DOSE-DEPENDENT PHARMACOKINETICS AND METABOLISM OF VALPROIC ACID IN NEWBORN LAMBS AND ADULT SHEEP

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ABSTRACT:

Dose-dependent pharmacokinetics and metabolism of valproic acid (VPA) were studied in newborn and adult sheep to assess age-related differences in plasma protein binding and metabolic elimination. Newborn lambs received either a 10- (n = 8), 50- (n = 5), 100- (n = 4), or 250-mg/kg (n = 4) VPA i.v. bolus. Individual adult sheep (n = 5) received all four doses in a random order with an appropriate washout period between experiments. Unbound or metabolic clearance of VPA was significantly higher in adult sheep at the two lower doses when compared with lambs, and similar to the lambs at the two higher doses. Plasma protein binding was nonlinear at all doses. Estimates of binding capacity (θm) at the saturable site were higher in adults (91.8 μg/ml) when compared with lambs (44.9 μg/ml), whereas the opposite trend was observed for binding affinity [Km = 9.6 μg/ml (adult) versus 3.2 μg/ml (lambs)]. Characterization of developmental differences in overall VPA metabolic elimination involved fitting of unbound VPA plasma concentration data to a two-compartment model with Michaelis-Menten elimination. This resulted in similar in vivo estimates of apparent Vmax [445.0 μg/min/kg (adult) versus 429.9 μg/min/kg (lambs)]. However, apparent Km estimates appeared to be higher in lambs [30.0 μg/ml (adult) versus 69.6 μg/ml (lambs)]. Similar findings were obtained from in vivo estimates of Vmax and Km for VPA glucuronidation obtained from VPA-glucuronide metabolite urinary excretion data. Thus, it appears that age-related differences in metabolic clearance may be related to differences in the apparent in vivo Km as opposed to Vmax of VPA glucuronidation.

Valproic acid (2-propylpentanoic acid; VPA1) is a broad spectrum anticonvulsant with a unique branched-chain fatty acid structure.

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1 Abbreviations used are: VPA, valproic acid; AIC, Akaike’s information criterion; AUCl acc. area under the curve of arterial plasma concentration-time profile; AUCluc. area under the curve of unbound drug; Cw, protein bound drug concentration; Clint, intrinsic clearance; Cltot, total body clearance of the total drug; Clunb, total body clearance based upon unbound drug concentrations; Cunb, unbound drug concentration; Cunb concentration at the midpoint of the urine collection interval; CV, coefficient of variation; fub, area weighted unbound fraction of the drug; fub, unbound fraction; t1/2a, time at the midpoint of the urine collection interval; Vdunb, steady-state volume of distribution; Vdint, steady-state volume of distribution based upon unbound drug concentrations; Vdint, steady-state volume of distribution corrected for the effects of saturable protein binding; 2-ene VPA, 2-n-propyl-2-pentenoic acid; 3-ene VPA, 2-n-propyl-3-pentenoic acid; 4-ene VPA, 2-n-propyl-4-pentenoic acid; 3-keto VPA, 2-n-propyl-3-oxopentanoic acid; 4-keto VPA, 2-n-propyl-4-oxopentanoic acid; 3-, 4-, and 5-OH VPA, 3-, 4-, and 5-hydroxy VPA, respectively; 2-PSA, 2-propylsuccinic acid; 2-PGA, 2-propylglutaric acid.

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(Davis et al., 1994). Despite cases of VPA-induced idiosyncratic hepatotoxicity, VPA remains the drug of choice for treating seizures of various etiologies in neonates, infants, and children due to its broad spectrum of activity and minimal cognitive side effects (Sarisjulis and Olivier, 1999). Studies examining VPA pharmacokinetics during the immediate newborn period in humans have reported extended elimination half-lives in newborns when compared with the adult (Levy and Shen, 1995). Similar findings have been observed in studies with other species, such as rats (Haberer and Pollack, 1994) and guinea pigs (Yu et al., 1985, 1987). Valproic acid is mainly eliminated by hepatic metabolism and exhibits a low hepatic extraction ratio. Thus, any developmental changes in plasma protein binding and/or metabolic capacity will influence VPA systemic clearance (Levy and Shen, 1995). The limited available data in human neonates indicate a low unbound VPA clearance in this population, suggesting low intrinsic metabolic clearance due to immature activity of the enzymes responsible for VPA elimination (Levy and Shen, 1995). In vivo developmental studies in rats (Haberer and Pollack, 1994) and guinea pigs (Yu et al., 1985, 1987) suggest that differences in both plasma protein binding and VPA metabolism contribute to age-related alterations in VPA disposition. Similarly in sheep, we observed a lower metabolic clearance and a higher unbound fraction in 10-day-old lambs when compared with adult sheep (Wong et al., 2000). The lower metabolic clearance in 10-day-old lambs observed in this study was attributed to age-related differences in VPA-glucuronidation (Wong et al., 2000).

Thus, the purpose of this study is to examine in more detail the role of plasma protein binding and metabolic elimination in determining...
VPA total body clearance in 10-day-old lambs and adult sheep. This was accomplished by conducting a dose-ranging experiment in these two age groups. Dose-dependent changes in VPA metabolism were also examined. Sheep were chosen for these studies due to their similarity to humans in terms of the main metabolic pathways (i.e., glucuronidation, β-oxidation, and P-450-catalyzed pathways) and VPA metabolites (i.e., VPA-glucuronide, 3-keto VPA (2-n-propyl-3-oxopentanoic acid)) previously observed in sheep (Kumar et al., 2000a). Furthermore, the use of chronically catheterized lambs and adult sheep overcomes limitations in the available sampling volume of biological fluids associated with smaller animal models allowing for more detailed studies.

Materials and Methods

Animals and Surgical Preparation. 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.

Adult sheep. Five nonpregnant Dorset Suffolk cross-bred ewes, with a body weight of 61.9 ± 7.3 kg (mean ± S.D.), were surgically prepared at least 3 days before 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). On the morning of the experiment, a Foley bladder catheter was inserted via the urethra of the ewe and attached to a sterile polyvinyl bag for cumulative urine collection.

Newborn lambs. A total of 21 Dorset Suffolk cross-bred lambs were used in this study. Lambs were divided into a 10-mg/kg group (n = 8), a 50-mg/kg group (n = 5), a 100-mg/kg group (n = 4), and a 250-mg/kg group (n = 4). All lambs were surgically prepared at least 3 days before the experiment under isoflurane (1%) anesthesia. Briefly, polyvinyl catheters (Dow Corning) were implanted in a carotid artery, a jugular vein, and the urinary bladder as described by Wong et al. (2000). On the day of the experiment, the lambs were moved to monitoring pens adjacent to and in full view of their mothers. The urinary bladder catheter was allowed to drain by gravity into a sterile reservoir.

While in the holding pens, lambs were fed Deluxe Lamb Milk Replacer (Canadian Nurs-ette Distributor Ltd., Canrose, AB, Canada) and had free access to hay, grain, and water.

Experimental Protocols. Adult sheep. Experiments involved administration of an i.v. bolus of VPA (sodium valproate, Sigma Chemical Co., St. Louis, MO) equivalent to 10, 50, 100, or 250 mg of VPA/kg of body weight (mean ewe body weight = 61.9 ± 7.3 kg) followed by a 5-day washout period, after which the next dose was administered. This continued until each ewe received one of each dose (i.e., a total of four experiments were performed on each animal). Doses were administered over 1 min via the femoral vein in a randomized order. Serial blood samples (~3 ml) were collected for adult sheep from the femoral artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 15, 24, 36, 48, 60, and 72 h following drug administration. For the 10- and 50-mg/kg doses, the experiments continued for only 36 and 48 h, respectively. Cumulative urine samples were also collected for the full duration of the experiment. The only exceptions were for four of the eight lambs in the 10-mg/kg group where urine collection was incomplete due to catheter failure; these were excluded from data analysis.

All doses were prepared in sterile water for injection and were sterilized by filtering through a 0.22-μm nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial. All blood samples collected were placed into heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ) and centrifuged at 2000g for 10 min. The plasma supernatant was removed and placed into clean borosilicate test tubes with polytetrafluoroethylene-lined caps. Plasma and urine samples were stored frozen at −20°C until the time of analysis.

Note: Some of the data from the lamb and adult 10-mg/kg VPA i.v. bolus experiments have been presented in an earlier manuscript (Wong et al., 2000).

