Kinetics of Valproic Acid Glucuronidation: Evidence for In Vivo Autoactivation

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List of Abbreviations: Cl: clearance; Cl_{int}: Intrinsic clearance; C_{mid}: Unbound plasma concentration at the midpoint of the urine collection interval; f_u: unbound fraction; k_{12}: rate constant from the central to the peripheral compartment in a two-compartment model; k_{21}: rate constant from the peripheral compartment to the central compartment in a two-compartment model; K_{m}^{app}: substrate concentration resulting in 50% of V_{max}^{app} (Michaelis-Menten constant); n: Hill coefficient; P450: cytochrome P450; S: substrate concentration; S_{50}^{app}: substrate concentration resulting in 50% of V_{max}^{app}; S.D.: Standard deviation; t_{mid}: time at midpoint of the urine collection interval; UDPGA: UDP-glucuronic acid; UGT: UDP-glucuronosyltransferase; ν: rate of an enzymatic reaction; V_d: volume of distribution; V_{max}^{app}: apparent maximum velocity of an enzymatic reaction; VPA: Valproic Acid; VPAG: Valproic acid-glucuronide.
ABSTRACT

Sigmoidal or autoactivation kinetics have been observed in vitro for both cytochrome P450 and UDP-glucuronosyltransferase catalyzed enzymatic reactions. However, the in vivo relevance of sigmoidal kinetics has never been clearly demonstrated. The current study investigates the kinetics of valproic acid-glucuronide (VPAG) formation both in vivo in adult sheep and in vitro in sheep liver microsomes (pool of 10). Following a 100 mg/kg i.v. bolus dose of valproic acid (VPA) to adult sheep (n=5), the majority of the dose was recovered in urine as VPAG (~79%). Eadie-Hofstee plots of VPAG formation rate (calculated from urinary excretion rate data for VPAG) were characteristic of autoactivation kinetics, and provided estimates of $V_{\text{max}} \text{app}$, $S_{50} \text{app}$ and $n$ of $2.10 \pm 0.75 \mu\text{mol/min/kg}$, $117 \pm 56 \mu\text{M}$ and $1.34 \pm 0.14$, respectively. Comparable estimates of $V_{\text{max}} \text{app}$ ($2.63 \pm 0.33 \mu\text{mol/min/kg}$), $S_{50} \text{app}$ ($118 \pm 53 \mu\text{M}$) and $n$ ($2.06 \pm 0.47$) describing overall VPA elimination from plasma were obtained by fitting VPA unbound plasma concentration-time data to a two-compartment model with elimination described by the Hill equation. Consistent with our in vivo observations, Eadie-Hofstee plots of VPAG formation in sheep liver microsomes were characteristic of autoactivation kinetics. To our knowledge, these data provide the first clear demonstration that autoactivation kinetics observed in vitro in liver preparations can translate to the in vivo situation at least under the certain experimental conditions and confirm its relevance.
The classical hyperbolic Michaelis-Menten equation has long been used to characterize the in vitro kinetics of enzyme-catalyzed reactions. More recently, cytochrome P450 (P450) catalyzed enzymatic reactions that exhibit sigmoidal kinetics have been described in the literature. Notable examples of this type of kinetic behavior include P450 3A4 catalyzed oxidation of testosterone (Ueng et al., 1997) and carbamazepine (Korzekwa et al., 1998). Rate versus substrate concentration profiles for sigmoidal kinetics have a characteristic initial lag at lower concentrations and result in characteristic curved Eadie-Hofstee plots. Such kinetic behavior is often attributed to autoactivation of enzymes that occurs with increasing substrate concentrations and are often described empirically using the Hill Equation (Houston and Kenworthy, 2000). Models involving the binding of multiple substrate molecules to the enzyme active site have been proposed to provide a mechanistic description of autoactivation kinetics (Ueng et al., 1997; Korzekwa et al, 1998).

More recently, examples of similar kinetic phenomenon have been reported for UDP-glucuronosyltransferases (UGT). The formation of estradiol-3-glucuronide, a UGT 1A1 selective reaction, was observed to have a better fit to the Hill equation in both human liver microsomes and recombinant UGT 1A1 (Fischer et al., 2000; Soars et al., 2003). Similar autoactivation kinetics have also been observed for the formation of the glucuronic acid conjugate of acetaminophen in human liver microsomes, and in recombinant UGT1A1 and UGT1A9 (Fischer et al., 2000; Court et al., 2001). The mechanism of autoactivation kinetics observed for UGT’s is currently unknown.

