Influence of enterohepatic recycling on the time course of brain-to-blood partitioning of valproic acid in rats

Jeannie M. Padowski and Gary M. Pollack

Curriculum in Toxicology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC (J.M.P., G.M.P.);
Division of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina, Chapel Hill, NC (J.M.P., G.M.P.);
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Corresponding author:
Dr. Gary M. Pollack
Washington State University
College of Pharmacy
PO Box 646510
Pullman, WA 99164-6510
Tel: 509-335-5637
Fax: 509-335-0162
Email: gary.pollack@wsu.edu

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ABSTRACT

A widely-used metric of substrate exposure in brain is the brain-to-serum partition coefficient ($K_{p,brain}; C_{brain}/C_{serum}$), most appropriately determined at distribution equilibrium between brain tissue and serum. In some cases, $C_{brain}/C_{serum}$ can peak and then decrease, as opposed to monotonically increasing to a plateau, precluding accurate estimation of partitioning. This “overshoot” has been observed with compounds which undergo enterohepatic recycling (ER) such as valproic acid (VPA). Previous simulation experiments identified a relationship between overshoot in the $C_{brain}/C_{serum}$ vs. time profile and distribution into a peripheral “compartment” (e.g., the ER loop). This study was conducted to evaluate model predictions of that relationship. Initial experiments tested the ability of activated charcoal, antibiotics, or Mrp2 deficiency to impair VPA ER in rats, thereby limiting the apparent volume of distribution associated with ER. Mrp2-deficiency (significantly) and antibiotics (moderately) interrupted VPA ER. Subsequently, brain partitioning was evaluated in the presence vs. absence of ER-modulation. Although overshoot was not eliminated completely, deconvolution revealed that overshoot was reduced in Mrp2-deficient and antibiotic-treated rats. Consistent with model predictions, overshoot was higher after antibiotic treatment (moderate ER-interruption) than in Mrp2-deficiency (substantial ER-interruption). Steady-state $K_{p,brain}$ was unaffected by experimental manipulation, also consistent with model predictions. These data support the hypothesis that $C_{brain}/C_{serum}$ may overshoot $K_{p,brain}$ based on the extent of peripheral sequestration. Consideration of this information, particularly for compounds which undergo significant extravascular distribution, may be necessary to avoid erroneous estimation of $K_{p,brain}$.
INTRODUCTION

A commonly-used metric for substrate exposure in brain is the brain-to-serum partition coefficient, \( K_{p,\text{brain}} \) (Summerfield et al., 2007). Accurate determination of exposure is imperative for CNS-targeted therapeutics, for which extensive exposure is desirable, and non-CNS-targeted compounds or non-therapeutic xenobiotics, for which exposure could produce neurotoxicity. A network of physical and biochemical barriers between the brain and systemic circulation (the blood-brain barrier; BBB) effectively attenuates CNS penetration of most compounds. Consequently, rate and extent of brain exposure are not easily predicted based upon physicochemical properties (Hammarlund-Udenaes et al., 1997; Liu et al., 2005). Experimental determination of penetration into brain therefore is particularly important.

\( K_{p,\text{brain}} \) reflects the net influence of multiple factors on extent of brain exposure and often is used in lead development screening for CNS drug discovery (Raub, 2006). Compounds with \( K_{p,\text{brain}}>1 \) are, somewhat arbitrarily, considered to exhibit “good” CNS exposure (Kalvass and Pollack, 2007). Tissue partitioning, which often is expressed as the tissue-to-serum concentration ratio at a fixed timepoint, is a time-dependent process; the \( C_{\text{tissue}}/C_{\text{serum}} \) ratio increases from 0 (at the time of administration) until distributional equilibrium (DE) between the target tissue and the systemic circulation is attained (Gibaldi, 1969). \( K_{p,\text{brain}} \) most appropriately is determined at DE, either as the ratio of areas under the concentration-time curve (AUC) in brain vs. serum or at steady-state (Lin et al., 1982; Dallas et al., 1994). For practical reasons, the \( C_{\text{brain}}/C_{\text{serum}} \) ratio under non-equilibrium conditions often is used as a surrogate for the “true” substrate partitioning.

Recent studies (Padowski and Pollack, 2011b; Padowski and Pollack, 2011a), demonstrated via mathematical simulation that distribution into a “deep” peripheral pharmacokinetic compartment (i.e., prolonged sequestration in a non-brain compartment) can impact distribution kinetics between brain and blood, resulting in a period of time during which
the $C_{\text{brain}}/C_{\text{serum}}$ ratio overshoots $K_{p,\text{brain}}$. This phenomenon has been reported for valproic acid (VPA), an antiepileptic drug that exhibits a rapid peak in $C_{\text{brain}}/C_{\text{serum}}$ which decreases, rather than increases, with time (Hammond et al., 1982; Golden et al., 1993). VPA undergoes significant enterohepatic recycling (ER). The acyl glucuronide of VPA is excreted via bile into the intestines, hydrolyzed to VPA by intestinal $\beta$-glucuronidase, and reabsorbed into blood. This ER loop sequesters VPA outside of the systemic circulation, essentially functioning as a large peripheral compartment (Pollack and Brouwer, 1991).

