Biochemical Pharmacology, Vol. 45, No. 3, pp. 735–742, 1993.
Printed in Great Britain.

0006-2952/93 $6.00+0.00

PROTEIN BINDING AND HEPATOBILIARY DISTRIBUTION OF VALPROIC ACID AND VALPROATE GLUCURONIDE IN RATS*

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(*Received* 20 *February* 1992; *accepted* 7 *October* 1992)

Abstract—The protein binding and hepatobiliary distribution of valproic acid (VPA) and its glucuronide conjugate (V-G) were examined in rats with a combination of *in vitro* and *ex vivo* protocols. VPA was moderately bound to proteins in both serum and hepatic cytosol, and the degree of binding was lower *ex vivo* than *in vitro*. V-G, which was more highly bound than VPA *ex vivo* in serum, may have displaced the parent drug from its binding sites when VPA was administered *in vivo*. Examination of *ex vivo* hepatic subcellular distribution revealed that VPA localization tended to be high in cytosol and low in the microsomal fraction; V-G appeared to be distributed evenly throughout the cell although V-G concentrations within the liver were very low. The steady-state elimination rate of VPA did not increase proportionately with increasing steady-state concentrations of unbound VPA in serum, consistent with saturable systemic elimination of the drug. In contrast, steady-state VPA elimination was related linearly to unbound cytosolic VPA concentrations. Moreover, a nonlinear relationship between the unbound concentrations of VPA in hepatic cytosol and serum was observed, consistent with saturable distribution of the unbound drug between the two compartments *in vivo*. These observations suggest that the nonlinear elimination of VPA in rats may be due to concentration-dependent penetration of the drug into the liver as opposed to saturable biotransformation.

Valproic acid (VPA)‖, an antiepileptic agent useful in the treatment of various types of generalized and partial seizures, is an aliphatic carboxylic acid that exists predominantly in the ionized form at physiologic pH. VPA is metabolized via β-oxidation (in hepatic mitochondria), ω-oxidation (in microsomes) and acyl glucuronidation [1, 2], and is excreted in bile primarily as the glucuronide conjugate (V-G) [3].VPA in the bloodstream gains access to hepatic metabolic and excretory systems by traversing the sinusoidal membrane of the hepatocyte. Hepatobiliary distribution of VPA and V-G may be influenced by hepatic transport systems, diffusional processes, and/or protein binding in serum, liver cytosol, and bile. Moreover, distribution of VPA between blood and hepatocyte may influence the hepatic clearance of this substrate.

VPA exhibits concentration-dependent binding to rat plasma proteins *in vitro* [4]. Although the binding of VPA to proteins in brain appears to be minimal [5], little is known concerning the potential for VPA protein binding in other tissues. Furthermore, the potential binding of V-G to serum and tissue proteins has not been addressed. Binding of VPA and V-G to hepatic proteins may be of particular importance. Such binding would, in part, control the accumulation of parent and metabolite in the liver, which is an important target for VPA-induced toxicity [6]. Moreover, if the affinity of hepatic proteins for VPA differs from that of proteins in serum, the partitioning of the drug between serum and liver may be nonlinear (i.e. VPA concentrations in hepatic tissue may not be directly proportional to concentrations in serum). Loscher and Nau [7] reported that the liver/plasma VPA concentration ratio in mice decreased with increasing plasma concentrations (from 2.75 at a plasma concentration of 6.9 μg/mL to 1.0 at a plasma concentration of 154 μg/mL). It is conceivable that the observed nonlinear partitioning resulted from differential binding in liver tissue and plasma.

If hepatic uptake of a low extraction ratio compound such as VPA is determined only by diffusional processes, the unbound steady-state concentration of VPA in the hepatic cytosol ($C_{ss\ \text{unbound, cytosol}}$) should be related to the unbound steady-state concentration in serum ($C_{ss\ \text{unbound, serum}}$) according to the following equation:

$$C_{ss\ \text{unbound, cytosol}} = C_{ss\ \text{unbound, serum}} \cdot (Cl_H'/Cl_i') \quad (1)$$

where Cl_H' is the hepatic clearance of unbound VPA (i.e. the clearance of unbound VPA from serum) and Cl_i' is the hepatic intrinsic clearance of unbound VPA (i.e. the clearance of unbound VPA from hepatic cytosol). Thus, partitioning between hepatic cytosol and serum could be predicted from *in vitro* binding data and a knowledge of the hepatic

* This work was presented in part at the 5th Annual Meeting of the American Association of Pharmaceutical Scientists, November 4–8, 1990, Las Vegas, NV.

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‖ Abbreviations: VPA, valproic acid; V-G, VPA glucuronide; C_{ss}, steady-state concentration; Cl_H', hepatic clearance of unbound drug; Cl_i', intrinsic hepatic clearance of unbound drug; V_{cytosol}, cytosolic volume; $T_{\max}$, maximum rate of serum-to-cytosol translocation; and NP_T, binding capacity.

extraction ratio. The present study was undertaken to explore the relationship between hepatic cytosol/serum partitioning and unbound VPA concentrations in serum, and to determine the subcellular distribution of VPA and V-G within the liver in rats.

