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ORIGINAL ARTICLE

Development and validation of a method for phenobarbital in serum: anticonvulsant pharmacotherapy monitoring

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Abstract

The therapeutic drug monitoring (TDM) is an important strategy for the effectiveness and safety of long-term pharmacotherapy, such as the use of phenobarbital as an anticonvulsant drug in epilepsy. In this sense, HLPC has been presented as a technique for the measurement of phenobarbital in serum. However, the ideal conditions for carrying out the method must be established for each laboratory reality. An analytical method using HPLC was developed and validated in order to identify and quantify Phenobarbital in blood. The chromatographic conditions were C-18 column (Shimpack XR-ODS 50L x 3.0), acetonitrile-water mobile phase (30:70, v v^{-1}), 0.2 mL min^{-1} flow and reading wavelength of 210 nm. Linearity was established in the range of 2.5 to 80 µg mL^{-1}, the linear correlation coefficient was 0.9981. The average of the coefficient of variation of the precision was 5.30%. The relative standard error of the accuracy was -2.17% and of the recovery coefficient was 97.83%. In all eleven patients, phenobarbital concentrations were below the therapeutic range. The tested method was selective, linear, precise, accurate and showed good recovery.

Keywords: Phenobarbital. Drug Monitoring. Validation Studies. HPLC.

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INTRODUCTION

Phenobarbital is an anticonvulsant drug that amplifies the inhibitory neurotransmission of Gamma-AminoButyric Acid (GABA). Many experts and authors criticize the use of phenobarbital for its therapeutic limitations in some types of seizures and the high risk of serious adverse effects such as sedation, megaloblastic anemia, osteomalacia and light hypersensitivity, besides the risk of metabolic tolerance and drug dependence. At high doses, there are frequent reports of cardiorespiratory depression and coma. However, phenobarbital, even today, is still widely used to prevent seizures, especially for its low financial cost (Brasil, 2018; Macnamara, 2012).

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Therapeutic drug monitoring (TDM) is a clinical practice that involves measuring drug concentrations in a patient's plasma or serum at a designated time point to provide guidance on the dosage regimen needed to maintain therapeutic concentrations. TDM is critical for drugs in the following situations: narrow therapeutic index, when there is no easy way to measure outcomes, in the event of severe side effects, when there is an association between drug concentrations and effect and/or side effects, in the existence of a large variation in drug exposure among patients receiving a fixed dose and when several endogenous and exogenous factors alter the drug exposure in a patient. Certain immunosuppressive drugs, antibiotics, antifungal drugs, antiviral drugs, antidepressants, cardiac drugs, and anticonvulsants are routinely monitored in patients (Junaid et al, 2019).

This procedure is not only limited to laboratory quantification of drugs in a given patient who uses it, but it also consists in associating such dosage to observed signs and symptoms in order to correlate the body levels of bioactive substance with the effects, which can be therapeutic or toxic, that are being observed in the user during the pharmacotherapy. The TDM is a method involving dose adjustment according to pharmacokinetics (PK) or pharmacodynamics (PD) to optimize patient outcomes, thereby individualizing treatment (Santos et al., 2014).

Antiepileptic drugs (AEDs) are widely used as long-term adjunctive therapy or as monotherapy in epilepsy and other indications and consist of a group of drugs that are highly susceptible to interactions. They have complex PK and PD, high inter-individual variabilities and, in the case of carbamazepine, phenobarbital, phenytoin and valproic acid, they also have a narrow therapeutic index. In addition, several AEDs are associated with cytochrome P450 enzyme metabolism, which can induce drug-drug interactions (Johannessen e Landmark, 2010; Food and Drug Administration, 2020).

One of the techniques that can assist in the therapeutic control of anticonvulsants, such as phenobarbital, is the High-Performance Liquid Chromatography (HPLC) technique. This physicochemical technique allows the separation and identification of substances in a sample. It can detect amounts as small as a picogram, depending on the goal of the analysis (Ezzeldin et al., 2013). HPLC is a simple, precise, accurate and cost-effective method and provides excellent recovery with high precision for a wide range of pharmaceutical compounds (Deeb et al., 2014; Domingues et al., 2016).

