Pharmacokinetic–Pharmacodynamic Relationships of (2*S*,3*S*)-Valnoctamide and Its Stereoisomer (2*R*,3*S*)-Valnoctamide in Rodent Models of Epilepsy

Nina Isoherranen,[1] H. Steve White,[3] Brian D. Klein,[3] Michael Roeder,[4] José H. Woodhead,[3] Volker Schurig,[4] Boris Yagen,[2] and Meir Bialer[1,5]

Received March 21, 2003; accepted April 30, 2003

Purpose. Racemic valnoctamide (VCD) is a central nervous system-active drug commercially available in Europe. VCD possesses two chiral centers and, therefore, it exists as a mixture of four stereoisomers. The purpose of this study was to evaluate the anticonvulsant activity of two VCD stereoisomers in comparison with VCD (racemate), valpromide (VPD), and valproic acid (VPA) and to study their pharmacokinetic–pharmacodynamic relationships.

Methods. The ability of racemic VCD, (2S,3S)-VCD, (2R,3S)-VCD and VPD to block partial seizures was studied in the 6Hz psychomotor seizure model in mice and in the hippocampal kindled rat. The ability of (2S,3S)-VCD and (2R,3S)-VCD to prevent generalized seizures was evaluated in the maximum electroshock (MES) and subcutaneous metrazole (sc Met) seizure tests. The PK of (2S,3S)-VCD, (2R,3S)-VCD, and VPD was studied in the mice utilized in the 6Hz model.

Results. All of the tested compounds were effective in the models tested. No significant difference in ED_{50} values was observed but the plasma and brain EC_{50} values of (2R,3S)-VCD in the 6Hz model at 32 mA stimulation were 2-fold higher than the EC_{50} values of (2S,3S)-VCD. An excellent pharmacokinetic–pharmacodynamic correlation was found between the plasma and brain concentrations of the VCD stereoisomers and their anticonvulsant effect in mice. Stereoselectivity was observed in clearance, volume of distribution, and in brain-to-plasma AUC ratio at a dose of 25 mg/kg, but the difference disappeared at higher doses as the clearance of the stereoisomers decreased and their half-life increased. For (2R,3S)-VCD the brain-to-plasma AUC ratio doubled at the tested dose range, while it remained constant for (2S,3S)-VCD.

Conclusions. Racemic VCD, VPD, (2R,3S)-VCD, and (2S,3S)-VCD are effective anticonvulsants in animal models of partial seizures and are more potent than VPA. The more favorable brain penetration of (2S,3S)-VCD and its lower EC_{50} value in the 6Hz test provides one advantage over (2R,3S)-VCD as a new antiepileptic drug.

KEY WORDS: anticonvulsant activity; stereoselectivity; valproic acid; valnoctamide; valpromide; pharmacokinetics.

INTRODUCTION

Valnoctamide (VCD, valmethamide or 2-ethyl-3-methyl pentanamide, Fig. 1) is a chiral amide analogue of the widely used, broad-spectrum antiepileptic drug (AED) valproic acid (VPA, Fig. 1) and is an isomer of valpromide (VPD, Fig. 1) a central nervous system-active drug, which is the primary amide of VPA. Racemic VCD is an over-the-counter drug in several European countries and it has been used for four decades as a mild tranquilizer (1). Results from several studies have found racemic VCD to be an efficacious anticonvulsant in several animal models (2–4). However, none of these studies has addressed the issue of VCD stereochemistry. Consequently, the objective of this study was to investigate the anticonvulsant activity and pharmacokinetics (PK) of (2S,3S)-VCD and (2R,3S)-VCD after administration of the individual stereoisomers and compare them with racemic VCD, its isomer VPD, and analogue VPA. The pharmacokinetic–pharmacodynamic (PK–PD) relationships of (2S,3S)-VCD and (2R,3S)-VCD were established in mice.

VCD is a chiral compound with two stereogenic carbons in its molecule. It is commercially available as a mixture of four stereoisomers, i.e., a mixture of two enantiomeric pairs, hereafter referred to as racemic VCD. The absolute configuration of all four VCD stereoisomers has been reported (5) and the PK of the four individual stereoisomers has been characterized after administration of the racemic VCD to healthy subjects, epileptic patients (6), rats, dogs, and mice (7). After administration of the racemic VCD, (2S,3R)-VCD was found to have a higher clearance and a larger volume of distribution in comparison with the other VCD stereoisomers regardless of the species examined (6,7). The stereoselective PK of VCD in healthy volunteers and epileptic patients made it of great importance to study stereoselectivity in the anticonvulsant activity and the PK of the individual stereoisomers after their separate administration and to investigate whether after a chiral switch one of the stereoisomers would be preferred over the racemate as a new AED.

Despite the good anticonvulsant activity of racemic VCD in the maximum electroshock seizure (MES) model, subcutaneous metrazole (sc Met) seizure test (8) and in the pilocarpine model of focal epilepsy (4), no study has evaluated the anticonvulsant activity of the individual stereoisomers. Furthermore, the PK of the individual stereoisomers after their separate administration has not been previously studied. Consequently, the purpose of this study was to characterize the anticonvulsant profile of (2S,3S)-VCD (Fig. 1) and (2R,3S)-VCD (Fig. 1) in two models of partial epilepsy (i.e., 6 Hz seizures and the hippocampal kindled rat model) and in two generalized seizure models (i.e., the MES and sc Met tests). The PK of (2S,3S)-VCD and (2R,3S)-VCD in mice, including their brain-to-plasma ratio, was evaluated. The time–courses of concentration and effect were concomitantly determined. The obtained PK parameters were also compared to the PK of VPD in mice.

[1] Department of Pharmaceutics, School of Pharmacy, Hebrew University of Jerusalem, Jerusalem, Israel.

[2] Department of Natural Products and Medicinal Chemistry, School of Pharmacy, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem, Israel.

[3] Anticonvulsant Drug Development Program, Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, Utah.

[4] Institute of Organic Chemistry, University of Tuebingen, Germany. B.Y. and M.B. are affiliated with the David R. Bloom Centre for Pharmacy, The Hebrew University of Jerusalem, Israel.

[5] To whom correspondence should be addressed. (e-mail: bialer@md.huji.ac.il)

0724-8741/03/0800-1293/0

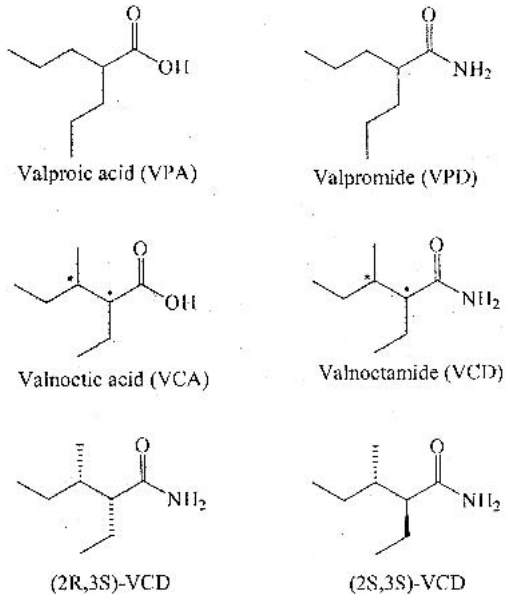

Fig. 1. Chemical structures of valproic acid (VPA), valpromide (VPD), valnoctic acid (VCA), valnoctamide (VCD), (2S,3S)-valnoctamide, and (2R,3S)-valnoctamide. The stars indicate the chiral center at position 2 and 3 of the VCD molecule.

