Anticonvulsant Profile of the Enantiomers of DL- HEPP

ABSTRACT

Aims: Epilepsy is a brain disorder that is characterized by recurrent seizures. It affects 1% of the global population and remains poorly managed in 30% of patients despite available antiepileptic drugs, which often cause significant side effects even in controlled cases, highlighting the urgent need for improved treatments. In this paper we report the resolution of DL-HEPP and the anticonvulsant activity and neurotoxicity of its enantiomers, as candidates for the treatment of refractory epilepsy.

Study Design: synthesis of DL-HEPP, separation of enantiomers (+)-HEPP and (-)-HEPP, and anticonvulsant profile and neurotoxicity of DL-HEPP and its enantiomers.

Place and Duration of Study: Biochemistry, Pharmacy and Organic Chemistry Departments, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, Mexico city. Duration of study three years.

Methodology: To investigate differences in biological activity between HEPP enantiomers, the racemate was resolved using (-) brucine and (+) phenylethylamine salts of the acids. The optically active acids were esterified with diazomethane and reacted with ammonia to yield (+)-HEPP and (-)-HEPP. Enantiomeric purity was confirmed (>99% ee) using proton magnetic resonance with Europium tris-[3-(trifluoromethylhydroxymethylene)-(+)camphorate] and chiral HPLC. Anticonvulsant activity of DL-HEPP and its enantiomers was tested in pentylenetetrazole (PTZ), β-mercaptopropionic acid (β-MPA), bicuculline (BIC), thiosemicarbazide (TSC), and maximal electroshock (MES) seizure models. The rotarod ataxia test was used to evaluate neurotoxicity. Time-to-peak drug effects were established prior to dose-response studies. The duration of the anticonvusant activity (+) and (-) HEPP was evaluated.

Results: Chiral HPLC confirmed enantiomeric purity (>99% ee) for HEPP enantiomers. ^{1}H and ^{13}C-NMR spectra revealed identical chemical shifts for DL-HEPP and its enantiomers, the addition of Eu[TFH-cam-d] highlighted the stereochemical differences. DL-HEPP and its enantiomers exhibited a similar significant anticonvulsant activity in seizures models tested, outperforming sodium valproate in PTZ test. Neurotoxicity was comparable among DL-HEPP enantiomers. Over time, (-) HEPP maintained the highest protection, while the anticonvulsant activity of (+) HEPP declined rapidly against PTZ induced seizures.

Conclusion: At the time of peak drug effect (30 min) there is no differences either in the anticonvulsant activity against PTZ, BIC, TSC and β-MPA induced seizures or in neurotoxicity between DL-HEPP and its enantiomers, which suggests that the optical resolution of DL-HEPP is not necessary for further preclinical studies.The protection of DL-HEPP and its enantiomers against

Comment [i-[1]: Please rephrase the paragraph & mention duration of study like from..... to.....

convulsant drugs that block GABA neurotransmission suggest that they act at the GABAergic system.

Key words: Anticonvulsants; DL-3-hydroxy-3-phenylpentanamide enantiomers; DL- HEPP; resolution; (-) HEPP; (+) HEPP; valproate; low affinity GABA binding sites

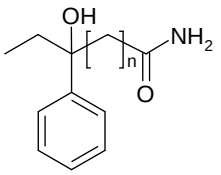
Comment [i-[2]: Arrange keywords alphabetically.

1. INTRODUCTION

Epilepsy is a brain disorder that is characterized by recurrent seizures that affects 1% of the population worldwide [1]. Despite the antiepileptic drugs (AEDs) available, at present 30% of patients with epilepsy continue to have seizures and, even among those considered controlled many unpleasant side effects are still endured [2]. There is clearly a need for more and better AEDs.

The mechanisms underlying AEDs therapeutic benefits may be summarized as follows: (a)by potentiating inhibitory mechanisms (predominantly the GABA system); (b) by inhibiting excitatory mechanisms (mainly the glutamate system); and (c) by inhibiting excessive neuronal firing (modulation of membrane cation conductance via sodium, calcium or potassium channels). In addition, several AEDs drugs operate through other or unknown mechanisms.

The compounds DL-2-hydroxy-2-phenylbutyramide (1, DL-HEPA), DL-3-hydroxy-3-phenylpentanamide (2, DL-HEPP) and DL-4-hydroxy-4-phenylhexanamide (3, DL-HEPB) have a broad profile of anticonvulsant activity (Fig. 1). They protect mice against seizures induced by pentylenetetrazol, maximal electroshock, bicuculline, 4-aminopyridine, thiosemicarbazide [3] and they also protected cats and rats against hippocampal kindling [4;5]. From these series 2 possesses the lowest toxicity [3; 6]. Compound 2 also protects against the γ-aminobutyric acid (GABA) withdrawal syndrome, a model of focal epilepsy, which has shown an extraordinary resistance to classic antiepileptics such as diazepam, one of the most effective agent for treating status epilepticus [7]. Compound 2 also produces a significant decrease of focal spike activity in the genetic absence epilepsy rats of the Strasbourg model (GAERS) [7]. Additionally, DL-HEPP also protects against picrotoxin and audiogenic seizures in mice and pilocarpine seizures in rats [8]. The profile of anticonvulsant activity of the homologous series of phenyl alcohol amides suggests that they are promising anticonvulsant drugs against epilepsy of the absence type [9;10;11] and they are currently undergoing preclinical development. The pharmacokinetic behavior of 2 has been tested in animals and healthy volunteers after the oral administration of single and multiple doses [12;13;14;15;16;17]. These studies showed that 2 has a rapid absorption, a long half-life, low protein binding and clinically adverse effects have been minor, so 2 shows great promise as a useful antiepileptic in drug therapy. However, further clinical investigation in humans is necessary to determine its use in clinical practice. In order to continue with the pharmacological evaluation of DL-HEPP is necessary resolve the racemate in order to study the differences in biological activity between its enantiomers. In this paper we report the resolution of DL-HEPP and the anticonvulsant activity and neurotoxicity of its enantiomers.



n	compound
0	DL-HEPA **1**
1	DL-HEPP **2**
2	DL-HEPB **3**

Figure 1. Structures of phenyl alcohol amides 1 to 3.