Valproic acid (2-propylpentanoic acid, VPA) is a broad spectrum anticonvulsant with a unique branched-chain fatty acid structure (Davis et al., 1994). Previous studies in sheep, demonstrated that VPA was primarily eliminated in this species via glucuronidation with ~ 70-80% of an administered intravenous dose recovered in urine as VPA-glucuronide (VPAG, Wong et al., 2001). The appearance of VPAG in sheep urine appeared to be formation rate-limited rather
than excretion rate-limited. Based upon these properties, we were able to assess the in vivo apparent $V_{\text{max}}$ and $K_{\text{m}}$ of VPA glucuronidation in sheep using either the disappearance of the parent compound from plasma or the excretion rate of VPAG in urine (Wong et al., 2001). In our preliminary studies examining VPAG formation in sheep liver microsomes, characteristics of autoactivation kinetics were observed (Kumar and Wong, unpublished results). As mentioned, sigmoidal kinetics has been previously observed in vitro for both P450 and UGT catalyzed reactions. However, the in vivo relevance of sigmoidal/autoactivation kinetics has never been clearly demonstrated. Valproic acid is unique in that high doses of the compound can be administered such that saturation of its elimination (glucuronidation) occurs in vivo, thus allowing for full characterization of kinetic parameters for its metabolism in vivo. This ability to characterize VPA glucuronidation kinetics in vivo provided a unique opportunity for us to investigate whether the autoactivation kinetics for VPA glucuronidation can be observed in vitro in liver microsomes and, if so, whether this would translate to the in vivo situation. Thus, the objective of the studies described in this manuscript was to investigate the occurrence of sigmoidal/autoactivation kinetics for VPA glucuronidation in vitro and in vivo in sheep.
METHODS

In Vivo Animal Experiments

Five Dorset Suffolk cross-bred ewes, with a body weight of 61.9 ± 7.3 kg (mean ± S.D.) were surgically prepared with a minimum of three days prior to experimentation. Polyvinyl catheters (Dow Corning, Midland, MI) were implanted in a femoral artery and vein (catheter i.d. 1.02 mm and o.d. 2.16 mm) as described by Kumar et al. (1999). Briefly, animals were administered an iv bolus of VPA (Sodium Valproate, Sigma Chemical Co., St. Louis, MO) at a dose of 100 mg/kg body weight over 1 min via the femoral vein. Blood samples (~3 ml) were collected via the femoral artery at 5, 15, 30, 45 min, and 1, 2, 4, 6, 9, 12, 15, 24, 36, 48, 60 and 72 h following drug administration. Blood samples were placed into heparinized Vacutainer® tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 2000 x g for 10 min. The plasma supernatant was harvested and placed into clean borosilicate test tubes with polytetrafluoroethylene-lined caps. Cumulative urine samples were collected via a Foley® bladder catheter for the following time intervals: 0-2, 2-4, 4-6, 6-9, 9-12, 12-15, 15-24, 24-36, 36-48, 48-60, and 60-72 hours post dose. Plasma and urine samples were stored frozen at -20°C until the time of analysis. All studies were approved by the University of British Columbia Animal Care Committee, and the procedures performed on sheep conformed to the guidelines of the Canadian Council on Animal Care.

Unbound plasma concentrations of VPA were determined ex vivo in all adult sheep plasma samples using an ultrafiltration procedure as described by Wong et al. (2001). Concentrations of VPA and its metabolites in plasma ultrafiltrate and urine were measured simultaneously using an established gas chromatographic-mass spectrometric (GC-MS) analytical method (Yu et al., 1995). The variability and bias of all analytes measured using this analytical method was determined to be
<15% in earlier assay validation studies (Yu et al., 1995). VPA and metabolite calibration and quality control standards as well as control (blank) biological fluid samples were run with each batch of study samples. Concentrations of the VPAG in urine were determined as described by Wong et al. (2001).