MATERIALS AND METHODS

Chemicals and Test Solutions

VPD and racemic VCD were obtained from Sanofi Pharma International, Paris, France. (2S,3S)-VCD and (2R,3S)-VCD were synthesized by previously reported methods (5). Chlorofom was obtained from J&T Baker, Deventer, Holland. Double distilled water was used throughout the study. For all the animal experiments, the compounds were suspended in 0.5% methylcellulose and administered intraperitoneally (i.p.) to mice in a volume of 0.01 mL/g body weight and in a volume of 0.004 mL/g body weight either orally (p.o.), or i.p. to rats.

Animals

All of the animal experiments adhered to the principles of care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Utah. Adult male CF#1 albino mice (18–27 g) were obtained from Charles River (Wilmington, MA, USA) and Sprague–Dawley rats from Simonsen (Gilroy, CA, USA).

Anticonvulsant Activity

The potential efficacy of VPD, racemic VCD, (2S,3S)-VCD, and (2R,3S)-VCD in preventing partial seizures was assessed in the 6-Hz psychomotor seizure test in mice and in hippocampal kindled rats. (2S,3S)-VCD and (2R,3S)-VCD were also evaluated in the MES seizure test and in the sc Met model of generalized seizures. VPD and racemic VCD were also investigated in the mouse subcutaneous bicuculline (s.c. Bic) test. A drop of 0.5% tetracaine in saline was applied to the eyes of the animals assigned to any electroshock test (i.e., MES or 6-Hz stimulation) before the test.

The 6-Hz "psychomotor" seizures characterized by stun, forelimb clonus, twitching of the vibrissae, and straub tail were induced according to previously described procedures (9,10). Animals in which none of these characteristics of the psychomotor seizures were observed were considered protected. Efficacy was assessed at three different current intensities (i.e., 22, 32, or 44 mA at 6 Hz), which was delivered through corneal electrodes for a total duration of 3 s. The median effective dose (ED_{50}) was determined at these three different current intensities at the time of peak anticonvulsant effect.

For the mouse MES test, a supramaximal current (50 mA, 60 Hz, 0.2 s) was delivered through corneal electrodes. Animals not displaying tonic hind limb extension were considered protected. The chemical tests employed included the s.c. Met test for (2S,3S)-VCD and (2R,3S)-VCD and the s.c. Bic seizure test for VPD and racemic VCD. The s.c. Met and s.c. Bic tests measured the ability of the test compounds to provide complete protection against threshold seizures (5 s of clonic activity) induced by that dose of the chemoconvulsant agent that induces clonic seizures in 97% of animals (CD97). All of these tests were conducted according to the previously described methods (11,12).

The compounds were also tested for their ability to prevent the expression of kindled seizures in the hippocampal-kindled rat. For hippocampal kindling, a bipolar stimulating electrode was stereotactically implanted in the ventral hippocampus (AP –5.2, ML 4.9, DV –5.0 from dura, flat skull) of adult male Sprague–Dawley rats (250–300 g) under ketamine–xylazine anesthesia. The rats were kindled according to previously described procedures (13). Briefly, 1 week after implantation of the electrodes, the rats were stimulated with suprathreshold trains (200 μA for 10 s, 50 Hz) every 30 min for 6 h on alternate days for a total of 5 stimulus days. At least 1 week after the fully kindled state was reached, the afterdischarge duration (ADD) and behavioral seizure score (BSS) was reassessed and a single dose of racemic VCD, VPD, (2S,3S)-VCD or (2R,3S)-VCD was administered i.p. in a randomized parallel design and their effect on BSS and ADD after 200 μA stimulation was assessed. The behavioral seizure was scored according to the following criteria as originally described by Racine: stage 1, mouth and facial clonus; stage 2, stage 1 plus head nodding; stage 3, stage 2 plus forelimb clonus; stage 4, stage 3 plus rearing; stage 5, stage 4 plus repeated rearing and falling (14). Animals not displaying stage 4 or 5 seizures were considered protected from seizure generalization. The total ADD in sec was determined from the EEG recording.

The effect of racemic VCD, VPD, (2S,3S)-VCD, or (2R,3S)-VCD on the afterdischarge threshold (ADT) in hippocampal kindled rats was evaluated in comparison with VPA in rats kindled according to the above described procedure. On the day of the test, the afterdischarge threshold of each rat was determined by increasing the current intensity stepwise until the rat displayed an electrographic afterdischarge with a duration of at least 4 s. The initial stimulation was conducted at 20 μA and increased in 10 μA increments every 1–2 min until an afterdischarge was elicited. The afterdischarge threshold for each rat was determined again at 0.5, 1, 2, and 4 h after i.p. dosing of one of the test compounds. The ADD and BSS were recorded at each time point and compared to the control values obtained before drug administration. The criteria for seizure scoring was the same as

described above for kindled seizures. Minimal motor impairment was determined in CF #1 mice after i.p. administration by the rotorod procedure as described previously (11).

Determination of the Median Effective Dose (ED_{50}) and Concentration (EC_{50}) and Median Behaviorally Impairing Dose (TD_{50})

For the determination of the ED_{50} (or TD_{50}) by the respective anticonvulsant procedures, doses of the racemic VCD, VPD, (2S,3S)-VCD, or (2R,3S)-VCD were varied (n = 8 animals/dose) until at least three points were established between the dose level that provided protection in 0% and 100% of the animals tested. These data were then subjected to probit analysis (15), and the ED_{50} (or TD_{50}) and 95% confidence intervals were calculated.

The median effective concentration (EC_{50}) was determined by subjecting the plasma concentrations after a specific dose and the percent of animals protected after that dose to probit analysis (15). The minimum effective plasma and brain concentration capable to protect all of the animals tested from seizures was extrapolated from the concentration–response curve.

The protective index (PI) was calculated from the quotient of TD_{50}/ED_{50} (same species, vehicle, and route of administration). The stereoselective index (SI) was calculated as the ratio between the ED_{50} value between (2S,3S)-VCD and (2R,3S)-VCD.

Pharmacokinetic–Pharmacodynamic (PK–PD) Study

To assess the PK–PD relationships of the VCD stereoisomers tested the plasma and brain concentrations of (2S,3S)-VCD and (2R,3S)-VCD were determined for the same mice used in the 6 Hz test following i.p. administration of 25, 50, 75, and 100 mg/kg of the test compounds. At varying time points (i.e., 5, 15, 55, 115, 175, and 235 min after dosing), a group of mice (n = 2–8 for time point) was subjected to the 6 Hz seizure test using a stimulation intensity of 32 mA and the degree of seizure protection was recorded. After the seizure test, mice were anesthesized using ketamine–xylazine and at deep anesthesia (at time points 10, 20, 30, 45, 60, 90, 120, 180, and 240 min after drug administration) blood was withdrawn by cardiac puncture and transferred into a heparinized test tube. Plasma was separated by centrifugation and stored in –20°C until analysis. The animal was then perfused via the heart with phosphate-buffered saline. The brain was removed and immersed in liquid nitrogen immediately after collection and stored at –20°C until analysis. For comparison, a similar PK study was conducted for VPD after an i.p. dose of 60 mg/kg. At time points 10, 20, 30, 45, 60, and 90 min after dosing four mice at each time point were sacrificed and blood and brain samples were collected as described above.

