

ONTOGENY OF VALPROIC ACID DISPOSITION AND METABOLISM: A DEVELOPMENTAL STUDY IN POSTNATAL LAMBS AND ADULT SHEEP¹

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ABSTRACT:

The ontogeny of valproic acid (VPA) disposition and metabolism was investigated in developing lambs and adult sheep (Dorset or Suffolk breed). Specifically, we wished to investigate the role of glucuronidation and β -oxidation on VPA elimination during development. Catheters were implanted in a carotid artery, a jugular vein, and the urinary bladder in 10-day-old (10 d; $n = 8$), 1-month-old (1 M; $n = 4$), and 2-month-old lambs (2 M; $n = 5$). In adult sheep ($n = 5$), catheters were implanted in a femoral artery and vein. After the administration of a 10 mg/kg VPA i.v. bolus, serial blood samples and cumulative urine samples were collected for 36 h in the adult ewes and for 72 h in the lambs. Due to saturable protein binding, age-related differences in VPA clearance were more ob-

vious when examining the total body clearance of unbound drug (Cl^{u}_{tb}). Mean Cl^{u}_{tb} increased significantly with age up to 2 months (10 d = 2.65 ± 1.16 ml/min/kg; 1 M = 5.11 ± 2.49 ml/min/kg; 2 M = 12.84 ± 3.88 ml/min/kg) before decreasing to adult levels (7.73 ± 2.64 ml/min/kg). Similarly, the urinary recovery of the major metabolite, VPA-glucuronide, was significantly less in 10 d lambs ($29.2 \pm 16.0\%$ of the dose) when compared with the adult and 2 M groups (both $\sim 74\%$ of the dose). No differences with age were observed in the portion of the dose excreted as the β -oxidation metabolite, 2-*n*-propyl-3-oxopentanoic acid. The results suggest that alterations in Cl^{u}_{tb} with age may be attributable to postnatal development of enzymes involved in VPA glucuronidation.

Valproic acid (2-propylpentanoic acid, VPA)² is a broad-spectrum anticonvulsant with a unique branched-chain fatty acid structure

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² Abbreviations used are: VPA, valproic acid; 10 d, 10-day-old lambs; 1 M, 1-month-old lambs; 2 M, 2-month-old lambs; AUC_{0-∞}, area under the curve of arterial plasma concentration-time profile; AUMC_{0-∞}, area under the first-moment curve; Cl_r , renal clearance; Cl^u_r , renal clearance for unbound VPA; Cl_{tb} , total body clearance of the total drug; Cl^{u}_{tb} , total body clearance based on unbound drug concentrations; C_{max} , maximal plasma concentration; f_p , area weighted unbound fraction of the drug; MRT, mean residence time of the total drug; MRT^u, mean residence time of the unbound drug; $t_{1/2\beta}$, terminal elimination half-life of total drug, $t^{u}_{1/2\beta}$, terminal elimination half-life of unbound drug; t_{max} , time of occurrence of maximal plasma concentration; Vd_{ss} , steady-state volume of distribution; Vd_{ss}^u , steady state volume of distribution corrected for the effects of saturable protein binding; Vd^{u}_{ss} , steady-state volume of distribution for the unbound drug; (E)-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-OH VPA, 3-hydroxy VPA; 4-OH VPA, 4-hydroxy VPA; 5-OH VPA, 5-hydroxy VPA; 2-PSA, 2-propylsuccinic acid; 2-PGA, 2-propylglutaric acid.

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(Davis et al., 1994). Although complex, VPA metabolism in man can be grouped into three main pathways: 1) glucuronidation, 2) mitochondrial β -oxidation, and 3) P-450-catalyzed oxidative metabolism (Fig. 1) (Baillie and Scheffels, 1995). Glucuronidation is the main route of elimination in man, accounting for approximately 10 to 70% of the administered dose (Gugler et al., 1977; Dickinson et al., 1989; Levy et al., 1990). β -Oxidation of VPA gives rise to the major plasma metabolites 2-*n*-propyl-2-pentenoic acid [(E)-2-ene VPA], (E,E)-2, 3'-diene, and 2-*n*-propyl-3-oxopentanoic acid (3-keto VPA; Siemes et al., 1993; Baillie and Scheffels, 1995). Of these, 3-keto VPA is the most prevalent in urine, accounting for approximately 10 to 60% of the administered dose (Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). In comparison with glucuronidation and β -oxidation, P-450-catalyzed oxidative metabolism plays a minor role in overall VPA elimination. However, two metabolites [i.e., 2-*n*-propyl-4-pentenoic acid (4-ene VPA) and (E)-2, 4-diene VPA], arising as a result of a distinct P-450-mediated desaturation reaction, have been hypothesized to be associated with the hepatotoxic effects of VPA (Rettie et al., 1987; Kasshun and Baillie, 1993). Thus, the study of their formation and subsequent fate has received considerable attention (Kasshun and Abbott, 1993; Kasshun and Baillie, 1993; Tang and Abbott, 1996).

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 (Sarisyulius and Oliver, 1999). Available data in the literature have shown that age-related alterations in VPA disposition exist in humans and other species. Information on VPA disposition is limited in human neonates due to obvious ethical

