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Research report

Probenecid-inhibitable efflux transport of valproic acid in the brain parenchymal cells of rabbits: a microdialysis study

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Abstract

Delivery of valproic acid (VPA) to the human brain is relatively inefficient as reflected by a low brain-to-unbound plasma concentration ratio (≤0.5) at steady state. Previous pharmacokinetic studies suggested that the unfavorable brain-to-plasma gradient is maintained by coupled efflux transport processes at both the brain parenchymal cells and blood–brain barrier (BBB); one or both of the efflux transporters are inhibitable by probenecid. The present study in rabbits utilized microdialysis to measure drug concentration in the brain extracellular fluid (ECF) of the cerebral cortex during steady-state i.v. infusion with VPA alone or with VPA plus probenecid. Probenecid co-infusion elevated VPA concentration in the brain tissue surrounding the tip of the microdialysis probe to a greater extent than in the ECF (230% versus 47%). Brain intracellular compartment (ICC) concentration was estimated. In control rabbits, the ICC concentration was 2.8±0.28 times higher than the ECF concentration. Probenecid co-infusion elevated the VPA concentration in the brain tissue surrounding the tip of the microdialysis probe to a greater extent than in the ECF (230% versus 47%). Brain intracellular compartment (ICC) concentration was estimated. In control rabbits, the ICC concentration was 2.8±0.28 times higher than the ECF concentration. Probenecid co-infusion elevated the ICC-to-ECF concentration ratio to 4.2±0.44, which confirms the existence of an efflux transport system in brain parenchymal cells. The ECF-to-unbound plasma concentration ratio was well below unity (0.029), indicating an uphill efflux transport of VPA across the BBB. Co-infusion of probenecid did not have a significant effect on VPA efflux at the BBB as evidenced by a minimal change in the ECF-to-unbound plasma concentration ratio. This study suggests the presence of distinctly different organic anion transporters for the efflux of VPA at the parenchymal cells and capillary endothelium in the brain. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Epilepsy: anticonvulsant drugs

Keywords: Valproic acid; Efflux transport; Organic anion transporter; Blood–brain barrier; Brain parenchyma

1. Introduction

Valproic acid (VPA), a branched medium-chain fatty acid, is widely used in the treatment of epilepsy and several psychiatric disorders such as manic depression and panic disorder. Studies in epilepsy patients undergoing brain surgery or lumbar spinal tap have revealed that VPA concentration in the brain cortex or cerebrospinal fluid (CSF) is only 1/10th that of total or free plasma concentration [17,29,31,33]. Poor distribution of VPA to the brain has also been observed in several animal species [11,12,14,16,21,28]. Evidence to date suggests that the low brain-to-plasma concentration ratio of VPA is due to avid efflux transport of VPA from the central nervous system (CNS) [1,6].

Adkison et al. [1] investigated the brain clearance of VPA through simultaneous ventriculocisternal perfusion of radiolabeled VPA and i.v. infusion of VPA in rabbits. Co-infusion of probenecid, an organic anion transport inhibitor, was used to probe the presence of transport processes. Extraction of radiotracer during transit through the ventricular space was not affected by probenecid co-treatment. Probenecid co-infusion did elevate the concentration of VPA in the brain tissue by almost twofold compared to control animals. Similar effects were observed in a later study with the unsaturated metabolite of VPA, E-\textsuperscript{2}-VPA [26]. These results led to an initial conclusion that removal of VPA or E-\textsuperscript{2}-VPA from the
CNS occurs via a probenecid-sensitive efflux transport at the BBB, and does not involve organic anion transport at the choroid plexus. However, further analysis of the data suggested a more complex scenario [26]. A critical observation relates to a lower effect of probenecid on the steady-state concentration of VPA or E-Δ²-VPA in the perfusate outflow at the cisterna magna relative to that in the brain tissue; i.e. an elevation in the brain-to-cisternal concentration ratio. An equivalent percentage increase in cisternal concentration, i.e. no change in brain-to-cisternal ratio, is expected if the exchange of VPA or E-Δ²-VPA between the intracellular compartment (ICC) and the brain extracellular fluid (ECF) occurs strictly via passive diffusion, and if ventricular fluid drug concentration is reflective of ECF drug concentration. Compartmental modeling suggested that the change in brain-to-cisternal gradient could be explained by blockade of probenecid-sensitive efflux transport of VPA or E-Δ²-VPA at both the BBB and the parenchymal cells.¹ In other words, the putative transport systems at the brain parenchymal cell membrane and BBB act in concert to remove VPA and its 2-unsaturated analog from the brain.

Multiple anticonvulsant mechanisms have been proposed for VPA, and can be broadly divided into actions at the intracellular sites versus actions at the neuronal membrane or the extracellular site. A long-standing hypothesis for valproate’s antiepileptic effect is its ability to increase inhibitory GABAergic neurotransmission in critical regions of the brain, mainly as a result of increase in GABA biosynthesis or inhibition of GABA catabolism (see review [15]). It has also been proposed that VPA limits the bursting activity of neurons, most likely by interfering with use- and voltage-dependent Na⁺ channels (see review [8]). Hence, the existence of an intracellular-to-extracellular gradient within the brain parenchyma is important with respect to which of the proposed anticonvulsant mechanisms of VPA is more likely to operate in vivo.

