Pharmacokinetics and Antiepileptic Activity of Valproyl Hydroxamic Acid Derivatives

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Purpose. To explore the utilization of seven novel hydroxamic acid derivatives of valproic acid (VPA) as new antiepileptics.

Methods. The study was carried out by investigating the pharmacokinetics of two active compounds in dogs and pharmacodynamics (anticonvulsant activity and neurotoxicity) of valproyl hydroxamic acid and six of its derivatives.

Results. Three valproyl hydroxamic acid derivatives: valproyl hydroxamic acid—VPA-HA, N-(1-hydroxyethyl)-valpromide—HEV and N-methoxy valpromide, showed better anticonvulsant activity than VPA at the maximal electroshock (MES) test. The remaining four compounds, O-valproyl-VPA-HA, N-valproyl-O-valproyl-VPA-HA, N-(1-methoxyethyl) valpromide and N-(1,2-dihydroxylpropyl)-valpromide were found to be inactive. Therefore, only the pharmacokinetics of the active compounds VPA-HA and HEV was studied.

Conclusions. In contrast to valpromide (VPD) which is biotransformed to VPA, VPA-HA and HEV were found to be stable *in vivo* to the biotransformation of the amide to its corresponding acid. VPA-HA and HEV showed improved anticonvulsant activity over VPA because of their greater intrinsic activity and not due to better pharmacokinetic characteristics. This paper discusses the structural requirements for active anticonvulsant valproyl hydroxamic acid derivatives.

KEY WORDS: valproic acid; valproyl hydroxamic acid derivatives; pharmacokinetics; antiepileptic activity; structural requirements.

INTRODUCTION

Valproic acid—VPA (I) is one of the four major antiepileptic drugs (1). While it has a broad antiepileptic spectrum of activity, two serious (although rare) side effects, teratogenicity and hepatotoxicity, have been associated with VPA therapy (1,2). Comparative analysis of the anticonvulsant potency and safety margin, utilizing the classical rodent models for anticonvulsant screening, shows that VPA is less potent than the other three major antiepileptics: phenobarbital, phenytoin, and carbamazepine. Consequently, there is a substantial need to develop improved derivatives of VPA (3).

Valpromide—VPD (II), is the primary amide of VPA (4). Studies in mice and rats showed that VPD is a non-teratogenic entity (5,6) which is more potent that VPA (4). However, the advantages of VPD over VPA in rodents have no clinical implications, as in humans VPD serves as a prodrug to VPA (4). Therefore, there is a need to develop stable VPD analogues which, unlike VPD, will not undergo biotransformation to VPA. In previous studies, we synthesized and developed a series of VPD isomers and VPD analogues with different aliphatic and cyclic moieties (7–9). In the current study, several novel derivatives of VPD were synthesized (compounds III–IX, Fig. 1), in which the iso-octanoyl side chain was retained and the structural changes were made in the structure of the substituents attached to the nitrogen of the amide moiety. The current study was designed to investigate the *in vivo* performance of the amides III–IX, to determine the structural requirements for anticonvulsant activity in this series of compounds, and to assess the relationship between the pharmacokinetics and pharmacodynamics of the active compounds which emerged from this study.

MATERIALS

All chemicals and solvents used were purchased from Aldrich, Milwaukee, Wisconsin, USA and were of analytical grade. Compounds III–IX were synthesized according to the following methods and their chemical structures and purity was confirmed by elemental microanalysis and NMR (which will be provided by the authors upon request):

Valproyl Hydroxamic Acid—VPA-HA (III)

Hydroxyl amine HCl (25 g, 0.37 mole), in water and triethylamine (54 ml) were stirred in a 500 ml, round-bottomed