**In Vitro Valproic Acid Glucuronidation Kinetics**

The glucuronidation kinetics of valproic acid were examined in pooled sheep liver microsomes (pool of 10) (Xenotech LLC, Lenexa, KS). The kinetics of VPA glucuronidation was determined under the following incubation conditions: VPA (5 to 640 µM), 0.5 mg/ml microsomal protein, 2.5 mM MgCl₂, 5 mM saccharlactone, alamethacin (10 µg/mg protein), and 3 mM UDPGA (Sigma-Aldrich Co., St. Louis, MO) in 100 mM phosphate buffer (pH 7.5). Total incubation volume was 200 µL. All contents of each incubation, with the exception of UDPGA, were pre-incubated for 5 minutes at 37°C. Following the pre-incubation period, reactions were initiated by the addition of UDPGA. Reactions were terminated at 20 minutes by the addition of 200 µL of ice-cold acetonitrile containing the internal standard (diclofenac at 2 µM final concentration; Sigma-Aldrich Co., St. Louis, MO) followed by thorough vortex-mixing. The resulting samples were centrifuged at 2000 g for 10 minutes at 4°C and 10 µL of the supernatant was injected into a liquid chromatograph coupled with a tandem mass spectrometer (LC-MS) for quantitation of VPAG. All reactions were performed in triplicate. In preliminary experiments, reactions performed under the described conditions were linear with respect to both microsomal protein concentration and incubation time (data not shown).
VPAG was quantitated using a modification of a LC-MS method previously described by Tong et al., (2005). The LC-MS instrument consisted of a Shimadzu LC-10 ADvp liquid chromatograph (Shimadzu Corporation, Columbia, MD) interfaced with a Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA). Samples were injected by a Shimadzu SIL-HTc autosampler (4°C) onto a Waters Symmetry C18 column (50 mm x 2 mm ID, Waters Corporation, Milford, MA). The mobile phase consisted of (A) 95% water and 5% acetonitrile with 5 mM ammonium acetate and (B) 95% acetonitrile and 5% water with 5 mM ammonium acetate. The LC pumps were programmed to pump 25% B from 0-1 min, followed by an increase to 90% B from 1-2 min, a hold at 90% B from 2-4 min, and a return to 25% B for column re-equilibration. The HPLC flow rate was 0.2 mL/min and the run time was 6 min. The mass spectrometer was operated in negative ion mode with MRM monitoring (VPAG: 319.1>143.2, IS: 293.9>250.0) at a collision energy of 17 eV and MS dwell time of 0.2 sec. Concentrations of VPAG were determined using a calibration curve prepared with authentic standard. Calibration curves (0.39 – 50 µM) for all assays performed were linear with r² values > 0.99 and CV’s <20% at LOQ and < 15% at all other concentrations.

**Determination of Unbound Valproic Acid in Sheep Liver Microsomes**

Unbound fraction of VPA in pooled sheep liver microsomes under in vitro incubation conditions was determined by ultracentrifugation using [14C]-VPA (valproic acid, [carboxyl-14C], sodium salt, American Radiolabeled Chemicals Inc., St. Louis, MO). Experiments were performed at 5 and 700 µM VPA in order to span the full range of concentrations used in the in vitro studies examining VPA glucuronidation kinetics. The determination of the VPA unbound fraction in sheep liver microsomes was performed under the following incubation conditions: VPA (5 and
700 µM), 0.5 mg/mL microsomal protein, 2.5 mM MgCl₂, alamethicin (10 µg/mg microsomal protein), and 5 mM saccharolactone in 100 mM phosphate buffer (pH 7.4). The total incubation volume was 4 mL. After an incubation period of 30 min at 37°C, 1 mL aliquot of each sample was transferred in triplicate to centrifuge tubes (11 x 34 mm PC tubes, Beckman Coulter Inc., Fullerton, CA) and samples underwent centrifugation at 100,000 rpm for 3 hrs using an Optima TLX ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). Radioactivity content was determined in the supernatant from the samples after centrifugation and in the original microsomal incubation prior to centrifugation using a Tri-Carb 3100 TR liquid scintillation analyzer (Perkin-Elmer, Boston, MA). The fraction unbound was calculated as the ratio of the average radioactivity measured in the supernatant layers (unbound fraction) to that in the initial microsomal incubate prior to centrifugation.

Pharmacokinetic and Enzyme Kinetic Analyses

In vivo estimates of apparent Hill ($V_{\text{max \ app}}$, $S_{50 \ app}$, $n$) parameters for overall VPA elimination from plasma were obtained through fitting of individual unbound plasma concentration–time profiles using SAAM II V1.2 (The SAAM Institute Inc, Seattle, WA). Unbound plasma concentration-time profiles were fit to a one or two compartment model with elimination described by either the Michaelis-Menten (Equation 1) or Hill equation (Equation 2).