According to the Brazilian Health Surveillance Agency (ANVISA) Resolution (RDC) Nº. 166 of July 24, 2017, the techniques used must contain minimum validation requirements. A validated method ensures the quality of chemical measurements, through its comparability, traceability and reliability (Brasil, 2017; Ribani et al., 2004).

Thus, the objective of this research was to validate a method for determining the phenobarbital level in blood serum using HPLC and apply it in blood samples from epileptic patients and chronic phenobarbital users.

METHODS AND MATERIALS

Type of research and population

This investigation was characterized as descriptive, analytical, cross-sectional, and documentary. The participants of the research were patients of the neurology service of Hospital Universitario Alcides Carneiro (HUAC), at Universidade Federal de Campina Grande (UFCG). The patients were diagnosed with epilepsy and had been treated with phenobarbital for more than a month at the time of data collection. Women known to be pregnant and patients who stopped using the drug in question were excluded.

The statistical analyses were performed using SPSS software (Statistical Package for the Social Science, version 17.0). An ethics committee (CEP-HUAC, process number 20110508) approved the research project.

Epidemiogical and clinical data

The instrument for collecting social and clinical data consisted of questionnaires prepared by the researchers and validated through a pilot study. Then, interviews were scheduled with patients who met the investigation inclusion criteria. The following variables were studied:

a) Sociodemographic profile: gender, age (<10; 10-19; 20-29; 30-39; 40-49; 50-59; 60-69; 70-79; >80) and level of education.
b) Related to the patient's clinical characteristics: associate pathologies, medication of chronic use (drug and dosage), habits (alcoholism, smoking, physical activity), and information about the latest seizure occurrence.
c) Related to the use of phenobarbital: presentation, dosage, treatment duration, adverse reactions, and association with other anticonvulsants.

From the collection of these data and the quantification of phenobarbital present in the blood of patients, using the HPLC technique, blood levels of phenobarbital were correlated with the incidence of seizures and / or adverse / toxic effects.

MATERIALS

Reagents and reference samples

Phenobarbital was acquired from Cristália laboratory (Brazil). Teuto Laboratory (Brazil) donated phenytoin (internal standard). The trichloroacetic acid (TCA) was purchased from Merck (Germany). Acetonitrile in HPLC grade was purchased from Merck (Darmstadt / Germany).

Preparation of stock solutions and work solutions

Standard stock solution of phenobarbital was prepared at a concentration of 1 $mg.mL^{-1}$. 10 mg were weighed and transferred to a 10 ml volumetric flask and the volume made up to methanol. The stock solutions were transferred to an amber flask and stored under refrigeration (2-8 ° C) for an established period of, at most, 30 days.

The working solutions were prepared from the stock solution, diluted in the mobile phase to obtain a concentration of 40 $\mu g.mL^{-1}$ for phenobarbital, and 50.0 $\mu g.mL^{-1}$ of phenytoin as internal standard.

Sample preparation

5 mL of blood were collected from each patient. To obtain the serum, blood samples were centrifuged for 15 minutes at 3000 rpm. 200 µL of serum were transferred to polypropylene tubes and it was added 200 µL of phenytoin internal-standard solution (1 mg mL^{-1}) and 50 µL of 15% TCA. This solution was homogenized on a vortex mixer for 30 seconds and subsequently centrifuged at 4400 rpm for 30 minutes at 4 °C. The supernatant was transferred to another tube and recentrifuged under the same conditions for five minutes. The supernatant was diluted in the mobile phase, filtered and 10 µL of it were injected into the HPLC.

Equipments and chromatographic conditions

QL-901 Vortex Stirrer (AAKER, China); BE6000 centrifugue (BioEng, Brazil); 5702R (Eppendorf, Germany).

The LC system used was a Shimadzu-Prominence UFLC XR (Shimadzu, Kyoto, Japan) equipped with two solvent flow pumps (LC-20AD), a SIL-20AC-AHT autosampler, a CBM-20A system controller, a SPDM20A-UV VIS detector with diode array and a CTO-20A column

oven. In order to collect data, the automatic integration of the peak areas was performed by LC-solution® software.

The method was carried out on a Shimpack XR-ODS (Shimadzu, Kyoto, Japan) analytical column (C-18 type, 3.0mm X 50mm I.D., particle size of 2.2 µm). The mobile phase for the chromatographic test consisted of acetonitrile: water (30:70, v v-1) at a flow rate of 0.2 ml min-1. The column temperature was maintained at 30 ° C and the wavelength was set at 210 nm.