Determination of Protein Binding. Unbound plasma concentrations of VPA were determined ex vivo in all adult sheep and postnatal lamb plasma samples by an ultrafiltration procedure at sheep body temperature (39°C). The procedure involved centrifuging at 1000g for 30 min using Centrifree micropartition devices (Amicon Inc., Danvers, MA). Plasma samples for the determination of unbound VPA concentrations were stored in separate aliquots to avoid repetitive thawing that could result in lipolysis and release of free fatty acids and hence competitive displacement of bound VPA from plasma binding sites (Haberer and Pollack, 1994).

Drug and Metabolite Assay. Concentrations of VPA and its metabolites in all biological fluids and plasma ultrafiltrate were measured simultaneously using an established gas chromatographic-mass spectrometric 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 VPA-glucuronide metabolite in both adult lamb and urine were measured using a base hydrolysis procedure described as follows. Urine samples were adjusted to pH 12.5, incubated at 60°C for 1 h, and the total VPA (unconjugated + conjugated) was quantified by the above gas chromatographic-mass spectrometric analytical method. The concentration of the VPA-glucuronide metabolite was estimated as the difference between total and unconjugated (unhydrolyzed) VPA concentrations. This described procedure was preferred over hydrolysis with β-glucuronidase because VPA-glucuronide has been shown to rearrange to at least six β-glucuronidase-resistant structural isomers via migration of the acyl moiety away from the C-1 position and subsequent ring opening, mutarotation, and lactone formation (Dickinson et al., 1984). These rearrangements are pH-, temperature-, and storage time-dependent (Dickinson et al., 1984). Hydrolysis with alkali, however, is capable of measuring total VPA-glucuronide despite these possible rearrangements (Dickinson et al., 1984).

Pharmacokinetic Analyses. Ex vivo protein binding data was analyzed by first calculating the bound VPA concentrations from the difference between the corresponding experimentally determined total and unbound concentrations. Rosenthal plots (bound/unbound concentration versus bound concentration) were constructed for identification of the multiplicity of binding sites. Bound versus unbound concentrations were then fitted using the nonlinear least-squares regression program ADAPT II (D’Argenio and Schumitzky, 1997) to a two-site binding model (a high-affinity saturable and a low-affinity linear site) according to the following equation:

\[
C_u = \frac{B_{max1} \cdot C_u}{K_{d1} \cdot C_u} + \frac{B_{max2} \cdot C_u}{K_{d2} \cdot C_u}
\]

(1)

where \(C_u\) and \(C_b\) are the corresponding bound and unbound concentrations, and \(B_{max1}\) and \(B_{max2}\) are the maximal binding capacities of the first and second binding site, respectively. \(K_{d1}\) and \(K_{d2}\) are the equilibrium dissociation constants of VPA at the first and second binding site, respectively. Adult sheep estimates of plasma protein binding parameters were obtained for individual animals and are presented as mean ± S.D. This was possible since a wide range of plasma VPA concentrations was achieved in each animal following the four dose-ranging experiments. For lambs, only a single estimate.
of the binding parameters could be estimated since each lamb only received one VPA dose, and therefore plasma concentration data from individual animals were insufficient to provide for individual estimates. Thus, VPA plasma concentration data for lambs were pooled together to generate estimates, and consequently plasma protein binding parameters are presented for lambs as an estimate followed by its coefficient of variation (CV) in parentheses. In vivo estimates of apparent Michaelis-Menten parameters ($V_{\text{max}}$, $K_m$) for overall VPA elimination were obtained through simultaneous fitting of unbound concentration-time data from the 50-, 100-, and 250-mg/kg experiments of an individual adult animal (more details as to why only the three higher doses were modeled will be provided under Results). A two-compartment model with Michaelis-Menten elimination provided the best “fit” of the data from all adult animals. Briefly, the first step involved generating microconstant estimates characterizing the movement of drug between the central and peripheral compartments for individual adult animals. This was accomplished by modeling of the unbound concentration-time data from their respective 10-mg/kg experiments to a standard two-compartment model. The resulting microconstant estimates were fixed for subsequent modeling involving simultaneous fitting of unbound concentration-time data from multiple experiments (i.e., 50-, 100-, and 250-mg/kg experiments). Model selection was based upon lower Akaike’s Information Criterion (AIC) and Schwarz Criterion values generated when using a two-compartment model with Michaelis-Menten elimination as opposed to a simpler one-compartment model with similar elimination characteristics (Wagner, 1993; Bourne, 1995). In contrast to adult sheep, individual neonatal lambs received only a single dose of VPA; therefore, individual animal estimates of apparent $V_{\text{max}}$ and $K_m$ could not be obtained. Instead, pooled plasma profiles (i.e., unbound plasma VPA concentrations at each time point were averaged) were constructed for each dose, and the resulting profiles were modeled as described for the adult above. Similar to adult sheep, a two-compartment model with nonlinear elimination provided the best fit for the pooled lamb data. CVs for all estimates of apparent $V_{\text{max}}$ and $K_m$ were <7% and <20%, respectively. Adult estimates for both parameters are presented as a mean ± S.D. The pooled estimate of $V_{\text{max}}$ and $K_m$ obtained for neonatal lambs is presented as the parameter estimate followed by its respective CV in parentheses. As for plasma protein binding, all data was modeled using ADAPT II (D’Argenio and Schumitzky, 1997).

In vivo estimates of apparent $V_{\text{max}}$ and $K_m$ for VPA glucuronidation were determined by constructing plots of urinary excretion rate of the glucuronide metabolite ($V$) versus the unbound VPA concentration at the midpoint of the urine collection interval ($C_{\text{mid}}^u$). Data was pooled from all experiments in adult sheep and in neonatal lambs resulting in one plot for adult sheep and one plot for lambs. Both plots were fit to a standard Michaelis-Menten equation as follows:

$$V = \frac{V_{\text{max}}}{K_m + C_{\text{mid}}^u} \times C_{\text{mid}}^u$$

(2)

where $V$ and $C_{\text{mid}}^u$ are as defined above, $V_{\text{max}}$ is the maximal formation rate of VPA-glucuronide, and $K_m$ is the Michaelis-Menten constant (Gibaldi and Perrier, 1982). Data were fit to eq. 2 using ADAPT II (D’Argenio and Schumitzky, 1997). CVs generated for $V_{\text{max}}$ and $K_m$ were <8% and <20%, respectively. The pooled estimate of apparent $V_{\text{max}}$ and $K_m$ obtained for both adult sheep and neonatal lambs is presented as the parameter estimate followed by its respective CV in parentheses.

Due to the nonlinear/saturable nature of VPA plasma protein binding, the parameters $f_p$ (area weighted unbound fraction of the drug) and $V_{\text{dul}}$ (steady state volume of distribution parameter corrected for the effects of saturable protein binding) were also calculated as follows:

$$f_p = \frac{\text{AUC}_{\text{unbound}}}{\text{AUC}_{\text{total}}}$$

(3)

$$V_{\text{dul}} = f_p \cdot V_{\text{dul}}^p$$

(4)

For drugs exhibiting saturable protein binding, the $V_{\text{dul}}$ when calculated using the “model-independent” approach (Gibaldi and Perrier, 1982) overestimates the “true” $V_{\text{dul}}$ and is concentration-dependent (McNamara et al., 1983). Similarly, $V_{\text{dul}}^p$ is constant only for a particular $f_p$ value and can be used to relate steady-state plasma concentrations to the amount of the drug in the body if steady-state unbound fraction of the drug is equal to $f_p$ (McNamara et al., 1983). Thus, both $V_{\text{dul}}$ and $V_{\text{dul}}^p$ are poor indicators of drug distribution. Instead, the $V_{\text{dul}}$ parameter is more reflective of shifts in drug mass into or out of the vascular space (i.e., information traditionally provided by the $V_d$ parameter) (McNamara et al., 1983). As with $V_{\text{dul}}$ above, this $V_{\text{dul}}$ parameter is also constant only for a particular $f_p$ or a steady-state plasma unbound fraction equivalent to $f_p$. All other pharmacokinetic parameters were calculated by standard methods as described in Gibaldi and Perrier (1982).

**Statistical Analysis.** All data are reported as mean ± S.D. Pharmacokinetic parameters were compared using either a t test for comparison between two groups or an analysis of variance followed by a Fischer’s least significant difference multiple comparison test for multiple group comparisons. The significance level was $p < 0.05$ in all cases.