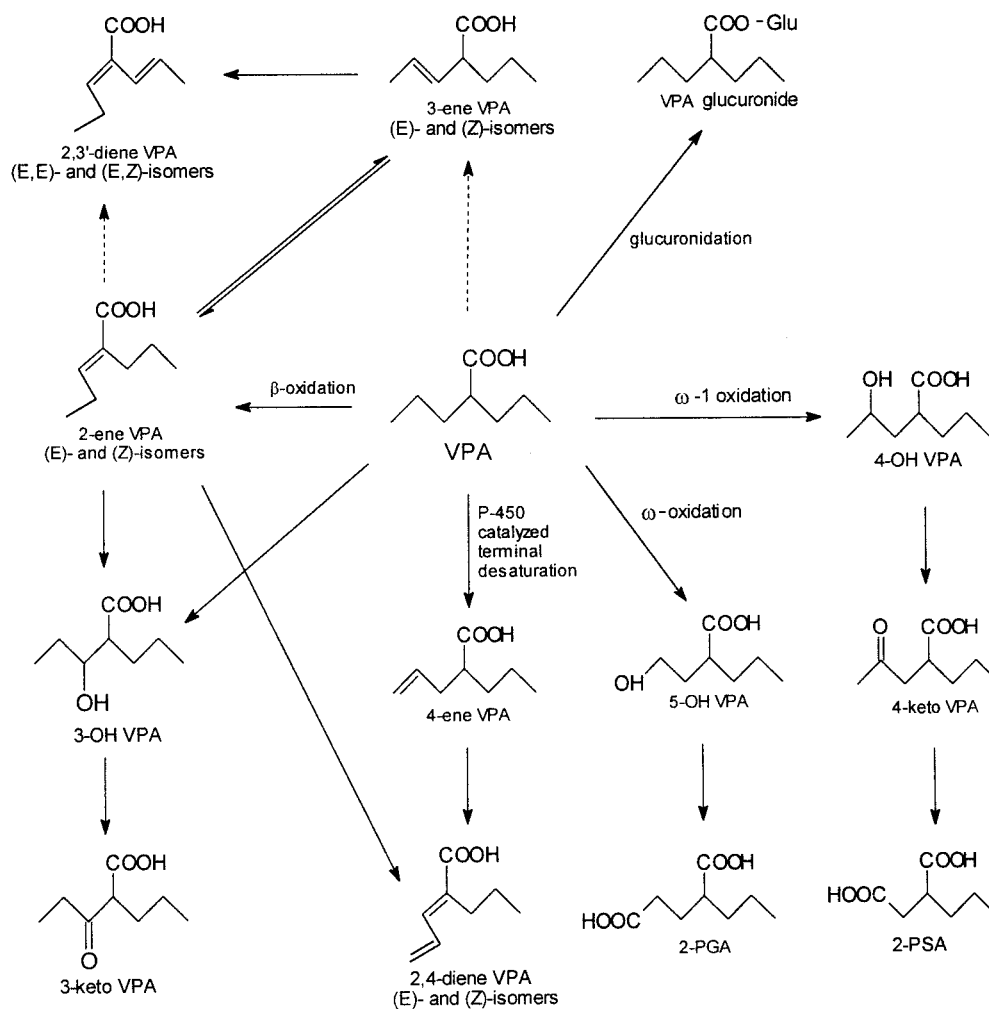


FIG. 1. Pathways of VPA metabolism.

Dotted arrows indicate pathways for which direct experimental evidence is lacking.

considerations. Data obtained from different studies indicate that in humans, VPA metabolic clearance appears low in neonates, increases to a maximal level in children before decreasing to adult values (Levy and Shen, 1995). More systematic *in vivo* studies have been conducted in rats (Haberer and Pollack, 1994) and guinea pigs (Yu et al., 1985, 1987), showing progressive increases in VPA metabolic clearance with age. However, these studies do not address the issue of developmental changes in VPA metabolism and renal elimination of both the parent compound and its metabolites.

The purpose of this study is to systematically investigate age-related alterations in the disposition of VPA and its metabolites in postnatal lambs. Specifically, we wished to study developmental changes in VPA glucuronidation and β -oxidation, as they appear to be the primary pathways responsible for VPA elimination in essentially all species previously examined (Nau and Loscher, 1984). As well, developmental changes in renal excretion were examined. In the past, we have studied VPA pharmacokinetics in pregnant and newborn sheep and have found that the main metabolic pathways (i.e., glucuronidation, β -oxidation, and P-450-catalyzed pathways) and metabolites (i.e., VPA-glucuronide, 3-keto VPA) observed in humans are present in sheep (Kumar, 1998). Finally, the use of chronically catheterized lambs and adult sheep overcomes limitations in the available sampling volume of biological fluids associated with smaller animal models, and thus allows 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 crossbred ewes, with a body weight of 61.9 ± 7.3 kg (mean \pm 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.

Postnatal Lambs. A total of 17 Dorset Suffolk crossbred lambs were used in this study. Lambs were divided into a 10-day-old group (10 d; $n = 8$), a 1-month-old group (1 M; $n = 4$), and a 2-month-old group (2 M; $n = 5$). All lambs were surgically prepared at least 3 days before the experiment under 1% isoflurane anesthesia. Briefly, polyvinyl catheters (Dow Corning) were implanted in a carotid artery and a jugular vein (catheter i.d. 1.02 mm and o.d. 2.16 mm). A third larger diameter catheter (i.d. 3.0 mm and o.d. 4.5 mm) was implanted in the urinary bladder via a lower abdominal midline incision. Catheters were flushed daily with ~ 2 ml of heparinized saline. After the recovery period (minimum of 3 days), 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) and had free access to hay, grain, and water.

Ampicillin (500 mg) was administered i.m. to both adult sheep and postnatal lambs on the day of the surgery and for 3 days postoperatively.

Experimental Protocols. All sheep experiments involved administration of an i.v. bolus dose of VPA (Sodium Valproate; Sigma Chemical Co., St. Louis, MO) equivalent to 10 mg VPA/kg b.wt. All doses were prepared in sterile water for injection and were sterilized by filtering through a 0.22- μ m nylon syringe filter (MSI, Westboro, MA) into a capped empty sterile injection vial. The dose was administered over 1 min via the jugular vein (lambs) or femoral vein (ewes). Postnatal lamb experiments were initiated at approximately 10 days, 1 month, or 2 months after birth, depending on which group the lambs had been assigned to. For postnatal lambs, serial blood samples (\sim 2 ml) were collected from the carotid artery at 5, 15, 30, 45, 60 min, and 2, 4, 6, 9, 12, 24, 36, 48, 60, and 72 h after drug administration. Serial blood samples (\sim 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, and 36 h after drug administration. Cumulative urine was also collected for both adult sheep and postnatal lambs for the full duration of the experiment (i.e., 72 h for lambs and 36 h for adult sheep). The only exceptions were the entire 1 month group ($n = 4$), four lambs in the 10 d group, and one lamb in the 2 M group, where urine collection was incomplete due to catheter failure; these were excluded from data analysis.