Gas Chromatographic Assay of Valpromide and Valnoctamide

An HP 5890 Series II gas chromatograph equipped with a 6860 autosampler and an HP5971 mass selective detector and HPChemstation data analysis software was used. The chromatographic separation was obtained using an HP5 Trace analysis column (5% Phenyl Methyl Siloxane, 25 m, 0.33-μm film thickness, 0.20-mm i.d. Hewlett Packard, Palo Alto, CA, USA). The injector operated on splitless mode and was held at 200°C. The oven temperature program included initial temperature 60°C (2 min), gradient of 15°C/min till 120°C, hold time of 5 min then second gradient of 40°C/min till 180°C was obtained and then held for 2 min. He (99.99% pure) was used as a carrier gas with a head pressure of 50 kPa.

The mass spectrometer transfer line temperature was set at 280°C and the detector was operated on the selected ion mode scanning for ions m/z 57, 72, 87, 100, and 114. For the analysis of VCD stereoisomers VPD (50 mg/L) was used as an internal standard and for VPD analysis VCD (50 mg/L) was used as an internal standard.

Plasma Samples

Twenty microliters of plasma was mixed with 20 μL of internal standard solution and 0.5 mL of 1 M NaOH. The aqueous solution was extracted using 1 mL of chloroform and 1 μL from the chloroform phase was injected to the gas chromatography apparatus.

Brain Samples

To 50 mg of whole brain tissue, 50 μL of internal standard solution and 1 mL of 1 M NaOH was added and the tissue was homogenized. The homogenate was extracted using chloroform and the mixture was centrifuged. One microliter of the chloroform phase was injected to the gas chromatograph.

The analytical method was validated according to published guidelines (16). Calibration curves for the VCD stereoisomers and for VPD in plasma and brain were constructed using least squares linear regression analysis with nine points between 0.5 and 200 mg/L in plasma and in brain using 10 points between concentrations 0.5 and 200 mg/kg. The variation of the slope between days (n = 3 for plasma and n = 4 for brain) was <5%. The accuracy and precision were assessed at concentrations 1, 3.25, 12.5, 50, 100, and 200 mg/L in plasma samples with seven replicates at each concentration and the inter-day precision was below 10% for all concentrations and inter-day accuracy was between 91 and 114%. In brain samples the accuracy and precision were assessed at concentrations 1, 5, 25, and 50 mg/kg (n = 8) and at 100 mg/kg (n = 5) during 4 days. The interday precision was below 10% for all concentrations and the interday accuracy was between 90 and 115% at all concentrations. The limit of quantification in plasma and brain samples was 1 mg/L and 1 mg/kg, respectively, and the recovery from plasma samples was 85–90% and from brain samples 72–77%.

PK Calculations

PK parameters were calculated by the classic noncompartmental methods based on the statistical moment theory (17) using Winnonlin standard edition 1.1 software package. The area under the plasma concentration-vs.-time curve (AUC) was calculated by the linear trapezoidal method with extrapolation to infinity. The mean residence time (MRT) was calculated from the quotient AUMC/AUC, where AUMC is the area under the concentration time product vs. time curve from zero to infinity. The clearance (CL) was calculated from the quotient dose/AUC and the volume of distribution at steady state (V_{ss}) from the product of

CL*MRT assuming complete absorption after i.p. dosing. The terminal half-life ($t_{1/2}$) was calculated from the quotient ln2/β where β is obtained from the linear fit of the log-concentration versus time curve. The goodness of fit was evaluated using ordinary least square. The volume of distribution at the terminal phase, Vβ, was calculated from the quotient D/AUC*β. A bioavailability of 100% was assumed for the i.p. administration.

Statistical Analysis

Results are presented as either the ED_{50} (or EC_{50}) and 95% confidence intervals, or as mean ± SD. Differences between the potency of the stereoisomers was evaluated by use of the one-tailed Z-test. The upper and lower limit of the 95% confidence interval of the log transforms provided by the probit analysis were used to calculate the standard error for the ED_{50} values and the ED_{50} values were then compared to determine statistical significance. Reduction in the kindled rat behavioral seizure score as compared to the pre-dosing control value was evaluated using two-tailed Mann–Whitney test. Difference in the afterdischarge threshold and ADD were evaluated using a two-tailed *t* test. The variance of the obtained AUC values was calculated as previously described (18,19). In brief, the SD of each AUC was calculated using individual concentration data points used for AUC calculation with the trapezoidal method and the SEM was determined for each of these data points. The SEM was multiplied by the time difference corresponding to the trapeze used, divided by two and squared. This procedure was repeated for each trapeze and the results summed. To the above sum a term was added that is equal to the last time interval of the AUC halved, added to 1/β, squared and multiplied by the squared SEM (of the last measured concentration). The square root of the overall sum described above gives the SD of the AUC. The differences in the AUC values were compared using two-tailed Z-test. A p value < 0.05 was considered significant in all tests.

The SI was calculated for the PK and PD parameters by dividing the value obtained for (2S,3S)-VCD by the value of (2R,3S)-VCD. According to the general theory, an SI value >1.2 when the higher value is divided by the lower value, is considered an indication of stereoselectivity in the PK or PD parameter evaluated (20). As we used a constant (2S,3S)-VCD to (2R,3S)-VCD ratio, an SI value <0.8 or >1.2 consequently, considered a meaningful difference between the stereoisomers.

RESULTS

Anticonvulsant Activity in Mice

The anticonvulsant spectrum of activity of (2S,3S)-VCD, (2R,3S)-VCD, racemic VCD, and VPD was characterized in mice and the results in three models of partial and two models of generalized seizures are summarized in Table I in comparison with VPA. The doses producing minimal motor impairment (TD_{50}) by these compounds are also summarized in Table I. (2S,3S)-VCD and (2R,3S)-VCD were both effective at non-toxic doses in the s.c. Met and 6 Hz seizure tests. Both substances were also effective against MES seizures, albeit at behaviorally toxic doses. No stereoselectivity was found in the anticonvulsant activity (ED_{50} values) in any of the mouse models. In the 6-Hz test the anticonvulsant potency of the stereoisomers decreased as the stimulation intensity was increased from 22 mA to 32 and then to 44 mA.