2. MATERIAL AND METHODS

The melting points were determined with a Mettler-Toledo apparatus FP-62 model. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum GX 2000 FT-IR spectrophotometer with attenuated total reflectance (ATR). The IR absorption frequencies are reported in cm^{-1}. The ^{1}H and ^{13}C NMR spectra were obtained in a Varian VNMRS-500 spectrometer, at 500 MHz (125.787 MHz for ^{13}C). The samples were dissolved in $CDCl_3$ using TMS as internal reference. HRMS data were obtained in a JEOL GCmateTM II spectrometer in electron impact (EI, 70 eV) mode. Optical rotations were measured on a Perkin Elmer 341 polarimeter equipped with a 1 dm cell at 589 nm (sodium-D-line). Analytical chromatography was performed with a HPLC modular liquid chromatograph Beckman System Gold equipped with a 166 variable wavelength detector, a 128 pump and an injector. A Chiracel column OJ (250 x 4.1 mm) packed with cellulose tris(4-methylbenzoate) (10 μm particle diameter) was used. Rotarod tests were performed on a Rotarod (M) 85052-4 Series. Maximal electroshock test was determined using a constant current electroshock unit Ugo Basile model 7801.

2.1 Chemistry

Compounds 5a, 5b, 6a, 6b, 7a and 7b were synthesized according the figure 2. The DL-(±)-hydroxyester, compound 4, was hydrolyzed to give the hydroxy acid DL-(±)-3-hydroxy-3-phenylpentanoic acid, compound 5, and resolved by means of its (-)-brucine and (-)-1-phenylethylamine salts. The (+) and (-) acids were then esterified with diazomethane to produce the hydroxyesters 6a and 6b which were reacted with ammonia to give the optically active isomers of HEPP (Fig. 2).

The addition of (-)-brucine to the racemic solution of 3-hydroxy-3-phenyl-pentanoic acid 5 afforded the diasteromeric insoluble salt of 5a that was collected by simple vacuum filtration, this salt was composed of (-)-brucine and the (+) enantiomer. The water soluble salt of 5b was composed of (-)-brucine and the (-) enantiomer. Both complexes were hydrolyzed separately with diluted HCl to produce 5a and 5b. The partially resolved acid 5b was treated with (-)-1-phenylethylamine to give 5b. The optically active acids liberated 5a and 5b were reacted with diazomethane to form the corresponding (+) and (-) methyl esters 6a and 6b. Amoniolysis of 6a and 6b formed the beta-hydroxyamides 7a and 7b which exhibited $[\alpha]_D^{20^\circ}$= +35.02^o and -34.97^o (c= 3, ethanol) respectively.

DL-(±)-Ethyl 3-hydroxy-3-phenylpentanoate (4)

The compound 4 was synthesized as described previously [18]. IR and NMR spectra were found to be identical with the ones described [19].

2.1.1 DL-(±) 3-Hydroxy-3-phenylpentanoic acid (5)

A solution of 44.4 g (200 mmol) of 4 containing 2 N KOH solution in 130 mL anhydrous methanol was stirred at room temperature for 7 h. After saponification, the methanol was evaporated at reduced pressure. The residue was taken up with 500 mL water, extracted with diethyl ether (100 mL x 3), and the organic phase was discarded. The aqueous phase was cooled and acidified (pH 2.5) with 35 mL 6 N HCl and extracted with diethyl ether (100 mL x 4). The combined ether extracts were washed with H_2O (30 mL x 2), saturated NaCl solution (30 mL x 2), and dried over Na_2SO_4 and concentrated in vacuo. The precipitate was recrystallized from water to afford 37.5 g (96.6 %) 5 as a white solid. Mp 123-124 ^{o}C (mp 121 ^{o}C [20]).

2.1.2 (+)-3-Hydroxy-3-phenylpentanoic acid (5a)

To a stirred solution of 24.7 g (127.3 mmol) of 5, 300 mL ethyl acetate at 60 °C was added 52.57 g (119.6 mmol) of (-)-brucine. The solution was heated under reflux for 10 min, cooled at -20 ^{o}C for 24 h and concentrated. The residue was treated with 50 mL hexane and the solid was filtered off and crystallized from 90 mL ethyl acetate. The brucine salt of 5a was filtered off, and the mother liquors were evaporated to dryness to obtain 26.9 g the brucine salt of 5b which was split with 31 mL 5% HCl in 350 mL diethyl ether. The ethereal layer was separated and 5b was extracted with acetone (150 mL x 2). The combined ethereal and acetone extracts were concentrated and the solid was crystallized from water to give 8.3 g (42.8 mmol) of partially resolved 5b, $[\alpha]_D^{20^\circ}$ = -15^o (c = 3.0, ethanol).

The crystalline brucine salt of 5a was heated with 80 mL ethyl acetate, cooled and filtered off several times to give the brucine salt of 5a (18 g), mp 118-119 ^{o}C, which was split with 21 mL 5 % HCl in 230 mL diethyl ether, the ethereal layer was separated and 5a was extracted with diethyl ether (100 mL x 2). The combined ether extracts were concentrated to give 5a as a white solid. The solid was crystallized from water to give 8 g (32.4 %) of 5a. Mp 92-93 °C; $[\alpha]_D^{20^\circ}$= +21.7° (c = 3.0, ethanol) (Mp 98 ^{o}C; $[\alpha]_D^{24^\circ}$= +22° (ethanol) [21].

2.1.3 (-)-3-Hydroxy-3-phenylpentanoic acid (5b)

5.84 g (30.1 mmol) of 5b partially resolved was mixed with 30 mL ethanol, 3.64 g (30.1 mmol) of (-)-1-phenylethylamine and heated at 40°C for 5 min. The mixture was concentrated to dryness and the solid was crystallized several times from ethanol to give the phenylethylamine salt of 5b (6 g) as white crystals, m.p. 174-

175 °C which was split with 5 mL 40 % NaOH, 24 mL water and 48 mL benzene. The aqueous layer was washed with benzene (22 mL x 4), cooled and the pH of the solution was lowered to 1 with 3.7 mL 37 % HCl. The precipitate was filtered off and the solid was crystallized from water to give 2.0 g (34.2 %) of 5b as a white solid. Mp 92-93 °C; $[\alpha]_D^{20^\circ}$ = -21.3º (c= 3.0, ethanol) ($[\alpha]_D$= -17.0º (c= 10.0, benzene) [22]); IR (ATR): ν= 1683, 3504 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 0.77 (t, 3H, J= 7 Hz), 1.77 (dq, 1H, J= 7 Hz, J= 14 Hz), 1.82 (dq, 1H, J= 7 Hz, J= 14 Hz), 2.83 (d, 1H, J= 16 Hz), 2.99 (d, 1H, J= 16 Hz), 6.25 (bs, 1H), 7.23 (d,d, 1H, J= 5 Hz, J= 10 Hz); 7.31 (d,d, 2H, J= 10 Hz), 7.35 (d, 2H, J= 5 Hz) ppm; ^{13}C-NMR (500 MHz, $CDCl_3$): δ= 7.72, 35.81, 44.52, 75.37, 125.44, 126.77, 128.22, 144.22, 177.38. HRMS EI:m/z calculated for $C_{11}H_{14}O_3$ (M+) 194.0943, found 194.0935 (M+).