The nonlinear relationship between VPA dose and concentration in rats has been attributed, in part, to saturable hepatic clearance of VPA [8]. The locus of this nonlinearity (i.e. specific enzyme or transport systems) has yet to be determined. An additional objective of this investigation was to assess whether the concentration-dependent hepatic clearance of unbound VPA over the concentration range examined *in vivo* in rats could be attributed to a factor(s) other than saturable metabolism, such as concentration-dependent hepatic translocation of VPA.

MATERIALS AND METHODS

Chemicals. VPA and cyclohexanecarboxylic acid were purchased from the Sigma Chemical Co. (St. Louis, MO) and the Aldrich Chemical Co. (Milwaukee, WI), respectively. All other reagents were obtained from commercial sources and were of the highest purity available.

Animals. Male Sprague–Dawley rats (290–317 g; Hilltop Laboratory Animals, Scottdale, PA) were housed individually in wire mesh cages, and maintained on a 12-hr light/dark cycle. Free access to food and water was allowed at all times prior to the experiment. Rats (N = 6) were anesthetized with urethane (1 g/kg i.p.), and silastic cannulas were implanted in the right jugular vein for blood sampling and the right femoral vein for drug administration. A polyethylene (PE-10) cannula was implanted in the bile duct. Rats remained anesthetized during surgery and for the duration of the study. Body core temperature was maintained at 37° throughout the study period by means of a rectal probe and a heating pad connected to a temperature regulator (YSI model 73A, Yellow Springs, OH). Rats received varying i.v. loading doses (2.85 to 57.0 mg/kg) and i.v. infusions (0.125 to 2.50 mg/min/kg) of VPA (in normal saline, pH 7.5), calculated based on previously published pharmacokinetic parameters of VPA in rats [9], to maintain steady-state serum VPA concentrations of approximately 10–400 μg/mL for 60 min. Blood was obtained every 15 min to assure that steady-state VPA concentrations were achieved, and bile was collected continuously. Animals were killed immediately following termination of the VPA infusion, serum was obtained and the liver was excised, blotted dry and weighed.

Subcellular distribution. The localization of VPA and V-G in liver subfractions (nuclear, mitochondrial, lysosomal, microsomal and cytosolic) was examined in rats after infusion of VPA to steady state by classic cell fractionation techniques according to the method of DeDuve *et al.* [10] with minor modifications as described by Meijer *et al.* [11]. Hepatic cytosol was obtained by homogenizing rat livers with 3 vol. of ice-cold 0.25 M sucrose (pH 7.0) in a Potter–Elvehjem homogenizer with a Teflon pestle. The nuclear fraction was removed by centrifugation at 600 *g* for 10 min. The mitochondrial and lysosomal fractions were obtained by centrifugation for 8.5 min at 6,780 *g* and 9 min at 35,000 *g*, respectively. The microsomal and cytosolic fractions were separated by ultracentrifugation (60 min at 100,000 *g*). To assess the purity of the various subcellular fractions, glucose-6-phosphatase, a microsomal marker enzyme, was analyzed by measuring inorganic phosphate production [12]; the activity of succinate dehydrogenase, a mitochondrial marker enzyme, was measured by the method of Shephard and Hübscher [13]; acid-phosphatase activity (a lysosomal marker) was measured with a kit purchased from the Sigma Chemical Co. Concentrations of VPA and V-G in subcellular fractions were normalized for the respective concentration in liver homogenate (to correct for varying steady-state VPA concentrations), and averaged across all animals.

In vitro *protein binding determinations.* Pooled serum and hepatic cytosol were obtained from naive animals. VPA (50–10,000 μg/mL in water, pH 7.5) was added in increasing amounts to both serum and cytosol to obtain concentrations ranging from 5 to 1000 μg/mL. Binding was assessed in aliquots of each sample by ultrafiltration with an Amicon (YMT membrane) micropartition system (W. R. Grace & Co., Danvers, MA). Each Amicon device was centrifuged (1000 *g*) at 37° for 0.5 to 2 min, depending on the time required to produce a 10% yield of the initial volume as filtrate, in a fixed angle rotor. In the *in vitro* experiments, a separate set of cytosolic samples was diluted with water to assess the influence of protein concentration on VPA binding.

Ex vivo *protein binding determinations.* Hepatic cytosol was prepared from VPA-treated rats as described above. The *ex vivo* binding of VPA and V-G to proteins in serum, cytosol and bile was determined as described in the *in vitro* experiment.

Assay methodology. VPA concentrations were determined in aliquots (50 μL) of serum, cytosol, ultrafiltrate, liver homogenate, subcellular fractions and bile by gas chromatography with flame ionization detection. Samples were acidified with 50 μL of 0.5 N HCl and extracted with ethyl acetate (200 μL) containing cyclohexanecarboxylic acid (50 μg/mL) as the internal standard. Following mixing (by vortex) and centrifugation, 1-μL aliquots of the organic phase were injected on the column. V-G concentrations were determined in biologic samples from the *ex vivo* study following base hydrolysis. An equal volume of 2.5 N NaOH was added to a second aliquot of the samples and incubated at 90° for 2 hr. Following cooling, samples were acidified with excess 4 N HCl and extraction of the liberated VPA proceeded as described above. Chromatographic separation was achieved on a 10 m (0.53 mm i.d.) fused silica capillary column with a free fatty acid (FFAP) bonded stationary phase. The column temperature was 120° and the injector/detector temperature was 150°. Carrier gas (helium) was delivered at 10 mL/min, and hydrogen and air flow rates to the flame ionization detector were 60 and 300 mL/min, respectively. Separate standard curves were constructed in each biologic matrix for both hydrolyzed and unhydrolyzed samples.