Table I. Anticonvulsant Activity and Behavioral Impairment of Racemic VCD, (2R,3S)-VCD, (2S,3S)-VCD, and VPD after Intraperitoneal Administration to Mice

	MES	s.c. Met	6 Hz 22mA	6 Hz 32mA	6 Hz 44mA	Behavioral impairment
	ED_{50} (mg/kg) (95% confidence interval) {Protective index}					TD_{50} (mg/kg)
VCD(rac)	58 (41–71) {1.3}	32 (22–45) {2.4}		37 (26–50) {2.5}	67 (61–72) {1.1}	77 (69–88)
(2S,3S)-VCD	132 (117–149) {1.0}	69 (63–72) {1.9}	25 (15–40) {5.1}	33 (23–45) {3.9}	80 (62–105) {1.6}	128 (108–155)
(2R,3S)-VCD	119 (98–147) {1.1}	67 (60–74) {1.9}	19 (15–25) {6.7}	48 (32–62) {2.6}	67 (54–79) {1.9}	127 (113–143)
VPD	56 (51–64) {1.4}	55 (45–63) {1.4}	19 (11–27) {4.3}	57 (47–65) {1.4}	66 (29–87) {1.2}	81 (74–91)
VPA[a]	263 (237–282) {1.5}	220 (177–268) {1.8}	42 (16–69) {9.5}	126 (95–152) {3.2}	310 (258–335) {1.3}	398 (356–445)
Stereoselective Index[b]	1.1	1.0	1.3	0.7	1.2	1.0

[a] Data from references 10 and 12.
[b] Stereoselective index: The ratio between the ED_{50} value of (2S,3S)-VCD and (2R,3S)-VCD.

Anticonvulsant Activity in the Hippocampal Kindled Rat

The ability of the test compounds to block the expression of partial seizures and to prevent secondary generalization was studied in the hippocampal kindled rat in order to get an idea of the potential of the VCD stereoisomers in a model of repeated seizures and chronic epilepsy. Both suprathreshold stimulation and threshold stimulation were employed to fully evaluate the anticonvulsant spectrum in this model. The time course of the anticonvulsant effect after an i.p. dose (40 mg/kg) of the VCD stereoisomers, racemic VCD and VPD at a suprathreshold stimulation of 200 μA is shown in Figure 2. At the suprathreshold stimulation, (2S,3S)-VCD prevented seizure generalization whereas (2R,3S)-VCD did not have any significant effect on either behavioral or electrographic seizures. Racemic VCD also blocked the partial seizures.

The effect of the valproylamides on kindled threshold seizures and seizure severity in the hippocampal kindled rat was also investigated. The time course of the effect of (2R,3S)-VCD, (2S,3S)-VCD, on the seizure threshold and afterdischarge duration at threshold stimulation in the kindled rat is shown in Figure 2 in comparison to racemic VCD, VPD and VPA. At the dose of 40 mg/kg i.p. all tested compounds attenuated the partial seizures evoked by threshold stimulation and caused significant reduction in behavioral seizure score. In addition, (2R,3S)-VCD and racemic VCD significantly elevated the afterdischarge threshold in the kindled rat and both VCD stereoisomers significantly shortened the afterdischarge duration.

PK of (2S,3S)-VCD, (2R,3S)-VCD, and VPD

The PK of (2S,3S)-VCD and (2R,3S)-VCD was studied in mice to evaluate possible stereoselectivity in PK and to establish the PK-PD relationship. The plasma and brain concentration vs. time curves obtained in mice after i.p. administration of (2S,3S)-VCD and (2R,3S)-VCD at doses of 25, 50, 75, and 100 mg/kg are shown in Figure 3 together with plasma and brain concentration vs. time curves of VPD following i.p. administration of 60 mg/kg. The calculated PK parameters are summarized in Table II. The PK of VCD was stereoselective as well as dose dependent.

The brain AUC of (2R,3S)-VCD was approximately half of that of (2S,3S)-VCD after a dose of 25 mg/kg ($p < 0.05$), but there was no difference after 50 mg/kg dose ($0.1 > p > 0.05$) and higher doses ($p > 0.1$) and, consequently, (2S,3S)-VCD had >2 times higher brain to plasma AUC ratio than (2R,3S)-VCD. The brain-to-plasma AUC ratio for (2S,3S)-VCD was dose independent (0.31–0.37) whereas for (2R,3S)-VCD, a 2-fold increase in the brain-to-plasma AUC ratio was observed at the dose range studied (from 0.16 to 0.35). The brain-to-plasma concentration ratio at individual time points following the different doses varied between 0.18 and 1.02 for (2S,3S)-VCD with an average at 0.45 between 0.11 and 0.90 for (2R,3S)-VCD with an average at 0.38.

Significant changes were observed in the PK parameters of each VCD stereoisomer with increasing doses. The CL decreased for both stereoisomers between the doses of 25 mg/kg and 50 mg/kg and the V_{ss} of (2R,3S)-VCD also increased with increasing doses (Table II). The plasma half-life of both stereoisomers nearly doubled between a dose range of 25 mg/kg and 100 mg/kg.

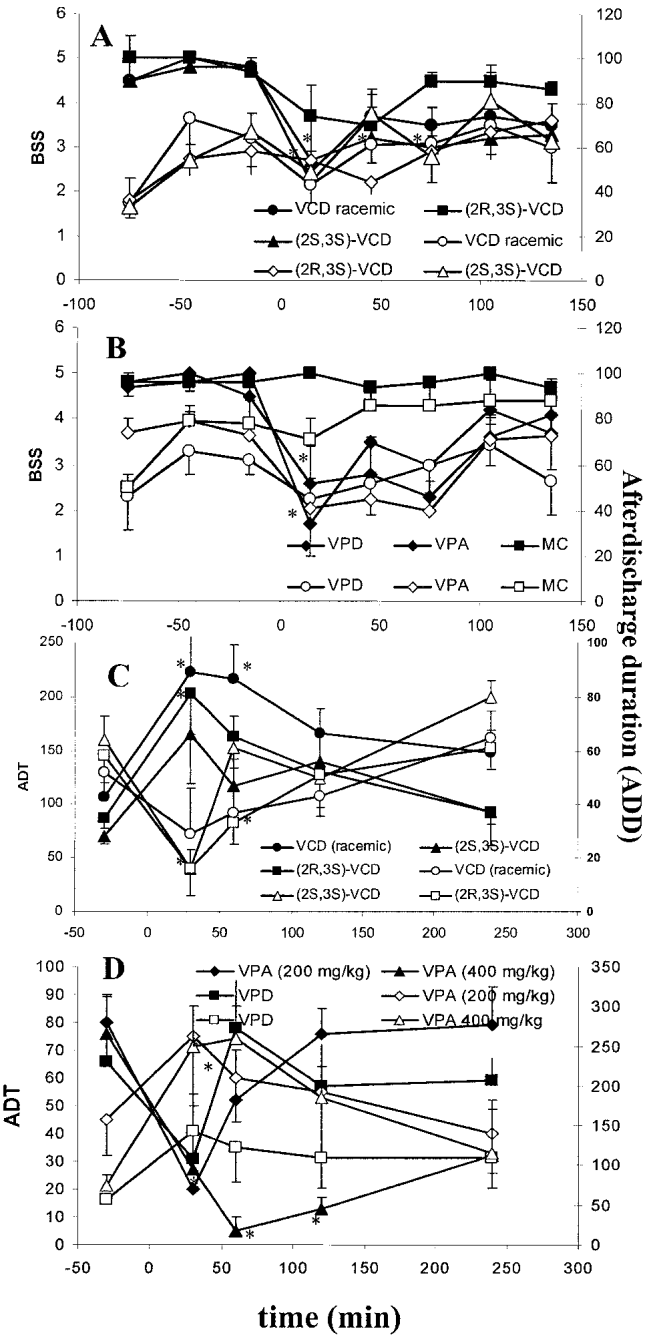

Fig. 2. Time course of the effect of racemic VCD (40 mg/kg), (2R,3S)-VCD (40 mg/kg), and (2S,3S)-VCD (40 mg/kg) (A) and VPD (40 mg/kg), VPA (250 mg/kg), and vehicle (methyl cellulose: MC) (B) on behavioral seizure score (BSS) and afterdischarge duration (ADD) at tested time points in hippocampally kindled rats at suprathreshold stimulation (200 μA) and of racemic VCD (40 mg/kg), (2R,3S)-VCD (40 mg/kg), (2S,3S)-VCD (40 mg/kg) (C) and VPD (40 mg/kg) and VPA (250 mg/kg) (D) on afterdischarge duration (ADD) and afterdischarge threshold (ADT) in the hippocampal kindled rats at threshold stimulation. Drugs were administered at time 0 min. The solid symbols are used for BSS and ADT and the open symbols for ADD. *indicates statistically significant difference compared to predosing baseline.