There have been two reports of studies attempting to measure brain ECF concentration of VPA in rats following i.v. injection or rapid infusion of VPA: one by microdialysis [34] and the other by VPA-selective microelectrode [19]. In the present study, we applied intracerebral microdialysis to measure the steady-state ECF concentration achieved during continuous i.v. infusion of VPA in rabbits. Brain tissue surrounding the dialysis probe was sampled and assayed for VPA content, which allowed an estimate of ICC concentration. In an effort to pinpoint the locations of the putative VPA transporter(s), we examined the effect of probenecid co-infusion on the steady-state concentration gradient of VPA between the brain ECF and plasma water (i.e. across the BBB), and between the ICC and the ECF within the brain parenchyma (i.e. across the parenchymal cell membrane).

¹Parenchymal cells refer to the cell types present in the brain parenchyma excluding the blood–brain barrier (i.e. neurons and glial cells).

2. Materials and methods

2.1. Animals

This study was approved by the University of Washington Animal Care Committee. Ten male New Zealand white rabbits (weight range 3.1–4.1 kg) were randomly assigned to two study groups designated as either the control group (n = 5) or the probenecid treatment group (n = 5).

2.2. Chemicals

VPA, probenecid, and Trypan Blue were purchased from Sigma Co. (St. Louis, MO). [²H]VPA was purchased from Moravek Biochemicals (Brea, CA).

2.3. Surgical preparation

Each rabbit was anesthetized with halothane (0.8–1.2% inspired) and nitrous oxide (66% inspired) in oxygen and mechanically ventilated via a small animal respirator (Harvard Apparatus, Dover, MA) through a tracheotomy tube. Expired CO₂ was monitored by a Novametrix model 1250 Capnogard end-tidal monitor (Wallingford, CT) and maintained within the normal range of 32±5 mmHg [30].

With the animal in the supine position, the left and right femoral veins were exposed and cannulated for the separate infusion of VPA and probenecid. An ear vein was cannulated for the administration of the muscle relaxant, pancuronium (0.5 mg/h). A femoral artery was cannulated to allow blood sampling for drug assay and blood gas and pH determinations. The femoral artery cannula was also used to determine arterial blood pressure and heart rate. The electrocardiogram was monitored using needle electrodes inserted bilaterally at the shoulder and thighs. Rectal temperature was monitored by a thermistor probe and maintained at 37.5–38.5°C by a servo-controlled heat lamp. Gold cup electrodes were placed over the right frontal and parietooccipital cortices to monitor the electroencephalogram (EEG) using a Lifescan Brain Activity System (Diatek Corp., San Diego, CA) with a band pass of 0.5–29.9 Hz. This system used aperiodic analysis to convert the analog EEG signal into a set of digital parameters [10]. Computer (Zenith Data Systems, Glenview, IL) analysis of the EEG was performed using a Lifescan Research System program. For each 60-s interval, the following values were determined: the power (calculated as amplitude squared) and number of waves in each of the standard frequency bins (delta, 0.5–3.0 Hz, alpha, 8–12 Hz, and beta, 12–30 Hz), total hemispheric power, and the activity edge (the frequency below which 95% of the hemispheric activity was present). The EEG was monitored to insure adequate anesthesia during the experimental procedure. It also allowed a measure of the neuropharmacological effects of VPA against VPA plus probenecid.
The animal was turned to the prone position and its head was affixed to a stereotaxic frame. A midline scalp incision was made and the scalp and muscle tissue reflected. A burr hole was drilled in the skull at 8 mm anterior and 2 mm lateral to the intersection of the sagittal and coronal sutures to allow placement of the microdialysis probe in the cerebral cortex of the frontal lobe. A nick was made in the dura using the tip of a 20 g needle. With the aid of a stereotaxic device, the microdialysis probe (CMA/12, 3 mm length, 0.5 mm outer diameter, Bioanalytical Systems, West Lafayette, IN) was placed over the burr hole and was lowered until the tip of the probe made contact with the surface of the brain (any accumulated CSF and/or blood was removed from the burr hole using the tip of a cotton swab prior to lowering the probe into the hole). Upon contact with the brain surface, the probe was slowly advanced into the brain tissue until the entire surface of the probe membrane was surrounded by brain tissue. The distance of probe insertion from the brain surface was between 3.5 and 3.7 mm.

