Technicon Type A standard membranes. When comparing the protein binding in patient sera to that in normal serum, the studies were carried out at 37°C. For each drug concentration, duplicate determinations using 500 μl of protein solution and buffer were performed. The time used for equilibration was 8.5 h. After equilibration, radioactivity was determined in duplicates with 0.1 ml of sample aliquots from both sides of the dialysis cell. The binding was related to the HSA concentration, which was determined after the dialysis by immunochemical quantitation according to Mancini *et al.* (16).

Evaluation of binding data. The binding data obtained with the different ligands were analyzed according to Scatchard (17). The equation

$$r/[D] = n \cdot K_a - r \cdot K_a$$

was used, where r is the number of moles of bound drug per mole of albumin, n is the number of binding sites, K_a is the association constant for the drug–albumin complex, and $[D]$ is the concentration of free drug.

Gel filtration of normal serum. This was carried out with Sephadex G-200 (92 × 3 cm) in 0.005 M phosphate buffer, pH 7.4, with 0.1 M KCl. The protein content of the fractions was analyzed by polyacrylamide gel electrophoresis (18) at pH 8.3.

RESULTS

Binding of valproate to serum protein fractions. Human serum from healthy individuals was fractionated on G-200. Fractions were taken out and their protein content was analyzed by polyacrylamide gel electrophoresis. The binding of valproate in the respective fractions was also determined by equilibrium dialysis. A summary of the results is given in Table 1. Significant binding of valproate was seen only in the fractions containing HSA, i.e., fractions 6–8.

Binding of valproate to HSA. The binding of valproate to HSA immobilized in microparticles (1 mg/ml or 15 μM) is illustrated by the Scatchard plot in Fig. 1. Figure 1 also includes the results obtained from equilibrium dialysis experiments with charcoal-treated HSA (1.24 mg/ml or 18.6 μM). As is evident from Fig. 1, the results obtained with the two different techniques are consistent

TABLE 1

Binding of valproate in fractions obtained from gel filtration of normal serum

The binding was determined by equilibrium dialysis at pH 7.4 at a valproate concentration of 8.8 μM. The numbering of the fractions is shown in Fig. 1. The protein content was obtained from polyacrylamide gel electrophoresis.

Fraction No.	Protein content			$OD_{280\,nm}$	Binding
	Immunoglobulins	Transferrin	Albumin		
					%
1	+			0.953	2.6
2	+			0.708	2.1
3	+			0.762	0
4	+	+		1.814	0
5	+	+	+	0.952	0.4
6		++	++	1.795	56.8
7		++	++	2.156	72.2
8			++	0.922	52.1

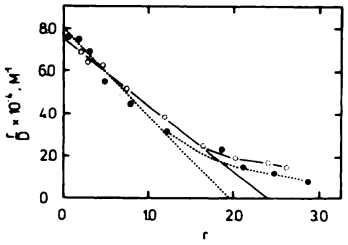


FIG. 1. *Scatchard plots for the binding of valproate to HSA immobilized in microparticles (○——○) and to charcoal-treated HSA as obtained from equilibrium dialysis (●——●)*

and indicate that there are two primary binding sites for valproate on HSA. The values of the association constants, K_a, are too close to be determined separately. The average K_a, determined from the straight portion of the Scatchard plots, where the influence from secondary binding is minimized, is 3.1×10^4 and 4.1×10^4 M^{-1}, from experiments with microparticles and equilibrium dialysis, respectively.

Binding of valproate to serum. The binding of valproate to human serum from healthy individuals was studied by equilibrium dialysis (Fig. 2). The study was made on one individual serum (A.K.) and on a pool of three sera, as described previously. The value for n is about 2 as obtained with isolated HSA, indicating that albumin is the predominant binding protein in serum. However, K_a is lower—1.3×10^4 M^{-1}—in human serum than with HSA. The binding degree of valproate in four individual sera is shown in Table 2.

Displacement of valproate from HSA. In this study a reference sample containing HSA in microparticles (1 mg/ml or 15 μM) and a labeled marker (valproate) was used. The study was made with two valproate concentrations, corresponding to 50 and 180%, respectively, of the molar concentration of HSA. In addition, the test samples contained different displacing agents, which were added to give drug:HSA molar ratios of 1:1, 5:1, and 10:1. Diazepam and warfarin significantly displaced valproate, while no effect was seen when phenytoin, carbamazepine, primidon, nitrazepam, or clonazepam was added (Figs. 3 and 4). The results were the same at both valproate levels. In separate experiments, we have shown that valproate does not displace digitoxin from HSA immobilized in microparticles (14).