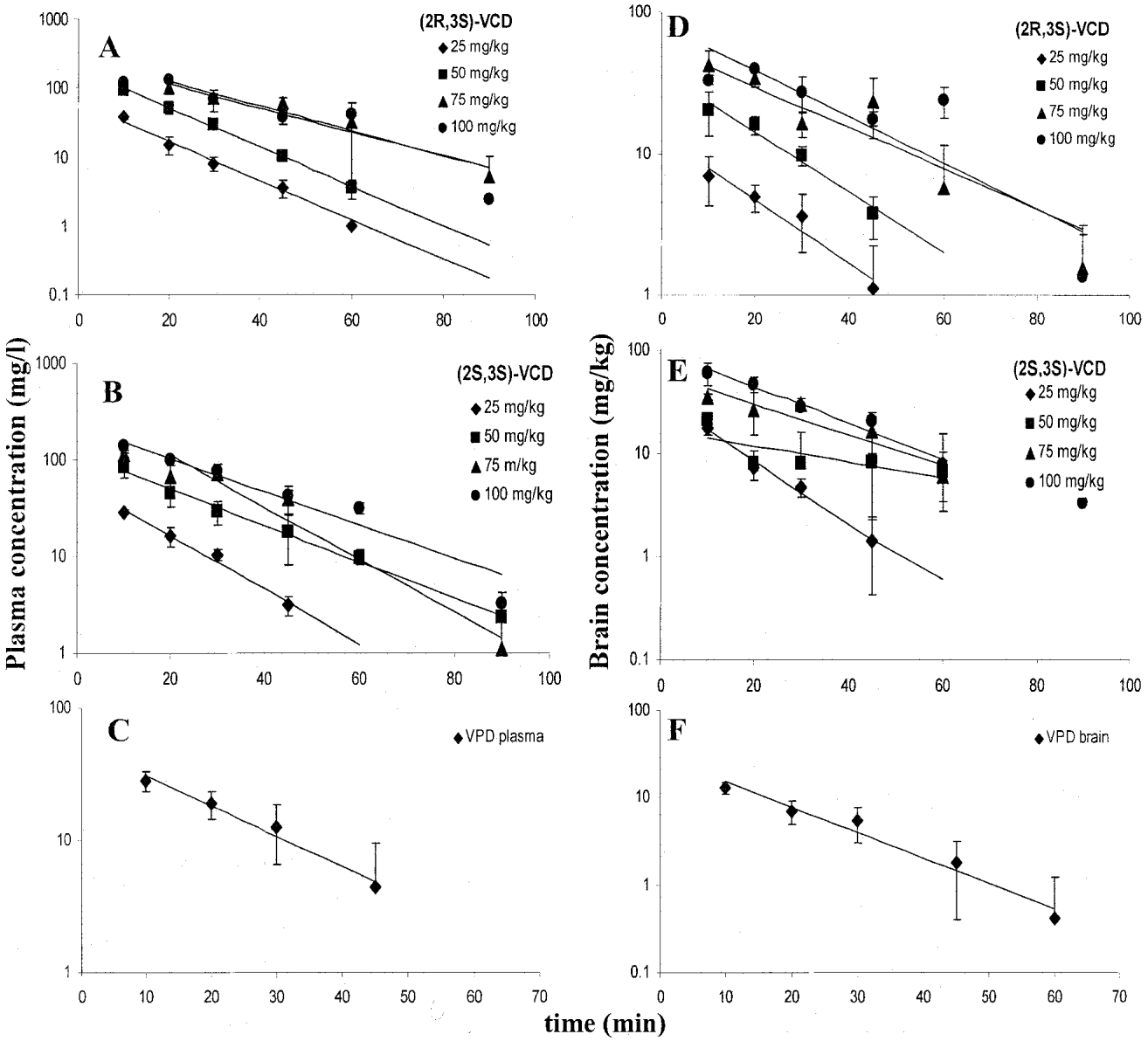

Fig. 3. Plasma concentration vs. time curves in mice for (2S,3S)-VCD (A) and (2R,3S)-VCD (B) after intraperitoneal (i.p.) administration of 25 mg/kg, 50 mg/kg, 75 mg/kg, and 100 mg/kg and VPD (60 mg/kg) (C) and brain concentration vs. time curves in mice for (2S,3S)-VCD (D) and (2R,3S)-VCD (E) after i.p. administration of 25 mg/kg, 50 mg/kg, 75 mg/kg, and 100 mg/kg and VPD (60 mg/kg) (F). The lines indicate the linear fit.

PK–PD Correlation

The animals used in the PK study were first tested in the 6-Hz model at 32 mA stimulation to enable PK–PD evaluation. The decline in plasma and brain concentrations with time correlated directly with a reduction in the antiepileptic efficacy. Figure 4 shows the concentration–response relationships after each dose for (2S,3S)-VCD and (2R,3S)-VCD. As such, the PK and PD could be combined and the brain and plasma EC_{50} values for the VCD stereoisomers were calculated. Unlike the ED_{50} values, the EC_{50} values in plasma and brain showed significant stereoselectivity (2S,3S)-VCD being significantly more potent than (2R,3S)-VCD ($p < 0.05$). The EC_{50} values of (2R,3S)-VCD were 50 mg/L (95% confidence interval 42–58 mg/L) and 15 mg/kg (95% confidence interval 12–19) in plasma and brain respectively. (2S,3S)-VCD had a plasma EC_{50} of 35 mg/L (95% confidence interval 27–43 mg/L) and a brain EC_{50} of 8 mg/kg (95% confidence interval 7–9 mg/kg). For (2S,3S)-VCD the minimum plasma and brain concentrations estimated to obtain 100% protection from the 6-Hz seizures (32 mA) were approximately 68 mg/L and 25 mg/kg, respectively. The minimum plasma and brain concentrations of (2R,3S)-VCD needed to obtain 100% protection were higher than the corresponding values for (2S,3S)-VCD (i.e., 125 mg/L and 41 mg/kg for plasma and brain, respectively).