2.1.4 (+)-Methyl 3-hydroxy-3-phenylpentanoate (6a)

To a stirred solution of 4.5 g (23.2 mmol) of 5a in 15 mL diethyl ether, was added a solution of 1 g (23.8 mmol) of diazomethane in 90 mL diethyl ether. The reaction mixture was stirred for 10 min liberating nitrogen. Then, it was concentrated at reduced pressure to obtain 4.8 g (99.5 %) of 6a. $[\alpha]_D^{20^\circ}$ = +2.2° (c= 3, ethanol) ($[\alpha]_D^{14^\circ}$ = +1.64° (c= 0.85, ethanol) [21]. IR and NMR spectra were found to be identical with the ones described [23].

2.1.5 (-)-Methyl 3-hydroxy-3-phenylpentanoate (6b)

This compound was obtained from 5b, following a similar procedure to the preparation of compound 6a. Yield 98.02 %, $[\alpha]_D^{20^\circ}$ = -2.2° (c = 3, ethanol). ($[\alpha]_D^{21^\circ}$ = -0.97° (c= 1.03, ethanol) [23]. NMR spectra were found to be identical with the ones described [24].

2.1.6 (+)-3-Hydroxy-3-phenylpentanamide (7a)

A mixture containing 4.8 g (23 mmol) of 6a, 15 mL ethanol and 15 mL 28% aqueous ammonia was cooled at 0 ºC and saturated with ammonia gas. The flask was closed with a rubber stopper and held at room temperature for 19 days. Then the mixture was cooled, and it was extracted with diethyl ether (15 mL x 4), dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was treated with 10 mL benzene, the precipitate was filtered off and the solid was crystallized from benzene to give 3 g (67.3 %) of 7a as a white solid. Mp 51-52 °C; $[\alpha]_D^{20^\circ}$= +35.02° (c = 3, ethanol); IR (ATR): ν= 1656, 3186, 3334 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 0.73 (t, 3H), 1.76 (dq, 1H, J= 6.5 Hz, J= 13 Hz), 1.82 (dq, 1H, J= 6.5 Hz, J= 13 Hz), 2.65 (d, 1H, J= 15 Hz), 2.70 (d, 1H, J= 15 Hz), 5.11 (br, 1H), 5.70 (br, 1H), 5.80 (br, 1H), 7.20 (tt, 1H, J= 9 Hz, J= 15 Hz), 7.23 (tt, 2H, J= 9 Hz, J= 15 Hz), 7.34 (dt, 2H, J= 9 Hz, J= 11 Hz) ppm; ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 7.98, 36.07, 46.06, 75.82, 125.4, 126.93, 128.4, 145.35, 175.29 ppm. HRMS EI:m/z calculated for $C_{11}H_{13}NO$ (M-H_2O) 175.0997, found 175.0995 (M-H_2O).

2.1.7 (-)-3-Hydroxy-3-phenylpentanamide (7b)

Compound 7b was obtained from 6b, following a similar procedure to the preparation of compound 7a. Yield 56.4 %. Mp 51-52 °C; $[\alpha]_D^{20^\circ}$ = -34.97° (c = 2.98, ethanol). IR, ^{1}H-NMR and ^{13}C-NMR spectra and HRMS data were identical to 7a.

2.1.8 DL-(±)-3-hydroxy-3-phenylpentanamide (2)

Compound 2 was synthesized as described previously [3]. Mp 101-102 °C (Mp 101-102 °C [3]).

Chromatographic determination of the enantiomeric purity
Optical purity of 7a and 7b was determined by chiral HPLC using a Chiracel OJ column (4.1 x 250 mm), eluting with n-hexane/2-propanol (85:15) at a flow rate of 0.9 ml/min; detection was at 221 nm. 20 µl of each enantiomer dissolved in n-hexane:2-propanol (85:15) (400 ng/ml) was injected into the column and the enantiomeric excess was determined.

2.1.9 Determination of the enantiomeric purity using Europium tris-[3-(trifluoromethylhydroxymethylene)-(+)camphorate] [Eu[TFH-cam-d]]

To establish the enantiomeric purity by ^{1}H-NMR (500 MHz), Eu[TFH-cam-d] (0.017 g, 0.02 mmol) was added either the racemic compound 2 (0.019 g, 0.01 mmol) or their enantiomers 7a and 7b (0.019 g, 0.01 mmol) dissolved in 0.5 ml $CDCl_3$. ^{1}H-NMR spectra were performed. Chemical shift differences (Δδ) were calculated by subtracting the low field signal to the high field signal of both enantiomers resolved in the spectrum.

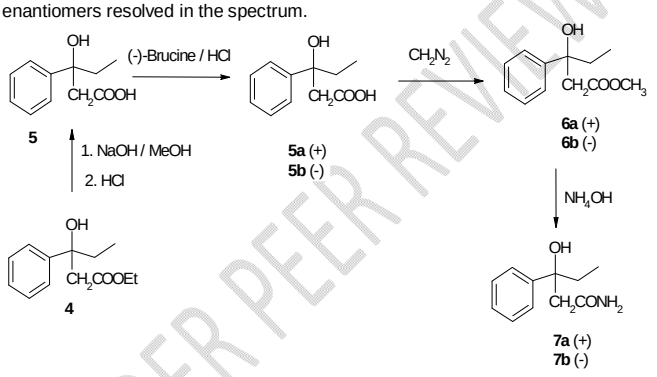


Figure 2. Synthesis of the enantiomers of HEPP.

2.2 Pharmacology

2.2.1 Animals and treatment

Male albino CD-1 mice (Universidad Autónoma del Estado de Hidalgo, México) weighing 25-30 g were housed in groups of 5, at room temperature (20-24 °C), with tap water and food (pellet type Lab Rodent Diet 5008; PMI Nutrition International, Brentwood, MO, USA) ad libitum, with a 12-h light-dark cycle (light on: 6.00 a. m.). Mice were used in the mouse anticonvulsant and rotarod tests. The experiments were carried out according to the National Institutes of Health animal care and use guidelines and were approved by our scientific research committee. Each treatment group and vehicle control group consisted of 7-10 animals.

2.3 Anticonvulsant activity [3]

The anticonvulsant activity of DL-HEPP and its enantiomers 7a and 7b was evaluated using models of pentylenetetrazol (PTZ), β-mercaptopropionic acid (β-MPA), bicuculline (BIC), thiosemicarbazide (TSC), and maximal electroshock (MES). DL-HEPP, 7a, and 7b were dissolved in a 10% polyethylene glycol-400 solution, while sodium valproate and PTZ were dissolved in water. All compounds were administered intraperitoneally (i.p.) except bicuculline which was injected subcutaneously (s.c). The convulsive doses causing seizures and death in 100% of the mice, were determined and used during the pharmacological evaluation. Before testing dose-response curves, the time to peak effect (TPE) for each anticonvulsant was determined.