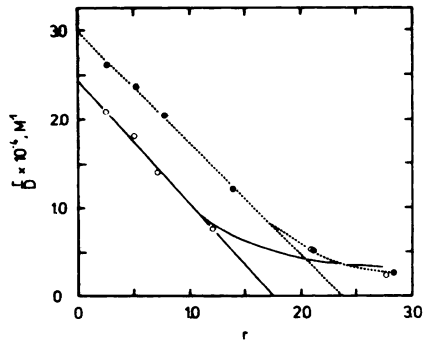


FIG. 2. *Scatchard plots for the binding of valproate to one individual normal serum, A.K. (○——○), and to a pool of three normal sera (●——●) as obtained by equilibrium dialysis*

TABLE 2

Binding of valproate and phenytoin in serum

Serum	Albumin concentration	Valproate		Phenytoin	
		Concentration	Binding	Concentration	Binding
	mg/ml	*μM*	*%*	*μM*	*%*
Epileptic patients					
N.S.	28.7	0	—	36	87.1
		230	90.1	36	84.8
		613	85.4	36	81.4
I.N.	33.8	0	—	25	89.5
		230	88.3	25	86.5
		613	86.7	25	83.2
A.N.	31.8	0	—	43	89.1
		230	—	43	87.0
		613	87.5	43	84.8
P.B.	32.2	0	—	24	87.0
		230	91.9	24	87.4
		613	89.0	24	84.7
Healthy individuals					
A.K.	30.8	230	92.1	—	
		613	87.0		
A.J.	35.7	230	93.4	—	
		613	90.7		
S.L.	35.7	230	93.1	—	
		613	90.1	—	
Y.O.	35.8	230	93.6	—	
		613	91.4	—	

Binding of phenytoin to HSA. The binding of phenytoin to HSA immobilized in microparticles (1.21 mg/ml or 18.2 μM) is illustrated by the Scatchard plot in Fig. 5. The concentration of ethanol was 2% in the final solution. The result indicates that HSA binds phenytoin primarily to one site with an association constant, K_a, of 1.7×10^4 M^{-1}, which agrees with earlier reported values (19). The poor solubility of phenytoin prevents studies at higher phenytoin concentrations and is the probable cause of the scattered data in Fig. 6.

Displacement of phenytoin from HSA. The study was performed as described previously for valproate except that the HSA concentration was 3 mg/ml or 45 μM and labeled phenytoin was used as the marker. Warfarin significantly displaced phenytoin. Up to an equimolar concentration relative to HSA, valproate did not displace phenytoin. However, at higher concentrations, when the first binding site is saturated, valproate will also compete

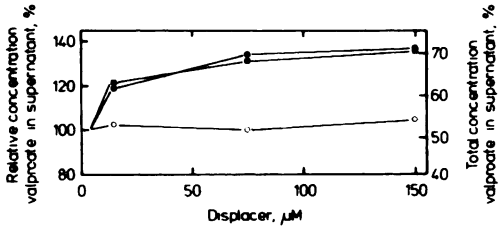


FIG. 3. *Displacement of valproate (7.5 μM) from HSA in microparticles (1 mg/ml or 15 μM) by phenytoin (○——○), diazepam (■——■), and warfarin (●——●), respectively, in 0.005 M phosphate buffer, pH 7.4, with 0.1 M KCl, at 25°C*

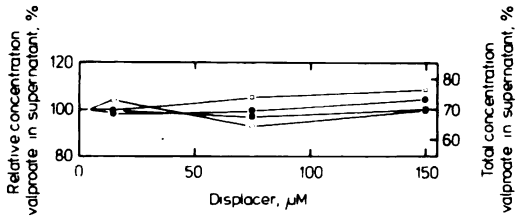


FIG. 4. *Displacement of valproate (7.5 μM) from HSA in microparticles (1 mg/ml or 15 μM) by carbamazepin (■—■), primidon (○—○), nitrazepam (□—□), and clonazepam (●—●), respectively, in 0.005 M phosphate buffer, pH 7.4, with 0.1 M KCl, at 25°C*