$$v = \frac{V_{\text{max \ app}} \times C}{K_{m \ app} + C}$$  \hspace{1cm} \text{Equation 1}

$$v = \frac{V_{\text{max \ app}} \times C^n}{S_{50 \ app} + C^n}$$  \hspace{1cm} \text{Equation 2}
where \( v \) is the rate of elimination, \( V_{\text{max}}^{\text{app}} \) is the apparent maximum rate of elimination, \( C \) is the substrate concentration, and \( K_m^{\text{app}} / S_{50}^{\text{app}} \) is the apparent substrate concentration resulting in an elimination rate equal to 50% of \( V_{\text{max}}^{\text{app}} \), and \( n \) is the Hill coefficient. A two-compartment model with elimination described by the Hill equation provided the best "fit" of the data from adult animals in comparison to a simpler one-compartment model with similar elimination characteristics. Model selection was based upon lower Aikake’s Information Criterion (Wagner, 1993). Mean estimates for parameters are presented as a mean ± SD.

*In vivo* estimates of kinetic parameters for VPA glucuronidation from urinary excretion data were determined by fitting \( v \) (urinary excretion rate of the glucuronide metabolite) vs. \( C_{\text{mid}} \) (unbound VPA plasma concentration at the midpoint of the urine collection interval) data to the Hill equation

\[
v = C_{\text{mid}}^n \times V_{\text{max}}^{\text{app}} / (S_{50}^{\text{app}} n + C_{\text{mid}}^n)
\]

\( C_{\text{mid}} \) and \( v \) are as defined above, \( V_{\text{max}}^{\text{app}} \) is the apparent maximal formation rate of VPAG, \( S_{50}^{\text{app}} \) is the substrate concentration resulting in 50% of \( V_{\text{max}}^{\text{app}} \), and \( n \) is the Hill coefficient. The fitting to the Hill equation rather than the classical Michaelis-Menten equation was determined based upon the distinct shape of the Eadie-Hofstee plots, and by minimization of the sum of squares of residuals and the standard error of parameter estimates when data were fitted to the Hill equation. Data sets were individually fit for each animal using GraphPad Prism V4.02 (GraphPad Software Inc., San Diego, CA).

Rate of VPAG formation vs. VPA incubation concentration from in vitro enzyme kinetic experiment using pooled sheep liver microsomes were fit to the Hill equation using GraphPad Prism V4.02 as described above for the in vivo urine data. VPA incubation concentration substituted for \( C_{\text{mid}} \) when fitting the data from the in vitro experiment.
Simulations

Simulations of the two-compartment model with elimination described by the Hill or Michaelis-Menten equation were performed using SAAM II V1.2. Values of $V_{\text{max}}^{\text{app}}$ (3.09 µmol/min/kg), $K_{\text{m}}^{\text{app}}$ or $S_{50}^{\text{app}}$ (208 µM) used in simulations were calculated from estimates previously reported by Wong et al. (2001). $V_d$ (0.272 L/kg), $k_{21}$ (1.90 l/hr), and $k_{12}$ (1.40 l/hr) values were mean estimates obtained from animals in the current study. Dose and $n$ were arbitrarily assigned values of 100 mg/kg and 2.00, respectively, in order to illustrate differences between elimination governed by the Michaelis-Menten and Hill equations.
RESULTS

Urinary recovery of VPA Dose

The recovery of the VPA and its metabolites in urine following a 100 mg/kg intravenous dose is presented in Table 1. Essentially, the entire VPA dose was recovered in urine from all five animals. VPAG was the main metabolite found in urine accounting for approximately 80% of the entire dose. Following the glucuronide metabolite, unchanged VPA accounted for the second largest portion of the administered dose recovered in urine (~7%). All other metabolites combined, on average, accounted for <10% of the administered dose (Table 1).

In Vivo Estimation of $V_{\text{max}}^\text{app}$, $S_{50}^\text{app}$, and $n$ of Overall VPA Elimination from Plasma

Unbound plasma concentration-time profiles were fitted to a two-compartment model with elimination from the central compartment governed by the Hill equation. Unbound concentration-time profiles from individual animals along with their model-predicted plasma profiles are presented in Figure 1. The in vivo estimates of apparent $V_{\text{max}}^\text{app}$, $S_{50}^\text{app}$, and $n$ of overall VPA elimination from plasma are presented in Table 2. These resulting parameters are hybrid constants that are largely reflective of overall metabolic elimination. These estimates are comparable to estimates generated using VPAG urinary excretion data (Table 2) consistent with glucuronidation being the primary path of VPA elimination in sheep. An attempt was made to fit the unbound plasma concentration-time profiles to a one or two-compartment model with elimination governed
by the Michealis-Menten equation. However, parameter estimates could not be obtained as these models failed to converge.