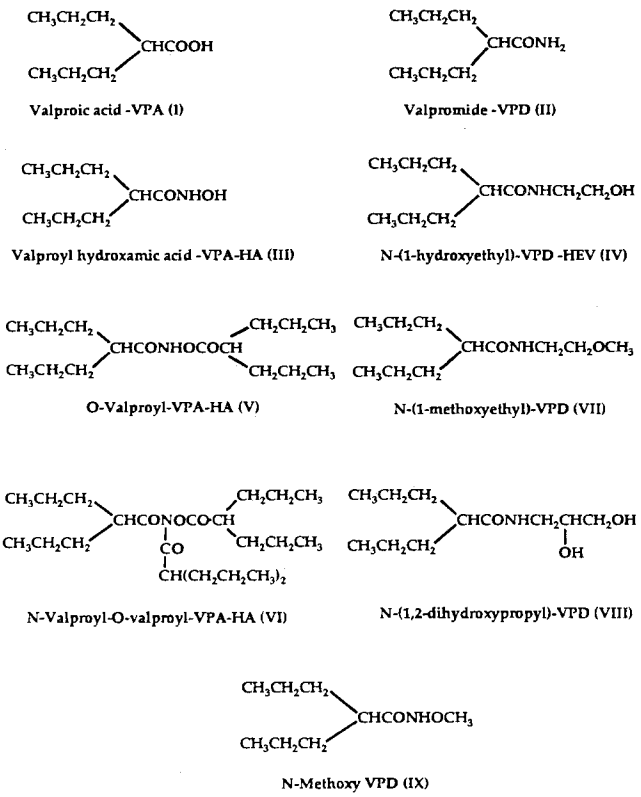

Fig. 1. The chemical structures of the valproic acid—VPA (I), valpromide—VPD (II), valproyl hydroxamic acid-VPA-HA (III) and N-(-hydroxylethyl)-VPD-HEV (IV), O-valproyl-VPA-HA (V), N-valproyl-O-valproyl-VPA-HA (VI), N-(1-methoxyethyl)-VPD (VII), N-(1,2-dihydroxypropyl)-VPD (VIII), and N-methoxy-VPD (IX).

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flask at 0°C for 30 min. Valproyl chloride (3 g, 18 mmole), in 30 ml of dry THF was added dropwise over a period of 15 min. The mixture was stirred at 0°C for an hour after the completion of the addition, then extracted three times with 100 ml of dichloromethane. The organic extracts were combined, dried with $MgSO_4$, filtered and evaporated. A crude white-brown solid was obtained. Recrystallization from a dichloromethane-hexane (2:3 v/v) mixture yielded 1.75 g (60%) of analytically pure VPA-HA. M.P.: 124–125°C.

2-Hydroxy-N-Ethyl Valpromide (HEV-IV)

Ethanol amine (6 ml, 0.1 mole), and 20 ml of dry dichloromethane were stirred in a 250 ml round-bottomed flask at 0°C for 15 min. Valproyl chloride (3.5 g, 20 mmole) in 20 ml of dichloromethane was added dropwise to this solution and stirring continued for an additional 30 min. The work-up of the reaction and isolation of the product was identical to that used for the synthesis of VPA-HA (III). Yield was 1.75 g (60%) of analytically pure HEV. M.P.: 58–60°C.

O-Valproyl, Valproyl Hydroxamic, Acid—O-Valproyl VPA-HA (V)

Hydroxyl amine HCl (13 g, 0.19 mole) and 80 ml of triethylamine were stirred in a 250 ml round-bottomed flask at 40°C for 30 minutes. The obtained salt triethylamine HCl was filtered. Valproyl chloride (6 g, 37 mmole) in 50 ml of dry dichloromethane was added dropwise to the above filtrate, kept at 0°C under nitrogen and stirring continued for 4 hr. The isolation of the products was identical to the procedure used for the synthesis of HEV (IV). Compound V was obtained by purification of the crude product on a silica gel column by flash chromatography using a 30% petrol ether/hexane mixture. An analytically pure O-valproyl VPA-HA (2.43 g, 46%) was obtained.

N-Valproyl, O-Valproyl, Valproyl Hydroxamic Acid—N-Valproyl, O-Valproyl-VPA-HA (VI)

Compound VI was synthesized in the same way as O-valproyl VPA-HA (V). The crude product was purified on a silica gel column using a mixture of 20% diethylether in hexane. An analytically pure, oily product (1.65 g, 30%) was obtained.

N-(1-Methoxyethyl)-Valpromide—N-1-Methoxyethyl-VPD (VII)

Compound VII was prepared from 2-methoxyl ethylamine (1.87 ml, 22 mmole) and valproyl chloride (3 g, 18 mmole), in a manner analogous to that described for the preparation of N- methoxy -VPD (IX). The crystallization from a mixture of dichloromethane and hexane yielded 1.67 g (50%) of white crystalline solid, M.P.: 60°–61°C.