Concentrations of V-G were estimated as the difference between VPA concentrations in the hydrolyzed and unhydrolyzed aliquots. Protein content was determined in each biologic sample according to the method of Lowry *et al.* [14]. Standard curves were constructed with bovine serum albumin in water. For protein binding determinations in cytosol, the unbound concentration measured in diluted cytosol was related to bound concentration normalized for protein content in diluted cytosol. In all other cases, cytosolic concentrations were corrected for dilutions during homogenization and fractionation of the liver.

Data analysis. The relationship between bound and unbound substrate concentrations in each biologic fluid was described with a saturable binding model:

$$C_{\text{bound}} = \sum_{i=1}^{n} \frac{N_i P_T K_i C_{\text{unbound}}}{1 + K_i C_{\text{unbound}}} \quad (2)$$

where n represents the number of classes of binding sites, $N_i P_T$ is the binding capacity of the i^{th} class of binding sites, and K_i is the corresponding association constant. The binding equation was fit to the data with the nonlinear least-squares regression program MINSQ (MicroMath, Salt Lake City, UT). Both one-site and two-site binding models were fit to the *in vitro* and *ex vivo* data; the optimal model fit was determined based upon a modified Akaike's information criterion [15] provided by the software. The total VPA concentration (C_{total}) may be expressed as:

$$C_{\text{total}} = C_{\text{unbound}} + \sum_{i=1}^{n} \frac{N_i P_T K_i C_{\text{unbound}}}{1 + K_i C_{\text{unbound}}} \quad (3)$$

Binding of VPA to proteins in serum and cytosol was determined *in vitro* and *ex vivo*; due to the lack of authentic material, the binding of V-G was determined only *ex vivo*.

The concentration of unbound VPA in hepatic cytosol will be dependent upon VPA flux between serum and cytosol as well as clearance of VPA from hepatic cytosol via biotransformation. The differential equation describing flux of VPA through hepatic cytosol is:

$$\frac{d[C_{\text{unbound, cytosol}}]}{dt} = \frac{T_{\max} \cdot C_{\text{unbound, serum}}}{K_m + C_{\text{unbound, serum}}} - \frac{Cl_I'}{V_{\text{cytosol}}} \cdot [C_{\text{unbound, cytosol}}] \quad (4)$$

where $T_{\max}$ is the transport maximum for *net* translocation of drug from serum to cytosol (i.e. contaminated by an indeterminate rate of efflux from cytosol to serum), K_m is the serum concentration of unbound VPA producing a net rate of serum-to-cytosol translocation equal to 50% of $T_{\max}$, and $C_{\text{unbound, cytosol}}$ and $C_{\text{unbound, serum}}$ represent the unbound concentrations of VPA in hepatic cytosol and serum, respectively. The intrinsic clearance of unbound VPA from hepatic cytosol (Cl_I') was estimated as the ratio of the VPA steady-state elimination rate (i.e. rate of infusion) to the unbound concentration of VPA in hepatic cytosol, assuming that steady-state conditions were achieved for VPA in cytosol as well as serum. The volume of hepatic cytosol (V_{cytosol}) was estimated to be 44% of wet liver weight [16]. At steady state, $C_{\text{unbound, cytosol}}$ is constant with respect to time. Thus, Equation 4 may be rearranged to yield the relationship between unbound VPA in hepatic cytosol and serum:

$$C_{ss\,\text{unbound, cytosol}} = \frac{T_{\max} \cdot C_{ss\,\text{unbound, serum}}}{[\frac{Cl_I'}{V_{\text{cytosol}}}](K_m + C_{ss\,\text{unbound, serum}})} \quad (5)$$

Equation 5 was fit to the $C_{ss\,\text{unbound, cytosol}}$ versus $C_{ss\,\text{unbound, serum}}$ data by nonlinear least-squares regression to obtain estimates of $T_{\max}$ and K_m for net VPA translocation from serum to the cytosolic compartment.

VPA evidenced apparent saturable clearance in the relationship between unbound steady-state serum concentrations and infusion rate. These data were fit with a standard Michaelis–Menten equation, assuming that the steady-state elimination rate was equal to the rate of infusion, to determine the $V_{\max}$ and K_m for clearance of unbound VPA from the systemic circulation. In all cases, the goodness of fit of a linear versus a nonlinear model was evaluated based upon the modified Akaike's information criterion.

Statistical comparisons. Orthogonal least-squares regression was used to examine the linearity of relationships between experimental variables. The significance of differences in unbound fraction between VPA and V-G was assessed with the paired Student's t-test. A one-way analysis of variance was employed to compare mean concentrations between various subcellular fractions. In all cases, the criterion for statistical significance was $P < 0.05$.