PTZ (100 mg/kg i.p.), TSC (20 mg/kg i.p.), and β-MPA (47.6 mg/kg i.p.)and BIC (2 mg/kg s.c.)were administered to four groups of 7-10 mice, assessing the suppression of tonic seizures and death as the endpoint. During the MES test, seizures were delivered by applying an electric current to the brain via electrodes placed on the ears. The shocks were delivered at a constant current of 20 mA, 100 Hz frequency, 0.4 ms pulse width, and 0.2 s duration. Testing was conducted at the TPE for DL-HEPP, 7a, 7b, and sodium valproate. The dose preventing tonic seizures in 50% of animals (ED_{50} value) was established through probit analysis. ED_{50}, TD_{50} values, and their 95% confidence intervals were determined using this method [25].

Comment [i-[3]: Mention the basis for dose calculation which you have administered to animals.

2.4 Effects in time

Groups of 10 mice were dosed i.p. with DL-HEPP, 7a and 7b, 100 mg/kg, and protection against convulsions and death produced by pentylenetetrazol, 100 mg/kg, i.p., was evaluated at different times.

2.5 Neurotoxic effects

Separate groups of mice were trained to stay on a rotarod that rotated at 10 rpm. The drum diameter was 2.54 cm. Four groups of 7-10 trained mice were dosed with the test compound or drug vehicle (10% polyethyleneglycol-400 solution) and were tested at TPE to measure the effect of the drug on motor performance. Animals which fell off before 120 s were considered ataxic. The dose at which 50% of the animals fell off the rotarod (TD_{50}) was determined by probit analysis[25].

Comment [i-[4]: Rephrase the praragraph.

2.6Protective index

It is calculated by dividing the TD_{50} value by the respective ED_{50} values as determined in either PTZ or MES tests. The protective index is considered to be an index representing the margin of safety and tolerability between ED_{50} and TD_{50} values [26].

3. RESULTS AND DISCUSSION

Comment [i-[5]: Plese rewrite results & discussion.

3.1 Chemistry

Melting point of compound 5, 123-124 ^{o}C, and those of their enantiomers 5a and 5b, 92-93 ^{o}C, were different. Similarly, the melting point of DL-HEPP, 101-102 ^{o}C, decreased to 51-52 ^{o}C in the enantiomers 7a and 7b. Examination of the ^{1}H and ^{13}C-NMR spectra of DL-HEPP and their enantiomers 7a and 7b in a

$CDCl_3$solution showed identical chemical shifts. This agrees with literature, where racemates, because of their different crystal structure have melting points which may be different from those of the pure enantiomers.
In order to study the enantiomeric purity of compounds 7a and 7b they were analyzed by using a chiral HPLC column to resolve the racemate. Figure 3A showed the chromatographic resolution of DL-HEPP exhibiting a retention time for 7a (12.08 minutes) and 7b (7.16 minutes). Figures 3B and 3C showed the chromatogram of pure enantiomers 7a and 7b, impurities were not detected and they have at least 99% ee.

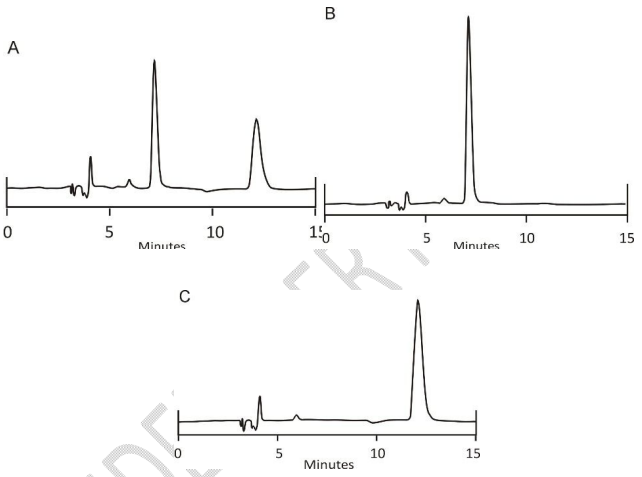


Figure 3. Chromatographic separation of the enantiomers of HEPP in a Chiracel OJ column. A) DL-HEPP; B)(-) HEPP; C) (+) HEPP.

The ^{1}H-NMR spectrum of DL-HEPP showed an AB coupled system for diasterotopic protons H-2 centered at 2.77 ppm; H-4 was observed as an overlapped system ABX3 at 1.82 ppm (Fig. 4a). The addition of Europium tris-[3-(trifluoromethylhydroxymetylene)-(+)camphorate] (Eu[TFH-cam-d]) to racemic 7a/7b split the methylene H-2 signals of isomer 7a at 3.20 ppm and for the isomer 7b at 3.08 ppm (Fig. 4b). Protons H-4 for (-) isomer were shifted at 2.18 ppm, same protons for (+) isomer were located at 2.10 ppm.
Figure 4c showed the proton NMR spectrum of isomer (+)-7a pure. The addition of 0.01 mmol of Eu[TFH-cam-d] to these compounds shifted protons H-2 to 3.08 ppm; methylene H-4 displayed two different signals, one at 2.15 ppm and other at 2.0 ppm for each diastereomeric proton. Due pseudocontact interaction between Eu[TFH-cam-d] and enantiomers (-) and (+) are different, isomer (+)-7a

pure in figure 4d displayed an AB coupled system for protons H-2 at 2.95 ppm and 2.82 ppm respectively, nevertheless, the diasterotopic methylene H-4 remained as a multiplet centered at 1.92 ppm.