The potential relationship between overshoot in VPA brain partitioning and ER is supported by two lines of evidence. VPA and two analogs, cyclohexanecarboxylic acid (CCA) and 1-methyl-1-cyclohexanecarboxylic acid (MCCA), differ in the fraction of the dose that undergoes ER ($\phi_{\text{ER}}$); the rank order of $\phi_{\text{ER}}$ corresponds to the rank order of overshoot in brain partitioning (Liu et al., 1992; Liu and Pollack, 1993; Liu and Pollack, 1994). This observation suggests that ER is quantitatively important in the overshoot phenomenon. Secondly, VPA ER is not complete until at least 60 days of age in rats (Haberer and Pollack, 1994), and the appearance of ER during postnatal development corresponds to the appearance of overshoot in the VPA brain partitioning profile (Padowski and Pollack, 2011a).

While simulation experiments and post-hoc evaluation of data support the hypothesis that overshoot in $K_{p,\text{brain}}$ can result from extensive peripheral distribution, this hypothesis has not been tested prospectively. VPA, by virtue of significant extravascular sequestration arising from ER, presents an interesting model compound for testing this hypothesis. Several methods for interrupting ER, and thus manipulating the apparent volume of the peripheral “compartment” due to ER, are available. Excretion of VPA-glucuronide in bile can be reduced by inhibiting the protein responsible for that excretion, Mrp2 (Wright and Dickinson, 2004); exteriorizing bile flow surgically diverts VPA glucuronide from the recycling loop (Nakajima et al., 2004). Liberation of VPA from the glucuronide conjugate in the intestines can be inhibited by killing gut flora that
provide β-glucuronidase (Gott and Griffiths, 1987; Takasuna et al., 1996). Finally, reabsorption of VPA into portal blood could potentially be limited by adsorbing VPA-glucuronide and/or liberated VPA in the gut on activated charcoal (Neuvonen et al., 1983). Moreover, if the efficacy of such ER-interrupting strategies differs, it becomes possible to test whether the magnitude of overshoot is related to the degree to which peripheral sequestration is changed, as predicted by simulations (Padowski and Pollack, 2011a).

The objective of this study was to test the hypothesis that reduction of the apparent volume of a peripheral compartment will reduce overshoot in $K_{p,\text{brain}}$. Initial experiments were conducted to evaluate the ability of activated charcoal, pretreatment with antibiotics, or genetic Mrp2-deficiency to ablate VPA ER. Next, the effect of ER interruption on the $C_{\text{brain}}/C_{\text{serum}}$ ratio vs. time profile was evaluated. Finally, negative control experiments were conducted to determine whether these treatments influenced aspects of VPA brain exposure in a manner unrelated to the interruption of ER.
MATERIALS AND METHODS

Chemicals and reagents. [4,5-3H] VPA (51 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA) and [14C] inulin (2.1 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). VPA sodium salt, CCA, and streptomycin sulfate were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents used in this study were of the highest grade available from commercial sources.

Animals. Male Sprague-Dawley rats (225-300 g), male Wistar rats (225-275 g), and male Mrp2-deficient rats [TR-; Abcc2 (-/-), 225-250 g] were purchased from Harlan (Indianapolis, IN). With the exception of jugular vein-cannulated rats, which were housed individually following surgery, all rats were housed 2-4 per cage on a 12-hr light-dark cycle, with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Interruption of VPA enterohepatic recycling. All rats were anesthetized with i.p. ketamine (160 mg/kg) and xylazine (8 mg/kg), and a silicone rubber cannula (0.047 in o.d., 0.025 in i.d.) was implanted in the right jugular vein, at least 24 hr before the experiment. On the day of experimentation, a single dose of VPA (75 mg/kg i.p.) was administered to all rats (n=20). Three approaches were evaluated for ability to interrupt VPA ER. Rats in the first treatment group (n = 3, Sprague-Dawley) received a 200-mg/mL suspension of activated charcoal (100-400 mesh) in water via oral gavage. Two doses (1200 mg/kg each) were administered, the first at the time of VPA administration and the second 2 hr later. Rats in the second treatment group (n = 5, Sprague-Dawley) received penicillin G (2 mg/mL) and streptomycin (4 mg/mL) in drinking water for 3 days (125 mg/kg/day penicillin G, 250 mg/kg/day streptomycin). At 24 hr prior to experimentation, an additional dose of penicillin G and streptomycin (40 and 80 mg/kg, respectively) was administered by oral gavage. The third treatment group (n = 4) consisted of rats deficient in Mrp2 function due to an autosomal recessive mutation on locus Abcc2. Two control groups were evaluated concurrently: the first (n
= 4, Sprague-Dawley) received vehicle treatment only (water administered by oral gavage) as a strain-specific control for the charcoal- and antibiotic-treated animals, and the second (n = 4, wild-type Wistar) as strain-specific controls for the Mrp2-deficient rats. Both control groups received VPA doses as described above prior to experimentation. Upon VPA administration, 0.2-0.3 mL blood samples (replaced with an equal volume of saline) were collected from each rat via the implanted cannula at 0.25, 0.5, 1, 1.5, 2.5, 4, 5, 6 and 8 hr post-dose. Serum was harvested from blood and stored at -20°C until analysis.

**Effect of ER interruption on VPA brain partitioning.** The VPA ER interruption experiment (see “Results”) indicated that partial interruption of VPA ER was achieved by pretreatment with antibiotics, and nearly complete interruption of VPA ER was observed in TR-rats. Thus, an experiment to evaluate the relationship between the shape of the VPA $C_{\text{brain}} / C_{\text{serum}}$ ratio vs. time profile and the degree of VPA ER was conducted using antibiotic-vs. vehicle-treated Sprague-Dawley rats, and Mrp2-deficient vs. wild-type Wistar rats. Penicillin G and streptomycin were administered in drinking water for 3 days prior to experimentation, and as a bolus 24 hr prior to experimentation, as described above. At the time of experimentation, all rats received a single dose of VPA (100 mg/kg, i.p.). This dose was selected to produce concentrations at the upper end of the linear range for VPA disposition in serum in rats (Liu and Pollack, 1993). Trunk blood and brain were collected by decapitation at 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, 180, 240 and 360 min post-dose from all Sprague-Dawley rats, and at 5, 10, 15, 25, 40, 60, 90 and 120 min post-dose from all Wistar rats (n = 3 per time point). Serum and brain samples were stored at -20°C until analysis.