DISCUSSION

All the tested valproylamides demonstrated a broad spectrum of anticonvulsant activity. No differences were found in the anticonvulsant potency between the investigated

Table II. Pharmacokinetic Parameters of (2R,3S)-VCD, (2S,3S)-VCD, and VPD Obtained after Intraperitoneal Administration to Mice

Dose (mg/kg)	CL (L/h/kg)	V_{ss} (L/kg)	$t_{1/2}$ (min)	MRT (min)	AUC_{plasma} (mg/L * min)	AUC_{brain} (mg/kg * min)	$t_{1/2\ brain}$ (min)	MRT_{brain} (min)	B/P[a]
(2S,3S)-VCD									
25	1.64	0.42	11 ± 2	16	916 ± 22	340 ± 19	10 ± 1	20	0.37
50	0.9	0.34	16 ± 2	21	3060 ± 153	945 ± 75	40 ± 11	64	0.31
75	0.98	0.36	15 ± 3	23	4575 ± 151	1466 ± 92	21 ± 5	34	0.32
100	1.03	0.43	17 ± 4	27	5826 ± 148	1992 ± 149	17 ± 2	30	0.34
(2R,3S)-VCD									
25	1.28	0.26	11 ± 1	12	1176 ± 30	193 ± 22	13 ± 1	25	0.16
50	1.04	0.26	11 ± 0.4	15	2881 ± 730	587 ± 155	14 ± 3	26	0.2
75	0.83	0.42	18 ± 1	31	5453 ± 451	1577 ± 151	21 ± 1	36	0.28
100	1.06	0.53	18 ± 4	30	5686 ± 208	1964 ± 148	20 ± 4	39	0.35
Stereoselective index									
25	1.3	1.6	1	1.3	0.8	1.8[b]	0.8	0.8	2.3
50	0.9	1.3	1.5[b]	1.4	1.1	1.6	2.8[b]	2.5	1.6
75	0.8	0.9	0.8	0.7	0.8	0.9	1	0.9	1.1
100	1	0.8	0.9	0.9	1	1	0.9	0.8	1
VPD									
60	3.6	1.19	13	19	954	302	10	22	0.32

[a] B/P; Brain-to-plasma AUC ratio.
[b] Significant difference between the stereoisomers.

amides but all of them were more potent than VPA. However their PI values in the various mouse models were similar to those of VPA and therefore, the overall anticonvulsant spectrum was similar to VPA. The great advantage of VCD is, however, the lack of teratogenicity (21) and probably also hepatotoxicity, which makes the VCD stereoisomers promising candidates as second generation VPA (22).

The ability of the investigated valproylamides to block the 6-Hz seizures and the kindled seizures suggests activity in patients with difficult to treat epilepsy because the 6-Hz model has been suggested to be a model of refractory epilepsy (10). Generally, in the 6-Hz model, there is a significant decrease in AED potency as the stimulation intensity is increased and in fact, all the old and new AEDs tested, except

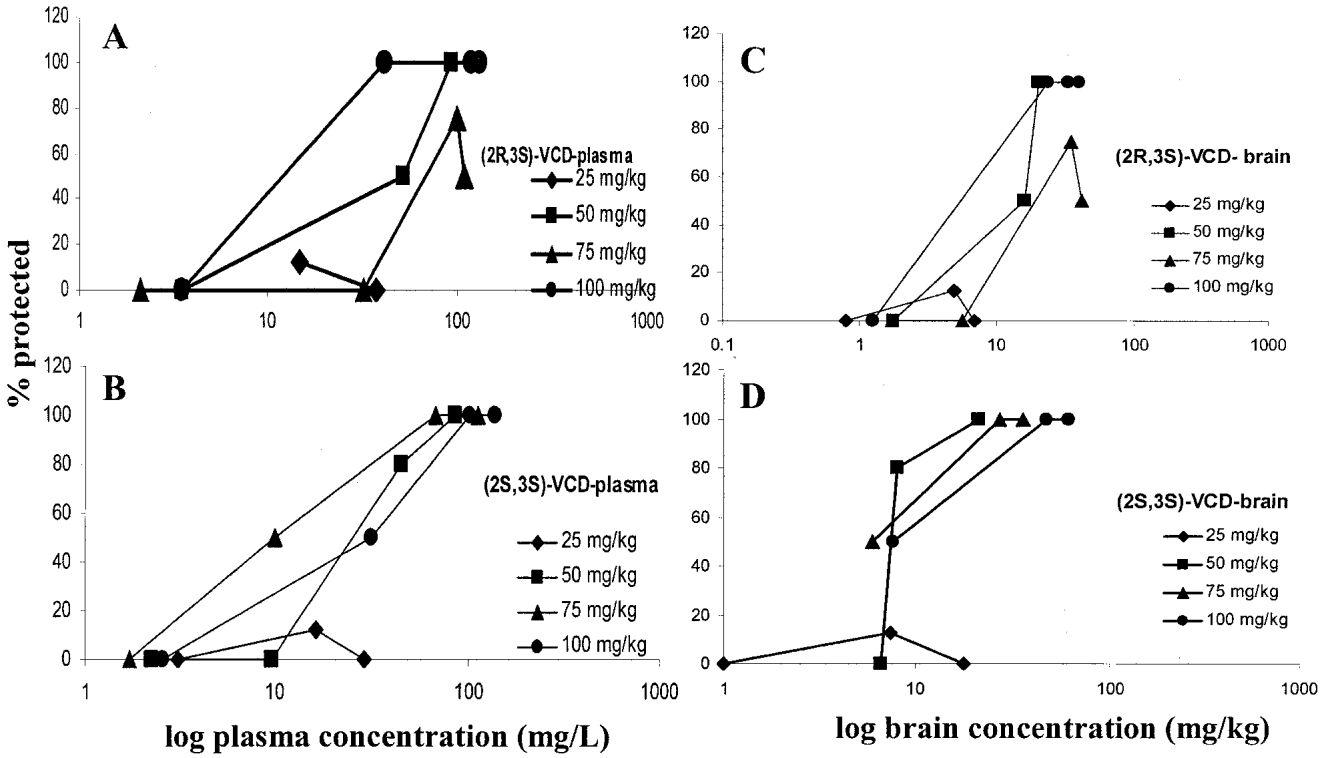

Fig. 4. The plasma concentration–response relationship of (2S,3S)-VCD (A) and (2R,3S)-VCD (B) and the brain concentration–response relationship of (2S,3S)-VCD (C) and (2R,3S)-VCD (D) in the 6-Hz model at 32 mA stimulation. The lines (from right to left) indicate the time course of the anticonvulsant effect (after each dose of 25, 50, 75, and 100 mg/kg) at the various time points after dosing (10, 20, 60, and 120 min).

high doses of levetiracetam (ED_{50} = 1089 mg/kg) and VPA (ED_{50} = 310 mg/kg) lose their efficacy at the 44 mA stimulus (10). The efficacy of the VCD stereoisomers at the three tested stimulus intensities in the 6-Hz model and the negligible increase in their ED_{50} values with stimulus intensity distinguishes them from most other AEDs. However, VPA and other amide derivatives of VPA were previously found to be effective at the 44 mA stimulus in the 6-Hz model at non-neurotoxic doses (23–25). This may indicate that VCD and other central nervous system-active valproylamides share a common mechanism of action.