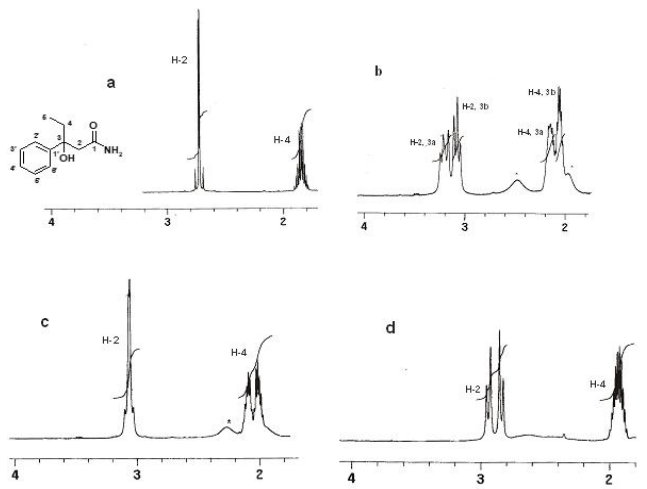


Figure 4. ^{1}H-NMR spectra (500 MHz): a) DL-HEPP; b) DL-HEPP added with 0.02 mmol Eu[TFH-cam-d]; c) (+) HEPP added with 0.01 mmol Eu[TFH-cam-d]; d) (-) HEPP added with 0.01 mmol of Eu[TFH-cam-d].
Time

3.1 Pharmacology

The anticonvulsant activity and neurotoxicity of DL-HEPP, 7a, 7b and sodium valproate after intraperitoneal administration is shown in table 1. The compound DL-HEPP has been previously shown to be endowed with anticonvulsant activity in several animal seizure tests but individual evaluation of each of these enantiomers 7a and 7b was still lacking. From the data of table 1 it can be seen that not only DL-HEPP but also each of its enantiomers exhibit interesting anticonvulsant protections in these seizure tests as potent as under valproate, a reference antiepileptic drug widely used in human clinics. With respect the PTZ test, compounds DL-HEPP, 7a and 7b showed a similar significant anticonvulsant activity (ED_{50}: 55, 61 and 50 mg/kg respectively) and sodium valproate was the least potent (ED_{50}: 120 mg/kg) (Table 1). Sodium valproate exhibited a 50% protection by the MES test at a dose of 237 mg/kg whereas the anticonvulsant activity of DL-HEPP and its enantiomers (+) 7a and (-) 7b was something different (ED_{50}: 138, 168 and 108 mg/kg respectively). There was no

differences in neurotoxicity between DL-HEPP and its enantiomers (+) 7a and (-) 7b (TD_{50}: 212, 223 and 207 mg/kg respectively). In this test sodium valproate was the least toxic (TD_{50}: 380 mg/kg) (Table 1).
In the β-MPA model, DL-HEPP showed an ED_{50} of 44.3 mg/kg, and values obtained for (+)7a (49.4 mg/kg) and (-)7b (41.9 mg/kg) were similar.
In the BIC model, there are no differences in anticonvulsant activity between DL-HEPP and its enantiomers. DL-HEPP shows an ED_{50} of 38.4 mg/kg, while (+)7a and (-)7b achieve values of 36.7 mg/kg and 35.8 mg/kg, respectively.
In the TSC model, DL-HEPP has an ED_{50} of 39 mg/kg, while the enantiomers show any significant differences: (+)7a has an ED_{50} of 51.2 mg/kg, and (-)7b, 48.6 mg/kg.
Compounds DL-HEPP, (+) 7a and (-) 7b showed protective indexes (PI: 3.85, 3.66 and 4.14respectively) better than sodium valproate (PI= 3.16) against PTZ induced seizures in mice (Table 1), which suggests that they could be effective against epilepsy of the absence type. However, in the MES test sodium valproate (PI= 1.6) had similar protective indexes than DL-HEPP, (+) 7a and (-) 7b (PI: 1.54, 1.32 and 1.91 respectively).

Table 1. Anticonvulsant activity and neurotoxicity of DL-HEPP and its enantiomers.

Compound	Pharmacological test[a]							
	ED_{50} (mg/kg) PTZ[e]	ED_{50} (mg/kg) β-MPA[g]	ED_{50} (mg/kg) BIC[h]	ED_{50} (mg/kg) TSC[i]	ED50 (mg/kg) MES[d]	TD50 (mg/kg) Rotarod ataxia	Protective index[b]	
							PTZ	MES
DL-HEPP	55 (51-60) [e]	44.3(37.6-52.2)	38.4(21.6-68.2)	39(30-50)	138(135-142)	212(209-216)	3.85	1.54
(+) HEPP	61 (55-68)	49.4(39.5-61.8)	36.7(29.1-46.3)	51.2(43.1-60.8)	168(164-172)	223(212-234)	3.66	1.32
(-) HEPP	50 (43-58)	41.9(36.4-48.2)	35.8(32.3-39.6)	48.6(45.3-52.2)	108(101-115)	207(203-210)	4.14	1.91
Valproato[f]	120(110-132)	N/T	N/T	N/T	237(221-253)	380(357-404)	3.16	1.60

[a]Test time: 30 minutes post-dosing to peak drug effect. [b]TD_{50}/ED_{50}. Seizures induced by: [c]pentylenetetrazol, [g]β-mercaptopropionic acid, [h]bicuculline, and [i]thiosemicarbazide, and [d]maximal electroshock. [e]95% confidence interval. [f]Test time: 30 minutes (TD_{50}) and 45 minutes (PTZ and MES) post-dosing. N/T: Not Tested

The anticonvulsant activity of (+) HEPP declined rapidly after the first two hours exhibiting 10% protection while (-) HEPP showed the highest activity (90% protection) and DL-HEPP showed an intermediate activity (50% protection) (Fig. 5). At 4 hours after administration the anticonvulsant activity of DL-HEPP and (-) 7b decreased to 20% and 70% protection respectively, at this time compound (+) HEPP had no protection. At 6 hours after injection the anticonvulsant activity of DL-HEPP and (-) 7b fell down to 10% and 20% protection respectively (Fig. 5).

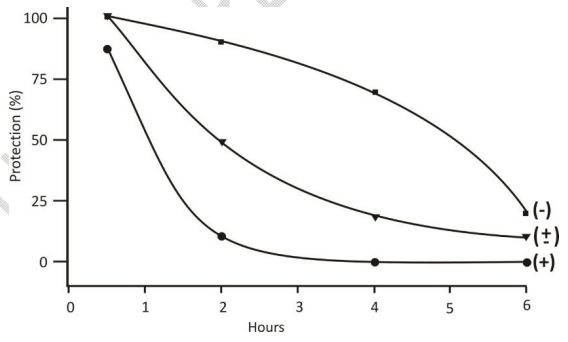


Figure 5. Anticonvulsant activity in time of DL-HEPP and its enantiomers against pentylenetetrazol induced seizures in mice.

Comment [i-[6]: Table in Microsoft word.

