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New nanocarried phenobarbital formulation: Maintains better control of pentylenetetrazole-Induced seizures

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ABSTRACT

This study aims to evaluate the efficacy of slow release phenobarbital in the control of convulsions triggered by pentylenetetrazole (PTZ), verifying the time of permanence in the anticonvulsant effect through behavior and electroencephalographic records. A total of 162 male Wistar rats weighing between 100 and 120 g were divided into two groups, one for behavior analysis (n = 90) and biochemistry, and another for the acquisition of electrocorticographic record (n = 72). Hepatic enzymes were measured by obtaining a blood sample from the animals studied by means of a biochemical analysis. The procedures for electrode implant and electrocorticographic recordings were performed. The intercalation of phenobarbital in layered double hydroxide (LDH) nanocarrier allowed us to evaluate a new slow release pharmaceutical formulation based on methodologies that have proven longer residence time and lower side effects. This study demonstrates that phenobarbital can be a new perspective pharmaceutical formulation.

1. Introduction

In the last decades, various nanomaterials have been studied as a perspective class for drug delivery systems [1–5]. Among them, layered double hydroxides (LDHs) has attracted the attention due to its ion exchange ability, more stable structure, good biocompatibility and large drug carrying, in addition to drug release [6–16]. LDHs have also been largely reported for biological and other broad applications [17–21]. Most studies have focused on the development of nano-system formulations for cancer treatment [10,22,23], inflammatory [7,15,24], cardiovascular and the fight against Acquired Immunodeficiency Syndrome virus (AIDS) [25–27]. LDHs can be described by the general formula $\left[M^{2+}_{x-1} + M^{3+}_{x}(OH)_2\right]^{x+}(A^{n-})_{x/n}\cdot mH_2O$ [28,29], where M^{2+} and M^{3+} are respectively divalent and trivalent cations, A^{n-} represents the interlayer charge of the ionic molecule, x is the molar fraction of the trivalent cation. m is the mole number of co-intercalated water molecules [30,31]. The molar fraction $\left[x = M^{3+}/(M^{3+} + M^{2+})\right]$ regulates the positive charge of the layers. Drug intercalation in LDHs occurs mainly when their molecules are in ionic form [32]. The properties of LDHs make it one of the most promising inorganic materials to be used as systems for storage and controlled release of numerous drugs.

Phenobarbital (5-phenyl-5-ethylbarbituric acid) was the first effective anticonvulsant drug, which was adopted for clinical usage in 1904. This drug is used to treat seizures [33–36]. Phenobarbital is a widely used drug because of its low cost and great indication. In developing countries, it is a drug indicated by the World Health Organization (WHO) as a first-line active ingredient [37,38]. This medicine is an anticonvulsant drug originated from barbiturate, which potentiates the GABA (gamma-aminobutyric acid) pathway in synapses, as well as antagonizing the glutamatergic pathway, acting as a CNS (Central Nervous System) depressant.

However, the drug intercalations in LDHs have been intensively studied, the intercalation of phenobarbital followed by *in vivo* evaluations has not yet been reported.

The research's aim was to test the use of intercalated phenobarbital in LDH nanocarrier, to compare it to non-intercalated

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phenobarbital through behavioral tests after acute pentylenetetrazole-induced seizures, and to confirm the results by electroencephalographic data.

2. Materials and methods

2.1. Chemicals

The following chemicals were used: $Mg(NO_3)_2{\cdot}6H_2O$, $Al(NO_3)_3{\cdot}9H_2O$ and NaOH (Cromoline, Brazil), Phosphate buffered saline (Sigma-Aldrich), ethyl alcohol and hydrochloric acid (Vetec Brazil).

The anesthetic ketamine was purchased from König (Santana de Parnaíba, SP, Brazil) and xylazine from Vallée (Montes Claros, MG, Brazil) while the local anesthetic lidocaine was obtained from Hipolabor (Sabará, MG, Brazil). Convulsant agent pentylenetetrazole (Sigma-Aldrich, St Louis, US); anticonvulsant compounds phenobarbital (Aventis-Pharma, Ribeirão Preto, SP, Brazil).