N-(1,2-Dihydroxylpropyl) Valpromide—N-(1,2-Dihydroxypropyl)-VPD (VIII)

3-amino-1,2-propandiol (9.1 g, 0.1 mole) was added to 50 ml dry dichloromethane in a 250 ml round-bottomed flask. Valproyl chloride (3 g, 18 mmole) in 20 ml of dry dichloromethane was added dropwise at room temperature. The mixture was left overnight with stirring. The isolation of the products was identical to the procedure used for the isolation of VPA-HA (III). Yield was 1.67 g (42%) of analytically pure 1,2-dihydroxy-N-ethyl-VPD. M.P.: 65°C.

N-Methyoxy Valpromide—N-Methoxy-VPD (IX)

Methoxylamine HCl (3.9 g, 36 mmole) in 15 ml of triethylamine and 30 ml of dry dichloromethane were mixed in a 250 ml round-bottomed flask at 0°C. Valproyl chloride (5 g, 30 mmole) in 30 ml of dry dichloromethane was added dropwise over 10 min. The mixture was stirred at 0°C for three hours after the addition. The isolation of the product was identical to the isolation of VPA-HA (V). Yield was 1.6 g (30%) of analytically pure N-methoxy-VPD. M.P.: 52°C.

Anticonvulsant Activity and Neurotoxicity

Compounds III–IX have been screened in mice and rats for their anticonvulsant activity and neurotoxicity at the NIH Epilepsy Branch (10). The screening procedure involved the following: 1) the maximal electroshock (MES) test which measures seizure spread; 2) the subcutaneous pentylenetetrazol (sc Met) test which measures seizure threshold; and 3) the rotorod ataxia test which assesses minimal neurotoxicity.

Animals

The pharmacokinetic studies with VPA-HA (III) and HEV (IV) were carried out on six mongrel dogs ranging in weight from 20.5 to 23.5 kg. In a randomized crossover design, each dog was intravenously injected with compounds III and IV (533–611 mg in 1 ml of DMSO) at a dose equivalent to 20 mg/kg of VPA. Both the PK and anticonvulsant screening adhered to the "Principles of Laboratory Animal Care".

Protocol

Venous blood samples (6 ml) were collected via an indwelling catheter at specific intervals following injection (0, 5, 10, 20, 30, and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, and 16 hr). The plasma was immediately separated by centrifugation at 3000 g for 15 minutes and stored at −20°. Before each assay, the plasma was allowed to reach room temperature, vortexed, centrifuged, and the residual clot removed. Plasma concentrations of VPA-HA and HEV were assayed by the aforementioned GC assays. Urine was collected systematically at one hour intervals for 10 hr and at two-hour intervals from 10 to 16 hr after dosing by means of an indwelling catheter. The urine volume at each time interval was recorded and an aliquot was frozen at −20°C until analysis. Urine levels of HEV and its potential metabolites HEV-glucuronide and VPA (I) where assayed by GC.

Assay for VPA-HA (III)

Plasma (0.5 ml) (containing VPA-HA) internal standard solution (10 μl of HEV solution, 1 mg/ml in chloroform) and 50 μl of HCl 1 N was vortexed, followed by the addition of 4 ml of tert. butyl methyl ether and by 30 seconds of additional vigorous vortexing. The mixture was centrifuged for 10 minutes at 3000 g and the organic phase was separated and evaporated

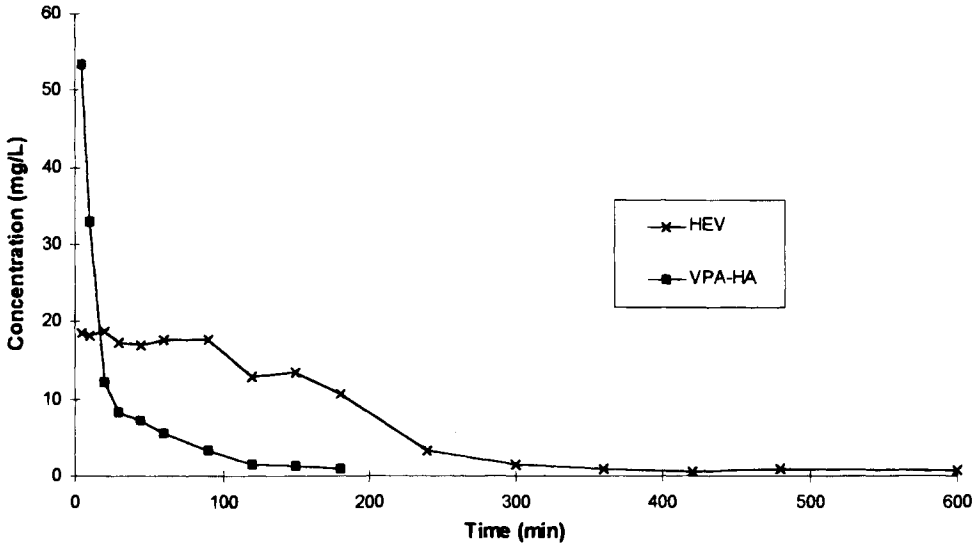

Fig. 2. Mean plasma levels of valproyl hydroxamic acid—VPA-HA (III) and N-(1-hydroxyethyl)-valpromide—HEV (IV) following their iv administration (at a dose equivalent to 20 mg/kg of VPA) to dogs.