**In Vivo Estimation of Apparent** $V_{\text{max}}^{\text{app}}$, $S_{50}^{\text{app}}$, and $n$ **of VPA Glucuronidation from Urinary Excretion Data**

A condition for estimating the apparent kinetic parameters of VPA glucuronidation using urine data is that the appearance of VPAG in urine is formation, as opposed to elimination rate-limited (Gilbaldi and Perrier, 1982). Metabolites demonstrating formation rate-limited urinary excretion exhibit plasma concentration vs time profiles that decline in parallel to those of the parent compound (Houston, 1986). In previous investigations with VPA using pregnant sheep, we attempted to measure the plasma concentrations of the VPAG by subjecting the samples to hydrolysis and subtracting the concentrations of the unconjugated VPA. However, it was evident from these attempts that the plasma concentrations of the VPA glucuronide conjugate were substantially lower than those of the parent and could not be reliably measured by our difference-based analytical approach (S. Kumar, unpublished results). This is consistent with the likely formation rate-limited urinary excretion of the VPA glucuronide. Furthermore, in the absence of a plasma profile, a plot of urinary excretion rate of VPAG versus $t_{\text{mid}}$ (i.e., time at the midpoint of the urine collection interval) can be used to estimate the slope of the terminal decline / half-life of the VPAG in plasma (Gilbaldi and Perrier, 1982). Figure 2 is a plot of mean urinary excretion rate of VPAG vs $t_{\text{mid}}$, together with a mean unbound VPA plasma concentration-time profile. The parallel decline of these two plots is also consistent with the urinary excretion of VPAG being formation rate-limited. This is consistent with our previous observation of
formation rate-limited urinary excretion of VPAG at intravenous doses of 250 mg/kg (Wong et al., 2001).

Eadie-Hostee plots of $v$ (urinary excretion rate of VPAG) vs. $v / C_{\text{mid}}$ (urinary excretion rate of VPAG/ the unbound VPA plasma concentration at the midpoint of the urine collection interval) for individual animals are shown in Figure 3. The plots from all animals were characteristically curved consistent with autoactivation kinetics described by the Hill equation. Plots of $v$ versus $C_{\text{mid}}$ from individual animals (Figure 3) were fitted to the Hill equation and the resulting estimates of $V_{\text{max}}^{\text{app}}$, $S_{50}^{\text{app}}$, and $n$ of VPA glucuronidation are shown in Table 2.

**Enzyme Kinetics of VPA Glucuronidation in Sheep Liver Microsomes**

Figure 4 shows a plot of VPAG formation rate ($v$) versus VPA incubation concentration and the corresponding Eadie-Hofstee plot in pooled sheep liver microsomes. The Eadie-Hofstee plot possessed a distinctive “hooked” profile characteristic of autoactivation kinetics. Estimates of $V_{\text{max}}^{\text{app}}$, $S_{50}^{\text{app}}$, and $n$ generated from fitting the data to the Hill equation are presented in Figure 4. The in vitro estimates of $S_{50}^{\text{app}}$ and $n$ were comparable to those estimated in vivo (Table 2).

Unbound fraction of VPA in sheep liver microsomes at VPA concentrations of 5 and 700 µM were 93 and 98%, respectively, suggesting negligible nonspecific binding in microsomes over the VPA concentration range used in our in vitro studies (5 to 640 µM). Hence, no correction was made for the binding of VPA to liver microsomal protein.
DISCUSSION

The in vitro kinetics of enzyme catalyzed reactions is commonly characterized by the hyperbolic Michaelis-Menten equation. Sigmoidal or autoactivation kinetics are distinguished from Michaelis-Menten kinetics by rate-substrate concentration profiles with initial lags at lower concentrations and result in characteristic curved Eadie-Hofstee plots. Sigmoidal kinetics is commonly described empirically using the Hill Equation (Houston and Kenworthy, 2000). The exact mechanistic explanation for various aspects of sigmoidal kinetics has yet to be fully elucidated. Early observations of sigmoidal kinetics for xenobiotic metabolizing enzymes were mostly in human liver microsomal and cDNA-expressed systems (Ueng et al., 1997; Korzekwa et al., 1998). Later work using dextromethorphan by Witherow and Houston (1999) demonstrated that sigmoidal kinetics could also be observed in hepatocytes. More recently, reports of similar kinetic phenomenon have been reported for UDP-glucuronosyltransferases (UGT) in both human liver microsomes and recombinant systems (Fischer et al., 2000; Soars et al., 2003; Fischer et al., 2000; Court et al., 2001). However, the in vivo relevance of autoactivation kinetics has never been clearly demonstrated.