2.2. Synthesis of nanocarriers and intercalation of phenobarbital

The LDH was obtained in Mg/Al molar ratio equal to 2.0 by coprecipitation method at constant pH (7.4 ± 0.2) [39,40]. $Mg(NO_3)_2{\cdot}6H_2O$ and $Al(NO_3)_3{\cdot}9H_2O$ salts dissolved in deionized water were added in hydroalcoholic solution under N_2 gas flow and stirring. The pH value was adjusted with drops wise of 2.0 M NaOH solution. The resulting solution was heated at 80 °C temperature during 24 h still under stirring. The final precipitate was washed with hydroalcoholic solution, dried for 12 h at 60 °C and, finally, macerated to obtain a fine powder to be stored properly. The ideal formula for this LDH of magnesium and aluminum cations can be represented as $\left[Mg^{2+}_{0,667} + Al^{3+}_{0,333}(OH)_2\right]^{0,333+}(NO_3^-)_{0,333}{\cdot}mH_2O$, with the molar ratio Mg/Al = 2 or the molar fraction $x = 0333$ $\left[x = Al^{3+}/(Al^{3+} + Mg^{2+})\right]$. Nitrate is the intercalated molecule and m, as already mentioned in the introduction, is the mole number of co-intercalated water molecules.

The procedure for the intercalation of phenobarbital in LDH is exactly the same as mentioned above, except that phenobarbital was added and solubilized in the hydroalcolic solution before being added with deionized water and the dissolved salts. Consequently, the nitrate and phenobarbital molecules were intercalated.

LDH sample resulting with intercalated phenobarbital was labeled as LDH-PBT. Pure phenobarbital sample (non intercalated) was labeled as PBT. In the present work, the molar ratio PBT/Al = 1.0, in order for the resulting LDH-PBT system to be electrically neutral.

2.3. Animals

An experimental quantitative study was carried out at the Laboratory of Pharmacology and Toxicology of Natural Products, which is part of the Biological Sciences Institute at Federal University of Pará. 162 male Wistar rats weighing between 100 and 120 g were used. The animals were kept in the Experimental Vivarium of the Laboratory of Pharmacology and Toxicology of Natural Products. This study was approved by the Animal Use Ethics Committee of the Federal University of Pará (CEUA/UFPA), and whose CEUA number is 3953260717.

2.4. Behavioral assessment

For the behavioral and biochemical analysis, 04 groups of rats were formed in the experiment, using a total of 90 animals, being LDH-PBT and PBT groups receiving a 10 mg/kg v.o., and the group with pentylenetetrazole using a 60 mg/kg dose via intraperitoneal (IP) [41], and control group receiving only the vehicle dose.

Group 1: (G1) (n = 9) Received a 60 mg/kg i.p. dose of pentylenetetrazole via IP to characterize seizure behavior over contact time, divided into the phases presented in Table 1.

After characterization of the seizure phase, the efficiency of the intercalated and non-intercalated phenobarbital was tested, taking into account the previously tested phases' deepening. Evolutionary characteristics of the acute convulsive condition demonstrated the tested drug's anticonvulsant.

Group 2 (G2): This group received 10 mg/kg v.o. of PBT sample for four days and the tests were performed at 12 (n = 9), 24 (n = 9) and 36 (n = 9) hours after the end of treatment. During this procedure, groups received a 60 mg/kg i.p. pentylenetetrazole dose application, and observation of seizures onset and onset of behaviors according to the phases' deepening.

Group 3 (G3): This group received 10 mg/kg v.o. of LDH-PBT sample for four days and the tests were performed at 12 (n = 9), 24 (n = 9) and 36 (n = 9) hours after the end of treatment, with subsequent application of pentylenetetrazole at a dose of 60 mg/kg i.p. During these procedures, the seizure-subjected animals' behavioral phases were analyzed.

Group 4 (G4): Received orally for 4 days in equivalent vehicle dose, and tests were performed at 12 (n = 9), 24 (n = 9) and 36 (n = 9) hours after its administration's discontinuation, followed by the 60 mg/kg i.p. pentylenetetrazole application. During these procedures, the seizure-subjected animals' behavioral phases were analyzed.

This experiment evaluated the efficacy of LDH-PBT and PBT samples, allowing to analyze whether the anticonvulsant effect was maintained after drug withdrawal, preventing or decreasing the intensity of pentylenetetrazole-induced seizures, as well as to verify if there is interference from the vehicle used for phenobarbital's slow release.

2.5. Electrocorticographic evaluation

For the electrocorticographic record acquisition, 72 animals were used - divided into 4 groups: **control group** receiving via oral inert vehicle in amounts equivalent to the treated groups for 4 days (n = 9); **PTZ group** receiving 60 mg/kg IP dose (n = 9); **LDH-PBT group** receiving 10 mg/kg v.o. for four days, with subsequent onset of seizures with PTZ (60 mg/kg i.p.) and tests done at 12 (n = 9), 24 (n = 9) and 36 (n = 9) hours; and **PBT group**, receiving oral dose of

Table 1
Characterization of seizure behavior over time of contact after application of 60 mg/kg i.p. pentylenetetrazole. Each phase includes the preceding phases' behaviors.