RESULTS

In the *in vitro* studies, unbound VPA concentrations ranged from 8.36 to 626 μg/mL in serum, and from 7.48 to 873 μg/mL in hepatic cytosol. The *in vitro* binding of VPA to proteins in serum and hepatic cytosol is displayed in Fig. 1. The data were well described by a saturable binding model (Eqn. 2); two independent classes of binding sites provided the optimal fit to the data. Parameter estimates for VPA binding to serum and hepatic cytosol are listed in Table 1. The binding capacity of cytosolic proteins was 2- to 4-fold higher than that in serum for both binding sites. The binding affinity for VPA to a cytosolic protein was approximately 10-fold higher at the high-affinity site, and 10-fold lower at the low-affinity site, as compared to the corresponding binding sites on serum proteins. The relationship between the *in vitro* binding of VPA to hepatic cytosolic proteins at two different VPA concentrations (25 and 100 μg/mL) and protein concentrations ranging form 4 to 40 mg/mL was examined by serial dilutions of hepatic cytosol (Fig. 2). Orthogonal least-squares regression of the data indicated a linear relationship ($r^2 > 0.93$) between VPA bound fraction and cytosolic protein at both VPA concentrations.

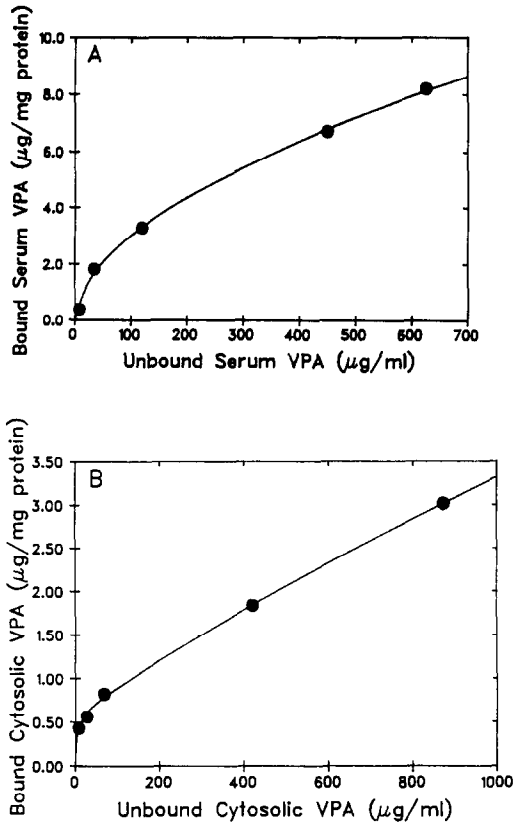


Fig. 1. Relationship between bound and unbound VPA following *in vitro* addition of VPA to (A) serum or (B) hepatic cytosol. The solid curve represents the fit of a saturable binding model (Eqn. 2) to the data assuming two independent classes of binding sites. Estimates of binding parameters are listed in Table 1.

Table 1. Model parameters for binding of VPA to rat serum and hepatic cytosol*

Parameter	Serum	Cytosol
In vitro		
N_1P_T† (mg/mL)	0.572 ± 0.116	1.21 ± 0.41
N_2P_T‡ (mg/mL)	0.015 ± 0.019	0.060 ± 0.006
K_1† (mL/mg)	3.78 ± 3.18	0.390 ± 0.180
K_2‡ (mL/mg)	32.1 ± 31.1	478 ± 264
Ex vivo		
NP_T (mg/mL)	0.229 ± 0.079	0.341 ± 0.224
K (mL/mg)	3.25 ± 1.86	6.70 ± 5.65

* Parameter estimates are expressed as means ± SD of the fit of a saturable binding model to 5 (*in vitro*) or 6 (*ex vivo*) data points.

† Total VPA binding capacity and affinity constant for the first class of binding sites.

‡ Total VPA binding capacity and affinity constant for the second class of binding sites.

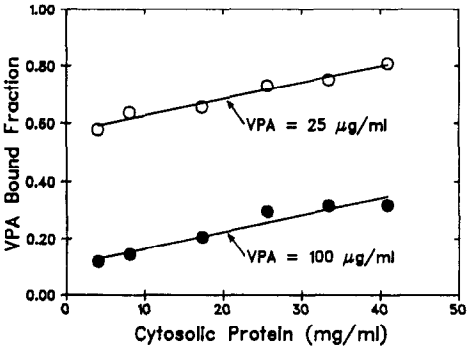


Fig. 2. Relationship between *in vitro* VPA binding and cytosolic protein at two VPA concentrations. The solid lines represent results of orthogonal least-squares regression; $r^2 > 0.93$ for both relationships.