**VPA brain partitioning under steady-state conditions.** In order to evaluate whether manipulations to interrupt VPA ER altered the overall degree of VPA partitioning into brain tissue, a steady-state administration experiment was conducted using antibiotic- and vehicle-treated Sprague-Dawley rats, and Mrp2-deficient and wild-type Wistar rats. VPA was administered in drinking water (6 mg/mL) to all rats for 3 days (~700 mg/kg/day) prior to
experimentation. This exposure condition was selected to produce steady-state VPA serum concentrations less than 100 mg/L, within the linear range of VPA disposition in rats. Antibiotic-treated rats received a combination of VPA, penicillin G and streptomycin in drinking water for 3 days, followed by a bolus dose of penicillin G and streptomycin by oral gavage as described above. At the end of the 12-hr dark cycle following 3 days of drug administration, trunk blood and brain were collected by decapitation. Serum and brain samples were stored at -20°C until analysis.

**VPA brain uptake index.** To identify any potential nonspecific alterations in BBB permeability to VPA resulting from manipulations to interrupt VPA ER, a brain uptake index experiment was conducted to compare uptake of VPA relative to the reference compound inulin in antibiotic- and vehicle-treated Sprague-Dawley rats, and Mrp2-deficient and wild-type Wistar rats. Penicillin G and streptomycin were administered in drinking water for 3 days prior to experimentation, and as a bolus dose 24 hr prior to experimentation, as described above. At the time of experimentation, all rats were anesthetized with i.p. ketamine (160 mg/kg) and xylazine (8 mg/kg). Brain uptake index was determined as described previously (Hardebo and Nilsson, 1979). Briefly, the right common carotid artery was exposed and injected (< 0.5 sec) with a 0.2-mL bolus of saline containing 1 µCi ³H-VPA (50 µg/mL VPA) and 0.5 µCi ¹⁴C-inulin. At 10 sec post-injection, brain tissue was collected by decapitation.

**Quantitation of VPA.** VPA in serum and brain tissue samples was determined by gas chromatography with flame ionization detection (Shimadzu GC2014 series) with a method modified from Liu et al. (Liu et al., 2005). Briefly, serum samples (50 µL), acidified 1:1 (v/v) with 2 N HCl, were extracted with 100 µL ethyl acetate containing CCA as an internal standard. Brain samples were homogenized 1:1 (w/v) in saline and 250 µL of the homogenate was acidified with 50 µL of 12 N HCL prior to extraction with ethyl acetate containing CCA. A 1-µL aliquot of the organic layer was injected onto a wide-bore fused-silica capillary column (30 m x 0.53 mm id) with free fatty acid phase as the bonded stationary phase and helium as the carrier
gas. For serum samples, isothermal chromatography was used, with the column oven maintained at 180°C and the injector and detector at 200°C. For brain samples, these same column oven, injector and detector temperatures were maintained for 5 min post-injection. The column oven temperature then was increased to 230°C, and the injector and detector to 250°C. Analyte vs. internal standard peak area ratios were determined using GC Solution software (Shimadzu). The assay detection limit was 0.5 µg/g for brain and 0.1 µg/mL for serum, and standard curves were linear through the relevant range of concentrations for these studies.

**Quantitation of 3H-VPA.** Upon collection, brain tissue was digested immediately with 3 mL of tissue solubilizer (Solvable; PerkinElmer Life Sciences) overnight at 37°C. Samples were mixed with 15 mL of scintillation cocktail (Ultima Gold; PerkinElmer Life Sciences) and stored at room temperature for 12 hr prior to simultaneous determination of total radioactivity (³H and ¹⁴C) (PerkinElmer 1600TR liquid scintillation analyzer). Brain tissue concentrations were corrected for residual blood contamination as described previously (Dagenais et al., 2000).

**Data analysis.** Data are presented as mean ± SD. For evaluation of VPA ER interruption methods, AUC₀⁻₈hr was calculated by the trapezoidal method. The contribution of ER to AUC₀⁻₈hr was determined by subtracting from the total AUC₀⁻₈hr the AUC prior to ER, extrapolated through infinity using non-compartmental analysis (WinNonlin 5.0.1, Pharsight) as described previously (Pollack and Brouwer, 1991). Due to the short half-life (< 20 min) associated with the pre-ER phase of VPA disposition, in the absence of ER VPA concentrations would become negligible well before 8 hr. Therefore, the AUC pre-ER extrapolated through infinite time is identical to the AUC pre-ER that would be calculated through 8 hr.

\[
φ_{ER} = \frac{AUC_{0-8hr} - AUC_{pre-ER}}{AUC_{pre-ER}}
\]

Eq. 1
The degree of overshoot in the $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile in treated and untreated rats was compared by calculating the AUC above the last measurable $C_{\text{brain}}/C_{\text{serum}}$ ratio value for each treatment group, by the trapezoidal method. Integrated $C_{\text{brain}}/C_{\text{serum}}$ was calculated by normalizing the AUC for the concentration ratio by the total sampling time ($t$) for each strain (1.25 hr for Sprague-Dawley, 1.5 hr for Wistar).