A good correlation between the anticonvulsant activity of amide derivatives of VPA in the 6-Hz model and in the kindled rat has been found previously (23–25). To further evaluate this correlation, the efficacy of racemic VCD, (2S,3S)-VCD and (2R,3S)-VCD was studied in comparison to VPD and VPA in the hippocampal kindled rat after threshold and suprathreshold stimulation. The investigated amides showed efficacy in the kindled rat after a 40 mg/kg i.p. dose whereas VPA had to be given at higher doses (250–400 mg/kg) to obtain a similar effect (Fig. 2). The results obtained confirmed the activity of VCD stereoisomers in rodent models against partial seizures and also agrees with the data previously obtained by Lindekens et al., who showed that racemic VCD and VPD (100 mg/kg) were equally effective and more potent than VPA in preventing pilocarpine-induced limbic seizures (4). No significant differences in the anticonvulsant activity were found between the VCD stereoisomers or between racemic VCD, VPD, and VCD stereoisomers.

Because of the marked similarity in the PD characteristics of the studied valproylamides, PK considerations are a major factor when selecting compounds for further development. For example, in humans, VPD is a prodrug to VPA and as such it offers no clinical advantage over VPA (26,27) but the VPD isomer VCD undergoes very limited amide-to-acid biotransformation to valnoctic acid (VCA) in humans and acts as a drug of its own and not as a prodrug to its corresponding acid (28). Great differences were found also in mice between the PK parameters of VCD stereoisomers and VPD, despite their equal anticonvulsant potency. VPD displayed a three-times greater CL than the VCD stereoisomers and its V_{ss} was three times lower than that obtained for (2S,3S)-VCD and (2R,3S)-VCD. The greater CL and lower V_{ss} of VPD observed in this mice study (compared to the VCD stereoisomers) is in agreement with previous rat data, where VPD displayed a two times higher CL than racemic VCD (29). There was no difference in the brain-to-plasma AUC ratio of VPD and (2S,3S)-VCD over the entire dose range but, after a dose of 60 mg/kg i.p. brain VPD concentrations were significantly lower than the brain concentrations of (2S,3S)-VCD or (2R,3S)-VCD obtained after a dose of 50 or 75 mg/kg, indicating that VPD has a greater intrinsic potency at the site of action.

Central nervous system-active amide derivatives of VPA, such as racemic VCD, VPD, and propyl isopropylacetamide, are generally three to five times more potent than VPA (3,4,25,27). One suggested reason for their better anticonvulsant potency is their better brain penetration compared to VPA. This assumption has been supported by data from rats where the brain-to-plasma AUC ratio for VPD, VCD, and propyl isopropylacetamide was found to be unity (~1) and only 0.16 for VPA (25,29). However, no studies have assessed the brain penetration of the valproylamides in mice. The mice brain-to-plasma AUC ratio of VPD and the two VCD stereoisomers, was remarkably less than unity and therefore, it appears that the brain penetration of VCD and VPD is poorer in mice than in rats. A similar phenomenon has also been observed with *N*-methyl-tetramethylcyclopropyl carboxamide, an amide analogue of VPA, which in mice displayed a brain-to-plasma concentration ratio of 0.3–0.7 (24), but in rats the AUC ratio was equal to unity. Interestingly, as previously observed for *N*-methyl-tetramethylcyclopropyl carboxamide (24), the brain-to-plasma AUC ratio of (2R,3S)-VCD was dose dependent. This may suggest that these amide derivatives of VPA are substrates for active transport across the mouse BBB. This is further supported by the observation that there was no stereoselectivity between (2S,3S)-VCD and (2R,3S)-VCD in the plasma AUC values but stereoselectivity was found in brain AUCs, indicating that there is stereoselective transport to the brain. Further studies will be required to confirm the existence of active transport.

Theoretically, as VCD has a chiral center at the α-position to the carbonyl (C-2), a chiral inversion (racemization) is possible. Stereoselective analysis of plasma samples with individual VCD stereoisomers obtained 30 min after i.p. administration (analyzed using a previously described assay; reference 6) showed lack of chiral inversion. None of the other stereoisomers could be detected in the plasma samples analyzed following administration of either (2S,3S)-VCD or (2R,3S)-VCD.

The effect compartment of the VCD stereoisomers is in the brain and in this study, both the plasma and brain concentrations were measured as part of the PK–PD correlation. The brain-to-plasma concentration ratio was not constant as a function of time, indicating that the PK–PD model best suited for VCD would be an indirect link model where there is a temporal dissociation between the plasma concentration-time-course and the anticonvulsant effect, most likely caused by distributional delay (30). The concentrations in the effect compartment (brain) irrespective of time of measurement agreed with the response observed, and the anticonvulsant effect declined in parallel with the brain concentration suggesting that the PD response of VCD can be characterized as a direct response, i.e., the observed effect is determined by the effect site concentration without a time lag and the response mechanisms in the brain mediate the effect rapidly enough to directly account for changes in concentration at the effect site (30).

The dose-dependent PK observed in this study can be extended through the literature. The PK of racemic VCD and VPD has been studied previously after toxicologically relevant doses in mice. The CL of the VCD stereoisomers, obtained in a previous study in mice was 0.4 L/h/kg (7), a value >50% lower than the values obtained in this study for the individual enantiomers. However, this difference may be due to the higher dose (300 mg/kg of racemic VCD) used in the previous study and the decrease in CL with increasing doses. The plasma half-life obtained in this study (11–18 min) was four times shorter than the half-life obtained for the VCD stereoisomers in a previous study (1.0 h) after a dose of 300 mg/kg of racemic VCD (7). Yet, a clear dose dependency can be observed in the half-lives reported in the literature. After a dose of 430 mg/kg, racemic VCD displayed a half-life of 2.3 h (21) and consequently, it appears that the increase in the

half-life observed here is part of a general phenomenon of VCD–PK in mice. The half-life of VPD obtained in this study following a dose of 60 mg/kg (13 min) was also shorter than the half-life obtained for VPD in a previous study (3.4 h) after a dose of 430 mg/kg (21). Dose-dependent PK is a likely explanation for the difference observed between these studies in the major PK parameters.

In conclusion, this study showed that (2S,3S)-VCD, (2R,3S)-VCD, VPD, and racemic VCD are broad spectrum anticonvulsants. Because of its lower EC_{50} value in the 6-Hz model and better brain penetration, (2S,3S)-VCD appears as a more promising new AED than (2R,3S)-VCD. Also (2S,3S)-VCD displayed a constant volume of distribution over the entire dose range studied, suggesting that it has a more predictable PK than (2R,3S)-VCD. The efficacy of (2S,3S)-VCD in the 6-Hz model, in the kindled rat and in the s.c. Met model suggests that it may be an effective AED in patients suffering from partial and generalized seizures.

ACKNOWLEDGMENTS

The authors wish to thank James P. Stables and Dr. Harvey J. Kupferberg from the NIH epilepsy Branch for the anticonvulsant screening and Leslie Hart and Timothy Pruess from the Anticonvulsant Drug Development program at the University of Utah for their skillful technical assistance. The assistance of Eyal Sobol in performing the chiral assay of the VCD stereoisomers is greatly appreciated. This study was supported by the Horowitz fund of the Hebrew University of Jerusalem and by a grant (360-106.13/94) from the German-Israeli Foundation (GIF) for scientific research and development and NINDS contract I-NO1-NS-4-2311 (HSW and JHW).