### 2.4. Microdialysis

The dialysate, which consisted of a degassed solution of \(^3\)H-VPA (1400 dpm/\(\mu\)l) and Trypan Blue (0.04%) in physiologic buffer (145 mM NaCl, 0.6 mM KCl, 2.0 mM \(K_2\)HPO\(_4\), 1.2 mM CaCl\(_2\), and 1.0 mM MgCl\(_2\), pH 7.4) [7], was loaded into a 1-ml gas-tight glass syringe. Retrodialysis with \(^3\)H-VPA allowed the determination of dialytic recovery of VPA (see Section 2.7). Trypan Blue was added to the dialysate to aid in visualizing the complete insertion of the probe membrane into brain tissue, to verify proper dialysate flow, and for marking the probe implantation site after probe removal. A Harvard syringe pump was used to pump the dialysate through 87 cm of fluoroethylene polymer (FEP) inlet tubing (i.d. 0.12 mm) (Bioanalytical Systems) at an elevated flow rate until the entire tube was cleared of any air bubbles. The flow rate was slowed to 30 \(\mu\)l/min and the inlet tubing was connected by a tube connector (Bioanalytical Systems) to the microdialysis probe, which had been prepared for use by soaking for 10 min in 70% ethanol and subsequently rinsed in physiological buffer. After flushing the probe with the dialysate, ~22 cm of outlet FEP tubing was connected to the probe. The dialysate was continuously perfused through the probe at 30 \(\mu\)l/min until all air bubbles had been cleared from both the tubing and the probe. The dialysate flow rate was then reduced to 1 \(\mu\)l/min and the probe was lowered into the brain.

The experiment began 45 min after the probe was inserted into the brain. At 10 min prior to the start of the experiment, a baseline arterial blood sample was drawn (5 ml). At the start of the study, the control group received an i.v. loading dose (7.5 mg/kg over 1 min) of VPA followed immediately by a constant rate i.v. infusion (125 \(\mu\)g/min/kg) that was designed to achieve a steady-state plasma target concentration of 60 \(\mu\)g/ml. The probenecid treatment group received the same VPA loading dose and constant rate infusion as the control group, along with an i.v. loading dose (60 mg/kg over 1 min) and a constant rate i.v. infusion (1 mg/min/kg) of probenecid. This probenecid dose was twice the dose used in our earlier studies [1] to ensure a significant increase (≥2-fold) in brain VPA concentration.

Dialysate samples were collected at 10-min intervals for 100 min after initiation of drug infusion for the analysis of unlabeled VPA. Beginning at 100 min, after steady state had been attained, dialysate samples were collected at 20-min intervals for six collection periods. Alternating 20-min collections of dialysate were analyzed for either unlabeled VPA or \(^3\)H-VPA. Arterial blood samples (1 ml) were taken at 15, 25, 35, 45, and 60 min and analyzed for plasma concentration of unlabeled VPA. Beginning at 90 min, the volume of each arterial blood sample was increased to 3 ml to allow for plasma protein binding determination as well as analysis of unlabeled VPA. Blood samples of 3 ml were collected at 90, 110, 130, 170, 190, and 210 min.

At 240 min after i.v. infusion of VPA or combined i.v. infusion of VPA and probenecid, the probe was removed from the brain. The halothane inhalation concentration was increased to 3.0%. The skin and subcutaneous tissue were removed to expose the top part of the skull. Saturated KCl was administered i.v. to euthanize the animal. An arterial blood sample was taken just before the injection of saturated KCl (i.e. a 240-min sample). A craniectomy was performed to expose the brain. The entire brain was removed from the cranium as quickly as possible (usually within 5 min), and rinsed with ice-cold saline. The Trypan Blue-marked tissue surrounding the probe implantation site was dissected out, divided into three portions of ~120 mg each, and immediately frozen on dry ice pending analysis of unlabeled VPA.

### 2.5. In vitro dialysis experiments

After removal from the rabbit brain, the microdialysis probe was placed in a beaker containing 3 \(\mu\)g/ml of VPA in 37°C dialysate buffer for the purpose of determining the relative recovery of VPA and the loss of \(^3\)H-VPA in vitro. Dialysate was continuously pumped through the probe at 1 \(\mu\)l/min. The probe was allowed to equilibrate in the VPA solution for 50 min prior to collection of dialysate samples. Dialysate samples were collected over 20-min intervals for six collection periods and were analyzed alternately for VPA or \(^3\)H-VPA. The in vitro loss and recovery determination was conducted to verify normal probe function.

### 2.6. Drug analysis

The free fraction of VPA in the plasma samples was
determined by ultrafiltration at 38°C using Centrifree® ultrafiltration devices (Amicon, Beverley, MA). The concentration of VPA in plasma and brain samples was analyzed by a capillary gas chromatographic assay described by Semmes and Shen [27]. A more sensitive assay, utilizing mass spectrometry, was performed for the measurement of subnanogram per ml VPA concentrations in dialysate samples. The dialysate assay used was a modified version of the assay previously described by Rettenmeier et al. [25]. Briefly, 10 μl of 1-methyl-1-cyclohexane carboxylic acid (MCCA) were added as the internal standard to each sample. Extraction of VPA and MCCA from the dialysate was accomplished by acidifying the sample with 500 μl of 1 N HCl, adding 3 ml of chloroform, and shaking for 15 min. The organic phase containing the VPA and MCCA was then dried over anhydrous magnesium sulfate powder and evaporated to ~50 μl under dry nitrogen. The VPA and MCCA were converted to trimethylsilyl (TMS) derivatives by adding 40 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heating to 65°C for ~40 min. The derivatized samples were then diluted to ~100 μl with chloroform and analyzed by gas chromatography/mass spectrometry (GC/MS).