Valproic acid presents a unique tool to examine the relevance of sigmoidal or autoactivation kinetics in vivo. Studies using rat hepatocytes over a wide concentration range (100 nM to 1.8 mM) indicate that the uptake of VPA into hepatocytes is linear, rapid, and does not involve carrier mediated transport (Booth et al., 1996). We have demonstrated that the excretion of VPAG in urine is formation rather then excretion rate-limited in the current study and in a previous study (Wong et al., 2001). Based upon these two properties, the in vivo excretion kinetics of VPAG are reflective of enzymatic processes involved in its formation rather than transport and/or distribution.
phenomenon. In a previous study, VPAG urinary excretion data from multiple in vivo experiments were pooled to generate a single rate versus substrate concentration plot (VPAG urinary excretion rate vs C_{mid}) (Wong et al., 2001). This profile was fitted to the Michaelis-Menten equation in order to provide in vivo estimates of apparent V_{max} and K_{m} for VPA glucuronidation. The sigmoidal features of rate-substrate concentration plots in our previous study were likely masked as a result of the data pooling process. Eadie-Hofstee plots from individual sheep are presented for the current study (Figure 3) and clearly display the “curved” shape that is characteristic of sigmoidal or autoactivation kinetics.

Since VPA is almost entirely eliminated via glucuronidation (Table 1), the disappearance of the unbound parent compound from plasma should be reflective of kinetic processes governing glucuronidation. VPA is a low clearance drug in sheep such that its total body clearance approximates f_{u} (unbound fraction of VPA) × Cl_{int} (intrinsic clearance) (Wilkinson and Shand, 1975). Thus, the clearance of unbound VPA is equal to its Cl_{int}. Cl_{int} is described by equation 3 for Michaelis-Menten kinetics and equation 4 for Hill kinetics.

\[
Cl_{\text{int}} = \frac{v}{C} = \frac{V_{\text{max}}}{K_{m} + C} \quad \text{Equation 3}
\]

\[
Cl_{\text{int}} = \frac{v}{C} = \frac{V_{\text{max}} \times C^{n-1}}{S_{50}^{n} + C^{n}} \quad \text{Equation 4}
\]

For compounds exhibiting Michaelis-Menten kinetics, Cl_{int} is linear and at its maximum value at concentrations that are << K_{m} where Cl_{int} approximates V_{max}/K_{m}. Cl_{int} decreases as substrate concentrations approach and exceed K_{m}. In contrast, the Cl_{int} of compounds exhibiting
autoactivation kinetics are not at their maximum at low substrate concentrations ($< S_{50}$). With increasing concentrations, the value of $Cl_{int}$ increases to a maximum, followed by an eventual decrease due to saturation of metabolic enzymes (Houston and Kenworthy, 2000). Figure 5 is a simulation to illustrate differences between elimination from a two-compartment model governed by the Michaelis-Menten and Hill equations. The distribution phase for both simulation profiles occurs between 0-2 hours post-dose. From ~ 2-5 hours, the shape of the unbound plasma concentration-time profile is slightly convex in both profiles characteristic of saturation of elimination processes. The difference between the two profiles becomes more obvious at lower concentrations after 6 hours. Elimination governed by the Michaelis-Menten equation results in a steeper terminal slope (i.e. shorter terminal $t_{1/2}$) in the unbound plasma concentration-time profile since the $Cl_{int}$ goes from being saturated at higher VPA concentrations to becoming first order and at its maximum at lower concentrations. In contrast, elimination governed by autoactivation kinetics results in a shallower terminal slope (i.e. longer terminal $t_{1/2}$) since $Cl_{int}$ is not at its maximum at low substrate concentrations. The degree of difference between the 2 equations is dependent on the value of $n$ providing all other parameters are kept constant. The unbound plasma concentration-time profiles presented in Figure 1 are similar in their shape to the simulation performed for the Hill equation displayed in Figure 5. As VPA is eliminated almost entirely by glucuronidation in sheep, $V_{max}^{app}$, $S_{50}^{app}$, and $n$ estimates from fitting unbound plasma concentration-time profiles were comparable to estimates derived from the VPAG urinary excretion data (Table 2).