Synthesis of optically pure 5a has been reported previously [21] and the absolute configuration was determined to be S $[\alpha]_D^{20°}$= +22° (ethanol). Since none of the reactions to produce 7a from 5a affected directly the chiral carbon atom and there is not acidic protons to promote racemization, the absolute configuration of 6a and 7a was deducted to be S by comparing the optical rotation obtained for 5a $[\alpha]_D^{20°}$= +21.7° (c = 3.0, ethanol). It is reported a specific rotation of +16° for the corresponding carboxylic acid 5a produced from the hydrolysis of 6a [23]. Compounds 6a and 6b has been synthesized from catalytic enantioselective aldol reaction of propiophenone [23; 24; 27]. It is published a specific rotation of +1.64° (77% ee) for the (+) hydroxyester 6a [27].It is reported a specific rotation of -0.97° for the (-) hydroxyester 6b [24]. For compounds 6a and 6b we reported specific rotations of $+2.2^{o}$ and -2.2^{o} respectively. Compounds 7a and 7b have not been reported previously. From the higher optical rotation values for compounds 6a and 6b respect those published previously and considering that compounds 7a and 7b have optical purities greater than 99% ee, it is assumed that compounds 6a and 6b reported in this paper have at least 99% ee. So, one of the advantage in the method reported in this paper respect the methods published previously for the preparation of (+) and (-) hydroxyesters [23;24;27] are the higher optical purities obtained.

The rapid onset of the anticonvulsant effect suggests that DL-HEPP and its enantiomers readily penetrates the blood-brain barrier. This finding agrees well with the low serum protein binding of HEPP as previously published [13]. The strong direct relationship between the concentrations of HEPP in plasma and/or brain and the anticonvulsant effect demonstrated that the parent compound is responsible for the anticonvulsant action [13]. When DL-HEPP was administered with diphenylhydantoin to rabbits, plasma HEPP levels decreased. The results suggested a pharmacokinetic interaction between diphenylhydantoin and HEPP, probably on the drug-metabolizing enzyme system in the liver [28]. As phenytoin acts as an enzyme inducer of microsomal P450, it is probable that DL-HEPP and its enantiomers might be metabolized by cytochrome P450, perhaps of the same genetic subfamily on which phenytoin acts as enzyme inductor. However, it will be necessary to perform HEPP metabolism studies with the racemate and its enantiomers inorder to determine the mechanisms involved in the biotransformation of this drug. The variation in the anticonvulsant activity in time between the enantiomers (+) 7a and (-) 7b could be due to differences in their metabolism or distribution.

The rotarod ataxia test was used to evaluate the neurotoxicity. In this test, the neurotoxicity of DL-HEPP, (+) 7a and (-) 7b was similar.

While the anticonvulsant activity of DL-HEPP and its enantiomers was similar in the PTZ assay at peak drug effect, in the MES model of epilepsy was something different. PTZ interacts with $GABA_A$ receptor [29;30] and sodium channel blockers like diphenylhydantoin are effective in the MES model of epilepsy [31;32]. It is reported that hydroxyphenylamides such as DL-2-(3-chlorophenyl)-2-hydroxynonanamide and its (-) and (+) enantiomers blocked sodium channels with inhibitory concentration 50 values of: 1.81, 1.88 and 2.61 mM respectively [33]. As DL-HEPP and its enantiomers are hydroxyphenylamides it may be possible that they also block sodium channels. This could explain their effect in the MES test.

Beta-mercaptopropionic acid (β-MPA) competitively inhibits glutamate decarboxylase (GAD), the enzyme that synthesizes GABA, causing convulsions [34]. It is also able to inhibit GABA release from rat brain slices in vitro, and this action may also contribute to its convulsive actions in vivo [35]. Thiosemicarbazide (TSC) is a carbonyl-trapping compound with combines with pyridoxal-5'-phosphate. It decreases the activity of GAD lowering GABA levels in the brain [36].GABA release studies show that DL-HEPP reverses GABA mediated inhibition of electrically and potassium chloride evoked exogenous [^{3}H]-GABA release from rat substantia nigra slices without having any effect on evoked release in the absence of GABA. DL-HEPP also counteracted the inhibition in electrically evoked release of [^{3}H]-GABA produced by the $GABA_A$ receptor antagonists picrotoxinin and bicuculline [10] according to the protection that DL-HEPP exhibits against picrotoxin and bicuculline induced seizures [3,8]. It is reported that DL-HEPP displaces [^{35}S]-tert-butylbicyclophosphorothionate, from picrotoxin sites on $GABA_A$ receptor complex in rat brain crude synaptic membranes obtained from rats treated with DL-HEPP (50 mg/kg) for 15 days [37]. From these results it was suggested thatDL-HEPP might be acting as a modulator at the $GABA_A$ receptor complex [10,37]. Preliminary microdialysis results show that DL-HEPP increases GABA levels in rat hippocampus, but this has to be confirmed by further experiments [38].The enantiomers of HEPP may increase GABA release enhancing the actions of GABA antagonizing BIC, β-MPA and TSC induced seizures. The mechanism underlying the anticonvulsant activity of DL-HEPP and its homologues DL-HEPA and DL-HEPB is not known. They protect against seizures induced by bicuculline, a $GABA_A$ receptor antagonist [3; 39; 40]. The protection of DL-HEPP against bicuculline induced seizures, which is an antagonist acting at the GABA recognition site of the $GABA_A$ receptor, suggested to examine its influence on GABA binding at this site. DL-HEPP did not displace [^{3}H]-GABA from high affinity GABA binding sites [10].

CryoEM high-resolution structure of $GABA_A$ receptor shows that low and high affinity GABA binding sites are structurally non-equivalent [41]. GABA binding to these sites leads to a conformational change that results in a concerted rotation of the extracellular domains (ECD), which can be facilitated by ligand binding at benzodiazepine site in a “lock and pull” mechanism. This ECD rotation leads to the opening of the Cl^- conducting pore [41].

The daily administration of DL-HEPP (50 mg/kg) for 15 days in rats increased the affinity for benzodiazepines in membranes obtained from cerebral cortex [37]. The increase in benzodiazepine binding produced by GABA is linked to a low affinity GABA binding site in $GABA_A$ receptor [42;43]. DL-HEPP enhances benzodiazepine modulation of GABA currents in oocytes from Xenopus laevis transfected with recombinant $\alpha1\beta3\gamma2$ $GABA_A$ receptor subunits [44]. In GWS, high GABA concentrations completely inhibited the paroxysmal activity owing to its previous withdrawal [45], in spite of the reduction in high affinity GABA binding sites in $GABA_A$ receptor [46]. In this model, GABA could bind to low affinity GABA binding sites resetting $GABA_A$ receptor function restoring GABA neurotransmission.

DL-HEPP is the only synthetic compound known that protects against GWS which is resistant to chemical anticonvulsants (such as phenytoin, barbiturates, ethosuximide, valproic acid and carbamazepine) including diazepam, the drug choice in case of epileptic status, even pentobarbital at anesthetic doses [47]. It is possible DL-HEPP binds to low affinity GABA sites in $GABA_A$ receptor and this could play an important role in its protection against GWS seizures. The low degree of stereochemical specificity in the anticonvulsant activity of the enantiomers of HEPP, may be a reflection of the absence of a chirality center in GABA and consequent lack of stereospecificity at GABA binding site.