$$\text{Integrated } C_{\text{brain}}/C_{\text{serum}} = \int_{0}^{t} (C_{\text{brain}}/C_{\text{serum}}) \, dt / t$$

Eq. 2

Brain uptake index was calculated as described in (Hardebo and Nilsson, 1979). In all cases, standard deviation on AUC measurements was calculated by the method of Bailer (1988). Statistical comparisons between groups were accomplished by t-test or ANOVA with post-hoc comparisons and correction for multiple comparisons, as appropriate, using SigmaStat software (Aspire Software International, Ashburn VA, USA). A p-value less than 0.05 was considered to be statistically significant. Deconvolution analysis of brain partitioning data was conducted with PCDCON software (http://www.boomer.org/pkin/soft.html).
RESULTS

**Interruption of enterohepatic recycling.** No interruption of VPA ER was observed in charcoal- vs. vehicle-treated Sprague-Dawley rats, based upon comparison of serum concentrations following a 75-mg/kg dose of VPA (Fig. 1a). Concentration-time profiles for the two groups were nearly superimposable, with appearance of a secondary peak in VPA concentration, a hallmark of the ER process, beginning at 2 hr and becoming maximal at approximately 4 hr post-dose. By subtracting the pre-ER AUC\(_{0-\infty}\) from the observed AUC\(_{0-8\text{hr}}\) for each treatment group, it was determined that the contribution of ER to the AUC\(_{0-8\text{hr}}\) did not differ significantly between the charcoal-treated (39.9 µg/mL*hr) and vehicle-treated (43.5 µg/mL*hr) groups; 91.8% of the AUC contributed by ER in vehicle-treated animals remained following charcoal treatment (Table 1). The fraction of the dose undergoing recycling through 8 hr (Φ\(_{\text{ER}}\); Eq. 1) also did not differ with charcoal treatment.

A partial interruption of ER was observed after antibiotic treatment. In antibiotic-treated rats, the beginning of a secondary VPA concentration peak occurred slightly later (4 hr) but at lower concentrations than in control Sprague-Dawley rats (Fig. 1b). This secondary rise in serum concentrations did not peak within the time frame of the experiment, but rose slowly through 8 hr, with concentrations remaining consistently lower than those in the control group. Antibiotic treatment substantially reduced the ER-contributed AUC relative to Sprague-Dawley controls (p<0.001), with antibiotic-treated rats exhibiting approximately 44% of the ER-contributed AUC observed in vehicle-treated animals (Table 1). The fraction of the dose undergoing recycling through 8 hr (Φ\(_{\text{ER}}\)) was also significantly reduced (p<0.001) with charcoal treatment. Antibiotic treatment also increased the pre-ER AUC significantly (Table 1), suggesting that systemic clearance of VPA in the absence of ER was reduced.

As illustrated in Fig. 1c, a nearly complete attenuation of ER was observed in Mrp2-deficient rats relative to wild-type Wistar controls. While no marked secondary VPA serum concentration peak was apparent in Mrp2-deficient rats, a minor contribution of ER (or some
other undefined distributional process) to the AUC$_{0-8hr}$ was calculated based on the functional definition underlying the area analysis, with ER accounting for 4.91 µg/mL*hr of the 94.4 µg/mL*hr AUC$_{0-8hr}$. Mrp2-deficient rats therefore exhibited only 10.4% of the ER-contributed AUC$_{0-8hr}$ observed in wild-type controls (Table 1). Loss of Mrp2 function increased the pre-ER AUC significantly (Table 1), suggesting that in the absence of ER, systemic clearance of VPA was reduced in Mrp2-deficient rats.

**Brain partitioning of VPA.** Because pilot studies indicated that brain concentrations fell below the lower limit of detection (0.5 µg/g) earlier than serum concentrations, the sampling schedule for this experiment was limited to 4 hr. Serum VPA concentrations (total and unbound) in antibiotic-treated and vehicle-treated control Sprague-Dawley rats, and in Mrp2-deficient and wild-type Wistar rats, are illustrated in Fig. 2. The beginning of a secondary VPA concentration peak was evident in Sprague-Dawley control animals by 120 min post-dose. A small apparent secondary rise in the concentration-time profile in antibiotic-treated rats was observed at 150 min post-dose, and no apparent secondary increase in concentration was observed for the Mrp2-deficient animals, consistent with the preceding experiment. The unbound fraction in serum varied from 0.3 to 0.5, and tended to increase with increasing concentration. Brain VPA concentrations determined in antibiotic- and vehicle-treated Sprague-Dawley rats, and in Mrp2-deficient and wild-type Wistar rats, following administration of 100 mg/kg VPA are depicted in Fig. 3.

Brain-to-blood partitioning of VPA, represented as C$_{\text{brain}}$/C$_{\text{serum}}$ vs. time, is illustrated in Fig. 4. Consistent with previous studies (Hammond et al., 1982; Golden et al., 1993), an early overshoot in the C$_{\text{brain}}$/C$_{\text{serum}}$ profile was apparent in vehicle-treated Sprague-Dawley rats (Fig. 4a). C$_{\text{brain}}$/C$_{\text{serum}}$ peaked at 10 min and then decreased throughout the remainder of the experiment, although the rate of decline decreased after approximately 45 min post-dose. In antibiotic-treated rats, an early overshoot also was observed, peaking within 10 min at a C$_{\text{brain}}$/C$_{\text{serum}}$ of 0.0810, which was slightly lower than that in control rats (0.0953). Notably, this
early peak in the concentration ratio decreased more rapidly in antibiotic-treated rats, and remained lower through 45 min, when the rate of change became low in both groups of animals.