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.

Determination of VPA Plasma 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., Danver, MA). Plasma samples for the determination of unbound VPA concentrations were stored in separate aliquots so as 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 using an established gas chromatographic-mass spectrometric analytical method (Yu et al., 1995). Concentrations of the VPA-glucuronide metabolite in both adult and lamb 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 in spite of these possible rearrangements (Dickinson et al., 1984).

Pharmacokinetic Analyses. Pharmacokinetic parameters were calculated by standard methods as described in Gibaldi and Perrier (1982). Terminal elimination half-life of the total ($t_{1/2\beta}$) and unbound ($t_{1/2\beta}^u$) VPA in plasma were obtained from a two-compartment model fitting of the data using the

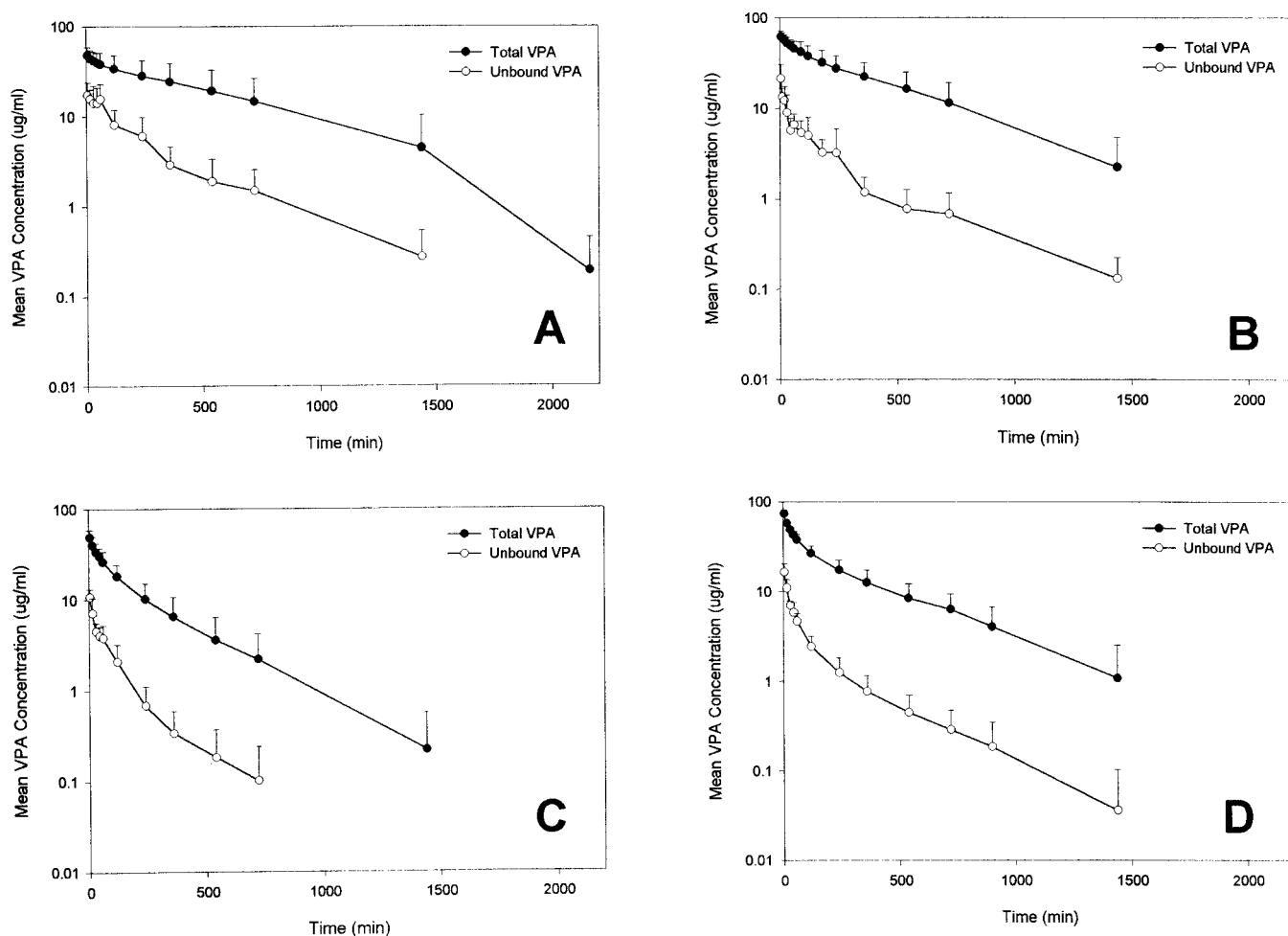


FIG. 2. Mean VPA (total and unbound) concentration versus time profiles in 10 d lambs (A), 1 M lambs (B), 2 M lambs (C), and adult sheep (D) after VPA i.v. bolus administration.

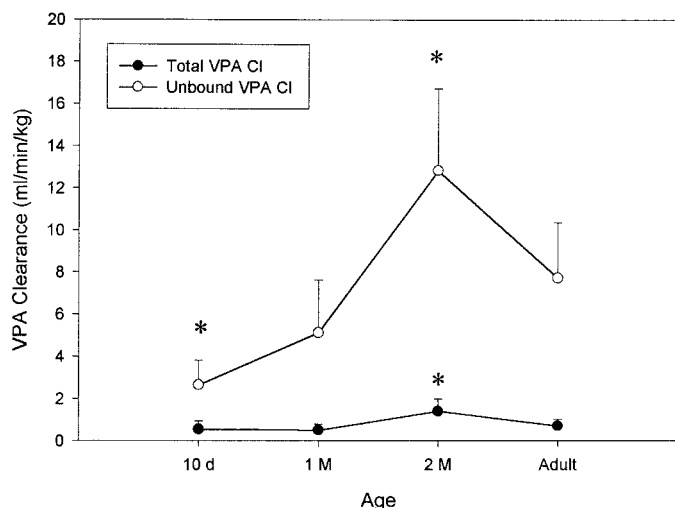


Fig. 3. Changes in mean VPA (unbound and total drug) total body clearance with age.