The protections of the enantiomers of HEPP against seizures induced by PTZ, β-MPA, TSC and BIC may suggest an activation of the $GABA_A$ receptor complex [48]. Although other mechanisms may be involved [8, 49].

At present, it is not yet clear what the implications of the activation of low affinity GABA binding sites will be for drug discovery. The activation of these sites may be useful for the treatment ofbenzodiazepines, barbiturates and alcohol withdrawal syndromes and in brain disorders such as epilepsy, autism and intellectual disability syndrome. Targeting these sites may open new avenues for drug research.

4. CONCLUSION

At the time of peak drug effect (30 min) there is no differences either in the anticonvulsant activity against pentylenetetrazol, bicuculline, thiosemicarbazide and β-mercaptopropionicacid induced seizures or in neurotoxicity between DL-HEPP and its enantiomers, which suggests that the optical resolution of DL-HEPP and its homologues is not necessary for further preclinical studies.The protection of the enantiomers of HEPP against convulsant drugs that block GABA neurotransmission suggest that they act at the GABAergic system.

ETHICAL APPROVAL

The study received approval from the Comité de Bioética de la Escuela Nacional de Ciencias Biológicas under protocol number CEI-ENCB-0032016, in compliance with the Mexican standard NOM-ZOO-062-00-1999, which outlines technical guidelines for the production, care, and use of laboratory animals in research.

REFERENCES

Comment [i-[7]: Please provide latestest citation.

1. McNamara JO. Emerging insights into the genesis of epilepsy. Nature.1999; 399(6738):A15-A22.
2. Dichter MA. Emerging insights into mechanisms of epilepsy: implications for new antiepileptic drug development. Epilepsia.1994; 35(4):S51-S57.
3. Meza-Toledo SE, Zenteno-García MT, Juárez-Carvajal E, Martínez-Muñoz D, Carvajal-Sandoval G. A new homologous series of anticonvulsants: phenyl alcohol amides. Syntheses and pharmacological evaluation. Arzneim. Forsch.1990; 40(II):1289-1291.
4. Solís H, Galindo-Morales JA, Bravo J, López E. Efecto de la DL-4-hidroxi, 4-etil, 4-fenil butiramida (HEPB) y de su homólogo propionamida (HEPP) sobre la excitabilidad neuronal. Arch. Neurosci.1996; 1:99-103. Spanish
5. Solís H, Jurado JL, Fernández-Guardiola A. La acción de la butiramida sobre el desarrollo del kindling y el kindling amigdalino ya establecido en el gato. In: Velasco-Suárez M, Escobedo-Rios F (Eds.), Neurobiología, Symposium Internacional. Progreso, México. pp. 83-94. 1979. Spanish
6. Chamorro G, Salazar M, Salazar S, Ulloa V, Carvajal G, Martínez D. Evaluation of the toxic and teratogenic potential of the anticonvulsant drug 4-hydroxy, 4-ethyl, 4-phenylbutyramide in mice. Arch. Med. Res.1994; 25:441-446.
7. Brailowsky S, Montiel T, Hernández E, Marescaux C, Vergnes M. Effects of 3-hydroxy, 3-ethyl, 3-phenylpropionamide (HEPP) on rat models of generalized and focal epilepsy. Epilepsy Res. 1992; 11:167-172.

8. Pages N, Maurois P, Bac P, Meza-Toledo S, Peralta-Cruz J, Chamorro-Cevallos G, Cristobal-Luna JM, Vamecq J. Anticonvulsive profile of two $GABA_B$ receptor antagonists on acute seizures mice models. Epilepsy Res. 2021; 174:106644.

9. Carvajal-Sandoval G, Juárez-Carvajal E, Cruz-Peinado C, Meza-Toledo SE. Synthesis and pharmacological evaluation of a new homologous series of (±)-p-fluoro-phenyl alcohol amide anticonvulsants. Arzneim. Forsch. 1998; 48:349-352.
10. Meza-Toledo SE, Bowery NG. Reversal of GABA-mediated inhibition of the electrically and potassium chloride evoked [^{3}H]-GABA release from rat substantia nigra slices by DL-3-hydroxy-3-phenyl pentanamide. Arzneim. Forsch. 2008; 58:53-61.
11. Meza-Toledo SE, Lira-Zárate F, Robles-Martínez EL, Peralta-Alvarez B, Cabrera-Cedillo F, Peralta-Cruz, J, Cruz-Peinado C, Miñón-López H. Synthesis of DL-fluorobenzenamides as anticonvulsants. Arzneim. Forsch. 2008; 58:155-159.
12. Gómez-Martínez LE. Gestational age dependency in the prenatal toxicity and in the disposition kinetics of the novel anticonvulsant HEPP (D,L-3-hydroxy-3-ethyl-3-phenyl propionamide) after subcutaneous administration in pregnant rats. Int. J. Toxicol. 2007; 26:237-246.
13. Gómez L, Cueva-Rolón R, Lehmann P. Disposition kinetics of HEPP in rats after intravenous, oral, and intraperitoneal administration. Correlation of plasma and brain levels with the anticonvulsant effect. Biopharm. Drug Dispos.1995a;16:77-89.
14. Gómez LEM, Lehmann FPA. Pharmacokinetics of the novel anticonvulsant HEPP after single intravenous administration of three different doses in dogs. Biopharm. Drug Dispos.1995b; 16:105-112.

15. González-Esquivel D, Pérez MR, Jung CH. Pharmacokinetics of HEPP, a new anticonvulsant, in healthy subjects and rabbits after multiple doses. Biopharm. Drug Dispos. 2004; 25:85-90.
16. González-Esquivel DF, Rubio-Donnadieu F, Carvajal SG, Jung CH. Single dose pharmacokinetics of HEPP, a new anticonvulsant in normal healthy volunteers. Biopharm. Drug Dispos.1998;19:583-587.
17. García AL, Medina RL, Pérez MR, Jung CH. Lack of interaction between HEPP, a new antiepileptic agent, and carbamazepine in rabbits. Biopharm. Drug Dispos. 2003; 24(5):205-209.
18. Barltrop JA, Acheson RM, Philpott PG, MacPhee KE, Hunt JS. Compounds of potential pharmacological interest. IV. Aryl and alkyl derivatives of 1-aminoindan. J. Chem. Soc. 1956; 2928-2940.
19. Fukuzawa SI, Hirai K. Lithium benzenetellurolate-induced Reformatsky-type reaction in the presence of cerium trichloride. J. Chem. Soc. Perkin Trans. 1993; 17:1963-1965.
20. Maroni-Barnaud Y, Gilard G, Montalla A, Perry M, Dubois JE. Condensations of the chloromagnesium derivative of tert-butyl acetate with ketones in ether. III. Synthesis of β-hydroxylated and β,δ-dihydroxylated derivatives in the cyclo-alkane and aromatic series. Bull. Soc. Chim. Fr.1966;10:3243-3249.
21. Mitsui S, Kudo Y. Absolute configurations and stereospecific hydrogenolysis of optically active 3-hydroxy-3-phenylvaleric acid and its derivatives. Chem. Ind. 1965; 381-382.
22. Mitsui S, Kudo Y. Asymmetric synthesis of 3-hydroxy-3-phenylvaleric acid. Tetrahedron 1967; 23:4271-4277.
23. Denmark SE, Fan Y, Eastgate MD. Lewis base catalyzed, enantioselective aldol addition of methyl trichlorosilyl ketene acetal to ketones. J. Org. Chem. 2005; 70:5235-5248.