**Results**

**Dose-Dependent Pharmacokinetics of VPA in Newborn Lambs and Adult Sheep.** Figure 1, A to D show mean semilogarithmic plots of VPA (unbound and total) concentration versus time for adult sheep following i.v. administration of a 10-, 50-, 100-, and 250-mg/kg VPA bolus. Figure 2, A to D show similar semilogarithmic plots of pooled VPA (unbound and total) concentration versus time data from newborn lambs. In a previous study, we observed that plasma protein binding was nonlinear following the administration of a 10-mg/kg i.v. bolus of VPA (Wong et al., 2000). In cases where binding is nonlinear, a parameter that can be used to assess overall changes in unbound fraction is the area weighted unbound fraction ($f_p^u$). Table 1 presents dose-dependent changes in $f_p^u$, $\text{AUC}_{0-\infty}^{u}$, (AUC of unbound VPA), and $V_{\text{dul}}$ for newborn lambs and adult sheep. As expected, $f_p$ increased with increasing dose for both age groups. At the three higher doses, $f_p$ values for 10-day-old lambs were similar to their corresponding adult $f_p$ estimates. This contrasts to what is observed at the lowest dose.

Dose-dependent changes in $\text{AUC}_{0-\infty}^{u}$ were examined as opposed to $\text{AUC}_{0-\infty}^{u}$ (AUC of total drug) since changes in $\text{AUC}_{0-\infty}^{u}$ are independent of changes in plasma protein binding. Increases in dose were associated with expected increases in $\text{AUC}_{0-\infty}^{u}$ for both age groups (Table 1). $\text{AUC}_{0-\infty}^{u}$ was significantly higher for 10-day-old lambs at the two lower doses; however, for the two higher doses the adult and lamb estimates were similar. For adult sheep, the increase in $\text{AUC}_{0-\infty}^{u}$ with increasing dose is nonlinear, suggestive of Michaelis-Menten elimination. In newborn lambs, $\text{AUC}_{0-\infty}^{u}$ increased linearly with dose until the 250-mg/kg dose, where its increase was nonlinear. Additional evidence of nonlinear elimination can be observed in the unbound VPA plasma profiles in Figs. 1 and 2 that displayed an increasing convex curvature with increasing dose. Although the plasma profiles for total VPA concentrations also showed signs of convexity, this could be a result of saturable protein binding rather than nonlinear elimination.

For drugs exhibiting saturable protein binding, the volume terms $V_d$ and $V_d^p$ do not provide any information on possible shifts of drug into or out of the vascular space. However, this information is provided by the $V_{\text{dul}}$; therefore, this term has been presented in Table 1 to examine changes in drug distribution with dose. The $V_{\text{dul}}$ for both age groups increased with an increase in dose, suggesting movement of drug out of the vascular space with increasing doses. In addition, for the three higher doses, $V_{\text{dul}}$ was significantly larger in lambs in comparison with adult sheep.

Figure 3A depicts changes in VPA total body clearance ($\text{CL}_{\text{tot}}$) for dose for newborn lambs and adult sheep. In both lambs and adult sheep, $\text{CL}_{\text{tot}}$ increases with dose to a maximum at the 100-mg/kg dose before decreasing at the highest dose. Figure 3B illustrates changes in VPA unbound clearance ($\text{CL}_{\text{un}}^{u}$). For adult sheep, the $\text{CL}_{\text{un}}$ decreases significantly with increasing dose, consistent with metabolic satura-
tion (Table 2). For neonatal lambs, CL\text{tu} decreases only following administration of the highest dose (Table 2). Adult CL\text{tu} is significantly higher than CL\text{tu} for newborn lambs at the two lower doses. However, at the two higher doses, CL\text{tu} is similar between the two age groups (Fig. 3B).

Ex Vivo Determination of Plasma Protein Binding Parameters. Rosenthal plots for a representative adult sheep and pooled lamb data are presented in Fig. 4, A and B. Plots for both adult sheep and newborn lambs were biphasic in nature. The initial steep declining portion of the biphasic Rosenthal plots suggests the presence of a high-affinity saturable binding site, whereas the relatively flat portion of the plots suggests the presence of a low-affinity linear (nonsaturable) site. Fitting the bound versus unbound concentration data to a two-site binding model with a saturable and a nonsaturable binding site (eq. 1) resulted in statistically better fits (lower AIC and Schwarz Criterion, smaller CV values for fitted parameters) when compared with a one-site binding model.

A scatter plot of bound versus unbound VPA plasma concentration data for an individual adult sheep (i.e., plasma data is pooled from the 10-, 50-, 100-, and 250-mg/kg experiments from a single animal) is presented in Fig. 4C. A similar plot was constructed for plasma data from all the newborn lamb experiments and is presented in Fig. 4D. The model-predicted lines based upon fitting to eq. 1 are also depicted, indicating a good fit of the data to the two-site binding model. Estimates of binding parameters for adult sheep and newborn lambs are presented in Table 3. From the data, it appears that binding capacity at the saturable binding site (\(B_{\text{max1}}\)) is higher in adult sheep. In contrast, the higher \(K_{d1}\) estimate in adult sheep suggests that binding affinity at the saturable site is lower when compared with lambs.

Total Protein Concentrations in Adult Sheep and Lamb Plasma. The total protein concentration in plasma from adult sheep (\(n = 5\)) and 10-day-old lambs (\(n = 18\)) was 73.8 ± 6.4 and 58.0 ± 4.6 mg/ml, respectively. Three lambs were excluded from total protein determination due to the lack of availability of remaining plasma following drug and metabolite analysis. Total protein concentration was significantly higher in adult sheep in comparison to 10-day-old lambs (unpaired t test, \(p < 0.05\)).

Dose-Dependent Changes in Urinary Excretion of VPA and Its Metabolites. Tables 4 and 5 present dose-dependent changes in the percentage of the total VPA dose recovered in urine as unchanged VPA and its metabolites in adult sheep and newborn lambs. Aside from the group of lambs receiving the 10-mg/kg dose, almost the entire dose (>90%) was recovered as either unchanged VPA or one of its metabolites (Tables 4 and 5). The majority of the dose in both age groups and at all doses was recovered as VPA-glucuronide. In adult sheep, 68.3 to 77.9% of the dose appeared in urine as the glucuronide metabolite, and there was no significant change in the recovery of this metabolite with dose (Table 4). In neonatal lambs, recovery of VPA-glucuronide in urine increased significantly from 29.2% at the lowest dose to 65.7 to 69.6% at the higher doses (Table 5). Recovery of the administered dose in urine as the unchanged drug appeared to follow no pattern with increasing dose. In adults, recovery of VPA in urine was significantly less at the 50- and 100-mg/kg doses than at the 10-mg/kg dose (Table 4). No such changes occurred with increasing dose in neonatal lambs (Table 5).

The major \(\beta\)-oxidation metabolite recovered in urine in both lambs and adult sheep was 3-keto VPA. For both age groups, the recovery of this metabolite in urine decreased significantly with increasing dose, suggestive of metabolic saturation of the \(\beta\)-oxidation pathway (Tables
4 and 5). At the 50- and 100-mg/kg doses, the percentage of the dose recovered as this metabolite in neonatal lambs was significantly higher than observed in adult sheep. The renal excretion of the other β-oxidation metabolites, (E)-2-ene and (E)-3-ene VPA, were limited in both adult sheep and lambs accounting for no more than 0.08% of the dose. Similar to 3-keto VPA, the recovery of 3-OH VPA decreased with increasing dose in neonates (Table 5). In adults, the mass balance of this metabolite appeared to follow no trend (Table 4).

The prominent metabolite formed by cytochrome P-450 pathways that was recovered in urine in both age groups appeared to be 4-OH VPA. In both newborn lambs and adult sheep, the urinary recovery of 4-OH VPA significantly increased with increasing dose, going as high as ∼11% of the dose in lambs (Table 5) and ∼7% of the dose in adults (Table 4). Similarly, in both age groups, 2-PGA increased significantly with increasing dose. All other P-450 metabolites (i.e., 5-OH, 4-ene and 4-keto VPA, and 2-PSA) accounted for less than 1% of the VPA dose at all dosing levels. Similar to (E)-2-ene and (E)-3-ene VPA, the recovery of 4-ene VPA in urine appeared to be particularly low, accounting for no more than ∼0.05% of the dose in any of the adult sheep or lamb groups.