Quantitative GC/MS analyses of VPA-TMS and MCCA-TMS derivatives were performed on a Carlo Erba 8000 gas chromatograph (Fisons Instruments, Manchester, UK) interfaced to a VG MD 800 Quadrupole mass spectrometer (VG BioTech, Cheshire, UK) using a fused silica capillary column (60 m×0.32 mm ID×0.25 μm film thickness) coated with a DB-1 stationary phase (J&W Scientific, Folsom, CA). The temperature of the ion source was 200°C. The temperatures at the injector and the GC interface were held at 250°C. Helium was used as the carrier gas, at a head pressure of 15 psi. The initial oven temperature was 40°C. After 1 min, the temperature was raised rapidly to 80°C. The temperature was then raised from 80 to 120°C at 2°C/min, followed by a 40°C/min gradient to 250°C. Molecular ions with mass-to-charge ratios of 201 and 199 were monitored that corresponded, respectively, to VPA and MCCA. Under these chromatographic conditions, VPA-TMS and MCCA-TMS eluted at ~15.7 min and 16.7 min, respectively. The concentration of VPA in samples was determined by comparing VPA-to-internal standard peak area ratios with standard curves, which were generated from the extracts of VPA standards.

Radioactivity of [3H]VPA in dialysate samples was assayed by liquid scintillation counting using a TriCarb 2000CA (Packard Instrument, Downers Grove, IL).

2.7. Data analysis

The method of retrodialysis was used to determine relative recovery of each probe. This method typically includes, in the perfusing dialysate, either a marker molecule of similar molecular weight and structure to that of the analyte, or radiolabeled analyte, as in the present study. The operating assumption is that the fraction of the marker molecule lost from the dialysate, as it is perfused through the probe, is equivalent to the fraction of the analyte recovered from the tissue surrounding the probe. The concentration of VPA in the ECF was calculated using the following equation:

\[
ECF = \frac{DC}{FL} \tag{1}
\]

where DC is the concentration of unlabeled VPA found in the dialysate sample and FL is the fraction of [3H]VPA lost from the dialysate. The ICC VPA concentration was estimated using Eq. (2):

\[
ICC = \frac{Br - fe \times ECF}{(1 - fe)} \tag{2}
\]

where Br is the concentration of VPA found in the brain tissue samples and fe is the fractional volume of ECF in cortical brain tissue, which has been determined to be ~0.18 [5].

Brain tissue-to-plasma concentration ratios of VPA were computed using the average total or free plasma concentration of unlabeled VPA at steady state. The steady-state plasma concentration in each rabbit was computed by averaging concentrations of samples taken from 90 min through 210 min. The mean steady-state dialysate and ECF values were computed by averaging dialysate samples and the corresponding ECF values from 90 min through 240 min, respectively. The steady-state ICC concentration of VPA for each rabbit was calculated using the mean ECF concentration and concentration of the brain tissue surrounding the dialysis probe sampled at the termination of the experiment.

All data are expressed as means±S.E. Data at serial time points from the control and probenecid-treatment groups were analyzed by repeated measure ANOVA, followed by post-hoc paired-wise comparisons. Statistical significance was defined as \( P \leq 0.05 \).

3. Results

3.1. Physiologic data

Table 1 compares the results of the physiologic data collected from the control and probenecid-treatment groups. Between-group comparisons indicated that there were no significant differences in any of the physiological parameters monitored. Within-group comparisons indicated a time-related decrease of blood pH and bicarbonate concentration in the probenecid group. Post hoc paired-wise comparisons of the data within the probenecid group indicated that only the mean pH at 220 min decreased significantly from baseline value.

The EEG data are displayed in Table 2. Baseline mean
value in the control group was not different from that in the PBD group; therefore, they were combined for the purpose of statistical analysis. Administration of VPA or VPA plus probenecid decreased power in the delta and theta frequency bins at the end of the study as compared to baseline values. Moreover, probenecid co-infusion increased power and number of delta waveforms, and decreased number of theta waveforms in comparison to values in the control group.

3.2. VPA pharmacokinetics

Fig. 1 illustrates the concentration–time profile of VPA in the plasma and dialysate during VPA infusion for one representative control rabbit. Plasma VPA concentration reached steady state within the first 25 min of the study and remained constant through 240 min. Cerebral dialysate VPA concentration reached steady state by 50 min, and remained relatively constant throughout the study.

3.3. Microdialysis probes

The mean fractional loss of \(^{[1]}\)H]VPA during retrodialysis in vivo was 0.28±0.014 for the control group and 0.26±0.014 for the probenecid treatment group (data not shown). The two mean values do not differ significantly, indicating that the probes used in each group had comparable dialytic characteristics.