(using N_2) to dryness. To the dry residue, 50 μl of the silylation reagent Sil-Prep© (Alltech Associate, Inc. 2051 Waukegan Road, Deerfield, Illinois 60015 USA) was added, the samples were incubated at 37°C for 5 minutes, vortexed, and 1 μl was taken for analysis by GC apparatus (Hewlett Packard 5890 A). GC conditions: The HP-1© capillary column (cross-linked silicon gum) 0.2 mm diameter, 12 meter length, 0.33 μm film thickness. Oven temp. was kept at 90°C for 5 min, and heated at the rate of 5°C/min to 185°C, injector temp. 150°C, detector temp. 300°C. Carrier gas nitrogen. The interday coefficience of variation (% CV) among replicates ranged from 5% to 12%, with 21% CV at the lowest limit of quantification (LOQ) of 2 mg/l.

Assay for HEV (IV)

Plasma (0.5 ml) (containing HEV) internal standard solution (2 μl of N-(1-methoxyethyl)-VPD (VII) solution, 1 mg/ml in chloroform) and 100 μl of HCl 0.1 N was vortexed, followed by the addition of 4 ml of tert. butyl methyl ether and by 30 seconds of additional vigorous vortexing. The mixture was centrifuged for 10 minutes at 3000 g and the organic phase was separated and evaporated (using vortex evaporator) to dryness. Chloroform (20 μl) was added to the dry residue, the mixture was vortexed, and 1 μl was taken for analysis by the GC apparatus (Packard model 437).

GC conditions: Packed column—10% OV-17 on GasChrom Q 80–100 mesh, 3 meters long, 1 mm diameter. Oven temp. 185°C; detector temp. 300°C, injector temp. 180°C. The carrier gas, nitrogen, had a flow of 25 ml/min. The interday coefficient of variation (% CV) among replicates ranged from 3% to 11%, with 23% CV at lowest limit of quantification (LOQ) of 1 mg/l.

Incubation of Urine Samples with β-Glucuronidase

To 0.5 ml urine sample, internal standard solution, 0.25 ml sodium acetate buffer, 0.2 N, pH 5, and 2235 units of the enzyme β-glucuronidase (Sigma) were added. The mixture was vortexed, sealed with parafilm and incubated at 37°C with shaking for 12 hr before extraction. Urine level monitoring of VPA and HEV following treatment with β-gluconidase was done according to a published GC assay for VPA (11) and the above-mentioned GC assay for HEV.

Pharmacokinetic (PK) Analysis

The linear terminal slope (β) of the ln C (the plasma concentration of VPA-HA or HEV) versus t (time) plot was calculated by the method of the least squares, utilizing at least six points in the descending portion of the curve. The terminal half-life of each compound (t1/2β) was calculated from the quotient: 0.69/terminal slope. The AUC (area under the C versus t curve) was established by using the trapezoidal rule with extrapolation to infinity by dividing the last experimental plasma concentration by the terminal slope. The total body clearance (CL) of each compound was calculated by using the quotient of the iv dose (D) and the AUC. The volume of distribution (Vβ) could then be calculated by using the quotient

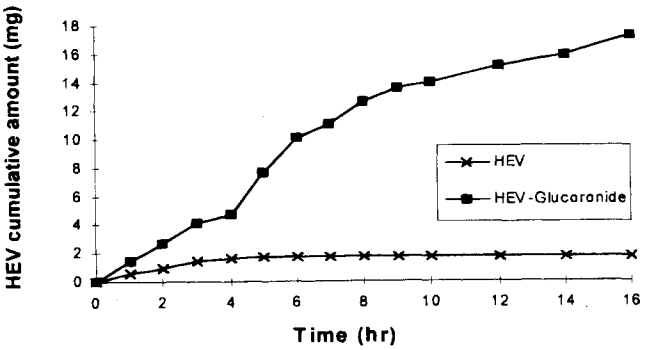

Fig. 3. Cumulative amount of N-(1-hydroxy-ethyl)-VPD—HEV (IV) and HEV glucuronide excreted in the urine of dog no. 2601.