*, denotes significant difference from and adult values ($P < .05$).

nonlinear least-squares regression software WinNonlin (Scientific Consulting, Inc., Apex, NC). Model fittings were carried out using a weighting factor of $1/\text{predicted } y^2$. Plasma area under the curve of arterial plasma concentration-time profile ($AUC_{0-\infty}$) and area under the first-moment curve ($AUMC_{0-\infty}$) of total and unbound drug were calculated by the linear trapezoid rule.

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

$$f_p = \frac{AUC_{0-\infty}(\text{unbound VPA})}{AUC_{0-\infty}(\text{total VPA})}$$

$$Vd_{ss}' = f_p \cdot Vd_{ss}^u$$

For drugs exhibiting saturable protein binding, the steady-state volume of distribution parameter (Vd_{ss}) when calculated using the 'model independent' approach (Gilbaldi and Perrier, 1982) overestimates the "true" Vd_{ss} and is concentration-dependent (McNamara et al., 1983). Similarly, steady-state volume of distribution for the unbound drug (Vd_{ss}^u) 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 Vd_{ss} and Vd_{ss}^u are poor indicators of drug distribution. Instead, the Vd_{ss}' parameter is more reflective of shifts in drug mass into or out of the vascular space (i.e., information traditionally provided by the Vd_{ss} parameter) (McNamara et al., 1983). As with Vd_{ss}^u above, this Vd_{ss}' parameter is also constant only for a particular f_p or a steady-state plasma unbound fraction equivalent to f_p .

Statistical Analysis. All data are reported as mean \pm S.D. Pharmacokinetic parameters were compared using ANOVA followed by a Fisher's Least

Significant Difference multiple comparison test. The significance level was $P < .05$ in all cases.

Results

Pharmacokinetics of VPA in Postnatal Lambs and Adult Sheep.

Average age of the postnatal lamb groups on the day of their experiments was 10.9 ± 1.4 days for the 10 d group, 30 ± 1.4 days for the 1 M group, and 60.0 ± 1.0 days for the 2 M group. Mean adult ewe body weight was 61.9 ± 7.3 kg, and mean lamb body weights were 5.8 ± 1.4 kg (10 d), 9.4 ± 1.7 kg (1 M), and 13.2 ± 2.1 kg (2 M). Figure 2, A–D, are semilogarithmic plots of mean VPA (unbound and total) concentration versus time for all age groups. After the administration of a 10 mg/kg VPA i.v. bolus, mean plasma levels at the first sampling time (i.e., 5 min after drug administration) were at or near the human therapeutic range (i.e., 50–100 $\mu\text{g/ml}$). Total and unbound VPA plasma profiles in lambs and adult sheep appeared to be biexponential in nature. In addition, VPA plasma protein binding was saturable (nonlinear) for all age groups. At the first sampling point, where measured VPA concentrations were the greatest (i.e., mean VPA concentration range 48–73 $\mu\text{g/ml}$), the mean unbound fraction was 0.37 ± 0.11 in 10 d lambs, 0.34 ± 0.12 in 1 M lambs, 0.22 ± 0.04 in 2 M lambs, and 0.22 ± 0.05 in adult sheep. Characteristic of nonlinear plasma protein binding, unbound fractions proceeded to decline with decreasing VPA concentrations in all animals such that at 12 h after drug administration (mean VPA concentration range 2–15 $\mu\text{g/ml}$), the mean unbound fraction in plasma was 0.11 ± 0.09 in 10 d lambs, 0.06 ± 0.01 in 1 M lambs, and 0.04 ± 0.02 in 2 M lambs and adult sheep.

Figure 3 depicts age-related changes in VPA total body clearance for both total (Cl_{tb}) and unbound (Cl_{tb}^u) drug increased significantly from 10 d values, reaching a maximum in 2 M lambs, before decreasing to adult levels (Fig. 3, Table 1). Due to the nonlinear nature of VPA plasma protein binding, differences in clearance were more evident when examining unbound drug concentrations (Fig. 3). Additional pharmacokinetic parameters for unbound and total VPA for all age groups are presented in Table 1. As with total body clearance, age-related alterations in $t_{1/2\beta}$ and mean residence time (MRT) were more pronounced for unbound VPA. Both $t_{1/2\beta}^u$ and MRT^u (mean residence time of unbound drug) were significantly longer for 10 d lambs than for other age groups. No differences in Vd_{ss} and Vd_{ss}' were observed between age groups; however, Vd_{ss} was significantly lower in the 10 d lambs. Furthermore, the f_p value was significantly higher in 10 d lambs when compared with the corresponding values from the other age groups.

VPA Metabolites in Postnatal Lamb and Adult Sheep Plasma.

Plasma samples collected from postnatal lambs and adult sheep were analyzed for VPA metabolites generated from fatty acid β -oxidation [(*E*)-2-ene, 2-*n*-propyl-3-pentenoic acid (*E*)-3-ene VPA], and 3-keto VPA], cytochrome P-450-mediated desaturation (4-ene VPA), and