Table 1
Comparison of physiologic parameters between the control and probenecid treatment groups (n=5 in each)\(^a\)

<table>
<thead>
<tr>
<th>Group/time</th>
<th>Mean arterial blood pressure (mmHg)</th>
<th>Heart rate (beats/min)</th>
<th>PaO(_2) (mmHg)</th>
<th>PaCO(_2) (mmHg)</th>
<th>pH</th>
<th>Arterial bicarbonate conc. (mEq/l)</th>
<th>Rectal temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>74±5</td>
<td>280±15</td>
<td>180±3</td>
<td>31±1</td>
<td>7.39±0.02</td>
<td>18.7±1.0</td>
<td>38.1±0.2</td>
</tr>
<tr>
<td>60 min</td>
<td>77±5</td>
<td>267±10</td>
<td>181±5</td>
<td>32±2</td>
<td>7.37±0.02</td>
<td>19.0±1.4</td>
<td>38.1±0.1</td>
</tr>
<tr>
<td>110 min</td>
<td>73±4</td>
<td>271±9</td>
<td>180±4</td>
<td>31±1</td>
<td>7.38±0.02</td>
<td>17.9±0.7</td>
<td>38.0±0.1</td>
</tr>
<tr>
<td>170 min</td>
<td>77±3</td>
<td>262±7</td>
<td>181±5</td>
<td>32±1</td>
<td>7.36±0.01</td>
<td>17.8±1.0</td>
<td>38.1±0.1</td>
</tr>
<tr>
<td>220 min</td>
<td>78±3</td>
<td>274±6</td>
<td>181±5</td>
<td>29±1</td>
<td>7.37±0.01</td>
<td>16.7±0.6</td>
<td>38.1±0.1</td>
</tr>
<tr>
<td>Probencid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>77±2</td>
<td>287±16</td>
<td>179±4</td>
<td>32±2</td>
<td>7.43±0.01 (_b^)</td>
<td>20.8±1.4</td>
<td>37.4±0.4</td>
</tr>
<tr>
<td>60 min</td>
<td>84±5</td>
<td>294±12</td>
<td>181±2</td>
<td>32±2</td>
<td>7.38±0.02 (_b^)</td>
<td>18.3±0.9</td>
<td>37.5±0.3</td>
</tr>
<tr>
<td>110 min</td>
<td>80±4</td>
<td>300±11</td>
<td>184±3</td>
<td>33±1</td>
<td>7.32±0.02 (_b^)</td>
<td>16.8±1.0</td>
<td>37.7±0.3</td>
</tr>
<tr>
<td>170 min</td>
<td>77±3</td>
<td>290±16</td>
<td>181±3</td>
<td>31±2</td>
<td>7.30±0.02 (_b^)</td>
<td>15.1±0.9</td>
<td>37.6±0.3</td>
</tr>
<tr>
<td>220 min</td>
<td>67±3</td>
<td>312±5</td>
<td>181±2</td>
<td>29±2</td>
<td>7.26±0.03 (_b^a)</td>
<td>13.0±0.7</td>
<td>37.7±0.3</td>
</tr>
</tbody>
</table>

\(^a\) Values represent mean±S.E.
\(^b\) Significant variation with respect to time, P<0.001.
\(^c\) Significantly different from baseline mean value, P<0.05.

Table 2
Electroencephalographic activity in the control and probenecid treatment groups\(^a\)

<table>
<thead>
<tr>
<th>Frequency bin</th>
<th>Combined baseline values (n=10)</th>
<th>Values at the end of the experimental period</th>
<th>Control (n=5)</th>
<th>Probencid (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta Power, (\mu)V×10(^7)/min</td>
<td>22.4±7 (_b^)</td>
<td>6.1±1.9 (_c^)</td>
<td>11.2±1.3 (_c^)</td>
<td>72±9 (_c^)</td>
</tr>
<tr>
<td>Waveform, number/min</td>
<td>47±9</td>
<td>41±8 (_c^)</td>
<td>84±21 (_c^)</td>
<td></td>
</tr>
<tr>
<td>Theta Power, (\mu)V×10(^7)/min</td>
<td>49.4±14 (_b^)</td>
<td>19.9±4.9</td>
<td>10.3±3.7</td>
<td>84±21 (_c^)</td>
</tr>
<tr>
<td>Waveform, number/min</td>
<td>150±23</td>
<td>169±25 (_c^)</td>
<td>84±21 (_c^)</td>
<td></td>
</tr>
<tr>
<td>Alpha Power, (\mu)V×10(^7)/min</td>
<td>9.3±3.1</td>
<td>3.5±1.1</td>
<td>3.0±0.7</td>
<td>89±16</td>
</tr>
<tr>
<td>Waveform, number/min</td>
<td>93±3.1</td>
<td>90±12</td>
<td>89±16</td>
<td></td>
</tr>
<tr>
<td>Beta Power, (\mu)V×10(^7)/min</td>
<td>4.9±2.1</td>
<td>1.7±0.7</td>
<td>2.0±0.7</td>
<td>152±48</td>
</tr>
<tr>
<td>Waveform, number/min</td>
<td>88±32</td>
<td>91±23</td>
<td>152±48</td>
<td></td>
</tr>
<tr>
<td>Total power, (\mu)V×10(^7)/min</td>
<td>87.2±24.3</td>
<td>31.2±7.9</td>
<td>26.6±2.9</td>
<td></td>
</tr>
<tr>
<td>Activity edge, Hz</td>
<td>12±3</td>
<td>12±1</td>
<td>13±2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Values represent mean±S.E.
\(^b\) Significant difference from experimental period, P<0.05.
\(^c\) Significant difference between groups, P<0.05.
Fig. 1. Typical time course of VPA concentration in the plasma and cerebral dialysate during a cerebral cortex microdialysis experiment in a control rabbit. At time zero, an i.v. priming dose (7.5 mg/kg) was administered and immediately followed by a constant rate infusion (125 µg/kg/min) that was maintained throughout the experiment. The respective mean±S.E. of steady-state plasma and dialysate concentration (from 90 min to the end of the study) was 52.8±2.9 µg/ml and 0.59±0.06 µg/ml.