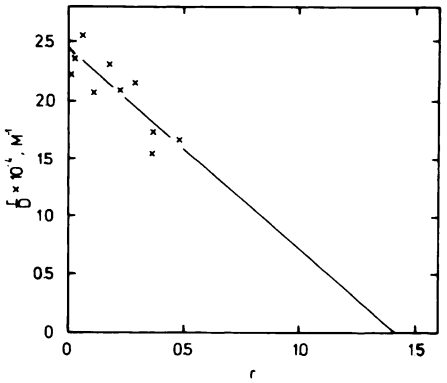


FIG. 5. *Scatchard plot for the binding of phenytoin to HSA immobilized in microparticles*

with phenytoin for its binding site (Fig. 6a). Nitrazepam, carbamazepin, primidon, and clonazepam showed no effects (Fig. 6b). The results with diazepam were the same as with nitrazepam.

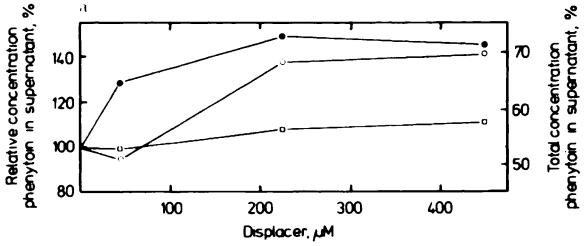
Binding of valproate and phenytoin in patient sera. The binding degree of phenytoin in patient sera was determined by equilibrium dialysis at 37°C with trace amounts of ^{14}C-labeled drug in the absence of valproate and after the addition of valproate to concentrations of 230 and 613 μM. In addition, the binding degree of valproate in the sera was determined separately with labeled valproate. The results are summarized in Table 2 and compared with the results obtained in sera from healthy individuals. As is evident, increasing valproate concentrations will decrease the binding of phenytoin (about 5%-units). On the other hand, the binding of valproate in the patient sera is only slightly impaired by phenytoin compared to the binding in the normal sera. However, a direct comparison is made difficult by the different albumin contents.

Computer calculations. The theoretical interaction between valproate and phenytoin in serum with a normal HSA concentration (40 mg/ml or 600 μM) was simulated with a computer, assuming that HSA is the only binding protein. For valproate, $n = 2$ and $K_a = 3.1 \times 10^4$ M^{-1} were used, and for phenytoin, $n = 1$ and $K_a = 1.7 \times 10^4$ M^{-1}. In the applied model, which was entirely based on the simple equilibrium equations defining the K_a values, phenytoin is competing with valproate for one of its binding sites. No secondary binding was accounted for, but within therapeutic concentrations of the drugs, such binding is small and will not affect the conclusions drawn. However, at the highest concentration levels used in the simulation, the calculated displacement may be somewhat overestimated. The variation of the free fraction of phenytoin with increasing concentrations of valproate can be seen in Fig. 7a at three different concentrations of phenytoin. The therapeutic range of valproate is 300–700 μM or 40–100 μg/ml (6). At the highest concentration (700 μM or 100 μg/ml), the free fraction of phenytoin was increased by almost 100%. In Fig. 7b the variation of the free fraction of valproate with increasing concentrations of phenytoin is shown at four different concentrations of valproate. Within the therapeutic range of phenytoin (30–80 μM or 7.5–20 μg/ml) (20), no significant displacement of valproate was seen.

DISCUSSION

Sodium valproate is strongly protein bound, and as is evident from the analysis of the binding capacity of the fractions obtained after gel filtration (Table 1), albumin is the principal protein binding the drug in human plasma. It is often administered in large doses together with other antiepileptic drugs, which also can be bound to HSA, e.g., phenytoin. Potentially, therefore, valproate can be involved in drug interactions on the protein binding level, which may be of pharmacokinetic significance. In the present work, we have specifically studied the possible interaction between valproate and phenytoin.

To be able to foresee any interactions between a pair of drugs, it is necessary to determine the site on the albumin molecule to which the drugs are bound. Furthermore, it is necessary to determine the association constants (K_a) of the drug–albumin complexes. Thus, the first prerequisite for a drug interaction to occur is that the drugs bind to the same site(s). HSA has at least three specific drug binding sites (14, 21, 22). It has earlier been shown that diazepam, digitoxin, and warfarin are essen-