The *ex vivo* binding of VPA and V-G to proteins in serum, hepatic cytosol and bile following *in vivo* infusion of VPA to steady state was examined. Concentrations of unbound VPA ranged from 6.83 to 315 μg/mL in serum, 4.14 to 55.4 μg/mL in hepatic cytosol preparations (19.8 to 234 μg/mL in cytosol corrected for dilution during preparation), and 62.0 to 529 μg/mL in bile. The relationships between bound and unbound VPA concentrations in serum and hepatic cytosol are shown in Fig. 3. The data were best described by a saturable binding model (Eqn. 2) with one class of binding sites. The parameter estimates for *ex vivo* binding to proteins in serum and hepatic cytosol are listed for comparison with *in vitro* parameters in Table 1. Binding capacities for VPA in both serum and hepatic cytosol were reduced 2- to 3-fold in rats receiving VPA infusions as compared to naive animals. Furthermore, the high-affinity binding site observed in the *in vitro* experiment was not apparent *ex vivo*. The net result of these changes was a lower degree of VPA binding *ex vivo* as compared to *in vitro*.

Total V-G concentrations in serum ranged from 10.6 to 365 μg/mL. The binding of V-G to serum proteins (Fig. 4) was best described by a saturable binding model (Eqn. 2) with one class of binding sites. Estimates (± SD) of the model parameters were: NP_T = 0.0753 ± 0.0131 mg/mL; K = 129 ± 106 mL/mg. V-G was not detected in the hepatic cytosol of rats infused with the three lowest doses of VPA. In the remaining rats, unbound cytosolic concentrations of V-G were below detectable limits; hence, the binding kinetics of the conjugate to cytosolic proteins could not be examined. In contrast to cytosol, V-G was localized predominantly in bile (biliary concentrations ranged from 1377 to 6599 μg/mL). V-G concentrations in serum, hepatic cytosol, and bile relative to the corresponding concentrations of VPA are depicted in Fig. 5A. In serum, V-G concentrations were similar to those of VPA. However, V-G appeared to be removed rapidly from hepatic cytosol (V-G/VPA ratio < 0.1) and accumulated to a significant extent in bile (V-G/VPA ratio > 20).

The unbound fraction of VPA in serum, cytosol, and bile, and the unbound fraction of V-G in serum

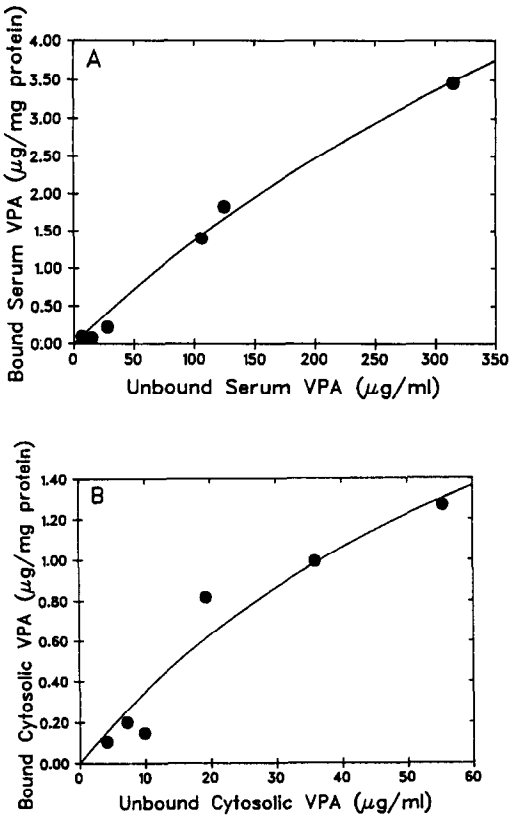


Fig. 3. Relationship between bound and unbound VPA *ex vivo* in (A) serum and (B) hepatic cytosol following *in vivo* infusion of VPA to steady state. The solid curve represents the fit of a saturable binding model (Eqn. 2) to the data assuming one class of binding sites. Estimates of binding parameters are listed in Table 1.

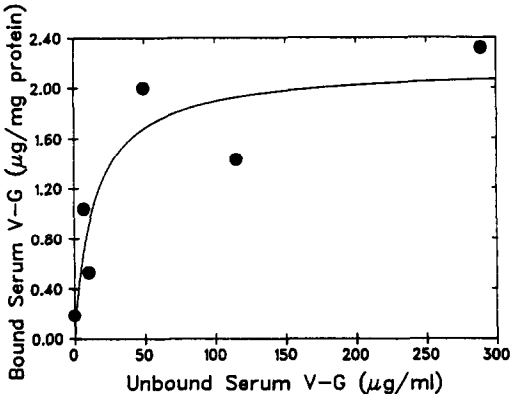


Fig. 4. Binding of V-G to serum proteins following administration of VPA *in vivo*. The solid curve indicates the fit of a saturable binding model (Eqn. 2) to the data assuming one class of binding sites. Estimates (mean ± SD) of binding parameters were: NP_T = 0.0753 ± 0.0131 mg/mL; K = 129 ± 106 mL/mg.