The relative recovery of VPA in vitro was 0.41±0.04 for probes used in control animals, and was not significantly different from the relative recovery for probes used in probenecid-treated animals (0.38±0.03). Our fractions of VPA recovery in vitro are comparable to the in vitro VPA value reported by Golden et al. [9] using microdialysis probes of similar construction to those used in this study. The loss of [³H]VPA in vitro was 0.46±0.04 for probes used in control animals, which was not significantly different from the value for probes used in probenecid-treated animals (0.42±0.01). The in vitro relative recovery value for VPA was not statistically different from the in vitro loss of [³H]VPA. This was in keeping with the operating assumption of retrodialysis, i.e. the fractional loss of tracer molecule is equivalent to the fractional recovery of analyte. The relative loss of [³H]VPA in vivo was ~60% of the in vitro loss, most likely due to restricted diffusion of VPA in tissue as compared to water; this is commonly observed in microdialysis [23].

3.4. Plasma, brain, and dialysate distribution of VPA

A comparison of the mean steady-state concentrations of VPA in plasma, dialysate, and brain tissue between the control and probenecid treatment groups is presented in Table 3. An average steady-state plasma VPA concentration of 60.4±3.5 µg/ml was reached in the control animals. With concurrent i.v. infusion of probenecid, steady-state VPA concentration was 63.6±6.1 µg/ml, which was not significantly different from that in the control group. Determination of the plasma protein binding of VPA for both groups showed that the unbound fraction of VPA in the plasma was not significantly different between control and probenecid treatment groups, 0.19±0.03 versus 0.23±0.02. The mean steady-state concentration of unbound VPA in the plasma was calculated to be 11.3±2.2 µg/ml for control animals and 14.9±2.3 µg/ml for probenecid-treated animals. The difference between the group means was not statistically significant. The mean steady-state tissue concentration of VPA at the probe site was lower than in plasma in both groups of animals. Co-treatment with probenecid resulted in a greater than twofold increase in brain tissue VPA concentration, from 4.15±0.24 µg/g to 9.34±1.37 µg/g. Steady-state

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>Probenecid (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (µg/ml)</td>
<td>60.4±3.5</td>
<td>63.6±6.1</td>
</tr>
<tr>
<td>Free fraction</td>
<td>0.19±0.03</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td>Free plasma (µg/ml)</td>
<td>11.3±2.2</td>
<td>14.9±2.3</td>
</tr>
<tr>
<td>Brain tissue (µg/g)</td>
<td>4.15±0.24</td>
<td>9.34±1.37</td>
</tr>
<tr>
<td>Dialysate (µg/ml)</td>
<td>0.49±0.04</td>
<td>0.72±0.07</td>
</tr>
</tbody>
</table>

* Values represent mean±S.E.
* Significantly different from control, P<0.05.
VPA concentration in the dialysate was much lower compared to either plasma or brain tissue in control animals (0.49±0.04 μg/ml), and increased significantly by probenecid treatment (0.72±0.07 μg/ml).

3.5. Estimated ECF and ICC VPA concentrations

The estimated mean steady-state concentrations of VPA in the ECF and the ICC in the control and probenecid groups are shown in Table 4. The ECF VPA concentration, calculated directly from the dialysate VPA concentration, was 1.72±0.16 μg/ml for control animals, and was significantly higher in the probenecid-treated animals, at 2.78±0.36 μg/ml. The mean estimated ICC VPA concentration for control animals was 4.69±0.27 μg/ml, which was almost threefold higher relative to the ECF VPA concentration. Treatment with probenecid significantly increased the mean ICC VPA concentration to 11.6±1.6 μg/ml.

Mean concentration ratios of brain tissue-to-plasma, brain tissue-to-ECF, ECF-to-plasma, ICC-to-plasma, and ICC-to-ECF are also presented in Table 4. The mean ratio of VPA concentration in the cerebral cortical to that in total plasma was well below unity for both the control group (0.069±0.0024) and the probenecid group (0.16±0.024).

The difference in the mean ratios between the two groups was statistically significant. When corrected for plasma protein binding, the brain tissue-to-free plasma VPA concentration ratio was still below unity for control animals, at 0.41±0.062, and increased by nearly twofold to 0.70±0.087 upon treatment with probenecid. The tissue-to-ECF VPA concentration ratio indicated that brain tissue, when assayed as a homogenate, had a VPA concentration that was 2.48±0.23 times higher than the VPA concentration in the ECF fraction in control animals. In probenecid-treated animals, this ratio was significantly increased to 3.66±0.36.