Phase - 0	No behavioral changes after PTZ application.
Phase - I	The animal has immobility, salivation, and tremors.
Phase - II	The animal has orofacial movements, raised tail and forelimbs' spasms.
Phase - III	The animal presents isolated clonic convulsions in the anterior limbs, without loss of the posture reflex.
Phase - IV	The animal has generalized clonic seizures, abundant salivation, and temporary loss of postural reflex.
Phase - V	The animal presents tonic-clonic seizures, cyanosis, loss of postural reflex, and death from respiratory arrest.

10 mg/kg v.o. for four days and tests done at 12 (n = 9), 24 (n = 9) and 36 (n = 9) hours, with subsequent PTZ-induced seizure outbreak (PTZ 60 mg/kg i.p.). From the groups mentioned above, electrocorticographic records were obtained to prove the drugs' brain activity during the experiment.

2.6. Liver function assessment

The liver enzymes were measured by obtaining a blood sample from the studied animals, being then analyzed at the Clinical Analysis Laboratory of the Institute of Biological Sciences at Federal University of Pará, through aliver function biochemical analysis by a Wiener Lab GROUP brand, CM 200 model.

2.7. Surgery for electrode placement

Animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (50 mg/kg i.p.) and xylazine hydrochloride (5 mg/kg i.p.). After abolishment of their corneal reflex, the animals were positioned in a stereotaxic apparatus. After surgical procedures to expose the skull, two bilateral holes were drilled into the rat skull with a dental drill. Stainless steel electrodes (tip exposure 1.0 mm diameter) were placed on the duramater above the frontal cortex at coordinates of bregma – 0.96 mm and ±1.0 mm lateral [42]. A screw was fixed in the occipital skull region, and the electrodes were fixed with dental acrylic cement using the screw as a base and ground for the electrodes.

2.8. Electroencephalographic records

After surgery, animals were kept in individual cages. Five days after surgery, the electrodes were connected to a digital data-acquisition system composed by a high impedance amplifier (Grass Technologies, P511), an oscilloscope (Protek, 6510) and a board for data acquisition and digitalization (National Instruments, Austin, TX). Data was continuously sampled at 1 kHz at a low pass of 3 kHz and a high pass of 0.3 Hz.

The recordings followed a standard protocol: 10 min of accommodation in carefully immobilized animal to avoid records interference. Basal electroencephalographic activity was recorded for 30 minuntes, which was used as a control treatment in EEG analyses. Thereafter, pentylenetetrazole - PTZ (60 mg/kg i.p.) was administered, and electrocorticographic activity was further recorded for 30 min. After the period of 30 min, as PTZ seizures can last for hours, the animals were sacrificed to avoid further distress [43].

2.9. Data analyses

Offline analysis was performed through a tool built using Python programming language (version 2.7). "Numpy" and "Scipy" libraries were used for the mathematical processing and "matplolib" library was used to obtain graphs and plots. A graphic interface was developed using the PyQt4 library. Spectrograms were calculated using Hamming window with 256 points (256/1000s). For power spectral density (PSD) each frame was generated with an overlap of 128 points per window. For each frame, the PSD was calculated by the Welch's average periodogram method. Frequency histograms were obtained by calculating the PSD of the signal using the Hamming window with 256 points without overlap, yielding a resolution of 1 Hz per bin. Each wave displayed in PSD is an average from a set of experiments. PSDs were calculated in each group and the means are shown by individual bins [43].

2.10. Statistical analyses

Normality and homogeneity of variances were verified using Kolmogorov-Smirnov and Levene's tests, respectively. Since residuals were normally distributed and variances were equal, comparisons among mean amplitude of tracings and control values were made using one-way ANOVA and Tukey counter-test. The minimum significance level was set at $p < 0.05$ in all cases. The software GraphPad® Prism 5 was used for statistical tests.

2.11. Quantification of phenobarbital in the LDH-PBT system

Quantitative analysis of the intercalated phenobarbital in the LDH-PBT system was carried out using absorbance measurement data in the ultraviolet region with maximum absorption wavelength at 242 nm. The absorbance experiments were performed by means UV–vis spectrophotometer, Shimadzu, model UV-2600.

The standard solution was prepared in a 100 mL volumetric flask, using: 4 mg phenobarbital, 20 mL ethyl alcohol, 10 mL 0.1 M NaOH and the volume made up to 100 mL with pH 7.4 buffer solution. From this standard solution, dilutions were performed to obtain the following concentrations: 04, 08, 12, 16, 20, 24, 28, 32, 36, and 40 mg/L. The standard solution preparation procedure and dilutions were performed in triplicate. After each procedure, 3 mL of the diluted solutions were transferred in increasing order of concentration to a quartz cuvette. The concentration of phenobarbital was calculated by regression analysis from calibration curve data of the solutions in different concentrations.