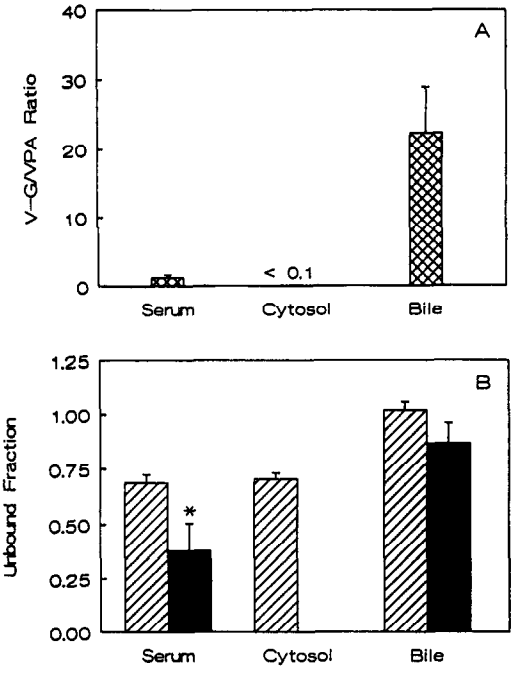


Fig. 5. (A) V-G concentrations normalized by VPA in serum, hepatic cytosol, and bile, and (B) unbound fraction of VPA (*hatched bars*) in serum, cytosol, and bile, and V-G (*solid bars*) in serum and bile following *in vivo* infusion of VPA to steady state. Unbound V-G was not detected in cytosol. Values are means ± SEM, N = 6. *P < 0.05, V-G vs VPA.

and bile, following *in vivo* infusion of VPA to steady state are presented in Fig. 5B. Neither VPA nor V-G was bound significantly to macromolecules in bile. Approximately 25% of the VPA present in hepatic cytosol was bound, which was similar to the bound fraction in serum. Interestingly, the unbound fraction of V-G in serum was significantly lower ($P < 0.05$) than that for VPA, consistent with the approximately 40-fold higher affinity of the glucuronide for proteins in serum.

The relationship between the hepatic cytosol-to-serum ratio for total VPA concentrations and unbound serum VPA, determined from data obtained *ex vivo*, was examined (Fig. 6). The cytosol-to-serum concentration ratio for total VPA decreased as steady-state concentrations of unbound VPA in serum increased. The model prediction based on *ex vivo* binding parameters in serum and hepatic cytosol is included in Fig. 6 for comparison with the observed data. In general, model predictions were in good agreement with the measured partitioning of total VPA between cytosol and serum. In contrast, *in vitro* binding parameters were unable to predict the observed cytosol-to-serum partitioning.

The nonlinear partitioning of VPA could not be attributed entirely to protein binding considerations. Unbound VPA concentrations in cytosol were not linearly related to unbound VPA serum concentrations in the *ex vivo* studies, as shown in

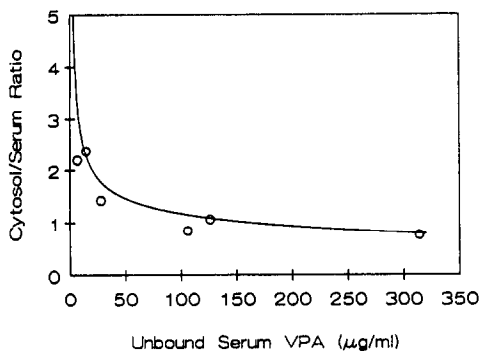


Fig. 6. Relationship between total VPA concentrations in hepatic cytosol and serum relative to the unbound VPA serum concentration. Data points represent values determined in animals treated with VPA. The solid curve indicates a model prediction (generated from Eqns. 3 and 5) based upon *ex vivo* binding parameters in serum and cytosol.

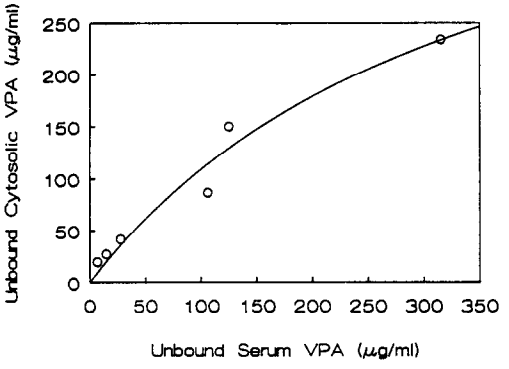
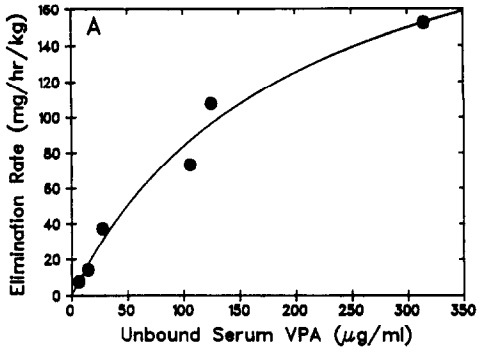


Fig. 7. Relationship between unbound VPA concentrations in hepatic cytosol and serum obtained from rats infused to steady state with VPA. The solid curve represents the fit of a saturable distribution model to the data with Equation 5. (T_{max} = 207 ± 73 μg/mL/min; K_m = 233 ± 125 μg/mL).