Wild-type Wistar rats also exhibited an early overshoot in the $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile (Fig. 4b). This overshoot peaked slightly later than that in Sprague-Dawley rats, at 25 min, with a $C_{\text{brain}}/C_{\text{serum}}$ value of 0.0733. An overshoot was observed in the $C_{\text{brain}}/C_{\text{serum}}$ profile for Mrp2-deficient rats, with a magnitude of overshoot similar to that in the control animals (0.0726). However, as with the antibiotic-treated Sprague-Dawley rats, this overshoot was truncated, with the partition ratio decreasing more quickly and remaining lower than that in the corresponding ratio in ER-unimpaired animals.

In both of the ER-interrupted groups, a brief (spanning 10-25 min in the Wistar group, and 5-15 min in the Sprague-Dawley group) early peak in the $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile was observed. In order to evaluate the relationship between the appearance of this peak and the VPA ER process, deconvolution analysis was conducted. Deconvolution was selected as an analytical approach because of the possibility that multiple processes unrelated to ER might influence the kinetics of brain-to-serum partitioning of VPA. By deconvolving data obtained in ER-interrupted rats from data obtained in ER-intact animals, it is possible to isolate the specific effect of ER on brain partitioning kinetics. Ideally, the influence of ER on these $C_{\text{brain}}/C_{\text{serum}}$ vs. time profiles would be evaluated by comparing brain partitioning profiles in the presence compared to the complete absence of ER and all other peripheral distribution mechanisms that might contribute to the overshoot phenomenon. However, the fact that VPA ER interruption was nearly complete in the Mrp2-deficient Wistar rats, while antibiotic treatment in Sprague-Dawley rats provided only partial interruption of ER, provided an opportunity to test the hypothesis that the magnitude of overshoot in the VPA $C_{\text{brain}}/C_{\text{serum}}$ ratio is related to the extent of VPA ER. Deconvolving the $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile in ER-ablated (or ER-diminished) rats from the profile in ER-intact controls allowed direct assessment of this hypothesis. As illustrated in Fig. 5, the deconvolved $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile in Wistar rats (Mrp2-deficient vs. control)
evidenced a clear peak, consistent with overshoot, approximately 30 min post-dose. The peak in the deconvolution profile represents the peak of the overshoot phenomenon resulting specifically from ER, and is unrelated to the time at which the brain-to-serum concentration ratio is maximal, which may be confounded by additional processes unrelated to ER. Sprague-Dawley rats (antibiotic treated vs. controls) also evidenced a peak at the same time. The area bounded by the partitioning rate vs. time profile in Wistar rats was approximately two times larger than that in Sprague-Dawley rats, consistent with the fact that Mrp2-deficiency essentially ablated ER (reducing the apparent volume of the recycling compartment to zero) while antibiotic treatment reduced ER by only about 50% (reducing the apparent volume of the recycling compartment by 50%).

As a result of the rapid decrease of brain VPA concentrations below the limit of detection, it was not possible to evaluate the brain partitioning profile through the attainment of distribution equilibrium. Thus, the magnitude of overshoot in the $C_{\text{brain}}/C_{\text{serum}}$ vs. time profile was quantitated through the last time point at which brain concentrations remained detectable by calculating the AUC (normalized for sampling time) for $C_{\text{brain}}/C_{\text{serum}}$ vs. time above the last measureable value for each treatment group. This final observed concentration ratio was used as a surrogate for the eventual (at distribution equilibrium) partition coefficient. The resulting AUC values (±SD), as calculated by the method for destructive sampling (Bailer, 1988) through the final sampling time, are presented in Fig. 6. Antibiotic-treated Sprague-Dawley (p<0.05) and Mrp2-deficient Wistar (p<0.01) rats exhibited significantly reduced overshoot areas, 32.7% and 21.3% of the size of the overshoot area calculated in each control group, respectively.

**VPA brain partitioning under steady-state conditions.** In order to rule out a generalized effect of ER-interrupting treatments on VPA brain partitioning, $C_{\text{brain}}/C_{\text{serum}}$ values were calculated for each treatment group under steady-state conditions. As depicted in Fig. 7, antibiotic- vs. vehicle-treated Sprague-Dawley rats evidenced similar serum and brain concentrations, and did not differ significantly in $C_{\text{brain}}/C_{\text{serum}}$. Although serum VPA
concentrations were significantly higher in wild-type vs. Mrp2-deficient Wistar rats, brain concentrations were also moderately higher (data not shown). Thus, $C_{\text{brain}}/C_{\text{serum}}$ did not differ significantly between the two groups.