**In Vivo Estimation of \(V_{\text{max}}\) and \(K_{\text{m}}\) of Overall VPA Elimination.** Unbound concentration versus time profiles from the 50-, 100-, and 250-mg/kg bolus experiments were modeled simultaneously to obtain in vivo estimates of apparent \(V_{\text{max}}\) and \(K_{\text{m}}\) values of overall VPA elimination. The 10-mg/kg experiments from both age groups were excluded from this modeling exercise due to our inability to recover the majority of the 10-mg/kg VPA dose administered to neonatal lambs (i.e., only ∼50% could be recovered). For the three larger doses we were able to recover the majority of the dose as known VPA metabolites derived from hepatic metabolism (i.e.,

![Fig. 2. Mean VPA [total (●) and unbound (○)] plasma concentration versus time profiles for newborn lambs (10 days old) following i.v. bolus administration of a 10- (A; n = 8), 50- (B; n = 5), 100- (C; n = 4), and 250-mg/kg (D; n = 4) dose of VPA.](image)

**Table 1.** Dose-dependent changes in \(f_u\), \(AUC_{0-\infty}^{ug}\), and \(V_{dss}\) in adult sheep and newborn lambs

<table>
<thead>
<tr>
<th>VPA Dose</th>
<th>(f_u)</th>
<th>(AUC_{0-\infty}^{ug}) mg - min/ml</th>
<th>(V_{dss}) l/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (n = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.09 ± 0.02*</td>
<td>1.4 ± 0.4*</td>
<td>0.13 ± 0.02*</td>
</tr>
<tr>
<td>50</td>
<td>0.27 ± 0.03*</td>
<td>12.1 ± 1.9*</td>
<td>0.13 ± 0.02*</td>
</tr>
<tr>
<td>100</td>
<td>0.41 ± 0.10*</td>
<td>32.8 ± 6.3*</td>
<td>0.13 ± 0.02*</td>
</tr>
<tr>
<td>250</td>
<td>0.61 ± 0.04*</td>
<td>191.1 ± 43.8*</td>
<td>0.18 ± 0.01*</td>
</tr>
<tr>
<td>Newborn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (n = 8)</td>
<td>0.21 ± 0.12**</td>
<td>4.5 ± 1.8**</td>
<td>0.14 ± 0.06*</td>
</tr>
<tr>
<td>50 (n = 5)</td>
<td>0.29 ± 0.03*</td>
<td>16.3 ± 1.5**</td>
<td>0.19 ± 0.02**</td>
</tr>
<tr>
<td>100 (n = 4)</td>
<td>0.44 ± 0.05*</td>
<td>34.9 ± 3.8*</td>
<td>0.28 ± 0.06**</td>
</tr>
<tr>
<td>250 (n = 4)</td>
<td>0.56 ± 0.05*</td>
<td>154.7 ± 32.4*</td>
<td>0.27 ± 0.004**</td>
</tr>
</tbody>
</table>

* Significant difference from the corresponding adult value as determined by t test (\(p < 0.05\)).

\(\alpha, \beta, \gamma, \delta\) Different groups as determined by Fischer’s LSD Multiple Comparison Test (\(p < 0.05\)).

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4 and 5). At the 50- and 100-mg/kg doses, the percentage of the dose recovered as this metabolite in neonatal lambs was significantly higher than observed in adult sheep. The renal excretion of the other β-oxidation metabolites, (E)-2-ene and (E)-3-ene VPA, were limited in both adult sheep and lambs accounting for no more than 0.08% of the dose. Similar to 3-keto VPA, the recovery of 3-OH VPA decreased with increasing dose in neonates (Table 5). In adults, the mass balance of this metabolite appeared to follow no trend (Table 4).

The prominent metabolite formed by cytochrome P-450 pathways that was recovered in urine in both age groups appeared to be 4-OH VPA. In both newborn lambs and adult sheep, the urinary recovery of 4-OH VPA significantly increased with increasing dose, going as high as ∼11% of the dose in lambs (Table 5) and ∼7% of the dose in adults (Table 4). Similarly, in both age groups, 2-PGA increased significantly with increasing dose. All other P-450 metabolites (i.e., 5-OH, 4-ene and 4-keto VPA, and 2-PSA) accounted for less than 1% of the VPA dose at all dosing levels. Similar to (E)-2-ene and (E)-3-ene VPA, the recovery of 4-ene VPA in urine appeared to be particularly low, accounting for no more than ∼0.05% of the dose in any of the adult sheep or lamb groups.

**In Vivo Estimation of \(V_{\text{max}}\) and \(K_{\text{m}}\) of Overall VPA Elimination.** Unbound concentration versus time profiles from the 50-, 100-, and 250-mg/kg bolus experiments were modeled simultaneously to obtain in vivo estimates of apparent \(V_{\text{max}}\) and \(K_{\text{m}}\) values of overall VPA elimination. The 10-mg/kg experiments from both age groups were excluded from this modeling exercise due to our inability to recover the majority of the 10-mg/kg VPA dose administered to neonatal lambs (i.e., only ∼50% could be recovered). For the three larger doses we were able to recover the majority of the dose as known VPA metabolites derived from hepatic metabolism (i.e.,
creatinine exhibit plasma profiles that decline in parallel to the plasma profile of parent compound (Houston, 1986). Thus, apparent plasma half-lives determined from the terminal slopes should be similar for both the parent compound and its metabolite. Due to the lack of plasma following drug (unbound and total) and metabolite analysis, we could not determine VPA-glucuronide levels in plasma. As an alternative, a plot of urinary excretion rate versus t_{mid} (i.e., time at the midpoint of the urine collection interval) was constructed (Gibaldi and Perrier, 1982). The apparent plasma half-life of the metabolite can be obtained from the terminal slopes of the semilogarithmic form of these plots (Gibaldi and Perrier, 1982). Since elimination rate-limited urinary excretion of the metabolite is likely to occur at the highest dose of VPA, we constructed semilogarithmic plots of excretion rate versus t_{mid} for the 250-mg/kg experiments (plots not shown). VPA-glucuronide half-lives of 4.70 ± 0.13 h (adult sheep) and 3.88 ± 0.62 h (10-day-old lambs) obtained from the terminal slopes of these plots were not significantly different from unbound VPA half-lives determined from plasma data (adult, 4.86 ± 2.50 h; 10-day-old lamb, 3.28 ± 0.34 h) (unpaired t test, p < 0.05). Thus, our assumption of formation rate-limited urinary excretion of the glucuronide metabolite appears to be reasonable.

Plots of V versus C_{\text{mid}} for adult sheep and neonatal lambs were fitted to eq. 2 and are presented in Fig. 6. The resulting estimates of V_{\text{max}} and K_{m} values of VPA-glucuronidation are presented in Table 7. As with V_{\text{max}} estimates of overall VPA elimination, the V_{\text{max}} estimates of VPA-glucuronidation were similar between the two age groups. However, K_{m} estimates appeared to be higher for neonatal lambs. In fact, K_{m} estimates of VPA glucuronidation for both adult and neonatal sheep (Table 7) are very similar to K_{m} estimates of overall VPA elimination (Table 6).

**Discussion**

Dose-Dependent Pharmacokinetics in Adult Sheep and Neonatal Lambs. The pharmacokinetics of VPA are unique, exhibiting both saturable/nonlinear plasma protein binding and saturable/capacity-limited metabolism at clinically relevant plasma concentrations. These characteristics are largely due to the high therapeutic doses of VPA in comparison with other drugs. Since VPA exhibits a low hepatic extraction and is mainly eliminated via hepatic metabolism (Levy and

**TABLE 2**

Changes in total and unbound VPA clearance in newborn lamb (10 days old) and adult sheep with increasing dose

<table>
<thead>
<tr>
<th>VPA Dose</th>
<th>CL_{t,tb} (ml/min/kg)</th>
<th>CL_{u,tb} (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.70 ± 0.31</td>
<td>7.74 ± 2.63</td>
</tr>
<tr>
<td>50</td>
<td>1.15 ± 0.29*</td>
<td>4.24 ± 0.82*</td>
</tr>
<tr>
<td>100</td>
<td>1.25 ± 0.22**</td>
<td>3.15 ± 0.63*</td>
</tr>
<tr>
<td>250</td>
<td>0.83 ± 0.13</td>
<td>1.37 ± 0.29*</td>
</tr>
<tr>
<td>Newborn (10 days old) (n = 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.55 ± 0.38</td>
<td>2.65 ± 1.16</td>
</tr>
<tr>
<td>50</td>
<td>0.94 ± 0.17**</td>
<td>3.21 ± 0.29</td>
</tr>
<tr>
<td>100 (n = 4)</td>
<td>1.31 ± 0.08**</td>
<td>3.42 ± 0.59</td>
</tr>
<tr>
<td>250 (n = 4)</td>
<td>0.81 ± 0.28</td>
<td>1.52 ± 0.12*</td>
</tr>
</tbody>
</table>

* Significantly lower than the 10-mg/kg group (p < 0.05).
** Significantly higher than the 10-mg/kg group (p < 0.05).