The described differences in shape of the unbound plasma concentration-time profile provide an explanation for our failed attempt to characterize the unbound VPA plasma-concentration time profiles using Michaelis-Menten elimination. Previously, we had assessed in vivo apparent $V_{max}$ and $K_m$ for VPA overall elimination using unbound plasma-concentration time profiles in sheep.
Characterization of VPA’s nonlinear elimination using Michaelis-Menten kinetics required the simultaneous fitting of unbound plasma concentration-time profiles from three doses (50, 100, and 250 mg/kg; Wong et al., 2001) in contrast to the single dose data (100 mg/kg) being fitted in the current study. The doses used in the previous study spanned a range over which metabolic saturation was obvious from the observed decreases in unbound VPA clearance with increases in dose (Wong et al., 2001).

Our in vitro experiment in pooled sheep liver microsomes demonstrates that the glucuronidation of valproic acid is characterized by sigmoidal or autoactivation kinetics and provides an explanation for our in vivo observations. To our knowledge, this is the first report of in vitro VPA glucuronidation exhibiting autoactivation kinetics. Sigmoidal kinetics that are observed in vitro may result as a consequence of in vitro incubation conditions where substrate depletion occurs due to non-specific binding to incubation matrix or an overabundance of enzyme. At the lowest concentration of VPA tested in our study (5 µM), ~2% of the substrate was converted to the glucuronide conjugate. In addition, non-specific VPA microsomal binding appeared to be negligible in the concentration range over which VPA glucuronidation kinetics were assessed. Based upon these conditions, our observations of sigmoidal kinetics in vitro are likely not an experimental artefact and are related to characteristic enzyme-substrate interactions.

Reports of enhanced rates of glucuronidation in humans at high doses of VPA (1000 mg) have been previously described in literature (Granneman et al., 1984). These enhanced rates could not be accounted for entirely by increases in unbound concentrations of VPA that occurred with increasing dose. The reported enhancement of VPA glucuronidation is consistent with autoactivation kinetic behavior and our in vivo observations in sheep. The in vivo kinetics of VPA
glucuronidation would be more difficult to fully characterize in humans as doses normally administered are far less than those employed in our current study.

To our knowledge, the current study is the first clear demonstration of the occurrence of autoactivation kinetics in vivo in any species. Since the uptake of VPA into hepatocytes has been shown to be rapid and linear over a wide concentration range (Booth et al., 1996) and the excretion of VPAG in sheep urine is formation rate-limited, our observations of sigmoidal kinetics in vivo are most likely reflective of the enzymatic processes involved in VPA glucuronidation. In agreement with this, our in vitro studies examining VPA glucuronidation using sheep liver microsomes clearly displayed the features of autoactivation kinetics.
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REFERENCES


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LEGENDS FOR FIGURES

Figure 1  Valproic acid plasma concentration-time profiles fitted to a two-compartment model with Hill equation elimination for animals A-E

Figure 2  Unbound VPA plasma-concentration time profile together with plot of VPAG urinary excretion rate vs $t_{mid}$ (time at the midpoint of the urine collection interval).

Figure 3  Plots of urinary excretion rate of valproic acid-glucuronide (V) versus unbound valproic acid plasma concentration ($C_{mid}$), and corresponding Eadie-Hofstee plots for Animal A (A, B), Animal B (C, D), Animal C (E, F), Animal D (G, H), and Animal E (I, J). Fit to the Hill equation is indicated by the dashed lines.

Figure 4  Rate of valproic acid-glucuronide formation versus valproic acid incubation concentration (A) and corresponding Eadie-Hofstee plot (B) in pooled (n=10) sheep liver microsomes. Fit to the Hill equation is indicated by the dashed line. Kinetic parameter estimates are presented as estimate ± SE. V is defined as the rate of valproic acid-glucuronide formation and S is defined as substrate (VPA) concentration in the figure.

Figure 5. Simulation of two-compartment model with elimination described by either the Michaelis-Menten or Hill equation. The following values were used for this simulation: Dose (100 mg/kg), $V_d$ (0.272 L/kg), $k_{21}$ (1.90 1/hr), $k_{12}$ (1.40 1/hr), $V_{max}^{app}$ (3.09 µmol/min/kg), $K_m^{app}$ or $S_{50}^{app}$ (208 µM), n (2.00).
Table 1  Recovery of VPA and its metabolites in urine following intravenous administration of a 100 mg/kg intravenous dose (n=5).