3. Results

Quantitative analyze of intercalated phenobarbital in the LDH was determined by means of calibration curve data from UV–vis absorbance measurements. The absorbance curve was plotted as a function of the phenobarbital concentration, as shown in Fig. 1. PBT intercalation in LDH lattice was verified in the proportion of 0.0397 g of phenobarbital per gram of material (397 mg/g), which allowed the behavioral and electroencephalographic test and verification. Doses were individually weighed in eppendorffs for administration to animals.

Taking into account the behavioral characteristics of acute pentylenetetrazole-induced seizure, we can classify 6 phases, as described in Table 1.

The animals were subjected to behavioral analysis during seizures and compared with the behavioral latency pattern observed for PTZ at a dose of 60 mg/kg i.p. In order to facilitate behavior-identifying scores' formation, the latencies for the onset

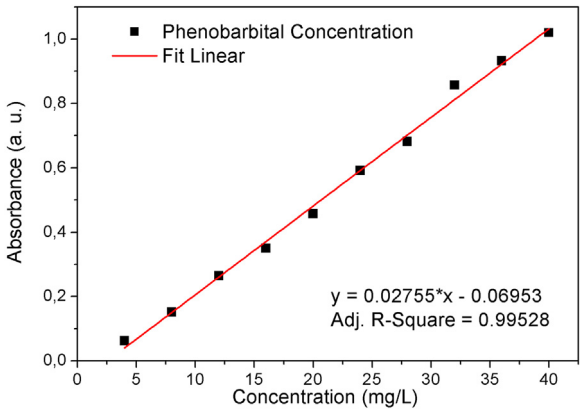

Fig. 1. Absorbance measurements recorded at room temperature for PBT solutions.

of seizure behavior were considered at the following depth degrees:

a) Forelimbs' spasms.
b) Isolated clonic seizures without loss of postural reflex.
c) Generalized clonic seizures with temporary loss of postural reflex.
d) Tonic-clonic seizures with loss of postural reflex.

After phenobarbital administration for a four days period, a seizure induction procedure was performed 12 h after the last dose of phenobarbital. It was observed that, in this condition, the seizure-induction did not have complete evolution, reaching phase II after the application of 60 mg/kg i.p. of PTZ. The behavioral characteristic observed in the animals during seizure's evolution was the spasm of the forelimbs, which showed that, pharmacologically, the absorption process was compatible between PBT and LDH-PBT samples in seizures control. These groups did not present statistical differences among themselves, but presented statistical differences to the PTZ and Control groups.

The permanence of phenobarbital in the body and the maintenance of high seizure threshold was observed after 24 h of phenobarbital withdrawal. In this case, both LDH-PBT and PBT samples had their activity proven after causing increased latency for onset of behavior, observed after induction of seizure. For the behavior of anterior limb spasms, which belongs to the end of phase II, there was no statistical difference between the LDH-PBT (84.22 ± 15.77 s) and PBT (77.89 ± 21.69 s) groups. But there was statistical difference between the groups that used phenobarbital for the control and PTZ groups (Table 3). In this case, the seizure evolved to subsequent phases presenting isolated clonic seizures without loss of postural reflex - that corresponds to a phase III characteristic. This behavior was observed with more than twice the latency for the LDH-PBT and PBT groups and there were no statistical differences between them. However, they maintained statistical differences for the control and PTZ groups (Table 2).

After 24 h of phenobarbital suspension, the animals presented phase IV behavioral characteristics, represented by generalized clonic seizures with temporary loss of postural reflex, presenting latencies for the PTZ group (150.1 ± 39.51 s) and control with clay (141.6 ± 38.46 s) without statistical differences between them. The LDH-PBT and PBT groups showed a latency of 316.4 ± 65.69 and 330.3 ± 78.4 s, respectively, showing no statistical difference between them, but maintaining differences for the control group, demonstrating a protection against PTZ-induced seizures' outbreak (Table 3).

With phenobarbital withdraw of for a 36 h period, the animals presented seizure evolution until phase V after PTZ induction, but the animals that received the intercalated phenobarbital presented higher latencies for the seizure onset, which indicated the drug's anticonvulsant effect's permanence, as can be seen in (Table 4).

The convulsive condition begins with forelimbs' spasms, with onset after PTZ administration latency time of 50.89 ± 5.92 s, with no statistical difference from the control group (48.11 ± 5.578 s).

Table 2
Average latency of forelimbs' spasm behavior observed after the 12 h suspension of phenobarbital treatment. Groups marked with different letters have statistical difference and groups marked with equal letters have no statistical difference. ANOVA followed by tukey test ($p < 0.05$) ($n = 9$).

Latency Treatment (seconds)	(Average ± DP)
PTZ	50.89 ± 5.925^{a}
LDH-PBT	155.7 ± 60.21^{b}
PBT	148.7 ± 53.46^{b}
Control	49.78 ± 9.217^{a}

Superscripts "a, b and c" are classes of values.