Table 1. Mean (±SD) Pharmacokinetic Parameters of VPA-HA (III) and HEV (IV) Obtained Following iv Administration (a Dose Equivalent to 20 mg/kg VPA) to Dogs in Comparison to VPA (I) and VPD (II)

Pharmacokinetic parameter	VPA-HA	HEV	VPA[b]	VPD[c]
t1/2 (hr)	0.64 ± 0.17	1.4 ± 0.8	1.6 ± 0.6	2.8 ± 0.5
AUC (mg/L·h)	19 ± 14	59 ± 16	58 ± 11	103 ± 18
CL (L/hr)	32 ± 12	10 ± 3	7.8 ± 1.8	4 ± 1.8
V_{ss} (L)	28 ± 14	23 ± 11	16 ± 5	16 ± 4
Vβ (L)	24 ± 13	22 ± 17	18 ± 9	16 ± 4
MRT (hr)	0.81 ± 0.24	2.2 ± 0.5	2.0 ± 0.6	3.8 ± 1.0
fm (%)		2.4 ± 1.0[a]		32 ± 12[d]

[a] Fraction metabolized to HEV glucuronide.
[b] Data taken from ref. 15.
[c] Data taken from ref. 16.
[d] Fraction metabolized to VPA.

of the clearance and the linear terminal slope. The volume of distribution at steady state (V_{ss}) and the mean residence time (MRT) were calculated by classical methods (12). The fraction excreted unchanged (fe) of HEV in the urine was calculated from the ratio of the cumulative amount excreted intact in the urine and the dose. The fraction metabolized (fm) of HEV to its urinary metabolite HEV-glucuronide was calculated from the quotient of the cumulative amount of the metabolite excreted in the urine and the dose. This was done by the assumption that the fe value of HEV-glucuronide is 100%. All of the pharmacokinetic parameters were calculated in a non-compartmental manner based on the statistical moment theory (12,13).

RESULTS

The mean plasma levels of compounds III and IV are presented in Fig. 2. VPA-HA (III) was eliminated more rapidly than HEV (IV) in a way that VPA-HA plasma levels were below its LOQ, 3 hours after dosing. Table 1 summarizes the mean PK parameters of compounds III and IV in comparison to VPA (I) and VPD (II). Fig. 3 describes the cumulative amount of HEV (IV) and HEV glucuronide excreted in the urine in one dog.

Anticonvulsant screening performed at the NIH Epilepsy Branch of compound III–IX in mice following ip administration of three doses, 30, 100, and 300 mg/kg showed that compounds V–VIII were inactive. Unlike these compounds, compounds III, IV, and IX demonstrated anticonvulsant activity in mice. Subsequently, VPA-HA (III), HEV (IV), and M-VPD (IX) were tested in mice and rats, following ip and oral administration, respectively, in order to determine their ED_{50} and TD_{50} values, as well as their protective indices—PI (the ratio between the TD_{50} and ED_{50} values). The PD (anticonvulsant activity and neurotoxicity) results of these active compounds in comparison to VPA (I) and VPD (II) are shown in Table 2.

DISCUSSION

In the literature, there is only one report on valproyl hydroxamic acid derivative (14). Johnson et al. evaluated o-allyl substituted hydroxamic acids as potent inhibitors of the hepatic glycine cleavage system (GCS) and its implications for glycine concentration in the brain (14). The most active compound *in vitro* was O-alkyl valproyl hydroxamic acid (14). Glycine hydroxamates derived from N-methyl hydroxylamine and N,O-dimethylhydroxylamine were both completely ineffective in inhibiting GCS (14).

In the present study, the valpromide (II) derivatives, with the structural changes of the substituents attached to the nitrogen, were synthesized and their pharmacodynamics and pharmacokinetics were investigated.