METHOD VALIDATION

Selectivity

For selectivity test it was used six serum samples from non-phenobarbital-users individuals, including one lipemic and another hemolyzed. The chromatographic test evaluated the presence of interfering peaks at retention times of the analytes of interest, the absence of these peaks indicates that the method is selective (Brasil, 2017).

Matrix Effect (EF)

Six serum samples following the Phenobarbital® standard were prepared at 40 µg mL^{-1}. The calculation of the matrix effect was done using the following equation:

$$EF = \frac{experimental\ value}{nominal\ value} \quad (1)$$

Experimental value - the phenobarbital peak area found in the sample subjected to the extraction process.
Nominal value - the phenobarbital peak area found after dilution in the mobile phase (Cassiano et al., 2009).

Linearity

Linearity was assessed at six levels, three times each level. Phenobarbital® solutions were added to drug-free plasma samples to obtain analytical solutions in the following concentrations: 2.5; 5.0; 10.0; 20.0; 40.0 and 80.0 µg mL^{-1}. The analytical curve was evaluated in three different days. The results were statistically analyzed by linear regression analysis by least squares method. The minimum acceptance criteria of the correlation coefficient were 0.99. The variation coefficient was lower or equal to 20% compared to the lowest nominal concentration, and less than or equal to 15% for other concentrations (Cassiano et al., 2009).

Sensitivity

The sensitivity was determined by limit of detection and the limit of quantitation. Solutions were analyzed for known and decreasing concentrations of Phenobarbital® standard. The values were based on the standard deviation of the slope of Phenobarbital® calibration curve (Ribeiro et al., 2008).

Accuracy and precision

Samples of five different concentrations were prepared each in quintuplicate. In this case we used the following concentrations (Brasil, 2017) (NOTE: the following acronyms are in Portuguese): LIQ - lower limit of quantitation - 2.5 µg mL^{-1}; CQB - quality control sample of low concentration - 7.5 µg mL^{-1}; CQM - quality control sample mean concentration - 40.0 µg mL^{-1}; CQA - quality control sample of high concentration 64.0 µg mL^{-1}; CQD - dilution quality control sample -100 µg $m^{-1}L$.

The results were analyzed in the same analytical run (intrarun) and in three different runs (interrun) (Ribeiro et al., 2008). The Relative Standard Error (E_R) using the following equation expressed accuracy:

$$E_R = \frac{(experimental\ value - nominal\ value) \times 100}{nominal\ value} \quad (2)$$

Where:
experimental value- peak area of the phenobarbital standard found in the spiked samples; nominal value- peak area of the phenobarbital standard diluted in the mobile phase.

The percentual coefficient of variation (CV%) using the following equation expressed precision:

$$CV = \frac{standard\ deviation \times 100}{experimental\ average\ concentration} \quad (3)$$

The acceptance criteria for accuracy was mean values of ± 15% of nominal value and ± 20% for LIQ. Precision was acceptable for a maximum CV of 15% and for LIQ values of ± 20% (Brasil, 2003, 2017).

Recovery

The recovery of the phenobarbital standard was assessed at five different concentrations (2.5; 7.5; 40.0; 64.0 and 100 μg mL^{-1}). To perform the calculation the following equation was used:

$$Recovery = \frac{experimental\ value \times 100}{nominal\ value} \quad (4)$$

Where:
experimental value is the response obtained for the analyte when it is added in the biological matrix and then extracted.

nominal value is the response obtained for the analyte in samples prepared in the mobile phase.

It is desirable that the analyte recovery values are near 100%, however, lower values are accepted, provided the recovery is precise and accurate. A variation of up to 15% of the recovery value determined for the analyte of interest is acceptable (Cassiano et al., 2009).

Robustness

The robustness of the analytical method was evaluated by analysis of the phenobarbital standard content on different chromatographic conditions. Changes were made in the following conditions of the test: ratio of mobile phase acetonitrile: water (25:75 and 35:75 v v^{-1}), temperature (27 ºC and 33 ºC) and reading wavelength (205 nm and 215 nm). Each parameter was changed individually while others were kept constant. These variations were evaluated for the selectivity, accuracy and precision criteria (Ribani et al., 2004).