For the PBT, this behavior's latency time was 68.44 ± 6.307 s, with statistical difference from control group, while the LDH-PBT group's average latency time 80.67 ± 5.723 s, showing statistical difference for both the PBT as well as for the other analyzed groups. For the behavior of isolated clonic seizures without loss of postural reflex, the LDH-PBT group had the highest latency for seizure behavior's onset, revealing statistical differences for all other groups. This indicates that the phenobarbital's slow release process influences both observed behaviors, corroborated by the fact that the LDH-PBT group had a higher average latency (Table 4).

Concerning the appearance of generalized clonic seizures with temporary loss of postural reflex, there was no statistical difference between LDH-PBT (213.3 ± 32.49 s) and PBT (211.6 ± 39.47 s) samples, indicating that the behavior observed in phase IV had a similar evolution for both forms of phenobarbital. However, the two phenobarbital preparations(preparações de fenobarbital) presented statistical difference from the control and PTZ groups (Table 4).

Regarding the behavior of tonic-clonic seizures with loss of posturalreflex, which composes the phase V and represents the most intense convulsive behavior, the LDH-PBT group presented higher latency time, showing statistical difference from the other groups (Table 4), which reveals that the intercalation process of phenobarbital in LDH lattice improves its performance at the same dose administered as non-intercalated phenobarbital.

The control groups' electrocorticographic tracings are characterized by a smaller amplitude (below 0.1 mV) in the spectrogram, and frequencies with higher energy intensity (below 10 Hz) can be observed (Fig. 2A). Basal record power averaged 0.2116 ± 0.08257 mV^2 / Hz $x10^{-3}$ (Fig. 3). The group that received PTZ presented electrocorticographic alterations compatible with convulsive condition, with irregularities and amplitude of record that reached 0.7 mV, presenting characteristic the energy elevation level in frequencies up to 50 Hz, observed in the spectrogram, corresponding to a significant change when compared to the control group (basal) (Fig. 2B). The PTZ group's power level was higher, with a mean of 6.844 ± 2.195 mV^2 / Hz $x\ 10^{-3}$, demonstrating a statistical difference from the control group (Fig. 3).

The group treated with intercalated phenobarbital (LDH-PBT 12) and non-intercalated phenobarbital (PBT 12) samples showed very similar electrocorticographic tracing characteristics with after PTZ seizure induction (Fig. 2 C and D). The groups' power analysis showed that the LDH-PBT 12 group presented means of 1.613 ± 0.709 mV^2/Hz x 10^{-3} and the PBT 12 group presented means of 1.91 ± 0.5688 mV^2/Hz x 10^{-3}, not showing statistic difference according to the values. However, the LDH-PBT 12 and PBT 12 groups showed statistical difference in relation to the control group and the PTZ group (Fig. 3).

The group that received PTZ 24 h after intercalated phenobarbital (LDH-PBT 24) treatment's withdrawal presented cortical record with triggers below 0.5 mV and higher energy intensity observed in the spectrogram (below 30 Hz) (Fig. 2 E and F). Regarding the record power, it was observed an average of 2.852 ± 0.4075 mV^2/Hz x 10^{-3}. On the group that received non- PBT 24 sample, the power level observed during recording was 3.736 ± 0.9467 mV^2/Hz x 10^{-3}, showing statistically significant differences between the PBT 24 and LDH-PBT 24 groups (Fig. 3).

In tests performed 36 h after ending phenobarbital treatment, groups LDH-PBT 36 and PBT 36 showed greater oscillations in tracing, indicating a decrease in the amount of drug that protects against PTZ-induced seizures (Fig. 2 G and H). In group LDH-PBT 36, the power presented an average of 3.811 ± 0.8631 mV^2/Hz x 10^{-3}, revealing a statistical difference for the PBT 36 group, with an average of 5.594 ± 0.8784 mV^2/Hz x 10^{-3}, showing, thus, that the slow release process of intercalated phenobarbital promoted greater protection for tested animals. This data is relevant for

Table 3
Average latencies for behaviors characteristic of seizure evolution 24 h after phenobarbital usage. Groups marked with different letters have statistical difference and groups marked with equal letters have no statistical difference. ANOVA followed by tukey test ($p < 0.05$) ($n = 9$).