To examine the equilibration kinetics of VPA across the BBB, the ECF-to-total plasma concentration ratio was calculated and was found to be somewhat higher for probenecid-treated animals (0.044±0.0048) than for control animals (0.029±0.0026). However, when plasma protein binding was taken into account, difference in the mean ECF-to-free plasma VPA concentration ratio between the control group (0.17±0.034) and the probenecid group (0.19±0.015) was no longer statistically significant.

The ICC-to-ECF VPA concentration ratio was calculated to examine the VPA concentration gradient between the brain ECF and ICC. On average, the control animals had an ICC VPA concentration that was 2.81±0.28 times higher than the extracellular VPA concentration. Treatment with probenecid increased this ratio to 4.24±0.44.

The ICC-to-free plasma VPA concentration ratio, which serves as an index of intracellular delivery, was computed to gain an appreciation of the overall effect of sequential exchange processes at both the parenchymal cell membrane and the BBB. The ICC-to-free plasma VPA concentration ratio was 0.46±0.068 for control animals and was significantly increased by probenecid treatment to 0.81±0.10.

4. Discussion

Microdialysis was used to measure the effects of probenecid on freely diffusible VPA concentration in the brain ECF of anesthetized rabbits under steady-state condition. Coupled with assay of drug concentration in brain tissue surrounding the probe site, this technique affords an estimate of the ICC concentration of VPA and provides an indirect means of studying the compartmentalization of drugs within the brain parenchyma.

The increase in brain concentration of VPA concentration by probenecid was in keeping with results from our previous study [1], in which rabbits were infused intravenously with VPA alone or in combination with probenecid, while simultaneously perfusing the ventricles with artificial CSF. We noted in this earlier study that upon co-infusion with probenecid the steady-state brain tissue concentration of VPA was elevated to a greater extent than was the cisternal outflow perfusate. This observation was unexpected if the exchange of drug between brain tissue and the ventricular fluid occurs strictly by passive diffusion across cell membrane barriers, through the ECF space, and across the ependyma. We had suggested that probenecid might have inhibited carrier-mediated transport of VPA from the ICC to the extracellular spaces, which resulted in intracellular retention of VPA and elevated VPA concentration in tissue relative to ventricular perfusate. Indeed our results from the present microdialysis study show that VPA is taken up by the cells of the brain parenchyma, and provide evidence in support of the existence of a probenecid-sensitive efflux transporter at the parenchymal cell membrane.

Parenchymal cells in the brain of control animals were
able to maintain a steady-state intracellular-to-extracellular concentration ratio of nearly 3.0. Apparent blockade of efflux transport with probenecid elevated this gradient to 4.2. The apparent concentration of VPA in the ICC relative to the ECF could be interpreted to mean that the parenchymal cells, or one or several of its constituent cell types (neurons or glial cells), have the ability to sequester VPA. Since VPA does not bind to brain homogenate constituents (unpublished data), sequestration can only occur by a concentrative, inward transport system at the parenchymal cell membrane. However, it is equally (or more) likely that the high ICC-to-ECF concentration ratio merely reflected a dynamic gradient created by facilitated release from the parenchymal cells followed by an efficient removal of VPA from the ECF via efflux transport at the BBB.

Our present finding indicates a very steep uphill gradient for VPA in the direction of ECF to plasma; i.e. 34:1 in terms of total plasma concentration or 5.9:1 in terms of free plasma concentration (see Table 4). This confirms our hypothesis of an active efflux transport at the BBB. In this regard, it is interesting that probenecid co-treatment appeared to have little effect on the ECF-to-free plasma VPA concentration ratio; efflux transporter at the BBB, unlike that at the parenchymal cell membrane, is not inhibitable by probenecid. This may imply that the transporter mechanisms at the two barrier sites are not identical. Alternately, probenecid may have inhibited both the influx (blood-to-ECF) transport as well as the efflux (ECF-to-blood) transport, such that there is minimal net perturbation in the bidirectional flux. It should be noted that some of probenecid’s effects may be related to the alteration in blood pH or bicarbonate. This is not likely, since the probenecid-induced elevation in dialysate concentration was observed throughout the study, whereas significant change in blood chemistry was only apparent toward the end of the 240-min infusion period.

Previous studies from our laboratory have already demonstrated the existence of carrier-mediated transport for the bidirectional movement of VPA across the blood–brain barrier [2, 20]. In addition to passive diffusion, the influx of VPA from blood into the brain is mediated by an organic anion exchanger that operates in a manner similar to the so-called para-aminohippurate (PAH) transporter at the renal proximal tubule, which recently has been cloned and characterized as hOAT1 [18, 24]. Studies with microvessels isolated from the rat brain provided evidence of energy-dependent uptake of VPA at the abluminal side of the brain capillary endothelium [20]. However, the molecular identity of this active, efflux transporter at the brain capillary endothelium is not known. Even less is known of the putative VPA transporter(s) in the parenchymal cells. Nilsson et al. [22] have shown that uptake of VPA into rat brain astrocytes in primary culture is mediated by a high affinity carrier system that is distinct from the GABA transporter. Whether a similar carrier transport system exists in neurons is not known. Also, the existence of outward, carrier-mediated transport of VPA in neurons and glial cells, as suggested by the effect of probenecid in the present microdialysis study, awaits further investigation.