>80% of the administered dose) in both age groups. Thus, the apparent V_{\text{max}} and K_{m} values estimated from the modeling of these doses are hybrid constants largely reflective of overall metabolic elimination. The unbound concentration versus time data from both age groups were modeled using a two-compartment model with Michaelis-Menten elimination based upon a better fit (lower AIC and Schwarz Criterion, smaller CVs for fitted parameters) with this model when compared with fitting to a one-compartment model with nonlinear elimination. Unbound concentration versus time profiles from adult sheep and neonatal lambs are presented in Fig. 5 along with their model-predicted plasma profile lines. Adult animals were individually modeled; however, for lambs the mean unbound plasma concentration versus time profiles at each dose were modeled. Estimates of apparent V_{\text{max}} and K_{m} values obtained from modeling are presented in Table 6. From the estimates, it appears that V_{\text{max}} is similar between the two age groups. However, K_{m} appears to be higher for lambs.

In Vivo Estimation of Apparent V_{\text{max}} and K_{m} of VPA Glucuronidation. A condition for estimating the apparent V_{\text{max}} and K_{m} values of VPA glucuronidation using urine data is that the appearance of VPA-glucuronide in urine is formation rate-limited as opposed to elimination rate-limited (Gibaldi and Perrier, 1982). In previous investigations with VPA using pregnant sheep, the glucuronide metabolite did not appear to accumulate substantially in plasma consistent with formation rate-limited urinary excretion (S. Kumar, unpublished results). Metabolites demonstrating formation rate-limited urinary excretion exhibit plasma profiles that decline in parallel to the plasma profile of parent compound (Houston, 1986). Thus, apparent plasma half-lives determined from the terminal slopes should be similar for both the parent compound and its metabolite. Due to the lack of plasma following drug (unbound and total) and metabolite analysis, we could not determine VPA-glucuronide levels in plasma. As an alternative, a plot of urinary excretion rate versus t_{mid} (i.e., time at the midpoint of the urine collection interval) was constructed (Gibaldi and Perrier, 1982). The apparent plasma half-life of the metabolite can be obtained from the terminal slopes of the semilogarithmic form of these plots (Gibaldi and Perrier, 1982). Since elimination rate-limited urinary excretion of the metabolite is likely to occur at the highest dose of VPA, we constructed semilogarithmic plots of excretion rate versus t_{mid} for the 250-mg/kg experiments (plots not shown). VPA-glucuronide half-lives of 4.70 ± 0.13 h (adult sheep) and 3.88 ± 0.62 h (10-day-old lambs) obtained from the terminal slopes of these plots were not significantly different from unbound VPA half-lives determined from plasma data (adult, 4.86 ± 2.50 h; 10-day-old lamb, 3.28 ± 0.34 h) (unpaired t test, p < 0.05). Thus, our assumption of formation rate-limited urinary excretion of the glucuronide metabolite appears to be reasonable.

Plots of V versus C_{\text{mid}} for adult sheep and neonatal lambs were fitted to eq. 2 and are presented in Fig. 6. The resulting estimates of V_{\text{max}} and K_{m} values of VPA-glucuronidation are presented in Table 7. As with V_{\text{max}} estimates of overall VPA elimination, the V_{\text{max}} estimates of VPA-glucuronidation were similar between the two age groups. However, K_{m} estimates appeared to be higher for neonatal lambs. In fact, K_{m} estimates of VPA glucuronidation for both adult and neonatal sheep (Table 7) are very similar to K_{m} estimates of overall VPA elimination (Table 6).
Shen, 1995), according to the well stirred model (Wilkinson and Shand, 1975) the CL<sub>tb</sub> of VPA can be described as

\[ CL_{tb} = f_u \cdot CL_{int} \quad (5) \]

where \( f_u \) is the unbound fraction and \( CL_{int} \) is the hepatic intrinsic clearance. Thus, saturation of plasma protein binding and metabolism influence VPA CL<sub>tb</sub> in opposite directions. Specifically, saturation of plasma protein binding increases drug free fraction, resulting in an increase in CL<sub>tb</sub>. In contrast, metabolic saturation acts to decrease CL<sub>tb</sub> by decreasing intrinsic clearance. Significant alterations in plasma protein binding occurred in our dose-ranging experiments as evidenced by the observed changes in \( f_p \) (a measure of overall drug free fraction) (Table 1). In addition, the nonlinear increases in AUC<sub>0→∞</sub> in both adult sheep and neonatal lambs, especially at the highest dose, provide evidence of metabolic saturation. Thus, dose-dependent alterations in VPA CL<sub>tb</sub> will be the result of relative changes in plasma protein binding and metabolism that occur with increasing dose.

For both adult sheep and neonatal lambs, we observed significant increases in VPA CL<sub>tb</sub> up to the 100-mg/kg dose (Table 2). At the highest dose (250-mg/kg), CL<sub>tb</sub> for both age groups fell to a level similar to that observed at the 10-mg/kg dose (Table 2). The observed increases in CL<sub>tb</sub> are a result of the observed increases in overall drug free fraction (i.e., \( f_p \)) with increasing dose (Table 1). The influence of plasma protein binding increases drug free fraction, resulting in an increase in CL<sub>tb</sub>. In contrast, metabolic saturation acts to decrease CL<sub>tb</sub> by decreasing intrinsic clearance. Significant alterations in plasma protein binding occurred in our dose-ranging experiments as evidenced by the observed changes in \( f_p \) (a measure of overall drug free fraction) (Table 1). In addition, the nonlinear increases in AUC<sub>0→∞</sub> in both adult sheep and neonatal lambs, especially at the highest dose, provide evidence of metabolic saturation. Thus, dose-dependent alterations in VPA CL<sub>tb</sub> will be the result of relative changes in plasma protein binding and metabolism that occur with increasing dose.

For both adult sheep and neonatal lambs, we observed significant increases in VPA CL<sub>tb</sub> up to the 100-mg/kg dose (Table 2). At the highest dose (250-mg/kg), CL<sub>tb</sub> for both age groups fell to a level similar to that observed at the 10-mg/kg dose (Table 2). The observed increases in CL<sub>tb</sub> are a result of the observed increases in overall drug free fraction (i.e., \( f_p \)) with increasing dose (Table 1). The influence of

\[
\begin{array}{cccc}
\text{Parameter} & \text{Adult}^{a} (n = 5) & \text{10-day-old}^{b} (n = 21) \\
B_{max 1} (\mu g/ml) & 91.8 \pm 24.3 & 44.9 \pm 15.0 \\
K_d 1 (\mu g/ml) & 9.6 \pm 5.9 & 3.2 \pm 8.3 \\
\frac{B_{max 1}}{K_d} & 0.23 \pm 0.06 & 0.33 \pm 0.69 \\
\end{array}
\]

\[a\] Adult estimates presented as mean \pm S.D.

\[b\] Lamb estimates presented as the estimate followed by the CV in parentheses.

FIG. 4. Rosenthal plot (A) and plots of the relationship between bound drug (\( C_b \)) versus unbound drug (\( C_u \)) in plasma (C) for a representative adult sheep (E6208) and 10-day-old lambs (B and D).