Behavior	Treatment	Average Latency (S) ± DP
Forelimbs' spasms	PTZ	50.89 ± 5.92^{a}
	LDH-PBT	84.22 ± 15.77^{b}
	PBT	77.89 ± 21.69^{b}
	Control	46.22 ± 6.457^{a}
Isolated clonic seizures without loss of postural reflex	PTZ	72.78 ± 16.01^{a}
	LDH-PBT	197.2 ± 58.59^{b}
	PBT	199.4 ± 84.96^{b}
	Control	73.64 ± 14.28^{a}
Generalized clonic seizures with temporary loss of postural reflex	PTZ	150.1 ± 39.51^{a}
	LDH-PBT	316.4 ± 65.69^{b}
	PBT	330.3 ± 78.4^{b}
	Control	141.6 ± 38.46^{a}

Superscripts "a, b and c" are classes of values.

Table 4
Shows the average latency for seizures 36 h after phenobarbital usage. Groups marked with different letters have statistical difference and groups marked with equal letters have no statistical difference. ANOVA followed by tukey test ($p < 0.05$) ($n = 9$).

Behavior	Treatment	Average Latency (S) ± DP
Forelimbs' spasms	PTZ	50.89 ± 5.92^{a}
	LDH-PBT	80.67 ± 5.723^{b}
	PBT	68.44 ± 6.307^{c}
	Control	48.11 ± 5.578^{a}
Isolated clonic seizures without loss of postural reflex	PTZ	72.78 ± 16.01^{a}
	LDH-PBT	116.6 ± 13.39^{b}
	PBT	87.89 ± 10.37^{a}
	Control	82.33 ± 13.31^{a}
Generalized clonic seizures with temporary loss of postural reflex	PTZ	150.1 ± 39.51^{a}
	LDH-PBT	213.3 ± 32.49^{b}
	PBT	211.6 ± 39.47^{b}
	Control	153.2 ± 28.75^{a}
Tonic-clonic seizures with loss of postural reflex	PTZ	203.3 ± 37.23^{a}
	LDH-PBT	281.4 ± 38.25^{b}
	PBT	227.7 ± 43.37^{a}
	Control	201.2 ± 20.80^{a}

Superscripts "a, b and c" are classes of values.

finding that, in relation to power, the PBT 36 group does not present statistical difference from the PTZ group.

Tests for the evaluation of liver enzymes of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) indicate hepatocyte membrane damage, which was observed for group receiving PBT samples. For animals receiving LDH-PBT samples there were no statistical differences in AST assessment relative to the control group (Table 5). There was a significant increase in AST and ALT for the PBT group compared to the control which may indicate liver damage.

4. Discussion

Phenobarbital has a long history of clinical use, which gives it an unrivaled level of information and safety about its efficacy and tolerability [33,35,44–46], not to mention its very low cost, being chosen as the anticonvulsant drug for this study due to all of these factors.

There are currently more than 20 drugs available for epilepsy treatment, however, none of them dramatically changed the long-term prognosis of this condition [47]. The cascade of events that triggers epilepsy is likely to vary greatly from individual to individual and, only by better understanding epileptogenesis, treatment can be directed to its specific mechanisms and then treatments that can modify the disease's natural history may be available. In this scenario, the use of slow drug delivery system, such as intercalated phenobarbital, may be observed as a possibility of better control of the epileptic episode and fewer side effects, visualized in this study through behavioral and electroencephalographic testing and verification after this drug's intercalation in LDH nanocarrier at a ratio of 0.0397 g per gram of material.

Layered double hydroxides consist of inorganic, two-dimensionally structured materials that are electrically charged and able to interact and carry organic molecules. They promote increased solubility, stability and alteration in the release of the intercalated molecule [11,32].

The LDH structure undergoes sustained release process due to the lamellar matrix's dissolution caused by acid attack or an ion exchange reaction in the organism [10]. Additionally, the confinement of substances in lamellar structures can increase their action time in the organism to maintain physical, chemical, therapeutic and toxicological properties of the active compound during storage. In addition to promoting substance stability and regulate their release into the organism [48]. This data is in accordance with the results of our study, since intercalated phenobarbital in LDH lattice had a longer time to control PTZ-induced seizures when compared to commercial phenobarbital.

Nowadays, more and more patent and scientific papers are focusing on the interleaving of biologically active products in LDH as a strategy for increasing substances' stability for modern therapies usage [49–51]. In this present paper, LDH nanostructure was chosen as carrier of phenobarbital, precisely because of their innovative characteristics. The control of chronic disease with