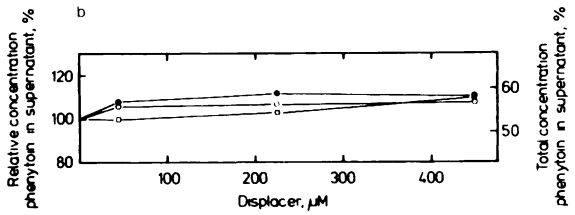


FIG. 6a. *Displacement of phenytoin (22.5 μM) from HSA in microparticles (3 mg/ml or 45 μM) by valproate (○—○), warfarin (●—●), and nitrazepam (□—□), respectively, in 0.005 M phosphate buffer, pH 7.4, with 0.1 M KCl, at 25°C*

FIG. 6b. *Displacement of phenytoin (22.5 μM) from HSA in microparticles (3 mg/ml or 45 μM) by carbamazepin (○—○), primidon (□—□), and clonazepam (●—●), respectively, in 0.005 μ phosphate buffer, pH 7.4, with 0.1 M KCl, at 25°C*

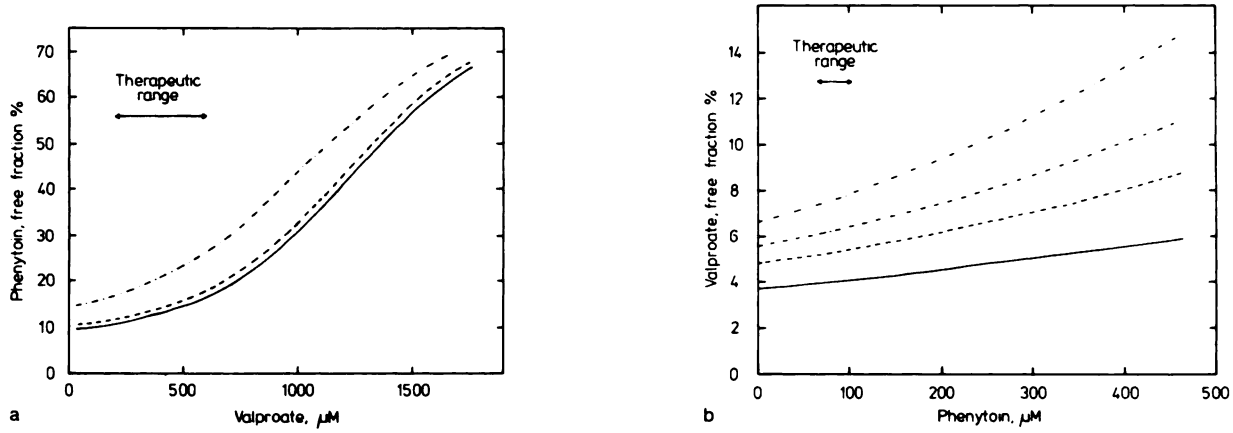


FIG. 7a. *The computer-calculated variation of the free fraction of phenytoin in serum with increasing concentrations of valproate*
The total concentration of phenytoin was constant: 40 μM or 10 μg/ml (——), 80 μM or 20 μg/ml (----), and 300 μM or 75 μg/ml (- - -).
FIG. 7b. *The computer-calculated variation of the free fraction of valproate in serum with increasing concentrations of phenytoin*
The total concentration of valproate was constant: 300 μM or 43 μg/ml (——), 500 μM or 72 μg/ml (- - -), 600 μM or 87 μg/ml (- - -), and 700 μM or 101 μg/ml (·····).

tially bound to single, separate sites on the albumin molecule. These three drugs have recently been used as markers to characterize the specificity of the different drug binding sites (14).

Valproate has two binding sites on isolated HSA as found with microparticles containing immobilized HSA and by equilibrium dialysis. The average association constant, K_a, is 3.1×10^4 M^{-1} (Fig. 1). In serum, the K_a is lower, 1.3×10^4 M^{-1}, probably due to endogenous binding inhibitors normally present in serum (15). These values are in good agreement with those reported earlier by Monks *et al.* (6). For phenytoin we found that K_a was 1.7×10^4 M^{-1} and that n was one (Fig. 5). The binding of phenytoin is thus weaker than that of valproate, and phenytoin is bound only to one specific primary site.