A and C are generated from plasma data pooled from all experiments (10, 50, 100, and 250 mg/kg) performed in E6208. B and D are similar plots generated from plasma data pooled from all 10-day-old lamb experiments (\( n = 21 \)). C and D, a model-predicted line obtained from a fit of the data to a two-site binding model is depicted.
metabolic saturation on VPA $\text{CL}_{\text{tb}}$ is only evident at the 250-mg/kg dose for both age groups. This is also the dose at which we observed the most substantial nonlinear increases in $\text{AUC}_{0-\infty}^{\text{VPA}}$ (i.e., a 2.5-fold increase in dose resulted in an ~6-fold and an ~4.5-fold increase in $\text{AUC}_{0-\infty}^{\text{VPA}}$ in adult sheep and neonatal lambs, respectively). In previous studies examining the dose-dependent pharmacokinetics of VPA in adult guinea pigs (Yu et al., 1987, 1993), rats (Liu et al., 1990; Liu and Pollack, 1993), and humans (Bowdle et al., 1980; Anderson et al., 1992; Gómez Bellver et al., 1993), the observed decreases in $\text{CL}_{\text{tb}}$ in these studies are indicative of metabolic saturation as the unbound clearance of VPA approximates hepatic intrinsic clearance (see eq. 5). Surprisingly, $\text{CL}_{\text{tb}}$ in neonatal lambs did not decrease significantly until the highest dose (Table 2). In contrast, a decrease in $\text{CL}_{\text{tb}}$ was observed with increasing dose in both 3-day-old and 21-day-old guinea pigs (Yu et al., 1987). However, in these experiments two of the three doses administered (i.e., 20, 200, and 600 mg/kg) were either similar or substantially larger than the highest dose (i.e., 250 mg/kg) used in our studies. The use of similar doses in lambs would likely result in a similar trend in $\text{CL}_{\text{tb}}$.

As expected, $\text{Vd}_{\text{ss}}$ increased with increasing dose in both adult sheep and neonatal lambs (Table 1). The modest increase in $\text{Vd}_{\text{ss}}$ is consistent with the VPA’s low tissue binding (Davis et al., 1994). Interestingly, the $\text{Vd}_{\text{ss}}$ in lambs is significantly larger than in adults at the 50-, 100-, and 250-mg/kg doses. The larger $\text{Vd}_{\text{ss}}$ observed in lambs may be related to the larger total body water content in the young (Moreselli et al., 1980). A similar phenomenon is observed in human neonates who exhibit higher volumes of distribution (i.e.,

### Table 4

<table>
<thead>
<tr>
<th>VPA or Metabolites</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
<th>250 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>VPA</td>
<td>12.2 ± 2.2</td>
<td>7.6 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3 ± 3.3</td>
</tr>
<tr>
<td>VPA-glu</td>
<td>73.8 ± 5.3</td>
<td>77.9 ± 5.9</td>
<td>78.7 ± 5.6</td>
<td>68.3 ± 6.5</td>
</tr>
<tr>
<td>(E)-2-ene</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>(E)-3-ene</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>0.012 ± 0.002</td>
<td>0.029 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-ene</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>2.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-keto</td>
<td>11.3 ± 6.5</td>
<td>3.5 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.07</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>4-keto</td>
<td>0.28 ± 0.08</td>
<td>0.25 ± 0.04</td>
<td>0.32 ± 0.16</td>
<td>0.54 ± 0.34</td>
</tr>
<tr>
<td>3-OH</td>
<td>0.53 ± 0.23</td>
<td>0.21 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.5 ± 2.6</td>
<td>7.1 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-OH</td>
<td>2.0 ± 0.6</td>
<td>3.7 ± 1.5</td>
<td>0.44 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51 ± 0.17</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.76 ± 0.30</td>
<td>0.52 ± 0.24</td>
<td>0.07 ± 0.01</td>
<td>0.14 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.14 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.6 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.5</td>
<td>1.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>101.5 ± 8.5</td>
<td>94.5 ± 6.3</td>
<td>95.0 ± 6.7</td>
<td>91.7 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly lower than the 10-mg/kg value ($p < 0.05$).
<sup>b</sup> Significantly lower than the 100-mg/kg value ($p < 0.05$).
<sup>c</sup> Significantly higher than the 10-mg/kg value ($p < 0.05$).

### Table 5

<table>
<thead>
<tr>
<th>VPA or Metabolites</th>
<th>10 mg/kg</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
<th>250 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>5.6 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6 ± 2.8</td>
<td>6.4 ± 1.9</td>
<td>7.0 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VPA-glu</td>
<td>29.2 ± 16.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.7 ± 8.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.6 ± 11.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.6 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(E)-2-ene</td>
<td>0.071 ± 0.076</td>
<td>0.019 ± 0.012</td>
<td>0.011 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(E)-3-ene</td>
<td>0.01 ± 0.02</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>4-ene</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>0.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-keto</td>
<td>12.6 ± 9.8</td>
<td>11.9 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-keto</td>
<td>0.04 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-OH</td>
<td>0.34 ± 0.05</td>
<td>0.26 ± 0.11</td>
<td>0.20 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-OH</td>
<td>0.6 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 1.6</td>
<td>8.6 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3 ± 5.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-OH</td>
<td>0.17 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56 ± 0.55</td>
<td>0.59 ± 0.38</td>
<td>0.65 ± 0.32</td>
</tr>
<tr>
<td>2-PSA</td>
<td>0.07 ± 0.05</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.04</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>2-PGA</td>
<td>0.46 ± 0.33</td>
<td>0.89 ± 0.50</td>
<td>0.73 ± 0.32</td>
<td>1.19 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>49.2 ± 25.7</td>
<td>92.3 ± 7.3</td>
<td>91.9 ± 7.1</td>
<td>91.0 ± 6.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly lower than the 10-mg/kg value ($p < 0.05$).
<sup>b</sup> Significantly lower than the 100-mg/kg value ($p < 0.05$).
<sup>c</sup> Significantly higher than the 10-mg/kg value ($p < 0.05$).
<sup>d</sup> Significantly lower than the 10-mg/kg value ($p < 0.05$).
saturate plasma protein binding to such an extent that we would have a wide enough range of bound ($C_b$) and unbound ($C_u$) drug concentrations required for appropriate characterization of plasma protein binding parameters. Examination of our Rosenthal (Fig. 4, A–B) and our $C_b$ versus $C_u$ plots (Fig. 4, C–D) provide obvious evidence that a two-site binding model is required to describe VPA plasma protein binding. This was not surprising since a similar model was used previously to characterize VPA plasma protein binding in pregnant sheep (Kumar et al., 2000b). The presence of a high-affinity saturable binding site and a low-affinity nonsaturable site has also been demonstrated in rats (Semmes and Shen, 1990; Haberer and Pollack, 1994; Slattum et al., 1996), guinea pigs (Yu and Shen, 1992), and humans (Riva et al., 1984; Scheyer et al., 1990). Our estimates of binding parameters for adult sheep (Table 3) appear to be reasonably similar to estimates obtained from humans (i.e., $B_{max1}$; $169 \text{ mg/ml}$ and $K_{d1}$; $6 –13 \text{ mg/ml}$; Riva et al., 1984; Scheyer et al., 1990). The $K_{d1}$ estimate obtained for adult sheep is consistent with observable saturation of VPA plasma protein binding at therapeutic concentrations (i.e., 50 –100 $\mu$g/ml).

A comparison of VPA binding parameters obtained from previous experiments in pregnant sheep and current estimates in nonpregnant animals. Kumar et al. (2000b) estimated a $B_{max1}$ of 62.8 $\mu$g/ml in pregnant sheep as opposed to $B_{max1}$ estimates of 91.8 ± 24.3 $\mu$g/ml in nonpregnant animals. A reduction of albumin (the primary protein involved in VPA plasma protein binding) concentrations that occurs with pregnancy has been suggested as one of mechanisms involved in reduced VPA binding in pregnant women (Nau et al., 1984; Riva et al., 1984; Nau and Krauer, 1986). A similar phenomenon may occur in pregnant sheep and result

![Fig. 5. Unbound VPA plasma concentration versus time profiles from a representative adult sheep (E6208) (A, B, and C) and mean unbound VPA plasma concentration versus time profiles from 10-day-old lambs (n = 8; D, E, and F) receiving a 50-mg/kg bolus (n = 5; A and D), a 100-mg/kg bolus (n = 4; B and E), and a 250-mg/kg bolus (n = 4; C and F). In all cases, a model-predicted line obtained from fit of the data to a two-compartment model with Michaelis-Menten elimination is depicted.](image-url)

### TABLE 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adult$^a$ (n = 5)</th>
<th>10-day-old$^b$ (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ ($\mu$g/min/kg)</td>
<td>445.0 ± 91.2</td>
<td>429.9 (2)</td>
</tr>
<tr>
<td>$K_m$ ($\mu$g/ml)</td>
<td>30.0 ± 12.6</td>
<td>69.6 (4)</td>
</tr>
</tbody>
</table>

$^a$ Adult estimates presented as mean ± S.D.

$^b$ Lamb estimates presented as the estimate followed by the CV in parentheses.

0.28–0.43 l/kg) in comparison with adults (i.e., 0.13–0.20 l/kg) (Levy and Shen, 1995).