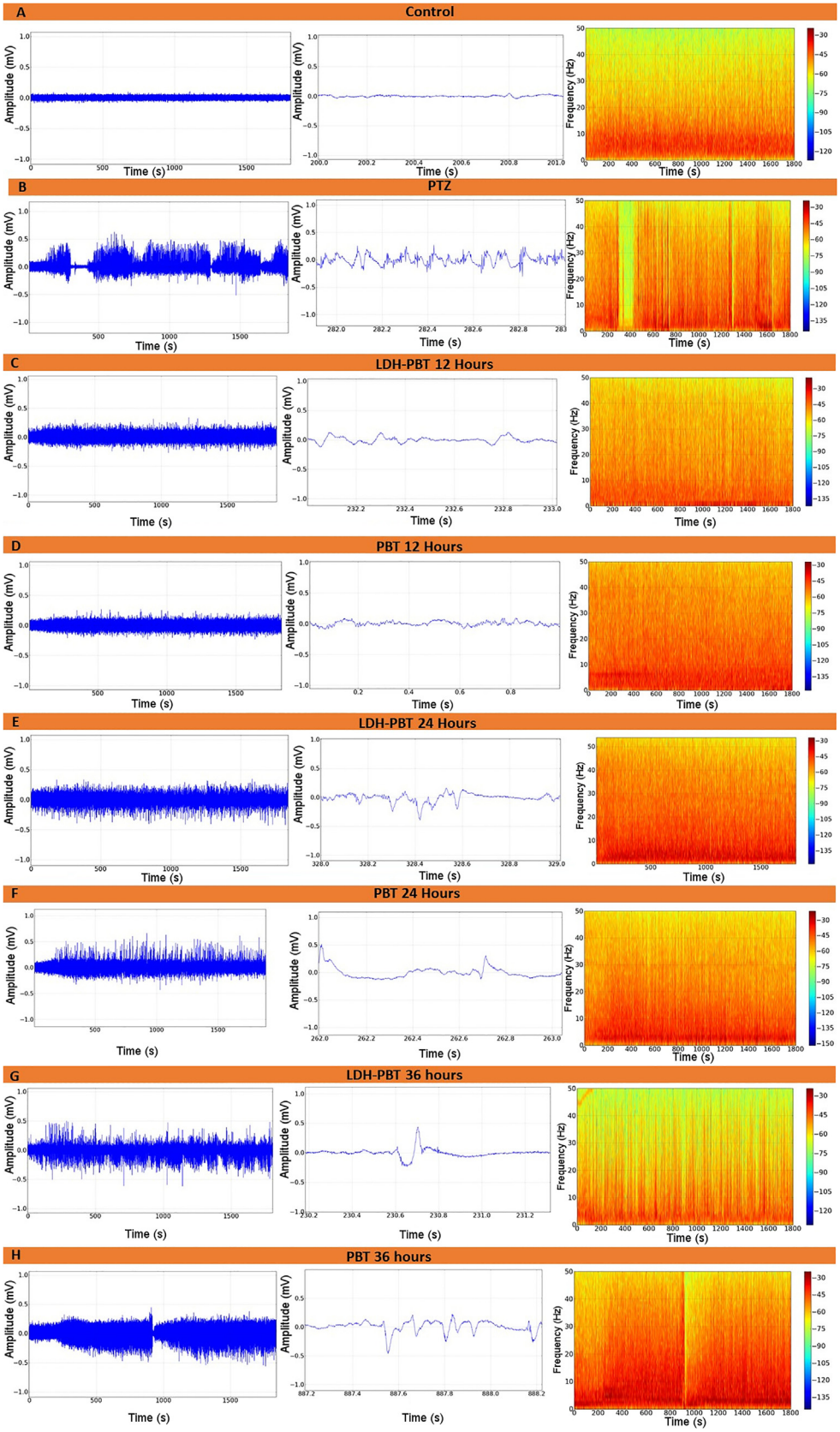

Fig. 2. Demonstration of electrocorticographic traces (ECoG) and spectograms,lasting 30 min after PTZ administration. Control electrocorticographic record (basal) (A), electrocorticographic recording of control after PTZ application (B), Electrocorticogram of animals receiving four LDH-PBT doses, with induction of seizure 12 h after treatment interruption (C), PBT electrocorticographic tracing with convulsive induction 12 h after treatment interruption (D), LDH-PBT group electrocorticogram with seizure induction 24 h after phenobarbital withdrawal (E),PBT group record with seizure induction 24 h after phenobarbital withdrawal (F), Record of LDH-PBT group with induction of seizure 36 h after phenobarbital withdrawal (G) and Registration of the PBT group with seizure induction 36 h after phenobarbital withdrawal (H).