Our present observation of higher VPA concentrations in ICC than in ECF differs from the conclusion reached in an earlier microdialysis study in rats. Wolf et al. [34] reported that 64% of VPA in the brain tissue was located in the ECF. However, the experimental technique differed between the two studies in several key respects. Firstly, the microdialysis probe was placed in the striatum of the rat in Wolf’s study, whereas the cerebral cortex of the rabbit was dialyzed in the present study. Given that a different mix of cell types is present at the two brain sites, regional differences may exist in the extent of parenchymal cell uptake. Secondly, there may be species differences in the transport mechanisms, which would account for the variation between rats and rabbits in the partitioning of VPA between the intracellular and extracellular compartments. Thirdly, retrodialysis was used to determine dialytic efficiency in the present study, whereas Wolf et al. calculated ECF VPA concentrations in two ways: by assuming the dialytic efficiency in vitro was identical to the in vivo efficiency, and by the method of Jacobson et al. [13] in which the extracellular concentration is estimated by measuring dialysate concentration at different flow rates and extrapolating to zero flow. Lastly, a critical difference between the two studies lies in the manner in which the VPA dose was administered. Wolf et al. [34] administered a single bolus dose of sodium valproate through a cannula implanted into the heart, and monitored the ECF VPA concentration over time as the blood VPA concentration declined rapidly. In contrast, blood or plasma as well as ECF concentration of VPA was maintained at steady state in the present study. It is quite conceivable that the uptake rate of VPA into parenchymal cells is sufficiently slow, such that only limited access into the parenchymal cells occurred in a non-steady-state situation. Also, the previous single dose study was complicated by transient high drug concentrations that led to saturation in VPA binding to plasma proteins and may have resulted in saturation of brain transport mechanisms. In contrast, maintenance of a constant ECF level of VPA in the present study allowed accumulation of VPA in the ICC, and avoided confounding pharmacokinetic complexities.

We believe results from a steady-state study are more indicative of VPA pharmacology during chronic drug therapy. This premise is supported by electrophysiologic studies conducted by Altrup et al. [3], which described early and late effects of VPA on snail neurons exposed to varying concentrations of VPA in vitro. Hyperpolarization accompanied by a reduction in frequency of paroxysmal depolarization shifts (PDS) over the first hour or so following extracellular drug application was thought to result from the action of VPA on the extracellular membrane site. The late effect (1–10 h post dosing), that of PDS decay, probably reflected an intracellular site of action, since intracellular application evoked the same response in an immediate fashion. Altrup et al. [3]...
concluded that the access of VPA to the intracellular sites of action is a relatively slow process. In fact, Löschner [15], in his recent review on VPA pharmacology, postulated that the sequence of early extracellular and late intracellular effects explains the well-known biphasic clinical effects of VPA in epileptic patients. We believe that the duration of VPA infusion in the present study (4 h) is sufficient to allow for the drug to fully equilibrate between the ECF and ICC of the rabbit brain. Moreover, the ECF-to-ICC distribution is crucial to our understanding of the in vivo importance of the various anticonvulsant mechanisms proposed for VPA.

Our incidental finding of EEG changes during VPA infusion or VPA and probenecid co-infusion warrants comment. The observation that VPA infusion decreased EEG power but not the number of waveforms is consistent with the EEG effect of VPA and Δ²-VPA reported previously [26]. In the present study, co-infusion of VPA and probenecid resulted in changes in both delta and theta waveforms that are qualitatively different from that observed with VPA infusion alone. The reported effects of probenecid on brain electrical activities are inconsistent. Bolander and Wahlstrom [4] reported that probenecid decreased the threshold for thiopental-induced EEG depression in rats. In contrast, Walker and Pratt [32] reported that probenecid did not change the incidence of low voltage electrical activity in unanesthetized fetal sheep. It is possible that the effect on theta waveform represented a pharmacodynamic interaction between VPA and probenecid. Furthermore, the effects of probenecid co-infusion could reflect a change in the pharmacology of VPA that occurred in response to the shift in intracellular-to-extracellular distribution of VPA in neurons. The idea of a qualitative change in the pharmacology of VPA as a result of drug–drug interaction at the level of membrane transporters is a novel concept that warrants further investigation. It is also possible that the effect on theta waveform may be secondary to probenecid's effect on blood pH (and possibly blood bicarbonate level).

In conclusion, microdialysis proved to be a powerful tool to investigate intracellular versus extracellular compartmentalization of VPA in the brain parenchyma. The concentration of VPA in the ICC relative to the extracellular pool suggests that intracellular anticonvulsant mechanisms (e.g. elevation in synaptic GABA pool) are largely responsible for the clinical efficacy of VPA. The results of this study, in combination with previous observations, also demonstrate a probenecid-sensitive efflux transport system in the parenchymal cells that works in tandem with the active efflux transport system at the blood–brain barrier in hindering an efficient delivery of VPA to the brain.

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