24. Adachi S, Harada T. Asymmetric Mukaiyama aldol reaction of nonactivated ketones catalyzed by allo-threonine-derived oxazaborolidinone. Org. Lett. 2008; 10:4999-5001.
25. Litchfield JT, Wilcoxon F. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 1949; 96:99-113.
26. Löscher W, Nolting B. The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. IV. Protective indices. Epilepsy Res.1991; 9:1-10.
27. Oisaki K, Zhao D, Kanai M, Shibasaki M. Catalytic enantioselective aldol reaction to ketones. J. Am. Chem. Soc. 2006; 128:7164-7165.
28. Medina L, García L, Pérez R, Fernández A, Jung H. Pharmacokinetic interaction in rabbits between a new anticonvulsant, DL-3-hydroxy-3-ethyl-3-phenylpropionamide, and phenytoin. J. Pharm. Pharmacol. 1998; 50:1393-1396.
29. Ramanjaneyulu R, Ticku MK Interactions of pentamethylenetetrazole and tetrazole analogues with the picrotoxinin site of the benzodiazepine-GABA receptor-ionophore complex. Eur. J. Pharmacol. 1984; 98:337-345.
30. Squires RF, Saederup E, Crawley JN, Skolnick P, Paul SM. Convulsant potencies of tetrazoles are highly correlated with actions on GABA/benzodiazepine/picrotoxin receptor complexes in brain. Life Sci. 1984; 35:1439-1445.
31. Willow M, Catterall WA. Inhibition of binding of [3H]batrachotoxinin A 20-alpha-benzoate to sodium channels by the anticonvulsant drugs diphenylhydantoin and carbamazepine. Mol. Pharmacol.1982; 22:627-635.
32. McNamara JO. Pharmacotherapy of the epilepsies. In: Brunton LL, Chabner BA, Knollmann BC (Eds.), Goodman & Gilman´s. The

pharmacological basis of therapeutics. McGraw Hill, New York, USA. pp. 583-607.2011.

33. Davis GC, Kong Y, Paige M, Li Z, Merrick EC, Hansen T, Suy S, Wang K, Dakshanamurthy S, Cordova A, McManus OB, Williams BS, Chruszcz M, Minor W, Patel MK, Brown ML.Asymmetric synthesis and evaluation of a hydroxyphenylamide voltage-gated sodium channel blocker in human prostate cancer xenografts. Bioorg. Med. Chem. 2012; 20:2180-2188.
34. Lamar CJr. Mercaptopropionic acid: a convulsant that inhibits glutamate decarboxylase. J. Neurochem. 1970; 17:165-170.
35. Fan SG, Wusteman M, Iversen LL. 3-mercaptopropionic acid inhibits GABA release from rat brain slices in vitro. Brain Res. 1981; 229:379-387.
36. Killam KF. Convulsant hydrazides II: comparison of electrical changes and enzyme inhibition induced by the administration of thiosemicarbazide. J. Pharmacol. Exp. Ther. 1957; 119:263-271.
37. Chávez JL, Martínez MD. Mecanismo de acción del anticonvulsionante DL-3-hidroxi, 3-etil, 3-fenil propionamida (HEPP). Arch. Neurosci. 1996; 1:173-177.
38. González-Esquivel DF. Personal communication.
39. Pérez de la Mora M, Tapia R. Anticonvulsant effect of 5-ethyl, 5-phenyl, 2-pirrolidinone and its possible relationship to γ-aminobutyric acid-dependent inhibitory mechanisms. Biochem. Pharmacol. 1973; 22:2635-2639.
40. Tapia R, Drucker-Colín RR, Meza-Ruiz G, Durán L, Levi G. Neurophysiological and neurochemical studies on the action of the anticonvulsant γ-hydroxy, γ-ethyl, γ-phenyl butyramide. Epilepsia1979; 20:135-145.

41. Masiulis S, Desai R, Uchanski T, Martin IS, Laverty D, Karia D, Malinauskas T, Zivanov J, Pardon E, Kotesha A, Steyaerd J, Miller KW, Aricescu AR. $GABA_A$ receptor signaling mechanisms revealed by structural pharmacology. Nature 2019; 454-461.
42. Olsen RW. Drug interactions at the GABA receptor-ionophore complex. Ann. Rev. Pharmacol. Toxicol. 1982; 22:245-277.
43. Gallager DW, Rauch SL, Malcolm AB. Alterations in a low affinity GABA recognition site following chronic benzodiazepine treatment. Eur. J. Pharmacol. 1984; 98:159-160.
44. Ernst M. Personal communication.
45. Brailowsky S, Kunimoto M, Silva-Barrat C, Menini C, Naquet R. Electroencephalographic study of the GABA-withdrawal syndrome in rats. Epilepsia 1990; 31:369-377.
46. Casasola C, Bargas J, Arias-Montaño JA, Calixto E, Montiel T, Galarraga E, Brailowsky S. Hippocampal hyperexcitability induced by GABA withdrawal is due to down-regulation of $GABA_A$ receptors. Epilepsy Res. 2002; 47:257-271.
47. Calixto E. GABA withdrawal síndrome: $GABA_A$ receptor, synapse, neurobiological implications and analogies with other abstinences. Neuroscience 2016; 313: 57-72.
48. Meza-Toledo SE, Cervantes-Espinoza JG, Vargas-Fernández E, dela Cruz-Marín A, Buendía-Pazarán JG, Chamorro-Cevallos G, Peralta-Cruz J. Anticonvulsant activity and neurotoxicity of the enantiomers of DL-HEPP. Afr. J. Pharm. Pharmacol. 2013; 7:2397-2405.
49. Meza-Toledo SE, Juárez-Carvajal E, Carvajal-Sandoval G. Synthesis of a homologous series of p-chlorophenyl alcohol amides, their anticonvulsant activity and their testing as potential $GABA_B$ receptor antagonists. Arzneim. Forsch. 1998; 48: 797-801.