**VPA brain uptake index.** To determine whether ER-interrupting conditions affected BBB permeability to VPA, the initial rate of VPA uptake relative to uptake of the brain vascular space marker inulin was compared between each strain of ER-interrupted and control rats (Fig. 8). In antibiotic-treated rats, $^{14}$C-inulin uptake was slightly lower than in vehicle-treated Sprague-Dawley rats, while $^{3}$H-VPA uptake was similar. The ratio of VPA to inulin uptake did not differ statistically significantly between the two groups. Although $^{14}$C-inulin uptake was somewhat lower in Mrp2-deficient vs. wild-type Wistar rats, $^{3}$H-VPA uptake was also slightly lower. Thus, the ratio of VPA to inulin uptake did not differ significantly between these groups.
DISCUSSION

**Interruption of VPA ER.** The present study demonstrated the various efficiencies of three different approaches to experimental ablation of VPA ER in rats. Oral administration of charcoal has been reported to eliminate more than half of the ER-related absorption of VPA in humans (Neuvonen et al., 1983), and administration of activated charcoal can be used to treat acute VPA overdose (Manoguerra et al., 2008). However, activated charcoal did not impact VPA ER in this study. Failure of this treatment to reduce VPA ER may have resulted from saturation of binding sites on the activated charcoal due to relatively high VPA concentrations under the conditions of the current experiment. A brief in vitro experiment demonstrated that the capacity of activated charcoal to bind VPA was saturable within a relevant range of VPA concentrations (data not shown). Although an increased dose of charcoal may have been effective, it was not viewed as a viable solution for the purposes of this experiment.

The 3-day regimen of oral penicillin G and streptomycin was selected based upon several reports documenting loss of enzyme-mediated hydrolysis of a β-glucuronide metabolite after exposing rats to 1 or 2 mg/mL penicillin and streptomycin in drinking water (Takasuna et al., 1996). In the current study, this treatment significantly reduced ER-associated systemic VPA exposure (by 56% relative to controls). Although other antibiotic regimens, including neomycin, bacitracin, and tetracycline, are efficient inhibitors of ER for various compounds, including VPA (Gott and Griffiths, 1987; Kojima et al., 1998; Takasuna et al., 2006), penicillin G and streptomycin were chosen because these compounds can be administered in drinking water (i.e., they do not influence water consumption in rats). This regimen furnished a simple and noninvasive means of reducing ER relative to other antibiotic administration routes. It also provided an intermediate degree of VPA recycling, less than in untreated controls but more than in Mrp2-deficient rats (Table 1).

The impact of lack of Mrp2 function on VPA ER was examined in Mrp2-deficient Wistar rats. VPA glucuronide is a substrate of Mrp2 (Khewawoot et al., 2007), and Mrp2 deficiency
almost completely eliminates active secretion of the acyl glucuronide metabolite of VPA across the canalicular membrane, with a nearly complete reduction of choleresis associated with movement of VPA glucuronide into bile (Wright and Dickinson, 2004). Absence of Mrp2 function resulted in a nearly-complete abolition of the VPA AUC attributable to ER relative to Mrp2-competent controls (Table 1), consistent with a central role of Mrp2 in the biliary excretion of VPA-glucuronide.

The fact that interruption of VPA ER by antibiotic treatment was incomplete, while VPA ER was essentially ablated in TR- rats, was viewed as an experimental advantage in that it allowed us to address that hypothesis that the magnitude of overshoot in VPA brain partitioning is related to the magnitude of ER. This hypothesis is different from, but compatible with, the broader hypothesis that overshoot in brain partitioning of VPA is a consequence of peripheral “distribution” into a recycling unit.

**Brain partitioning of VPA.** As predicted based upon previous simulations (Padowski and Pollack, 2011b; Padowski and Pollack, 2011a) and the abundance of examples in the literature (Hammond et al., 1982; Liu et al., 1992; Golden et al., 1993; Liu and Pollack, 1993; Haberer and Pollack, 1994; Liu and Pollack, 1994; Padowski and Pollack, 2011a), overshoot in the brain-to-serum concentration ratio was observed in Sprague-Dawley and wild-type Wistar rats as an early peak, followed by a decline in the C<sub>brain</sub>/C<sub>serum</sub> vs. time profile (Fig. 4). Although antibiotic treatment and Mrp2-deficiency elicited moderate and nearly complete ablation of VPA ER, notable, although brief, peaks were observed at early time points in both ER-reduced groups. This observation suggests that mechanisms other than ER also contribute to the VPA overshoot phenomenon.

Previous simulation experiments (Padowski and Pollack, 2011a) predicted that increasing the apparent volume of a peripheral compartment would result in a proportionate increase in the duration of overshoot. Visual comparisons of the brain partitioning profiles in the current study are consistent with these predictions. In contrast, simulations predicted that a
“ceiling” would exist for the magnitude of overshoot. In the current study, the overshoot magnitude was similar in partially-ER-interrupted (Sprague-Dawley) and completely-ER-interrupted (Wistar) rats (Fig. 6), suggesting that overshoot magnitude is maximum at a peripheral volume represented by 50% of normal valproate recycling.

Due to deficiencies associated with simple visual comparisons, deconvolution analysis was performed to isolate the influence of ER on the brain-to-serum concentration ratio vs. time profile. Deconvolution abolished the initial early peak (Fig. 5), indicating that this peak was a component of both profiles and was independent of ER. This analysis suggests that a mechanism unrelated to ER may be responsible for the brief overshoot observed in the ER-interrupted rats. The mechanism leading to this non-ER related overshoot is unclear, and will require additional experimentation to explore. However, it could be related to a process as simple as distribution into another (non-ER-related) peripheral compartment, resulting in peripheral sequestration that is much less substantial than that associated with ER. Moreover, deconvolution analysis confirmed that incomplete interruption of ER (antibiotic treatment) had a smaller effect on the C_{brain}/C_{serum} vs. time profile than nearly-complete ablation of ER (Mrp2-deficient rats).