METHOD APPLICATION

The developed method was applied to 11 serum samples obtained from patients with epilepsy and chronic users of phenobarbital, treated at neurology department of Hospital Universitario Alcides Carneiro (HUAC). The samples were subjected to the extraction process

described in the Sample preparation section. It was made a correlation between blood levels of phenobarbital and the incidence of seizures and adverse or toxic effects of these drugs in patients.

The analytical tests were performed in the "Laboratório de Ensaios Cromatográficos (LEC)" that is part of the "Laboratório de Desenvolvimento e Certificação de Biomateriais do Nordeste (Certbio)", Department of Pharmacy, State University of Paraiba (Universidade Estadual da Paraíba - UEPB).

RESULTS AND DISCUSSION

According to the Brazilian Institute of Geography and Statistics, Paraíba is divided into four mesoregions considering political, social and economic aspects: Mata Paraibana, Agreste Paraibano, Borborema and Sertão Paraibano (Instituto Brasileiro de Geografia e Estatística, 2010). In this research, 50 patients were interviewed. The vast majority (90%) came from the mesoregion of agreste Paraibano, especially from the municipality of Campina Grande, with patients still coming from municipalities in Borborema (8%) and Sertão da Paraíba (2%). This finding reflects the regional impact of the service provided by HUAC. Thus, one can estimate the relevance of implementing a therapeutic monitoring service for anticonvulsants at HUAC as a strategy for optimization of individual pharmacotherapy.

As for gender, there was a slight predominance of women (58%). This higher percentage of women with epilepsy can be related to the population studied, since, according to the 2010 Census of the Brazilian Institute of Geography and Statistics, the city of Campina Grande has 52.70% of the female gender (Instituto Brasileiro de Geografia e Estatística, 2010).

The age of the patients interviewed in the study showed a slight predominance of children up to 10 years old, with a relative balance in the other age groups. This age profile is confirmed by the fact that although seizures can occur at any age, they occur mainly in early life. Studies state that until the age of 20 years, 78% to 90% of individuals who will be epileptic have already started their seizures and among children, 60% had their first seizure by the age of three, mostly occurring in the first year of life (Calvano et al, 2010). Thus, once most patients were children or young people, the importance of the service proposed is emphasized by the fact that it addresses people who will still live with the disease for a long time or who are in the most productive phase of their lives, including economically.

Regarding education, individuals who studied up to elementary school predominated (64%). This result is compatible with that found in other studies (Calvano et al, 2010). This can be explained by the profile of patients seen in public hospitals who are, for the most part, poor which makes it difficult for the individual to continue in the educational system. In a population-based study on the prevalence and incidence of epilepsy worldwide using meta-analytical techniques the prevalence of epilepsy did not differ with respect to gender, age group and education with this study (Fiest et al., 2017).

The frequency of seizures in patients ranged from one seizure every two years to thirty-five seizures per day. This variability in the frequency of crises among users of phenobarbital constitutes an indication of difficulties in pharmacotherapeutic control. According to the Ministry of Health, the patient is considered crisis-free when they have not occurred for at least two years, while being treated with an unchanged dose of the drug. Using this criterion, 50% of patients have controlled seizures. Official guides state that, ideally, patients should be treated with a single antiepileptic drug (AED). If the initial treatment is not successful, monotherapy should be used, using another drug. In the case of persistent failure, a combination of two anticonvulsant drugs can be attempted (Brasil, 2018).

When looking at the sample data, 74% of patients are on monotherapy. This fact is in accordance with the recommendations of the guidelines (Brasil, 2018). In addition, among patients undergoing monotherapy, 59.5% present with controlled crises; while among the 26% who are under polytherapy, only 23.07% have their crises controlled. The most common associate drug is carbamazepine, occurring in 66.66% of cases.

It is noteworthy that, when correlating these two variables (association with other anticonvulsants and crisis control), an Odds Ratio of 4,400 is obtained. This means that the chance of a monotherapy patient presenting a controlled crisis is 4.4 times greater than a patient undergoing polytherapy, which confirms the inefficiency of simultaneous use of more than one anticonvulsant to control epileptic seizures. The analysis of these data indicates that the treatment received by the patients, although it seems appropriate, can be optimized with the aid of laboratory dosage data of the blood concentration of phenobarbital in such patients.