### Age-Related Differences in VPA Plasma Protein Binding

Plasma protein binding exhibits nonlinear characteristics in adult and neonatal lambs following the administration of a clinical dose (i.e., 10 mg/kg) of VPA (Wong et al., 2000). One of our goals of the dose-ranging study was to saturate plasma protein binding to such an extent that we would have a wide enough range of bound ($C_b$) and unbound ($C_u$) drug concentrations required for appropriate characterization of plasma protein binding parameters. Examination of our Rosenthal (Fig. 4, A–B) and our $C_b$ versus $C_u$ plots (Fig. 4, C–D) provide obvious evidence that a two-site binding model is required to describe VPA plasma protein binding. This was not surprising since a similar

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**Fig. 5.** Unbound VPA plasma concentration versus time profiles from a representative adult sheep (E6208) (A, B, and C) and mean unbound VPA plasma concentration versus time profiles from 10-day-old lambs (n = 8; D, E, and F) receiving a 50-mg/kg bolus (n = 5; A and D), a 100-mg/kg bolus (n = 4; B and E), and a 250-mg/kg bolus (n = 4; C and F). In all cases, a model-predicted line obtained from fit of the data to a two-compartment model with Michaelis-Menten elimination is depicted.
in pregnant and nonpregnant sheep (7.6 and 9.6 m
gm). The two-fold higher than the corresponding estimate in 10-day-old lambs
(1989). Since from their binding sites (Kearns and Reed, 1989; Reed and Besunder,
and free fatty acids, which can act to displace albumin-bound drugs
adults (Kearns and Reed, 1989; Reed and Besunder, 1989). Further-
protein binding for adult sheep and neonatal lambs were similar
in the lower observed $B_{max1}$ estimate. $K_{d1}$ estimates were similar in
pregnant and nonpregnant sheep (7.6 and 9.6 µg/ml, respectively).
Estimates of the linear component (i.e., $B_{max1}/K_{d2}$) of VPA plasma
protein binding for adult sheep and neonatal lambs were similar
(Table 3). However, both the binding capacity ($B_{max1}$) and affinity
($K_{d1}$) of the high-affinity saturable binding site of the two age groups
appeared to be different. $B_{max1}$ estimates from adult sheep were
two-fold higher than the corresponding estimate in 10-day-old lambs
(44.9 µg/ml). The lower binding capacity may play a role in the
higher $f_p$ previously observed in 10-day-old lambs when compared
with adult sheep (Wong et al., 2000). Similar apparent reductions in
plasma protein binding have been observed in neonates for several
anticonvulsants (including VPA), antibiotics, and analgesics (Kearns
and Reed, 1989; Reed and Besunder, 1989; Levy and Shen, 1995).
Causes for differences in binding include lower plasma levels of total
protein, albumin, and α1-glycoprotein in neonates compared with
adults (Kearns and Reed, 1989; Reed and Besunder, 1989). Further-
more, neonates exhibit an increased level of unconjugated bilirubin
and free fatty acids, which can act to displace albumin-bound drugs
from their binding sites (Kearns and Reed, 1989; Reed and Besunder,
1989). Since $B_{max1}$ is a measure of binding capacity, the presence of
binding inhibitors (i.e., unconjugated bilirubin and free fatty acids)
would have no effect on this parameter. Thus, it is likely that the lower
$B_{max1}$ observed in neonatal lambs is a result of lower total protein and
albumin concentrations. Our measurements of total protein in adult
and neonatal sheep plasma support this explanation, since we ob-
served significantly lower concentrations of total protein in neonatal
plasma in comparison with the adult. Similar increases in binding
capacity of VPA with age were observed in developmental studies in
rats (Haberer and Pollack, 1994) and guinea pigs (Yu et al., 1985).
Surprisingly, the binding affinity of VPA appeared to be higher in
neonatal lambs (Table 3). As mentioned, in the young there is gen-
erally a higher concentration of substances such as unconjugated
bilirubin and free fatty acids, which can act as inhibitors of VPA
binding (Kearns and Reed, 1989; Reed and Besunder, 1989). The
presence of such inhibitors influences binding by decreasing binding
affinity (i.e., increase in $K_{d1}$). In addition, the lingering presence of
fetal albumin in neonates usually results in a decrease in binding
affinity (Moreselli, 1976). Consistent with the expected situation,
binding affinity increases with age in rats (Haberer and Pollack,
1994). An explanation for the unexpected results observed in sheep
may be the presence of a rumen in these animals. In newborn rumin-
ants, blood glucose and fatty acid concentrations are similar to
monogastric animals. As the animal ages and the rumen develops,
glucose levels in the blood fall to half of what is observed in nonrumin-
ants. In contrast, volatile fatty acid concentration in blood in-
creases substantially due to their production in the rumen and subse-
quent absorption (Annison and Lewis, 1959). Higher concentrations
of fatty acids in adult sheep plasma may act as binding inhibitors,
resulting in the observed reduction in VPA binding affinity (i.e.,
increase in $K_{d1}$) in adult sheep.

**Dose-Dependent Changes in VPA Metabolism.** The interpreta-
tion of VPA metabolite mass balance data from the dose-dependent
studies is complex, as the contributions to overall elimination of the
different metabolic pathways are not entirely independent of each
other. At high concentrations, saturation of primary pathways of
elimination results in the “shunting” of the administered dose to
normally minor routes of elimination. Our mass balance data are
consistent with metabolic saturation of β-oxidation. For both age
groups, we observed an overall decrease in the percentage of the
administered dose recovered as β-oxidation metabolites [i.e., (E)-2-
enone, (E)-3-ene, 3-OH, and 3-keto VPA], with 3-keto VPA being
the primary metabolite at all doses (Tables 4 and 5). A similar phenom-
енon has been previously observed in dose-ranging studies in humans
(Granneman et al., 1984; Dickinson et al., 1989). In contrast, the
contribution of ω-oxidation (5-OH VPA and 2-PGA) and ω-1-oxida-
tion (4-OH and 4-keto VPA, and 2-PSA) to VPA elimination in-
creased with increasing dose (Tables 4 and 5). Of the metabolites
derived from P-450 metabolism, the increase in the percentage of the
dose recovered as the ω-1-oxidation metabolite, 4-OH VPA, was the
greatest, suggesting increased formation of P-450 metabolites. As
mentioned, dose dependence has not been previously observed for ω-
and ω-1-oxidation in humans; however, the doses used in these experiments were substantially lower than the doses used in our sheep studies. The urinary excretion of 4-ene VPA was minor for both adult and neonatal sheep and appeared only at the higher doses. By far the main pathway of VPA elimination is glucuronidation. In adult sheep, no significant change was observed in the recovery of VPA with increasing dose. In fact, VPA-glucuronide accounted for 68.3 ± 6.5 to 78.7 ± 5.6% of the administered VPA at all doses (Table 4). A different situation was observed in lambs. At the 10-mg/kg dose, 29.2% of the administered VPA was recovered as the glucuronide metabolite. This percentage significantly increased to 65.7 to 69.6% at the three higher doses (Table 5). Previous dose-ranging experiments in adult rats (Dickinson et al., 1979) and humans (Granneman et al., 1984; Dickinson et al., 1989; Anderson et al., 1992) showed a pattern similar to neonatal lambs wherein VPA-glucuronidation appeared to increase with dose. The observed differences in changes in VPA-glucuronidation with dose in adult rat and humans in comparison with adult sheep could be a result of species differences in VPA-glucuronidation. Species differences in the relative contribution of other routes of elimination (i.e., especially β-oxidation) may also play a role since mass balance data for each metabolic pathway is not independent of other routes of elimination.