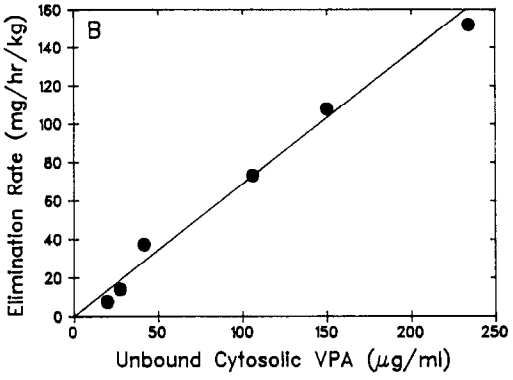


Fig. 8. Relationship between the total VPA elimination rate at steady state and unbound concentrations of VPA in (A) serum and (B) hepatic cytosol. For serum, the solid curve represents a fit of the Michaelis–Menten equation to the data (V_{max} = 238 ± 26 mg/hr/kg; K_m = 175 ± 49 μg/mL). For cytosol, the solid line represents the results of orthogonal least-squares regression (Y = 0.689·X + 0.071, r^2 = 0.985).

Fig. 7. The partitioning of unbound VPA into cytosol decreased as serum concentrations increased, and data were well described by a saturable distribution model (T_{max} = 207 ± 73 μg/mL/min; K_m = 233 ± 125 μg/mL).

The relationship between the total VPA elimination rate at steady state (i.e. rate of infusion) and unbound concentrations of VPA in serum and hepatic cytosol was examined (Fig. 8). The elimination rate was not linearly related to steady-state unbound serum VPA concentrations over the range examined. These data were consistent with a saturable elimination process. The Michaelis–Menten equation was fit to the serum data to obtain the following parameter estimates: V_{max} = 238 ± 26 mg/hr/kg; K_m = 175 ± 49 μg/mL. In contrast, the elimination rate was linearly related to steady-state cytosolic unbound VPA concentrations: Y = 0.689·X + 0.071, r^2 = 0.985. The hepatic intrinsic clearance of unbound VPA remained constant (0.671 ± 0.193 L/hr/kg) over the concentration range examined in hepatic cytosol.

The activities of several marker enzymes in liver homogenate and subfractions were examined to assess the purity of the various fractions. Glucose-6-phosphatase activity was enriched 3.5-fold in microsomal fractions and 2.5-fold in lysosomal fractions relative to homogenate. Succinate dehydrogenase activity was enriched 4.5-fold in mitochondrial fractions relative to homogenate. Acid phosphatase activity was enriched 5.2- to 6-fold in lysosomal fractions, 4.6-fold in microsomal fractions, and 2.5- to 2.8-fold in cytosolic and mitochondrial fractions, respectively, relative to homogenate. In all cases, activities of the marker enzymes in fractions other than those indicated were less than or equal to that in the homogenate, demonstrating adequate separation of the subfractions.

Concentrations of VPA, normalized for the concentration in liver homogenate, varied significantly across subcellular fractions due to localization in cytosol and relatively low microsomal content (Table 2). Concentrations in the nuclear, mitochondrial and lysosomal fractions were similar to those in the liver homogenate. In contrast, no

Table 2. Hepatic subcellular distribution of VPA and V-G*

Subcellular fraction	VPA†	V-G
Nuclear	0.922 ± 0.234 (6)	0.76 ± 0.23 (4)
Mitochondrial	0.934 ± 0.264 (6)	1.58 ± 1.05 (5)
Lysosomal	0.873 ± 0.233 (6)	2.46 ± 1.63 (6)
Microsomal	0.397 ± 0.250 (5)	2.53 ± 2.43 (3)
Cytosolic	2.60 ± 0.60 (6)	1.90 ± 1.02 (3)

* Data are expressed as means ± SD; concentrations of VPA or V-G in each fraction were normalized for the respective concentration in whole liver homogenate. Numbers in parentheses represent the number of rats in which VPA or V-G was detected in a given fraction.

† Significantly different across fractions, $P < 0.01$.

statistically significant differences in normalized V-G concentrations were observed between fractions ($P > 0.1$). One potential reason for the lack of apparent differential distribution of V-G between subcellular fractions is that the concentrations of V-G within the liver were quite low. V-G concentrations in the initial liver homogenate ranged from 1.5 to 25 μg/g tissue across the different dosage rates employed; in some rats, V-G was below detectable limits in at least one subcellular fraction.

DISCUSSION

VPA was moderately bound *in vitro* to proteins in serum (approximately 20–60%, depending upon concentration) and hepatic cytosol (approximately 15–70%). This moderate degree of binding to rat serum proteins is comparable to previous reports [4, 17]. In both matrices, the degree of binding decreased with increasing VPA concentration, consistent with a saturable binding isotherm for this drug [4]. *In vitro* binding of VPA to proteins in serum and hepatic cytosol was described most accurately by an equation incorporating two independent classes of saturable binding sites: a high-capacity, low-affinity site and a low-capacity, high-affinity site. The low-capacity binding site in cytosol had a much higher affinity for VPA than the low-capacity binding site in serum. VPA binding to hepatic cytosolic proteins was linear over an order of magnitude of protein concentrations (Fig. 2), suggesting that the binding parameters estimated in diluted cytosol were representative of VPA binding to cytosolic proteins within intact hepatocytes.