The liquid chromatography method used in this work was suitable for the determination and quantification of phenobarbital (PNB) in blood serum of users. After performing various tests, the following optimal chromatographic conditions were obtained: [acetonitrile: water] (30:70, v v^{-1}) mobile phase, flow of 0.2 mL min^{-1}, column temperature of 30 °C and reading wavelength of 210 nm with a total duration of 13 min. Under these conditions, the retention times for PNB and internal standard were 4.471 and 8.948 min, respectively. After analyzes of six plasma samples (including lipemic and hemolyzed samples) of different volunteers, only one peak of endogenous origin was identified at 1.48 min, with no significant interference in the analyte investigated leading to conclude that the method is selective (Figure 1).

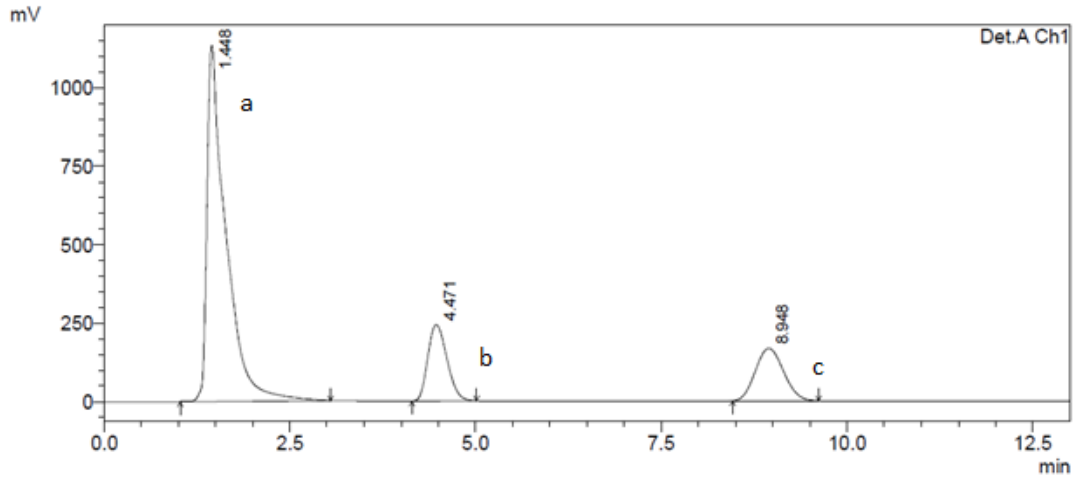

Figure 1. Chromatographic separation of clinical serum sample containing the Phenobarbital® standard (concentration of 40 µg mL^{-1}) and Phenytoin® Internal Standard (concentration de 50 µg mL^{-1}). a – endogenous interferent, b –Phenobarbital® standard, c – Phenytoin® Internal Standard

Validation of methods that use biological matrix demands the calculation of the EF. In this work, the analysis of the six samples showed that the average EF was 0.98, suggesting that there is suppression of ionization and the coefficient of variation of the responses was 9.30%, which is an acceptable value according to the literature (Cassiano et al., 2009).

Linearity was determined in the concentration range of 2.5 to 80.0 µg mL-1, as it encompasses the therapeutic range of the drug (10-30 µg.mL^{-1}) (MacNamara, 2012). The calibration curve found corresponds to the mathematical model established by the relationship between the instrumental response (area/height of the chromatographic band) and the analyte concentration, which is represented by the equation y = 5954.4x + 3352.5 and linear correlation coefficient (r) 0.9981. The coefficients of variation were less than 15% (Table 1). Therefore, the method application was satisfactory and reliable from the point of view of reproducibility. This indicates that the method is suitable for the analysis (Brasil, 2017).