Although mathematical descriptors of the overshoot phenomenon have been developed previously (Padowski and Pollack, 2011b; Padowski and Pollack, 2011a), these metrics of the magnitude (peak C_{brain}/C_{serum} value as a percentage of the K_{p,brain} value) and duration (time at which the system reaches a stable plateau value, K_{p,brain}) of the overshoot could not be utilized in the current study. Both metrics depend upon confirming the plateau C_{brain}/C_{serum} value (i.e., K_{p,brain}, which is calculated after attainment of distribution equilibrium). Despite good sensitivity of the VPA assay, (0.1-0.5 µg/mL limit of detection), brain concentrations could not be quantified beyond 2 hr post-dose. A survey of reported VPA brain partitioning profiles failed to yield an example of attainment of distribution equilibrium after a bolus dose of VPA.
An additional consequence of the inability to determine the plateau $K_{p,brain}$ for VPA in the current study is the inability to measure total overshoot in each treatment group. Comparison of the measurable portions of the overshoot area between ER-inhibited and control groups necessarily over-predicts the ER-interruption-associated difference between the two groups, although, depending on the true $K_{p,brain}$ value, the magnitude of this over-prediction could be large or small. Although $K_{p,brain}$ for VPA was measured in all treatment groups under steady-state conditions, this value, while relatively consistent across groups, was not used as the baseline $K_{p,brain}$ value for comparison of overshoot area in the brain partitioning vs. time profiles because they were generated under fundamentally different conditions, and may have been affected by the well-established non-stationary kinetics of VPA (Arens and Pollack, 2001).

**Nonspecific effects of ER-interrupting conditions on VPA CNS kinetics.** The current study was intended to compare the effect of manipulation of the apparent volume of a peripheral pharmacokinetic compartment (as degree of ER) on the kinetics of brain partitioning. In order to rule out nonspecific effects of ER-interrupting conditions, two endpoints were evaluated. One endpoint was the steady-state $K_{p,brain}$ value. Although no experimental evidence in the literature would suggest that steady-state $K_{p,brain}$ values should differ in the presence vs. absence of ER, a previous simulation study (Padowski and Pollack, 2011a) illustrated several situations under which a small effect (typically <10% difference) might be observed. No statistical differences between $K_{p,brain}$ in ER-interrupted vs. control rats were observed in the present study, consistent with predictions. The other endpoint evaluated for potential nonspecific influence of the ER interruption was initial uptake clearance into brain, which was assessed with the brain uptake index technique. No significant differences were observed between antibiotic-and vehicle-treated Sprague-Dawley rats. Therefore, we can conclude that changes in the $C_{brain}/C_{serum}$ ratio vs. time profile were likely due to changes in peripheral distribution kinetics of VPA and not to non-specific effects of VPA translocation across the BBB.
Although VPA-glucuronide is a substrate for Mrp2 in liver, mediating biliary excretion of the conjugate (Wright and Dickinson, 2004), VPA does not appear to be a substrate for this transport system (Baltes et al., 2007; Luna-Tortos et al., 2010). Thus, we would not anticipate a direct influence of Mrp2-deficiency on the kinetics of brain-to-serum partitioning of VPA. This expectation is consistent with the results of both the steady-state (Fig. 7) and brain uptake index (Fig. 8) experiments. VPA does appear to undergo carrier-mediated translocation at the blood-brain and blood-CSF barriers by monocarboxylic acid (Terasaki et al., 1991; Takasuna et al., 2006) and/or organic anion transporting systems (Fischer et al., 2008). However, there is no evidence that either Mrp2-deficiency or antibiotic treatment would affect VPA transport into or out of the CNS by these systems or by either passive diffusion or electrostatic effects known to impede flux of anions across the endothelial cells that compose the BBB (Yuan et al., 2010).

Conclusions. The current study demonstrated that overshoot in the VPA \( \frac{C_{\text{brain}}}{C_{\text{serum}}} \) vs. time profile occurs in the presence of active ER, and that attenuation of ER (and thus reduction of peripheral distribution volume) reduced the degree of overshoot. The nearly complete ablation of VPA ER in Mrp2-deficient vs. wild type Wistar rats did not completely abolish overshoot. The persistence of a diminished magnitude of overshoot in the nearly-complete absence of ER may be due to non-ER-related peripheral sequestration of VPA (e.g., binding to proteins on tissue) or to another as-yet identified mechanism. Future simulation or experimental studies may be able to probe the origin of the brief, early overshoot peak in ER-interrupted animals. Finally, although the present study is consistent with simulations (Padowksi and Pollack, 2011), the identification of additional CNS-distributing compounds with significant ER-related overshoot would be desirable.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Padowski and Pollack.

Conducted experiments: Padowski and Pollack.

Contributed new reagents or analytic tools; Not applicable.

Performed data analysis: Padowski and Pollack.

Wrote or contributed to the writing of the manuscript: Padowski and Pollack.
REFERENCES


acid, and 1-methyl-1-cyclohexanecarboxylic acid in the bile-exteriorized rat. *Drug Metab Dispos* 20:810-815.


FOOTNOTES

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Reprint requests should be addressed to Dr. Gary M. Pollack at Washington State University College of Pharmacy, PO Box 646510, Pullman, WA 99164-6510 (gary.pollack@wsu.edu)

Current address:

Jeannie M. Padowski, School of Medicine, University of Washington, Seattle, WA
Gary M. Pollack, College of Pharmacy, Washington State University, Pullman, WA
FIGURE LEGENDS

Figure 1.
Serum VPA concentrations in (a) Sprague-Dawley control (filled symbols) and charcoal-treated (open symbols) rats, (b) Sprague-Dawley control (filled symbols) and antibiotic-treated (open symbols) rats, and (c) Wistar wild-type (filled symbols) and Mrp2-deficient (open symbols) rats following a 75-mg/kg bolus dose of VPA. Symbols indicate sample mean, and bars represent SD. Lines are included to emphasize temporal relationships.