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>% of VPA dose recovered</th>
<th>Total % Recovered</th>
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<tbody>
<tr>
<td></td>
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<td>80.3</td>
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<tr>
<td>E</td>
<td>6.0</td>
<td>70.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.0 ± 1.6</td>
<td>78.7 ± 5.6</td>
</tr>
</tbody>
</table>

* includes the following VPA metabolites; 2-n-propyl-2-pentenoic acid, 2-n-propyl-3-pentenoic acid, 2-n-propyl-4-pentenoic acid, 2-n-propyl-3-oxopentanoic acid, 2-n-propyl-4-oxopentanoic acid, 3-hydroxy VPA, 4-hydroxy VPA, 5-hydroxy VPA, 2-propylsuccinic acid, 2-propylglutaric acid.
Table 2. Summary of Hill equation parameters estimates for overall VPA elimination from plasma and for valproic acid-glucuronide formation in urine.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>V\textsubscript{max}\textsuperscript{app} (µmol/min/kg)</th>
<th>S\textsubscript{50}\textsuperscript{app} (µM)</th>
<th>n</th>
<th>V\textsubscript{max}\textsuperscript{app} (µmol/min/kg)</th>
<th>S\textsubscript{50}\textsuperscript{app} (µM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.43</td>
<td>40.3</td>
<td>2.83</td>
<td>1.90</td>
<td>71.9</td>
<td>1.54</td>
</tr>
<tr>
<td>B</td>
<td>2.31</td>
<td>109</td>
<td>2.13</td>
<td>1.44</td>
<td>104</td>
<td>1.37</td>
</tr>
<tr>
<td>C</td>
<td>2.43</td>
<td>106</td>
<td>1.90</td>
<td>1.82</td>
<td>86.0</td>
<td>1.35</td>
</tr>
<tr>
<td>D</td>
<td>2.91</td>
<td>180</td>
<td>1.56</td>
<td>1.94</td>
<td>111</td>
<td>1.18</td>
</tr>
<tr>
<td>E</td>
<td>3.06</td>
<td>154</td>
<td>1.90</td>
<td>3.40</td>
<td>215</td>
<td>1.25</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.63 ± 0.33</td>
<td>118 ± 53</td>
<td>2.06 ± 0.47</td>
<td>2.10 ± 0.75</td>
<td>117 ± 56</td>
<td>1.34 ± 0.14</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Estimated from fitting unbound VPA plasma concentration-time profiles to a 2-compartment model with elimination characterized by the Hill equation (Figure 1)

\textsuperscript{b} Estimated from fitting plots of valproic acid-glucuronide urinary excretion rate of vs. unbound VPA plasma concentrations to the Hill equation (Figure 3)
Figure 1

A, B, C, D, E: Unbound VPA Plasma Concentration (μM) vs. Time (hours).
Figure 2: Graph showing the relationship between time and plasma concentration of unbound VPA and urinary excretion rate of VPAG. The x-axis represents time normalized by t_{mid} (hours), while the y-axis on the left shows unbound VPA plasma concentration in μM, and the y-axis on the right shows VPAG urinary excretion rate in μmol/min/kg. The graph includes error bars indicating variability in the data.
Figure 3

(A) $V$ (nmol/min/kg) vs. $C_{mid}$ (µM VPA)

(B) $V/C_{mid}$ (nmol/min/kg / µM VPA)

(C) $V$ (nmol/min/kg) vs. $C_{mid}$ (µM VPA)

(D) $V/C_{mid}$ (nmol/min/kg / µM VPA)

(E) $V$ (nmol/min/kg) vs. $C_{mid}$ (µM VPA)

(F) $V/C_{mid}$ (nmol/min/kg / µM VPA)

(G) $V$ (nmol/min/kg) vs. $C_{mid}$ (µM VPA)

(H) $V/C_{mid}$ (nmol/min/kg / µM VPA)

(I) $V$ (nmol/min/kg) vs. $C_{mid}$ (µM VPA)

(J) $V/C_{mid}$ (nmol/min/kg / µM VPA)
Figure 4

Panel A: Enzyme activity as a function of VPA concentration. The data points show a sigmoidal curve, indicating saturation kinetics. The parameters are:

- $V_{max}^{app} = 334 \pm 43 \text{ pmol/min/mg}$
- $S_{50}^{app} = 132 \pm 24 \text{ µM}$
- $n = 1.52 \pm 0.07$

Panel B: The V/S ratio plotted against VPA concentration. The V/S ratio reaches a maximum at an intermediate VPA concentration, indicating an allosteric mechanism. The data points are dispersed along a curve, suggesting a complex interaction.

V (pmol/min/mg)

VPA Concentration (µM)

V/S (pmol/min/mg / µM VPA)
Figure 5.