Table 1. Areas of the Phenobarbital® peaks in the analytical curve

Concentration	Areas	Average of areas ± standard deviation	CV (%)
2.5 μg mL^{-1}	16154.0000	14955.7778 ± 1042.5626	6.97
	14457.3333		
	14256.6667		
5.0 μg mL^{-1}	40924.6667	37961.3333 ± 2604.9030	6.86
	36926.3333		
	36033.0000		
10.0 μg mL^{-1}	54343.0000	50163.6667 ± 3670.6187	7.32
	48685.3333		
	47463.0000		
20.0 μg mL^{-1}	136027.3333	132051.6667 ± 3462.4583	2.62
	130433.3333		
	129695.6667		
40.0 μg mL^{-1}	261608.3333	246689.3333 ± 13020.6151	5.28
	240846.6667		
	237614.0000		
80.0 μg mL^{-1}	489519.6667	476117.0000 ± 14789.9997	3.11
	460249.6667		
	478583.6667		

* CV – coefficient of variation.

The limits of quantification and detection (LOD) used to demonstrate the sensitivity of the method were estimated from the calibration curve. The result of the detection limit was 7.95 μg ml^{-1} and the quantitation 11.76 μg ml^{-1}, indicating that the method has adequate sensitivity for the proposed analysis since the therapeutic range is 10 – 30 μgmL^{-1}.

Precision and accuracy determine the errors of an analytical measure and are the main criteria used to judge the quality of a bioanalytical method. Table 2 shows the calculation of intra- and interday accuracy and precision. The method was considered precise, with the average of the coefficient of variation equal to 5.30% and accurate with the average of E_R being -2.16% (Brasil, 2003, 2017).

Table 2. Values obtained of accuracy, precision and recovery of the method in the evaluation of five concentration levels

Concentration	Intraday									Interday		
	CV (%)			E_R (%)			Recovery			CV (%)	E_R (%)	Recovery (%)
	Day I	Day II	Day III	Day I	Day II	Day III	Day I	Day II	Day III			
LIQ (2.5 μg mL^{-1})	10.74	11.45	4.29	-3.06	-10.15	-0.20	96.93	89.84	99.79	4.29	-5.58	94.41
CQB (7.5 μg mL^{-1})	7.91	4.05	2.84	1.80	-10.44	4.03	101.80	89.55	104.03	8.76	-1.59	98.40
CQM (40 μg mL^{-1})	2.69	2.66	2.34	-1.35	-0.36	7.32	98.64	99.63	107.32	4.28	1.84	101.84
CQA (64 μg mL^{-1})	0.71	2.57	2.28	-3.71	-8.98	-3.32	96.28	91.01	96.67	5.17	-5.31	94.68
CQD (100 μg mL^{-1})	2.89	2.27	0.72	-5.63	-5.09	11.97	94.36	94.90	111.97	3.99	-0.18	99.81
Average	4.98	4.60	2.49	-2.39	-7.00	3.36	97.60	92.98	103.95	5.30	-2.16	97.83

* LIQ – lower limit of quantitation. CQB – quality control sample - low concentration. CQM – quality control sample - average concentration. CQA – quality control sample - high concentration. CQD – dilution quality control sample. CV – coefficient of variation. E_R – relative standard error.

The method had a good recovery of the phenobarbital standard with mean values of 97.83% in the analyzed samples (Table 2). These results are considered suitable, since recovery values between 70 and 120% are accepted in most analytical validation procedures, provided that they are accurate and precise. Recovery values above 100% are explained by the matrix effect. The recoverable values found are consistent with the literature findings, which usually range from 63 to 113% (Dural et al, 2020; Oliveira et al., 2013). Regarding the internal standard, it was also precise (average CV of 8.23%), accurate (E_R average was -6.29%) and with good recovery (average of 93.70%).

The robustness measures the method's ability to remain selective, accurate and precise front to small changes in the experimental conditions that may occur during routine analysis (Chierentin & Salgado, 2013). One can vary the proportion of the mobile phase, column temperature, wavelength, etc.

The method was considered robust only for temperature changes when it suffered modification at 27 °C and 33 °C. When assessing the wavelength at 205 nm and 215 nm, the method remained precise and selective, but was not accurate (Table 3).

Table 3. Robustness assessment regarding precision and accuracy

	T - 27°C	T - 33°C	ʎ - 205 nm	ʎ - 215 nm	MP 35:65 v v-^{1}
CV % (precision)	5.72	1.95	2.33	2.74	1.95
EPR % (accuracy)	8.04	14.29	39.55	-13.79	22.72

* T - temperature. ʎ - wavelength. MP - mobile phase. CV - coefficient of variation. E_R - relative standard error.