In Vivo Estimation of Apparent $V_{\text{max}}$ and $K_m$ of Overall VPA Elimination and VPA Glucuronidation. The mass balance data presented in Tables 4 and 5 clearly show that VPA glucuronidation is the main pathway responsible for VPA elimination in both adult sheep and neonatal lambs. In fact at 50-, 100- and 250-mg/kg doses, the majority of the administered dose was recovered as this metabolite in both neonatal lambs (~65–70%) and adult sheep (~70–80%). Thus, the estimated apparent in vivo $V_{\text{max}}$ and $K_m$ for overall VPA metabolic elimination is largely reflective of VPA-glucuronidation. This would explain the similar information obtained from the $V_{\text{max}}$ and $K_m$ estimates using plasma data and urine data. Estimates from both methods suggest that $V_{\text{max}}$ is similar in both age groups (Tables 6 and 7). The higher estimates of $V_{\text{max}}$ obtained from plasma data (445.0 μg/min/kg for adult sheep and 429.9 μg/min/kg for lambs) in comparison with the urine data (288.5 μg/min/kg for adult sheep and 326.5 μg/min/kg for lambs) may be a consequence of the fact that VPA-glucuronidation does not account for elimination of the entire dose. The presence of other routes of elimination will also influence the parameter estimates obtained from plasma data. However, urine data directly monitor the production of the specific metabolite of interest. Apparent $K_m$ estimates from the two methods were surprisingly similar with both, suggesting that $K_m$ is higher in lambs. A similar phenomenon was observed in a study examining acetaminophen glucuronidation in adult and fetal sheep microsomes (Wang et al., 1986). In this study, estimates of $K_m$ obtained from microsomes were higher for the fetus than for the adult. This difference in $K_m$ was attributed to either a different form of UDP-glucuronyltransferase being present in fetal microsomes or age-related differences in enzyme function resulting from differences in the immediate environment of the UDP-glucuronyltransferases (Wang et al., 1986). It is possible that the higher $K_m$ estimate observed for VPA glucuronidation in neonatal lambs is a result of similar causes since the neonatal lamb is an early phase in the transition from the fetal to the adult situation.

The Michaelis-Menten parameter $V_{\text{max}}$ is defined as the maximum enzyme capacity and is related to the total concentration of enzyme. The other Michaelis-Menten parameter, $K_m$, is the Michaelis-Menten constant and has an inverse relationship to enzyme affinity (Rowland and Tozer, 1980). Therefore, the apparent $V_{\text{max}}$ and $K_m$ values from our dose-ranging studies suggest that metabolic capacity is similar in the two age groups of interest. However, since $K_m$ was estimated in vivo, it is not possible to determine whether a difference in $K_m$ is related to developmental changes in enzyme affinity and/or enzyme function. A higher apparent $K_m$ in 10-day-old lambs explains the relative changes in $CL_{\text{int}}$ with increasing dose in both age groups. Earlier we stated that VPA $CL_{\text{int}}$ approximates intrinsic clearance. Intrinsic clearance is related to $V_{\text{max}}$ and $K_m$ by the following equation:

$$CL_{\text{int}} = \frac{V_{\text{max}}}{K_m + C_u}$$  \hspace{1cm} (6)

where $CL_{\text{int}}$ is the intrinsic clearance and $C_u$ is the unbound drug concentration (Rowland and Tozer, 1980; Gibaldi and Perrier, 1982). At lower drug concentrations, the denominator of eq. 6 approximates $C_u$, and $CL_{\text{int}} \approx V_{\text{max}}/K_m$. Since $K_m$ is smaller in adult sheep, VPA $CL_{\text{int}}$ estimates should be higher for adults than for lambs in this first scenario. At higher concentrations, the denominator of eq. 6 approximates $C_u$, and $CL_{\text{int}} \approx V_{\text{max}}/C_u$. Therefore, as concentrations increase, the $CL_{\text{int}}$ in adult and neonatal sheep should become increasingly similar since $V_{\text{max}}$ is similar for both age groups. Our $CL_{\text{int}}$ data follow this exact pattern, with adult $CL_{\text{int}}$ values being significantly higher at the two lower doses (Fig. 3). By the 100-mg/kg dose, the adult $CL_{\text{int}}$ estimates have decreased to the point where they are no longer different than the lamb values. The $CL_{\text{int}}$ estimates from both age groups remain similar at the highest dose (Fig. 3).

Differences observed in the recovery of VPA-glucuronide in adult (73.8% of the dose) and neonatal lambs (29.2% of the dose) following the 10-mg/kg VPA dose are also consistent with a higher $K_m$ in neonatal lambs. VPA metabolism is essentially a competition between various metabolic pathways. Therefore, a decrease in the ability of one pathway to eliminate VPA will result in the elimination of the compound by one of many alternate metabolic routes. Thus, the higher apparent $K_m$ of VPA glucuronidation in lambs would allow a larger portion dose to be eliminated by other processes than would occur in adult sheep. With increases in dose, elimination pathways tend to saturate, which would allow a larger portion of VPA to be glucuronidated. This theory is consistent with our data, as VPA glucuronidation in neonatal lambs increases substantially with an increase in dose. In fact, at the 50-mg/kg dose, almost the entire dose is recovered (92.3 ± 7.3%), with the majority being VPA-glucuronide (65.7 ± 8.5% of the dose). We have yet to recover ~50% of the dose in 10-day-old lambs following the administration of the lowest dose (i.e., 10 mg/kg). Thus, the presence of a high-affinity, low-capacity VPA elimination process would explain our results. The nature of this process remains to be identified.

$V_{\text{max}}$ and $K_m$ values of VPA glucuronidation have been previously estimated in adult guinea pigs using both in vitro (i.e., 5% liver homogenate and microsomes) and in vivo methodologies (Yu et al., 1993; Yu and Shen, 1996). In these studies, in vitro $V_{\text{max}}$ estimates of ~260 and ~170 μg/min/kg were obtained using liver homogenate and microsomes, respectively. A similar apparent $V_{\text{max}}$ estimate of ~220 μg/min/kg was obtained in vivo in dose-ranging drug infusion experiments. $K_m$ estimates obtained for guinea pigs from these studies were similar (~45 μg/ml (5% liver homogenate), ~23 μg/ml (microsomes), and ~22 μg/ml (in vivo)) (Yu et al., 1993; Yu and Shen, 1996). These adult guinea pig estimates are comparable with our estimates obtained for adult sheep using urine data ($V_{\text{max}} = 288.5$ μg/min/kg and $K_m = 30.0$ μg/ml; Table 7). $K_m$ estimates from both adult sheep and guinea pigs are within the clinical range of unbound drug (Yu, 1984).
Valproic Acid Dose-Dependent Pharmacokinetics and Metabolism

Estimation of in vivo apparent \( V_{\text{max}} \) and \( K_{\text{m}} \) of overall VPA elimination has also been assessed using plasma data from developing rats (Haberer and Pollack, 1994). The \( V_{\text{max}} \) (~302 \( \mu \text{g/min/kg} \)) and \( K_{\text{m}} \) (~100 \( \mu \text{g/ml} \)) for 10-day-old rats in this study are comparable with our own estimates obtained from lamb plasma data (\( V_{\text{max}} \) = 429.9 \( \mu \text{g/min/kg} \) and \( K_{\text{m}} \) = 69.6 \( \mu \text{g/ml} \); Table 6). However, \( V_{\text{max}} \) estimates in slightly older rats (i.e., 20 days and 60 days) were substantially larger [i.e., 975 \( \mu \text{g/min/kg} \) (20-day-old) and 4460 \( \mu \text{g/min/kg} \) (60-day-old)] than even our adult estimates. It must be mentioned that these estimates were obtained following single bolus dose experiments. Reliable estimation of in vivo \( V_{\text{max}} \) and \( K_{\text{m}} \) requires dose-ranging experiments at three to four dose levels with at least some doses wherein saturation kinetics is evident (Metzler and Tong, 1981; Gibaldi and Perrier, 1982). Although not nearly as reliable, estimates can still be obtained from single dose studies exhibiting some degree of saturation kinetics. However, signs of saturation kinetics (i.e., nonlinear terminal slope of semilogarithmic plasma concentration versus time plot) were only present in the plasma profiles of the 5- and 10-day-old rats in these studies. Thus, it is difficult to assess the validity of the estimates obtained from the older rats (i.e., 20- and 60-day-old) and make comparisons with our own adult data.

In summary, developmental differences exist in both plasma protein binding and metabolism of valproic acid. VPA plasma protein binding capacity was higher in adult sheep, whereas binding affinity appeared to be lower. The unexpected lower binding affinity in adult sheep may be attributed to higher variable fatty acid levels in plasma of adult ruminants in comparison with lambs and monogastric animals. Differences in apparent \( K_{\text{m}} \) rather than the metabolic capacity of the glucuronidation pathway appeared to be primarily responsible for the differences in \( CL_{\text{u,v}} \) previously observed between adult and neonatal sheep (Wong et al., 2000). Since mass balance data from an earlier study (Wong et al., 2000) suggest that rapid changes in VPA glucuronidation occur during the time period between 10 days and 2 months of age, future dose-ranging studies in 1- and 2-month-old lambs would provide further insight on developmental changes in VPA-glucuronidation.

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References