In contrast to the *in vitro* experiment, *ex vivo* binding of VPA in serum and hepatic cytosol was described most accurately by one class of binding sites; the *ex vivo* estimates of binding affinity for VPA were similar between the two matrices, but were lower than the corresponding parameters obtained in the *in vitro* experiment. The protein binding of substrates typically is examined *in vitro*, after the addition of known concentrations of a substrate to naive serum. Products of *in vivo* biotransformation that bind significantly to proteins in serum and/or tissues have the potential to displace the parent compound from its binding site(s). For example, the metabolite mono-*N*-dealkyldisopyramide increases the unbound fraction of disopyramide in a competitive manner [18]. In the presence of such a metabolite–parent binding interaction, discrepancies between the results of protein binding studies conducted *in vitro* and *ex vivo* would be anticipated.

Following *in vivo* infusion of VPA to steady state, V-G was bound more highly than VPA to serum proteins (Fig. 5B). Furthermore, V-G was bound to only one class of binding sites in serum. These data are consistent with the hypothesis that V-G may displace VPA from the high-affinity, low-capacity serum binding site *in vivo*. Several acyl glucuronides have been found to bind irreversibly to proteins, including albumin [19]. The nature of the apparent binding of V-G to serum proteins, and the potential influence on the binding of the parent compound, remain to be determined.

Other investigators have noted that oxidative VPA metabolites bind to proteins. Semmes and Shen [4] reported that the monounsaturated product of mitochondrial VPA metabolism, E-Δ^2-VPA, binds to rat plasma proteins; the binding affinity of the metabolite was an order of magnitude higher than that of VPA. Porubek *et al.* [20] reported that the 2-*n*-propyl-4-pentenoic acid metabolite of VPA (Δ^4-VPA, a product of microsomal oxidation) binds covalently to protein in various tissues of the rat. The potential for the various Phase I and Phase II metabolites of VPA to bind to serum, cytosol, or tissue proteins and displace the parent drug may have important pharmacologic implications, and may explain the inability of *in vitro*, as opposed to *ex vivo*, binding parameters to predict accurately drug disposition *in vivo*.

Since VPA binds significantly to proteins and is metabolized by both mitochondrial and microsomal enzyme systems, a differential distribution of the drug within the hepatocyte was anticipated. In general, VPA appeared to localize preferentially in cytosol, with a lower content in the microsomal fraction (Table 2). Cytosolic localization may be due to the relatively high-affinity binding observed in both the *in vitro* and *ex vivo* binding studies. Low concentrations of VPA in the microsomal fraction may be due to a low degree of binding to microsomal proteins or to rapid biotransformation in this compartment. In contrast to the parent drug, V-G did not display differential distribution between the various subcellular fractions, but, as anticipated for this polar conjugate, was localized almost exclusively in bile. Due to the exceedingly low concentrations of V-G within the liver, it was not possible to determine the degree of binding of the conjugate to cytosolic proteins.

The distribution of VPA between hepatic cytosol and serum was not governed solely by passive diffusion. Total concentrations of VPA in hepatic cytosol were high relative to serum, and the cytosol-to-serum ratio decreased as unbound VPA serum concentrations increased. A nearly identical non-linear VPA partitioning between liver and plasma has been reported in mice [7]; the liver/plasma VPA concentration ratio 30 min after an i.p. dose of the

drug decreased from 2.5 to 1.0 as the total VPA concentration in plasma increased from 6.9 to 154 μg/mL. In the present study, the cytosol/serum concentration ratio decreased from 2.2 at a total serum concentration of 12.2 μg/mL to 0.84 at a total serum concentration of 158 μg/mL. Thus, the nonlinear hepatic partitioning of VPA does not appear to be restricted to the rat, and may be a general phenomenon. Binding to cytosolic proteins undoubtedly plays an important role in determining the hepatic distribution of VPA.

Dose-dependent elimination of VPA in the rat has been reported [8, 21] and attributed to a saturable metabolic pathway. In the rat, urinary excretion of conjugated VPA (primarily the ester glucuronide) accounted for approximately 24 and 53% of the dose at 15 and 150 mg/kg VPA, respectively [8]. Glucuronidation is a high-capacity system that is not readily saturated in the rat liver [22]. In the present investigation, VPA elimination appeared to be saturable when the elimination rate was related to steady-state serum concentrations of unbound VPA. Indeed, a standard Michaelis–Menten equation was adequate to describe these data, and the calculated parameters (V_{max} and K_m) were in agreement with those derived previously following a 150 mg/kg i.v. bolus dose administered to rats with exteriorized bile flow [21]. In contrast to serum concentrations, the VPA elimination rate was related linearly to unbound cytosolic VPA concentrations. Thus, the intrinsic hepatic clearance of unbound VPA remained constant (approximately 0.7 L/hr/kg) over the concentration range examined. These results suggest that the nonlinear relationship between VPA dose and systemic concentrations in rats may be due to nonlinear distribution of VPA into the hepatocyte rather than to saturable metabolism. The K_m estimated for serum-to-cytosol translocation of VPA (233 ± 125 μg/mL) was similar to that estimated for removal of VPA from the systemic circulation (175 ± 49 μg/mL), suggesting that concentration-dependent translocation of VPA from serum to hepatocyte may be the rate-limiting step in systemic elimination of the drug.

Acknowledgements—This work was supported in part by the National Institutes of Health, Grants GM 41935 and ES 05774, and Training Grant T32 ES07126.

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