The interaction studies, which were made with albumin immobilized in microparticles, showed that valproate can bind to both the diazepam and the warfarin site (Fig. 3). Moreover, the results indicate that valproate primarily binds to the diazepam site and secondarily to the warfarin site. This conclusion is based on the fact that the displacement of valproate is approximately the same with diazepam and warfarin, in spite of the higher binding constant of warfarin. Thus, less valproate (to be displaced) is bound to the warfarin site. This means that the K_a for the binding of valproate to the diazepam site is slightly higher and that to the warfarin site is slightly lower than 3.1×10^4 M^{-1}. However, the difference is not sufficient to allow the constants to be separately determined from the Scatchard plots. The single binding site of phenytoin was shown to be identical to the warfarin binding site (Fig. 6a) and thus the same as the second valproate site. The results are summarized in Fig. 8.

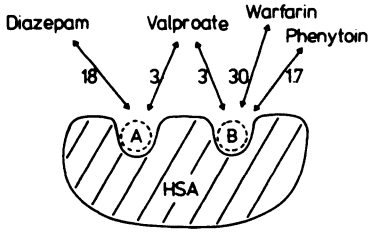


FIG. 8. *The binding of drugs to two sites on human serum albumin (HSA)*
The numerical values given denote the association constant of the respective drug × 10^{-4} M^{-1}.

Monks *et al.* (6) have reported that valproate competitively displaces phenytoin but that phenytoin displaces valproate only at higher concentrations in normal human sera *in vitro*. The results agree with those obtained in this study with sera from epileptic patients treated with phenytoin. In these sera, the binding of phenytoin successively decreased when valproate was added. The binding of valproate, on the other hand, was only slightly less than that in normal individuals, when the different albumin content is considered.

The mechanism behind the findings can conceivably be interpreted as follows: Phenytoin binds to only one primary site on HSA and this is identical to the second valproate site. The binding of phenytoin to HSA is somewhat weaker than that of valproate (a lower K_a value). The normal molar therapeutic serum concentration is about 10 times higher for valproate than for phenytoin and is about as high as the molar concentration of albumin (600 μM). At low concentrations of the drugs, most of the small amount of valproate which phenytoin displaces from their common binding site will instead bind to the other valproate site, i.e., the displacement will not result in a significantly increased fraction of free valproate. On the other hand, valproate will competitively displace phenytoin from its single binding site even at moderate concentrations. The displacement will be larger, for both drugs, at higher serum concentrations.

The consequences of these findings can easily be simulated mathematically. With the constants obtained in this work, the theoretical free fractions of the respective drug in serum at increasing concentrations of the displacing drug was calculated by computer (Figs. 7a and b). Within the normal therapeutic interval, phenytoin does not significantly increase the free fraction of valproate, while valproate displaces phenytoin from its single binding site. Thus, at a total phenytoin concentration

of 40 μM, valproate at the highest therapeutic concentration (700 μM) will increase the free fraction of phenytoin from 9 to almost 18%, i.e., 100%. At a total valproate concentration of 500 μM, 80 μM phenytoin increases the free fraction of valproate from about 4.8 to 5.3%, i.e., only about 10%. (It should be noted that the calculations were based on the assumption that no other inhibitors are present and that the HSA concentration is 40 mg/ml or 600 μM.)

In conclusion, it is evident that phenytoin is not able to significantly displace valproate from its binding sites, even at higher concentrations. However, drugs with higher binding constants reaching higher plasma concentrations may displace valproate. Valproate is almost exclusively metabolized in the liver and has a very small volume of distribution (V_d = 8–12 liters) and a blood flow-independent hepatic clearance (4, 5, 23, 24). The clearance of the drug is thus of the restrictive type, and changes in the free fraction may then alter the pharmacokinetic properties. Changes in the free fraction may partly explain the poor correlation between daily dose and total plasma concentration (23, 24).

Valproate itself, in turn, may have profound effects on the free fraction of other drugs binding to the warfarin and/or diazepam binding sites on HSA, essentially due to its high plasma concentration. For drugs like phenytoin, having a moderate apparent distribution volume (V_d about 40–50 liters (25)), the pharmacokinetic consequences will probably not be important, as long as the metabolism is not concentration dependent. The increased clearance (due to the increased f_u) will be compensated for by an increase in the V_d, so that the half-life in plasma ($t_{1/2} = (V_d \times 0.693)/Cl$) will largely remain constant. If V_d, on the other hand, is small (about 10 liters), as it is for valproate, the displacement should lead to shorter half-lives since the relative increase in the V_d is smaller than that in the *Cl*. It should be stressed, however, that drug monitoring in *all* these cases should be based on determinations of the concentration of *free* drug and on a knowledge of the mechanisms determining the elimination kinetics of the drug in question.

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