TABLE 1

VPA pharmacokinetic parameters in 10 d, 1 M, 2 M, and adult sheep

Age	Total VPA					Unbound VPA				f_p
	Cl_{tb}	$t_{1/2\beta}$	MRT	Vd_{ss}	Vd_{ss}'	Cl_{tb}^u	$t_{1/2\beta}^u$	MRT ^u	Vd_{ss}^u	
	ml/min/kg	h	h	l/kg	l/kg	ml/min/kg	h	h	l/kg	
10 d (n = 8)	0.55 ± 0.38	7.0 ± 3.4	10.0 ± 4.9 ^a	0.22 ± 0.08	0.14 ± 0.06	2.65 ± 1.16 ^a	10.6 ± 8.5 ^b	6.7 ± 2.8 ^b	0.79 ± 0.32 ^b	0.21 ± 0.12 ^b
1 M (n = 4)	0.50 ± 0.27	5.2 ± 1.1	7.2 ± 1.9	0.19 ± 0.07	0.14 ± 0.07	5.11 ± 2.49	2.5 ± 1.2	3.2 ± 1.6	1.48 ± 0.32	0.10 ± 0.01
2 M (n = 5)	1.40 ± 0.58 ^a	2.6 ± 0.7	3.5 ± 1.1	0.30 ± 0.06	0.16 ± 0.03	12.84 ± 3.88 ^a	1.5 ± 0.5	1.8 ± 0.6	1.54 ± 0.39	0.11 ± 0.03
Adult (n = 5)	0.70 ± 0.31	4.2 ± 2.0	5.7 ± 2.8	0.24 ± 0.03	0.13 ± 0.02	7.73 ± 2.64	1.7 ± 0.7	2.1 ± 0.9	1.47 ± 0.51	0.09 ± 0.02

^a Statistically different from adult age group ($P < .05$).

^b Statistically different from 1 M, 2 M, and adult age groups ($P < .05$).

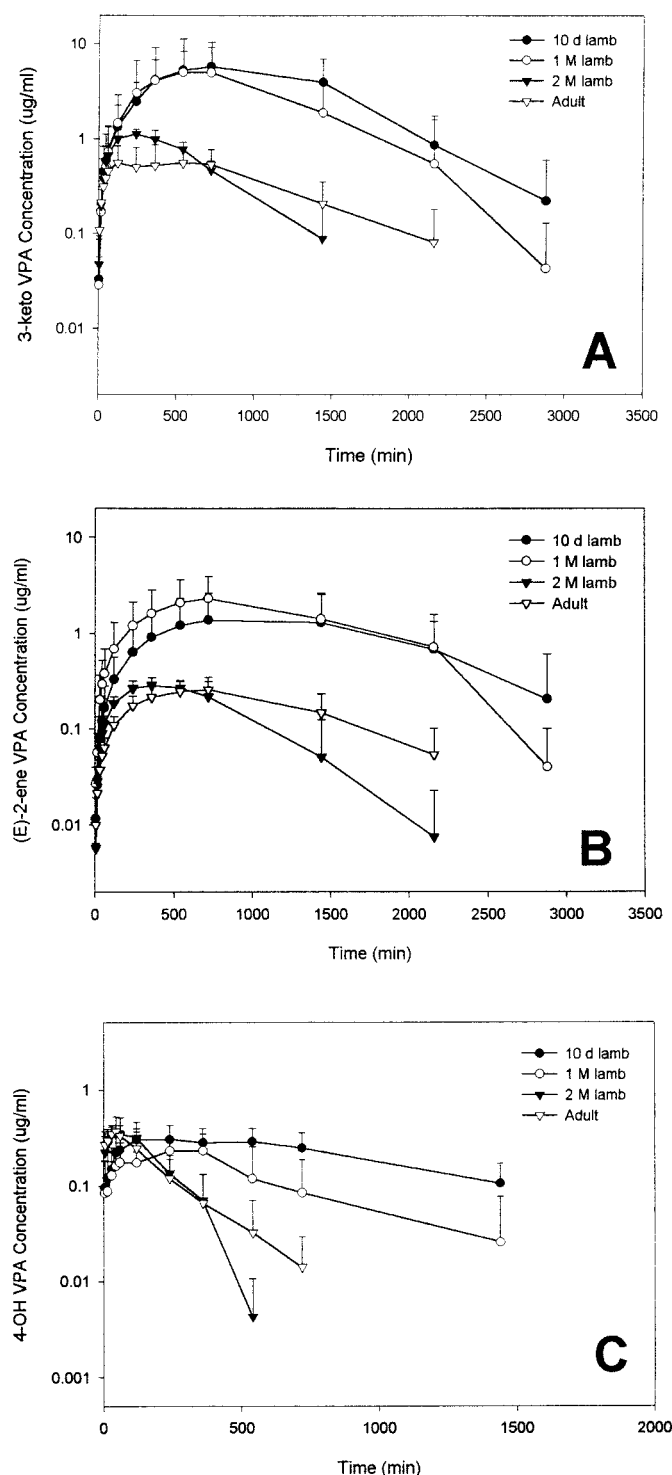


FIG. 4. Mean metabolite concentration versus time profiles in 10 d, 1 M, 2 M, and adult sheep for 3-keto VPA (A), (E)-2-ene VPA (B), and 4-OH VPA (C).

hydroxylation and subsequent oxidation [3-hydroxy VPA (3-OH VPA), 4-hydroxy VPA (4-OH), 5-hydroxy VPA (5-OH), 2-*n*-propyl-4-oxopentanoic acid (4-keto VPA), 2-propylsuccinic acid (2-PSA), and 2-propylglutaric acid (2-PGA)]. The diunsaturated metabolites (see Fig. 1) were not observed in both preliminary experiments in this study or in previous studies with fetal, newborn, and pregnant sheep (Kumar, 1998), and thus were not monitored. The main metabolites observed in lamb and adult sheep plasma were the β -oxidation me-

tabolites 3-keto and (E)-2-ene VPA. This was followed by the cytochrome P-450-generated 4-OH VPA. Figure 4, A-C, shows mean plasma profiles of these three metabolites for lambs and adult sheep after i.v. bolus administration of the drug. The maximal plasma concentration (C_{max}), time of occurrence of the maximal plasma concentration (t_{max}), and $AUC_{0-\infty}$ for the three mentioned metabolites are presented in Table 2. In general, metabolite profiles appeared to be more similar between 10 d and 1 M lambs in comparison to 2 M lambs and adult sheep. All three metabolites could be detected for a longer period of time in the younger animal groups (10 d and 1 M). The C_{max} of 3-keto and (E)-2-ene VPA were both significantly higher in 10 d and 1 M lambs when compared with 2 M lambs and adult sheep (Table 2). Also, the mean t_{max} values of 3-keto, (E)-2-ene, and 4-OH VPA were significantly longer for the 10 d group than for all the other age groups. Mean 3-keto $AUC_{0-\infty}$ values were significantly larger (~7- to 10-fold) for the two younger age groups (i.e., 10 d and 1 M). Similarly, $AUC_{0-\infty}$ estimates for (E)-2-ene and 4-OH VPA were also significantly larger in the two younger groups (i.e., ~13- to 16-fold for (E)-2-ene VPA and ~3- to 5-fold for 4-OH VPA). Accurate estimates of $AUC_{0-\infty}$ could not be obtained for 5-OH, (E)-3-ene, 4-ene, and 4-keto VPA due to their low plasma concentrations (i.e., C_{max} values of $<0.2 \mu\text{g/ml}$). Plasma concentrations of 3-OH VPA, 2-PGA, and 2-PSA were almost always below the lower limit of quantification of the assay (Yu et al., 1995).