It was also determined that the change in the gradient of the mobile phase is a condition that must remain fixed, because the method has lost selectivity. Using the mobile phase acetonitrile: water (25:75 v v-1), the phenobarbital retention time was 2.03 min (Figure 2) co-eluting with the biological matrix interferent. The mobile phase acetonitrile: water (35:65 v v-1) remained selective (retention time 3.58 min), but it is not necessary. Therefore, the method is susceptible to these changes, which must be adequately controlled (Cassiano et al., 2009).

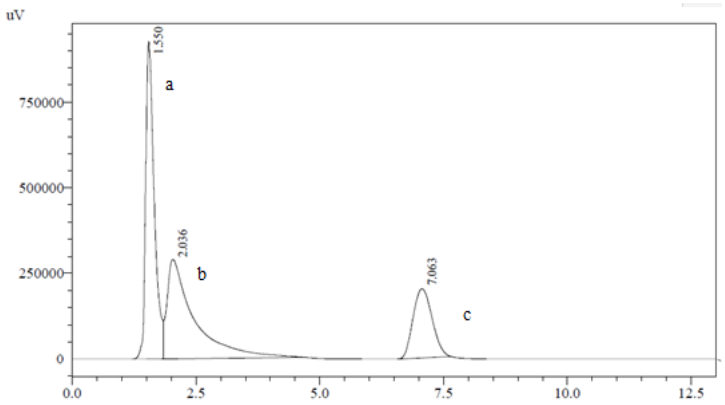

Figure 2. Chromatogram of the clinical serum sample containing the Phenobarbital® standard (concentration of 40 µg ml^{-1}) and Phenytoin® Internal Standard (concentration of 50 µg mL^{-1}), using mobile phase acetonitrile: water (25:75, v v^{-1}). Stationary Phase: C-18 column (Shimpack XR-ODS 50L x 3.0 P N 228-41606-92). a – endogenous interferent, b – Phenobarbital® standard, c – Phenytoin® Internal Standard

The proposed method was applied to eleven patient serum samples. All samples tested showed phenobarbital concentration below the detection limit (7.95 µg ml^{-1}), therefore, below therapeutic range (10 – 30 µg mL^{-1}), confirming other studies (Oliveira et al., 2013; Hahn et al., 2013). Subsequently, a second sample of these patients was collected and analyzed, and the concentration of phenobarbital remained below the detection limit.

Among these eleven patients, the most prevalent age group was 30-39 years (36.3%) with an average age of 30.5 years. The average time of phenobarbital use in these patients was 14.59 years.

When observing the clinical and epidemiological data of patients, it was found that most patients had been using phenobarbital for more than five years and do not have seizures under control (81.82%) and that in 72.72% of the cases the dose of phenobarbital is in accordance with the recommendations of the Brazilian National Therapeutic Form (Brasil, 2018). Moreover, 90.9% of the patients reported having developed adverse effects and 63.4% use more than one drug (63.64%).

According to the Brazilian Ministry of Health, the patient is considered free from seizures if they do not occur for at least two years during treatment with unchanged dose of the drug (Brasil, 2018). In this study only 18.18% of patients have their seizures controlled, showing that there is a clinical laboratory relationship, that is, it is necessary to change the dose used by patients who do not have control of seizures.

The correlation between these patients and the duration of treatment with phenobarbital indicates that only 18.18% of patients using phenobarbital for more than five years have controlled seizures. When correlating the data with the association of medications, it is observed that 54.54% of patients using polytherapy do not have their seizures controlled (Table 4).

Table 4. Relationship between control of seizures with phenobarbital usage time, drug combination and proper dosage

		Over five years of use		**Drug combination**		**Appropriate dose**	
		Yes	No	Yes	No	Yes	No
Control of seizures	Yes	18.18%	0%	9.09%	9.09%	18.18%	0%
	No	63.63%	18.18%	54.54%	27.27%	54.54%	27.27%

The phenobarbital dose indicated for the treatment of epilepsy ranges 5 to 8 mg Kg^{-1} (daily) for children and from 60 to 180 mg Kg^{-1} (daily) for adults (Brasil, 2018). According to this criterion, 72.72% of the patients are prescribed an appropriate dose of phenobarbital, however the serum concentration is below the therapeutic range in all patients, what is confirmed by 54.54% of the patients that despite having a theoretically appropriate dose do not have their seizures controlled; and the average time of last seizure (one year and ten months). This low concentration could be caused by enzyme induction caused by phenobarbital in cytochrome system CYP450 3A4. This process of induction is slow (7-10 days), so dose adjustments are important in the presence of chronic administration of phenobarbital to ensure the effectiveness of treatment (MacNamara, 2012).