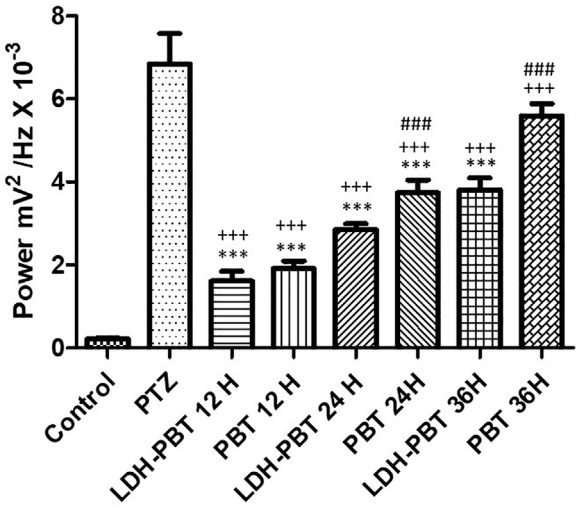

Fig. 3. Power of records after withdrawal of LDH-PBT and PBT samples at 12, 24 and 36 h. (***) statistical difference for the PTZ group,(+++)statistical difference for the basal control group, (###) statistical difference between the groups receiving LDH-PBT and PBT samples. ANOVA followed by tukey test ($p < 0.05$) ($n = 9$).

Table 5
Liver enzyme activity after LDH-PBT and PBT samples usage. Groups highlighted with different letters have statistical difference and groups highlighted with equal letters have no statistical difference. ANOVA followed by tukey test ($p < 0.05$) ($n = 9$).

Liver Enzymes	Control	LDH-PBT	PBT
AST (mg/dL)	1003 (± 7.881)[a]	106.9 (± 7.167)[a]	119.1 (± 5.231)[b]
ALT (mg/dL)	5300 (± 6.946)	5400 (± 9.220)	5856 (± 5.247)

Superscripts "a, b and c" are classes of values.

fewer oscillations in plasma concentrations may favor epilepsy's treatment using intercalated phenobarbital.

In this present study, through electrocardiographic tracing (ECoG) and spectrograms, it was observed significant statistical changes between LDH-PBT (Fig. 2G) and PBT (Fig. 2H) samples after 36 h of these drugs' usage. Compared to PBT, LDH-PBT has been shown to offer greater protection against seizure after this period, showing this intercalated drug's longer residence time in the body.

Liver enzymes ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase) are used to assess liver damage [52]. It is now known that most drugs used to treat epilepsy may induce some change in the values of these enzymes, such as phenobarbital, valproic acid, phenytoin, felbamate, carbamazepine, among others [53–55].

Phenobarbital is a widely used antiepileptic drug, but with important side effects - liver failure being the most severe - and may go from only increasing liver enzymes to death, even [56]. In this study's results, the use of phenobarbital promoted an increase in liver transaminase values, corroborating the results found in other animal studies [57,58].

The use of non-intercalated and intercalated phenobarbital did not increase the ALT enzyme in relation to the control group, maintaining the enzyme close to normality standards. However, when analyzing the AST enzyme, the use of the PBT sample induced a significant increase in AST compared to the control group, which may suggest a certain hepatic impairment. The result with the LDH-PBT sample showed no difference from the control group, which may be justified by the fact that the drug is released slowly in the organism, avoiding rapid absorption, with its gradual level increase in blood. Slow release causes increased drug safety, leading to lower enzyme induction [49], which is an important side effect caused by phenobarbital.

5. Conclusion

The synthesis of LDH structures and the phenobarbital intercalation through the coprecipitation method were successfully performed. The quantification of the intercalated phenobarbital in sample was performed by measurements of UV–vis spectroscopy.

Data from the experimental model of PZT-induced seizures showed that LDH-PBT had a better anticonvulsant effect, due to its maintenance in the organism. The results of the electrocorticographs and spectrograms revealed that the LDH-PBT sample has longer protection against seizures than the PBT sample. Assessment tests for liver enzyme activity showed that using the PBT sample induced increase in AST. While the use of the LDH-PBT sample showed no difference from control group data, indicating slow release of phenobarbital in the organism. Therefore, assays in vivo have shown a higher protective effect of LDH-PBT sample for a longer time (36 h).

Pharmacological studies of phenobarbital release in vivo indicated that the compound LDH-PBT is a perspective material for potential application as controlled drug delivery systems, as it maintains the slow release and better control of convulsions experimentally induced by PTZ.

Author statement

Lorena Cristina Nunes de Almeida and Beatriz de Andrade Marques: Surgery for electrode placement, electroencephalographic data records and analysis.

Rafaela Laranjeira Silva and Akira Otake Hamoy: Liver function assessment, behavioral effects and electroencephalographic data analysis.

Frank Sales Nunes Brito: Synthesis of LDH.

Moisés Hamoy: Supervision, conceptualization, methodology, validation and formal analysis of the electroencephalographic experiments. Writing - review & editing.

Vanessa Jóia de Mello: Supervision, validation and formal analysis of the liver function assessments.

Rosivaldo dos Santos Borges: Phenobarbital intercalation in LDH lattice.

Marcos Anicete-Santos: Supervision, conceptualization, methodology and validation of the synthesis of LDH and intercalation of phenobarbital. Writing - review & editing.

Elson Longo: Supervision, conceptualization, resources, funding acquisition, project administration. Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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