Urinary Excretion of VPA and its Metabolites. Urinary recovery of the VPA dose as the parent compound and its metabolites for 10 d, 2 M, and adult sheep is presented in Table 3. The majority of the administered dose was recovered in urine as VPA, VPA-glucuronide, 3-keto VPA, and 4-OH VPA. All other metabolites (3-OH, 5-OH, (E)-2-ene, (E)-3-ene, 4-ene, 4-keto VPA, 2-PSA, and 2-PGA) individually accounted for less than 1% of the administered dose in all age groups. When added together, these minor metabolites combined to account for $1.1 \pm 0.6\%$ of the dose in 10 d lambs, $1.4 \pm 0.2\%$ of the dose in 2 M lambs, and $2.2 \pm 0.9\%$ of the dose in adult sheep. For both 2 M lambs and adult sheep, essentially the entire dose was recovered in urine during the experimental period with the major component being in the form of the glucuronide metabolite (Table 3). However, for 10 d lambs, only ~50% of the dose could be accounted for. The percentage of the dose recovered as the parent compound was significantly lower in lambs when compared with adult sheep. Similarly, recovery of the parent compound as VPA-glucuronide and 4-OH VPA was significantly less in 10 d lambs. No differences were found in the percentage of the dose excreted in urine as 3-keto VPA.

Figure 5A depicts age-related alterations in renal clearance for unbound (Cl_r^u) and total VPA (Cl_r). As with total body clearance, changes in VPA renal clearance were more apparent when examining unbound drug concentrations. Consistent with the mass balance data mentioned above, renal clearance estimates for unbound VPA from both lamb groups (10 d $Cl_r^u = 0.28 \pm 0.22 \text{ ml/min/kg}$ and 2 M $Cl_r^u = 0.39 \pm 0.21 \text{ ml/min/kg}$) were significantly lower than adult values ($0.97 \pm 0.43 \text{ ml/min/kg}$). Figure 5B displays changes in Cl_r of 3-keto and 4-OH VPA with age. Similar to unbound VPA, Cl_r of both of these metabolites increased significantly with age, reaching adult levels by 2 months after birth. Renal clearance values were not calculated for other metabolites due to either their low plasma concentrations and/or trace levels excreted in urine.

Discussion

The results of this study indicate that VPA plasma protein binding is nonlinear in nature at therapeutic concentrations for all age groups. This is consistent with previous observations in sheep (Kumar, 1998), rats (Haberer and Pollack, 1994), guinea pigs (Yu and Shen, 1992),

TABLE 2
 C_{max} , t_{max} , and $AUC_{0-\infty}$ of VPA metabolites in plasma

Metabolite	Age			
	10 d (n = 7)	1 M (n = 4)	2 M (n = 5)	Adult (n = 5)
3-keto VPA				
C_{max} ($\mu\text{g/ml}$)	6.83 \pm 3.05 ^a	5.66 \pm 6.10 ^a	1.20 \pm 0.12	0.67 \pm 0.26
t_{max} (h)	13.3 \pm 4.9 ^a	8.5 \pm 4.1	3.6 \pm 1.7	7.0 \pm 3.7
$AUC_{0-\infty}$ ($\mu\text{g}\cdot\text{h/ml}$)	150.4 \pm 69.7 ^a	103.7 \pm 103.3 ^a	14.4 \pm 4.1	13.8 \pm 6.7
(E)-2-ene VPA				
C_{max} ($\mu\text{g/ml}$)	1.76 \pm 1.45 ^a	2.32 \pm 1.60 ^a	0.30 \pm 0.05	0.27 \pm 0.08
t_{max} (h)	18.4 \pm 7.0 ^b	11.3 \pm 1.5	6.8 \pm 3.0	12.0 \pm 2.1
$AUC_{0-\infty}$ ($\mu\text{g}\cdot\text{h/ml}$)	47.2 \pm 44.1 ^a	57.1 \pm 39.6 ^a	3.5 \pm 1.4	6.8 \pm 2.8
4-OH VPA				
C_{max} ($\mu\text{g/ml}$)	0.34 \pm 0.14	0.28 \pm 0.10	0.42 \pm 0.09	0.40 \pm 0.14
t_{max} (h)	4.6 \pm 4.2 ^a	3.3 \pm 2.2	0.75 \pm 0.25	0.55 \pm 0.21
$AUC_{0-\infty}$ ($\mu\text{g}\cdot\text{h/ml}$)	7.4 \pm 2.7 ^a	4.7 \pm 2.8 ^a	1.5 \pm 0.5	1.6 \pm 0.8

^a Statistically different from 2 M and adult age groups ($P < .05$).