Among the patients who did not use the proper dose of phenobarbital, 66.67% are using a dose above the indicated and 33.33% below the indicated.

The most prevalent adverse effects found in patients were sedation (63.63%) and dizziness (54.54%). We also identified ataxia, fatigue, headache, depression, behavior disorders, cognitive and concentration impairment, these effects agree with literature (Brasil, 2018; MacNamara, 2012).

Analyzing the relationship between the presence of adverse effects and other characteristics, it is observed that: 72.72% of patients with adverse effects have been using phenobarbital for more than five years; 63.64% of patients reported having an adverse effect, despite using a theoretically appropriate dose of phenobarbital; in 72.72%, seizures are not controlled and have an adverse effect; 54.54% use polytherapy and have adverse effects (Table 5). The presence of adverse effects was reported by 90.90% of patients. However, all patients have blood concentrations of phenobarbital below the therapeutic range, leading to the hypothesis of failure in the reporting of adverse effects.

Table 5. Relationship between occurrence of adverse effects with phenobarbital usage time, proper dosage, seizures control and drug combination

		Over five years of use		Appropriate dose		Seizures control		Drug combination	
		Yes	No	Yes	No	Yes	No	Yes	No
Adverse effect	Yes	72.72%	18.18%	63.64%	27.27%	18.18%	72.72%	54.54%	36.36%
	No	9.09%	0%	18.18%	0%	0%	9.09%	9.09%	0%

Association of drugs was observed in 63.64% of the cases, mainly with carbamazepine (27.27%). This differs from the recommended official guidelines, which indicate that patients should be treated with a single antiepileptic drug. If the initial treatment is not successful, monotherapy should be continued, however, using another medication. In case of persistent failure, the combination of two anticonvulsant drugs should be attempted (Brasil, 2018).

Among the risk factors for epilepsy, the most prevalent was the use of alcohol followed by traumatic brain injury (TBI), with most cases having no definite cause. The data obtained here agree with other studies (Calvano et al., 2010).

The prevalence of seizures in alcohol abusers who use phenobarbital is at least three times that in non-alcoholic individuals and excessive alcohol intake has an intimate relationship with the occurrence of epileptic crises. It should be noted that the consumption of alcohol concomitantly with phenobarbital can potentiate the sedative effect of the anticonvulsant, since ethanol is also an inhibitor of the Central Nervous System. On the other hand, ethanol is also an enzyme inducer, including the biotransforming enzymes of phenobarbital, which can result in impairment of its biological actions thereby impairing its anticonvulsant efficacy.

These data on alcohol use and epileptic crises show once again the importance of therapeutic monitoring with these patients, even associated with educational actions, since alcohol consumption is something common in the population and that can influence the treatment and control of seizures.

CONCLUSIONS

The developed method shows appropriate confidence limits for the application in studies of biological samples containing phenobarbital. The sample preparation was based on simple liquid-liquid extraction. The chromatographic run was relatively short time when compared with the tests described in literature. In addition to that, it does not suffer interference from other drugs commonly used by patients, proving its selectivity, precision, and accuracy. The chromatographic run was relatively short time when compared with the tests described in literature. In addition to that, the method proved its selectivity, precision, and accuracy.

In this work, it was shown that the method is suitable for clinical application, being used to dose phenobarbital in blood serum of 11 patients under treatment with this medicine. The concentration found in all patients was subtherapeutic and showed correlation with their clinical conditions. Thus, it is noted that the procedure can be used as a routine test to quantify phenobarbital in human serum for therapeutic monitoring studies. In this sense, therapeutic drug monitoring is useful for diagnosis of subtherapeutic treatment, individualization of dose or effective therapeutic dose adjustment.

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Author contributions

PADM: conceptualization, methodological analyzes, statistical analysis; PCDS, methodological analyzes, statistical analysis; LPAS: collecting and storing samples; writing; SRM: supervision, writing; SMLF: conceptualization, supervision, writing.