PK analysis in dogs showed that VPA-HA (III) had the highest clearance value. Its mean clearance was 3 to 4 times larger than that of HEV (IV) and VPA (I), and 8 times larger than that of VPD (II). The volume of distribution of VPA-HA

Table 2. Anticonvulsant Activity Data of Valpoyl Hydroxamic Acid (VPA-HA-III), N-methoxy-VPD (M-VPD-IX) and Ethyl-2-hydroxy Valpromide (HEV-IV) Compared to Valproic Acid (VPA) and Valpromide (VPD), Obtained Following ip Administration to Mice and Oral Administration to Rats (po)

Test	Mice					Rats
	VPA-HA	M-VPD	HEV	VPA	VPD	VPA-HA
MES, ED_{50} (mg/kg)	100	80	105	200	56	79
sc Met, ED_{50} (mg/kg)	>150	>180	225	146	55	—
Neurotoxicity, TD_{50} (mg/kg)	148	157	254	283	81	180
PI, MES	1.5	2.0	2.4	1.4	1.4	2.3
PI, sc Met	1.5	1.1	1.1	1.9	1.5	—

[a] MES = Maximal electroshock; sc Met = Chemically induced shock obtained following subcutaneous injection of metrazol; ED_{50} = Effective dose in 50% of the animals; TD_{50} = Neurotoxic dose in 50% of the tested animals; PI = Protective index—the ratio to the TD_{50} to the ED_{50}.

and HEV was about 1 l/kg and was similar to that of VPA and VPD. Consequently, VPA-HA had the shortest half-life.

Unlike VPD, neither VPA-HA (III) nor HEV (IV) were metabolized *in vivo* to VPA. Thus, the substitution of a hydrogen attached to the nitrogen in the VPD molecule by a hydroxyl or a β-hydroxyethyl moiety prevented the biotransformation of the amide to the valproic acid. In this regard, VPA-HA and HEV can be considered as metabolically stable analogues of VPD, like the VPD isomers valnoctamide (VCD) and propylisopropylacetamide (PID) (7–9).

Both VPA-HA and HEV had better anticonvulsant activity than VPA, but lower activity than VPD or its metabolically stable isomers VCD or PID (3). Thus, the ED_{50} values of VPA-HA and HEV were between those of VPA and VPD. Comparative pharmacodynamic analysis shows that the substitution of the hydroxyl group in VPA by a hydroxylamine moiety improved its anticonvulsant potency, in comparison to VPA, in both mice and rats (Table II). HEV (IV) is a homologous compound to VPA-HA (III), in which the hydroxyl moiety was shifted from the nitrogen atom by two carbons. This structural change did not affect the anticonvulsant activity of these two homologous compounds in mice, but reduced the neurotoxicity of HEV (IV) as compared to VPA-HA (III), resulting in a compound with a better safety margin. Both VPA-HA were more active than VPA in the electrically induced shock (MES) test, but were less active than VPA in the metrazol-induced shock (sc Met) test. Anticonvulsant activity in the MES test on rodents indicate that these compounds may have potential in the treatment of different kinds of partial epilepsy.

Substitution of a hydroxyl hydrogen in the VPA-HA (III) by a methyl group resulted in N-methoxy-VPD (IX), another molecule with increased anticonvulsant activity, and PI values as compared to VPA (Table II). In contrast, substitution of a hydroxyl hydrogen in the anticonvulsant active compound HEV (IV) by a methyl group led to molecule VII, which showed no anticonvulsant activity. Substitution of the hydroxyethyl substituent attached to the nitrogen of VPD by 1,2-dihydroxypropyl moiety led to N-(1,2-dihydroxy propyl)-VPD (VIII), with no anticulvulsant activity.

O-acylation of VPA-HA (III) produced an ester of VPA-HA (V), and N,O diacylation of VPA-HA (III) led to an ester amide of VPA-HA (VI). Both transformations drastically decreased the anticonvulsant activity of VPA-HA, leading to the inactive compounds (V and VI). Esterification of VPA with a series of aliphatic alcohols caused anticonvulsant inactivation of the parent compound (15). A similar phenomenon was observed in this study where VPA-HA (III) was esterified by valproic acid, leading to the inactive O-valproyl VPA-HA (V).

Analysis of HEV (IV) in comparison to VPA (I) showed that substitution of OH by ethyl hydroxylamine improved the pharmacodynamics of the molecule without affecting its pharmacokinetics. The substitution of OH in VPA (I) by hydroxylamine produced VPA-HA (III), a more active compound than VPA (at the MES test) despite its unfavourable pharmacokinetic profile (large CL and short t1/2 values).

In conclusion, the two new active valproyl hydroxamic acids, VPA-HA (III) and HEV (IV), showed better anticonvulsant activity than valproic acid because of their better intrinsic potency and not due to better pharmacokinetic characteristics. In addition, the present results have shown that unlike VPD (II), VPA-HA (III) and HEV (IV) are not biotransformed *in vivo* to VPA.

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