REFERENCES

1. W. Stepansky. A clinical study in the use of valmethamide, an anxiety reducing drug. *Curr. Ther. Res.* **2**:144–147 (1960).
2. J. P. Chambon and A. Perio. Valnoctamide: pharmacological data suggesting anticonvulsant activity. *Neurosci. Lett.* **S5**:327 (1980).
3. A. Haj-Yehia and M. Bialer. Structure–pharmacokinetic relationships in a series of valpromide derivatives with antiepileptic activity. *Pharm. Res.* **6**:683–689 (1989).
4. H. Lindekens, I. Smolders, G. M. Khan, M. Bialer, G. Ebinger, and Y. Michotte. *In vivo* study of the effects of valpromide and valnoctamide in the pilocarpine rat model of focal epilepsy. *Pharm. Res.* **17**:1408–1413 (2000).
5. M. Roeder, O. Spiegelstein, V. Schurig, M. Bialer, and B. Yagen. Absolute configuration of the four stereoisomers of valnoctamide (2-ethyl-3-methyl valeramide), a potentially new stereospecific antiepileptic and CNS drug. *Tetrahedron Asymm.* **10**:841–853 (1999).
6. S. Barel, B. Yagen, V. Schurig, S. Soback, F. Pisani, E. Perucca, and M. Bialer. Stereoselective pharmacokinetic analysis of valnoctamide in healthy subjects and in patients with epilepsy. *Clin. Pharmacol. Ther.* **61**:442–449 (1997).
7. O. Spiegelstein, B. Yagen, G. Bennett, R. H. Finnell, S. Blotnik, and M. Bialer. Stereoselective pharmacokinetic analysis of valnoctamide- a CNS-active chiral amide analogue of valproic acid in dogs, rats and mice. *Ther. Drug Monit.* **22**:574–581 (2000).
8. A. Haj-Yehia and M. Bialer. Structure-pharmacokinetic relationships in a series of short fatty acid amides that possess anticonvulsant activity. *J. Pharm. Sci.* **79**:719–724 (1990).
9. W. C. Brown, D. O. Schiffman, E. A. Swinyard, and L. S. Goodman. Comparative assay of antiepileptic drugs by "psychomotor" seizures and minimal electroshock threshold test. *J. Pharmacol. Exp. Ther.* **107**:273–283 (1953).
10. M. E. Barton, B. D. Klein, H. H. Wolf, and H. S. White. Pharmacological characterization of the 6Hz psychomotor seizure model of partial epilepsy. *Epilepsy Res.* **47**:217–227 (2001).
11. E. A. Swinyard, J. H. Woodhead, H. S. White, and M. R. Franklin. General principles: experimental selection, quantification and evaluation of antiepileptic drugs. In R. H. Levy, R. H. Mattson, B. S. Meldrum, K. J. Penry, F. E. Dreifuss (eds.), *Antiepileptic Drugs,* 3rd ed. Raven Press, New York, 1989, pp. 85–102.
12. H. S. White, J. H. Woodhead, K. S. Wilcox, J. P. Stables, H. J. Kupferberg, and H. H. Wolf. General principles: discovery and preclinical development of antiepileptic drugs. In R. H. Levy, R. H. Mattson, B. S. Melddrum, E. Perucca (eds.), *Antiepileptic Drugs*, 5th ed. Lippincott Williams and Wilkins, Philadelphia, Pennsylvania, 2002, pp. 36–48.
13. E. W. Lothman, R. A. Salerno, J. B. Perlin, and D. L. Kaiser. Screening and characterization of antiepileptic drugs with rapidly recurring hippocampal seizures in rats. *Epilepsy Res.* **2**:367–379 (1988).
14. R. J. Racine. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroenceph. Clin. Neurophysiol.* **32**: 281–294 (1972).
15. D. J. Finney. *Probit Analysis*, 3rd ed. Cambridge University Press, Cambridge, United Kingdom, 1971.
16. V. P. Shah, K. K. Midha, J. W. A. Findlay, H. M. Hill, J. D. Hulse, I. J. McGilveray, G. McKay, K. J. Miller, R. N. Patnaik, M. L. Powell, A. Tonell, C. T. Viswanathan, and A. Yacobi. Bioanalytical method validation—A revisit with a decade of progress. *Pharm. Res.* **17**:1551–1557 (2000).
17. M. Gibaldi and D. Perrier. *Pharmacokinetics*, 2nd ed. Marcel Dekker, New York, 1982.
18. A. J. Bailer. Testing for the equality of area under the curves when using destructive measurement techniques. *J. Pharmacokin. Biopharm.* **16**:303–309 (1988).
19. J. Yuan. Estimation of variance for AUC in animal studies. *J. Pharm. Sci.* **82**:761–763 (1993).
20 R. H. Levy and A. V. Boddy. Stereoselectivity in pharmacokinetics: a general theory. *Pharm. Res.* **8**:551–555 (1991).
21. M. Radatz, K. Ehlers, B. Yagen, M. Bialer, and H. Nau. Valnoctamide, valpromide and valnoctic acid are much less teratogenic in mice than valproic acid. *Epilepsy Res.* **30**:41–48 (1998).
22. N. Isoherranen, B. Yagen, and M. Bialer. New CNS-active drugs which are second-generation valproic acid: can they lead to the development of a magic bullet? *Curr. Opin. Neurol.* **16**:203–211 (2003).
23. N. Isoherranen. J. H. Woodhead, H. S. White, and M. Bialer. Anticonvulsant profile of valrocemide (TV1901): A new antiepileptic drug. *Epilepsia* **42**:831–836 (2001).
24. N. Isoherranen, H. S. White, R. H. Finnell, B. Yagen, J. H. Woodhead, G. D. Bennett, K. S. Wilcox, M. Barton, and M. Bialer. Anticonvulsant profile and teratogenicity of N-methyl-tetramethylcyclopropyl carboxamide: a new antiepileptic drug. *Epilepsia* **43**:115–126 (2002).
25. N. Isoherranen, B. Yagen, J. H. Woodhead, O. Spiegelstein, S. Blotnik, K. S. Wilcox, R. H. Finnell, G. D. Bennett, H. S. White, and M. Bialer. Characterization of the anticonvulsant profile and enantioselective pharmacokinetics of the chiral valproylamide propylisopropyl acetamide in rodents. *Br. J. Pharmacol.* **138**:602–613 (2003).
26. M. Bialer, A. Rubinstein, J. Dubrovsky, I. Raz, and O. Abramsky. A comparative pharmacokinetic study of valpromide and valproic acid after intravenous administration in humans. *Int. J. Pharm.* **23**:25–33 (1985).
27. M. Bialer. Clinical pharmacology of valpromide. *Clin. Pharmacokinet.* **20**:114–122 (1991).
28. F. Pisani, A. Haj-Yehia, A. Fazio, C. Artesi, G. Oteri, E. Perucca, D. L. Kroetz, R. H. Levy, and M. Bialer. Carbamazepine-valnoctamide interaction in epileptic patients: *In vitro/in vivo* correlation. *Epilepsia* **34**:954–958 (1993).
29. S. Blotnik, F. Bergman, and M. Bialer. Disposition of valpromide, valproic acid, and valnoctamide in the brain, liver, plasma, and urine of rats. *Drug Metab. Dispos.* **24**:560–564 (1996).
30. H. Derendorf and B. Meibohm. Modeling of pharmacokinetic/pharmacodynamic (PK/PD) relationships: concepts and perspectives. *Pharm. Res.* **16**:176–185 (1999).