^b Statistically different from 1 M, 2 M, and adult age groups ($P < .05$).

and humans (Scheyer et al., 1990). Thus, for VPA, unbound drug clearance is more reflective of metabolic clearance. A species comparison of pharmacokinetic parameters revealed that in general, VPA clearance is slightly higher in sheep when compared with humans. Clearance estimates from human neonates 6 to 9 days of age (Cl_{tb} : 0.1–0.2 ml/min/kg and Cl_{tb}^u : 0.7–1.2 ml/min/kg), and newborns ~1 month of age (Cl_{tb} : 0.2–0.5 ml/min/kg and Cl_{tb}^u : ~4 ml/min/kg) were lower than their corresponding lamb values (Table 1) (Irvine-Meek et al., 1982; Gal et al., 1988). Furthermore, similar findings were observed in comparisons between adult humans (Cl_{tb} : 0.1–0.3 ml/min/kg and Cl_{tb}^u : 1–3 ml/min/kg; Davis et al., 1994; Levy and Shen, 1995) and sheep (Table 1). The trend observed in previous investigations in developing rats (Haberer and Pollack, 1994) and guinea pigs (Yu et al., 1985, 1987), was a detectable increase in VPA metabolic clearance with age. In contrast, we observed an increase in Cl_{tb} and Cl_{tb}^u with age to reach a maximum at 2 months of age, before decreasing to adult levels. This pattern of change is similar to what is observed in humans where metabolic clearance is lowest in newborns, reaches a maximum from 2 to 36 months of age, and then decreases to adult values (Levy and Shen, 1995). The relatively lower VPA clearance in 10 d lambs is reflected in the longer $t_{1/2\beta}$ and MRT estimates observed for both unbound and total drug. Similarly, reported VPA elimination half-lives in human neonates (17–80 h) are substantially longer than for adult subjects (9–18 h) (Davis et al., 1994; Levy and Shen, 1995). Vd_{ss} and Vd_{ss}' estimates for all age groups were similar to reported values in humans (0.13–0.20 l/kg) (Davis et al., 1994; Levy and Shen, 1995). The significantly lower Vd_{ss}^u value for the 10 d lambs is likely related to their higher f_p value because the two parameters are inversely related (McNamara et al., 1983).

By far the most prevalent metabolites observed in sheep plasma were the β -oxidation metabolites, 3-keto and (E)-2-ene VPA followed by the P-450-mediated hydroxylation metabolite, 4-OH VPA. The presence of 3-keto and (E)-2-ene VPA as the two most prominent metabolites in plasma is similar to previous observations in humans, dogs, rats, and mice (Nau and Loscher, 1984). All three metabolites appeared to persist longer in the two younger animal groups as evident by their significantly larger $AUC_{0-\infty}$ values. Also, 3-keto and (E)-2-ene VPA C_{max} values for 10 d and 1 M lambs were significantly greater (~5- to 10-fold) when compared with 2 M lambs and adult sheep (Table 2). Although detailed data on VPA metabolism is unavailable in human neonates, similarities exist between our data and information available on plasma serum profiles from human epileptic children (Nau et al., 1991; Siemes et al., 1993). Similar to the two

younger animal groups, higher concentrations of β -oxidation metabolites (i.e., 3-keto, 2-ene, 2,3'-diene, and 3-ene VPA) were observed in children <2 years of age in comparison to children >2 years of age. Also, 4-OH VPA concentrations, although not significant, were slightly higher in children <2 years of age. The higher observed $AUC_{0-\infty}$ values for the 3-keto, (E)-2-ene, and 4-OH VPA in the younger lambs may be related to either an increased formation and/or a reduced elimination of the mentioned metabolites.

As a component of this study, we examined postnatal development in VPA renal elimination. As mentioned, VPA Cl_r increased progressively with age. The mechanisms responsible for the tubular secretion of organic acid are not fully functional in late gestational lamb (Jones and Stapleton, 1992). We have observed in previous studies, using pregnant sheep, that the urinary excretion of acidic compounds such as indomethacin (Krishna et al., 1995) and VPA (Kumar, 1998) by the fetal lamb is limited. Thus, the postnatal increase in VPA Cl_r may be a result of the development of mechanisms responsible for the tubular secretion of organic acids. This study indicates that by 2 months of age, VPA Cl_r has not yet reached adult levels (Fig. 5A). The significantly lower Cl_r^u in 10 d and 2 M lambs is reflected in the lower percentage of the dose recovered as the parent compound in postnatal lamb urine when compared with adult sheep (Table 3). Renal excretion of unchanged VPA accounts for a larger percentage of the administered dose in sheep (~12% in adult) in comparison with humans (~2–3%) (Levy and Shen, 1995). This observed difference has been previously attributed to species differences in VPA renal clearance (Kumar, 1998). Despite playing a larger role in sheep, renal excretion of the unchanged drug remains a minor route of elimination.

VPA-glucuronide is by far the major metabolite recovered in urine, accounting for ~74% of the dose in adult sheep. This is at the high end of the range of values previously reported for humans (10–70%) (Gugler et al., 1977; Dickinson et al., 1989; Levy et al., 1990). The percentage of the dose metabolized via glucuronidation appears to increase with age. Our data in 10 d lambs indicates that a significantly smaller portion of the dose is recovered as VPA-glucuronide (i.e., ~29%) in younger animals. This is in excellent agreement with previous studies in 1-day-old lambs, where ~28% of the administered dose was recovered as the glucuronide metabolite (Kumar, 1998). A similar phenomenon was observed in studies involving acetaminophen, where the percentage of the dose that was glucuronidated in newborn lambs (46 \pm 11%) was less in comparison to the adult (64 \pm 4%) (Wang et al., 1990). By 2 months of age, recovery of VPA-glucuronide in urine accounts for ~75% of the administered dose,

TABLE 3

Recovery of VPA and its metabolites as a percentage of the total dose in 10 d, 2 M, and adult sheep after administration of a 10 mg/kg i.v. bolus

Drug or Metabolite	% of Administered Dose Recovered in Urine		
	10 d (n = 4)	2 M (n = 4)	Adult (n = 5)
VPA	5.6 ± 3.1 ^a	3.0 ± 1.3 ^a	12.2 ± 2.2
VPA-glucuronide	29.2 ± 16.0 ^b	74.6 ± 3.2	73.8 ± 5.3
3-keto	12.6 ± 9.8	13.2 ± 5.8	11.3 ± 6.5
4-OH VPA	0.6 ± 0.4 ^b	1.6 ± 0.3	2.0 ± 0.6
Other ^c	1.1 ± 0.6	1.4 ± 0.2	2.2 ± 0.9
% of Dose Recovered	49.1 ± 25.6	93.7 ± 9.63	101.5 ± 8.5

^a Statistically different from adult age group ($P < .05$).