Figure 2.
Total (left) and unbound (right) serum VPA concentrations in (a) control (filled symbols) and antibiotic-treated (open symbols) Sprague-Dawley rats and (b) wild-type (filled symbols) and Mrp2-deficient (open symbols) Wistar rats following a 100-mg/kg bolus dose of VPA. Symbols indicate sample mean, and bars represent SD. Lines are included to emphasize temporal relationships.

Figure 3.
Brain VPA concentrations in (a) control (filled symbols) and antibiotic-treated (open symbols) Sprague-Dawley rats and (b) wild-type (filled symbols) and Mrp2-deficient (open symbols) Wistar rats following a 100-mg/kg bolus dose of VPA. Symbols indicate sample mean, and bars represent SD. Lines are included to emphasize temporal relationships.

Figure 4.
Brain partitioning of VPA in (a) Sprague-Dawley control (filled symbols) and antibiotic-treated (open symbols) rats and (b) Wistar wild-type (filled symbols) and Mrp2-deficient (open symbols)
rats following a 100-mg/kg bolus dose of VPA. Symbols indicate sample mean, and bars represent SD. Lines are included to emphasize temporal relationships.

**Figure 5.**
Results of deconvolving \( \frac{C_{\text{brain}}}{C_{\text{serum}}} \) vs. time data in ER-interrupted rats from control animals. Solid line indicates Mrp2-deficient/control Wistar rats; dashed line indicates antibiotic-treated/control Sprague-Dawley rats. The deconvolved partitioning rate is the rate of change in the \( \frac{C_{\text{brain}}}{C_{\text{serum}}} \) ratio. The area under the partitioning rate vs. time profile is a unitless number that serves as a metric of the degree of overshoot, and was 7.95 x 10^{-4} for Mrp2-deficient/control Wistar rats and 3.38 x 10^{-4} for antibiotic-treated/control Sprague-Dawley rats.

**Figure 6.**
Area under the \( \frac{C_{\text{brain}}}{C_{\text{serum}}} \) vs. time profile normalized for sampling duration (Eq. 2) following a 100-mg/kg bolus dose of VPA. Filled bars indicate ER-competent control (vehicle-treated Sprague-Dawley and vehicle-treated wild-type Wistar) and open bars indicate ER-interrupted (antibiotic-treated Sprague-Dawley and Mrp2-deficient Wistar) rats. Error bars indicate SD; asterisk indicates statistically significant difference within each strain (p<0.001).

**Figure 7.**
Brain to blood partition ratios calculated following administration of VPA to steady-state. Filled bars indicate ER-competent control (vehicle-treated Sprague-Dawley and wild-type Wistar) and open bars indicate ER-interrupted (antibiotic-treated Sprague Dawley and Mrp2-deficient Wistar) rats. Error bars indicate SD.

**Figure 8.**
Percent uptake of the injected dose of (a) $^{14}$C inulin and (b) $^3$H VPA, and (c) ratios of VPA to inulin uptake, determined by the brain uptake index method. Error bars indicate SD.
### Table 1. Influence of experimental manipulations on the enterohepatic recycling of VPA in rats.

<table>
<thead>
<tr>
<th></th>
<th>Sprague-Dawley</th>
<th>Wistar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Charcoal</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
</tr>
<tr>
<td><strong>AUC 0-8 hr</strong></td>
<td>124 (6.54)</td>
<td>133 (14.3)</td>
</tr>
<tr>
<td><strong>AUC pre-ER</strong></td>
<td>80.1 (4.73)</td>
<td>92.8 (8.11)</td>
</tr>
<tr>
<td><strong>AUC ER</strong></td>
<td>43.5 (4.44)</td>
<td>39.9 (11.5)</td>
</tr>
<tr>
<td><strong>φ ER</strong></td>
<td>0.352 (0.0274)</td>
<td>0.298 (0.0605)</td>
</tr>
<tr>
<td>% Control</td>
<td>91.8</td>
<td>44.1</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD).

- AUC _0-8 hr_, area under the concentration-time profile calculated by the trapezoidal method; AUC _pre-ER_, area under the concentration-time profile prior to the secondary increase in concentrations and extrapolated through infinite time; AUC _ER_, difference between AUC _0-8 hr_ and AUC _pre-ER_; φ _ER_, fraction of the dose undergoing ER.

†Significantly different among Sprague-Dawley groups, ANOVA, p<0.05

**Significantly different from respective control, p<0.01

***Significantly different from respective control, p<0.001
Figure 1

(a) Graph showing serum VPA (mg/L) over time (hr) with error bars.
(b) Graph showing serum VPA (mg/L) over time (hr) with error bars.
(c) Graph showing serum VPA (mg/L) over time (hr) with error bars.
Figure 4

Graphs showing the ratio of $C_{\text{brain}} / C_{\text{serum}}$ over time (in minutes) for two different conditions labeled (a) and (b). The graphs display data points with error bars indicating variability.
Figure 5

Partitioning Rate (min\(^{-1}\) x 10^4)

Time (min)
Figure 6

Integrated $C_{\text{brain}}/C_{\text{serum}}$ for Sprague-Dawley and Wistar rats.
Figure 8

(a) % Uptake $^{14}$C Inulin

(b) % Uptake $^3$H VPA

(c) VPA : Inulin Ratio

Sprague-Dawley  Wistar