^b Statistically different from 2 M and adult age groups ($P < .05$).

^c Includes 3-OH, 5-OH, (E)-2-ene, (E)-3-ene, 4-ene, 4-keto VPA, 2-PSA, and 2-PGA.

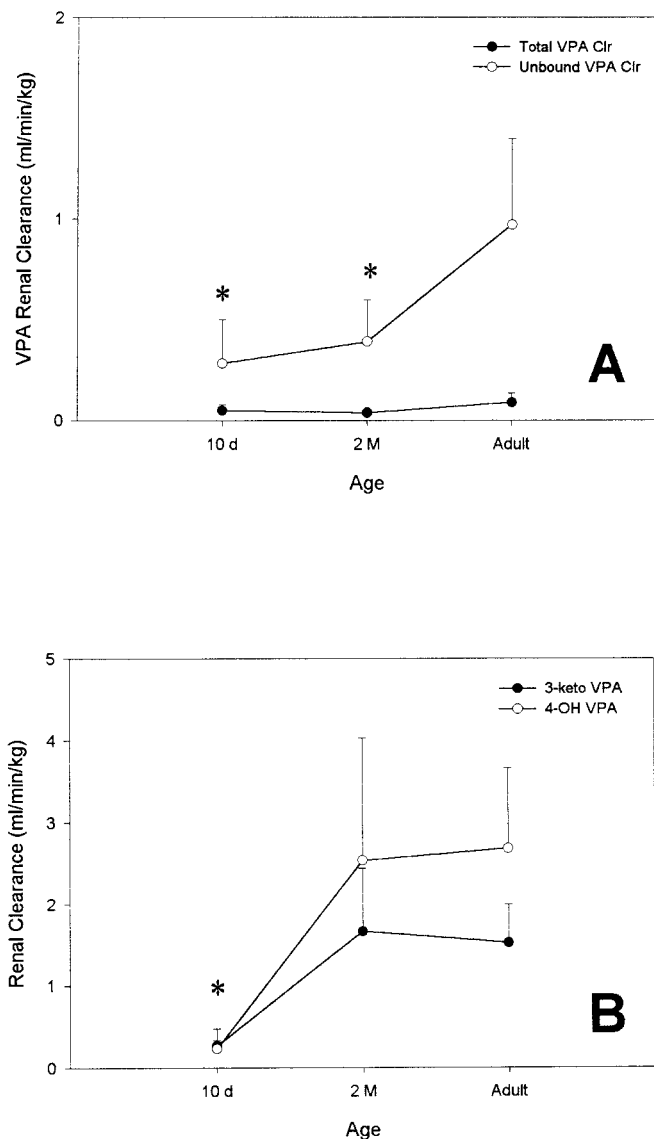


FIG. 5. Changes in renal clearance with age in 10 d, 2 M, and adult sheep for total and unbound VPA (A) and 3-keto and 4-OH VPA (B).

*, denotes significant difference from the adult value ($P < .05$).

suggesting a rapid postnatal development of the glucuronidation pathway responsible for VPA metabolism (Table 3). This increase in VPA glucuronidation coincides with the significant increases in unbound and total VPA clearance mentioned above. Although the effects of advancing age on VPA glucuronidation have been investigated pre-

viously using rat hepatic S9 fractions (Chen et al., 1996), there are no detailed studies examining the role of glucuronidation in overall VPA elimination early on in development. Our data suggests that glucuronidation may play an important role in changes in VPA disposition that occur during the first 2 months of life.

Approximately 11% of the administered dose was recovered in adult sheep urine as the β -oxidation metabolite, 3-keto VPA. In contrast with VPA-glucuronide, this is at the lower end of the range of values previously reported for humans (10–60%) (Dickinson et al., 1989; Levy et al., 1990; Sugimoto et al., 1996). Recovery of the dose in nonpregnant sheep urine as 3-keto VPA is substantially larger than corresponding values in pregnant sheep (~1.6%) (Kumar, 1998). This difference may be related to reductions in β -oxidation activity associated with pregnancy (Grimbert et al., 1993). No developmental changes with age were observed when examining mass balance data for the 3-keto metabolite. In fact, a similar percentage of the administered VPA dose was recovered in studies involving 1-day-old lambs (~11%) (Kumar, 1998). Thus, it appears that β -oxidation activity is substantially developed early on in life. This observation is consistent with findings that β -oxidation activity increases dramatically during the first few hours after birth and may reach levels exceeding that of the adult within 1 day (Krahlting et al., 1979; Duee et al., 1985; De Vivo et al., 1991). In contrast, the percentage of the dose recovered in urine as 4-OH VPA increased ~3-fold from 10 d lambs to adult sheep, suggesting postnatal development of cytochrome P-450-mediated metabolism. Despite the observed increases, the cytochrome P-450 pathway remains relatively unimportant in terms of overall VPA elimination.

Overall, the entire administered dose was essentially recovered in 2 M lambs and adult sheep. In contrast only ~50% of the dose was excreted in urine by 10 d lambs. This is similar to the observations in 1-day-old lambs where ~30 to 50% of the total VPA dose could not be accounted for (Kumar, 1998). Thus, it is possible that additional routes of metabolism, which are responsible for the elimination of a significant portion of the administered VPA dose, are present during the newborn period.

Examination of the Cl_r of 3-keto and 4-OH VPA revealed age-related increases in this parameter. By 2 months of age, the Cl_r values of these two metabolites appear to be at adult levels. The reduced Cl_r of these metabolites in 10 d animals may play a role in the significantly larger metabolite AUC_{0-∞} values observed for 3-keto and 4-OH VPA in the younger animals.

In summary, age-related alterations in VPA clearance for unbound and total drug in developing lambs appears to follow a pattern similar to what is observed in humans. The mass balance urine data suggests that these changes in clearance may be largely related to postnatal development of enzymes involved in VPA glucuronidation. Further investigations are needed to fully understand the impact of age-related alterations in glucuronidation on VPA elimination.

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