Phenobarbital loaded microemulsion: development, kinetic release and quality control

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This study aimed to obtain and characterize a microemulsion (ME) containing phenobarbital (PB). The PB was incorporated in the proportion of 5% and 10% in a microemulsion system containing Labrasol®, ethanol, isopropyl myristate and purified water. The physicochemical characterization was performed and the primary stability of the ME was evaluated. An analytical method was developed using spectrophotometry in UV λ = 242 nm. The kinetics of the in vitro release (Franz model) of the ME and the emulsion (EM) containing PB was evaluated. The incorporation of PB into ME at concentrations of 5 and 10% did not change pH and resistance to centrifugation. There was an increase in particle size, a decrease of conductivity and a change in the refractive index in relation to placebo ME. The ME remained stable in preliminary stability tests. The analytical method proved to be specific, linear, precise, accurate and robust. Regarding the kinetics of the in vitro release, ME obtained an in vitro release profile greater than the EM containing PB. Thus, the obtained ME has a potential for future transdermal application, being able to compose a drug delivery system for the treatment of epilepsy.


O objetivo deste trabalho foi obter e caracterizar uma microemulsão (ME) contendo fenobarbital (FEN). O FEN foi incorporado na proporção de 5% e 10% em um sistema microemulsionado composto por labrasol®, etanol, miristato de isopropila e água purificada. Foi realizada a caracterização físico-química e avaliada a estabilidade preliminar da ME. Desenvolveu-se um método analítico por espectrofotometria em UV λ = 242 nm. Foi avaliada a cinética de liberação in vitro (em modelo de Franz) da ME e da emulsão (EM) contendo FEN. A incorporação do FEN em ME nas concentrações de 5 e 10% não alterou o pH e a resistência à centrifugação. Houve aumento do tamanho da partícula, redução da condutividade e alteração do índice de refração em relação à ME placebo. A ME manteve-se estável nos ensaios de estabilidade preliminar. O método analítico demonstrou ser específico, linear, preciso, exato e robusto. Na cinética de liberação in vitro, a ME obteve um perfil de liberação in vitro superior a EM contendo FEN. Desta forma, a ME obtida tem potencial para uma futura aplicação transdérmica, podendo compor um sistema de liberação de fármacos para tratamento da epilepsia.


INTRODUCTION

Approximately 50 million people worldwide are living with epilepsy (Gomes et al., 2002). It affects between 7 and 14 of each 1,000 people (WHO, 2015). It is one of the most common serious neurological disorders worldwide, which can be successfully controlled with one single antiepileptic. Uncontrolled seizures may result in injuries, embarrassment, anxiety, unpredictability, and occasionally may induce death (Dalmora et al., 2010).

Phenobarbital (PB – Figure 1) is a barbiturate that acts as a non-selective Central Nervous System depressant. It is widely used as an antiepileptic drug for it is complete and very effective drug and has advantages such as low cost, broad spectrum of action and ease of use (Dalmora et al., 2010). It is a centenarian drug and is still the best cost-benefit pharmacological treatment for epilepsy, especially in developing countries (Brodie, Kwan, 2012).

![Chemical structure of antiepileptic drug PB.](image)

**FIGURE 1** - Chemical structure of antiepileptic drug PB.

Studies have shown that the pharmacokinetics of PB in children and young adults is age-dependent, and may have an absolute bioavailability of 48.9% among the pediatric population (Yukawa, Higuchi, Aoyama, 1992; Marsot et al., 2013). In addition, comparing oral administration with the intramuscular route, there is a difference of about 60% in terms of bioavailability in newborns (Yukawa et al., 2011). Newborns present a late and incomplete oral absorption of PB when compared with intramuscular administration (Jalling, 1975). Furthermore, the narrow therapeutic margin of this anticonvulsant is one of the aggravating factors for the treatment of epileptic seizures, since the usual doses may lead plasma concentrations to toxic levels.

Recently, a great attention has been paid to the development of drug delivery systems using lipid microemulsions (ME) as carrier agents of drugs with a low aqueous solubility, such as PB (Üstündağ Okur et al., 2011). The drug penetration rate into the skin can be increased by the use of permeation enhancers (Williams, Barry, 2004), and MEs are known for increasing the availability of certain drugs into the deeper layers of the skin.

MEs are systems in which the internal phase is a dimensionally restricted microenvironment, with particular properties. It may bond or associate molecules with different polarities (Oliveira et al., 2004). They are generally characterized as spherical aggregates, and have diameters smaller than 1,400 Å, typically 100 Å (Yacubian, 2002). They can be defined as thermodynamically stable, isotropic and as transparent systems with two immiscible liquids (usually water and oil) stabilized by a film of surfactant compounds (with or without co-surfactants) located in the oil/water interface (Lawrence, 1994; Üstündağ Okur et al., 2011).

In the literature, several PB quantification methods are described, such as titration (ANVISA, 2010), spectrophotometry (Boeris, Luco, Olsina, 2000), high performance liquid chromatography (HPLC) (Dalmora et al., 2010; ANVISA, 2010), and immunoassays (Spiehler et al., 1976; Pastore, Ofuchi, Nishiyama, 2007). Several pharmaceutical formulations were described, such as oral solution (Yska et al., 2000) and tablets (Goicóchea, Olivieri, 1998). It is necessary, therefore, to develop an analytical method for determining PB in ME.

Most publications on PB quantification use biological samples. They generally consist of simultaneous quantifications with structurally similar drugs (Boeris, Luco, Olsina, 2000) and/or metabolites (Romer, Donaruma, Zuman, 1977) in blood. The Brazilian Pharmacopoeia describes an analytical method using spectrophotometric to determine drugs in solutions, which preconizes the solubilization of the drug in a borate buffer with a pH = 9.6 (ANVISA, 2010).

Djabri, Guy and Delgado-Charro (2012) showed that determining PB by iontophoresis was capable of penetrating skin layers. PB administered by organogel and oil formulation was capable of permeating the skin of healthy cats (Gasper et al., 2015). In a recent study conducted by our research group, Figueiredo et al. (2015) demonstrated that PB in nanocarriers reduced seizures and the death rate in the rats submitted to seizures using a model induced by pilocarpine. Further, the transdermal application of the formulation was capable of reducing oxidative stress and neuronal damage to the hippocampus of animals.

In the market, an oral formulation containing cyclosporine in microemulsion (Sandimmun Neoral®) is used in immunosuppressive therapy. In addition, drugs are being marketed in adhesives for transdermal administration such as estradiol (Dermestriol®), fentanyl (Durogesic®), scopolamine (Scopoderm®), among others. Formulations containing microemulsions for transdermal administration are patent-protected worldwide (Figueiredo et al., 2013).
Knowing that MEs are known for increasing the availability of certain drugs into the deeper layers of the skin and that the PB is a candidate drug for transdermal permeation, this study aimed to use MEs containing PB for transdermal use as a strategy for the treatment of epilepsy. In addition, its preliminary stability and physicochemical characterization were analyzed, an analytical method for the determination of PB in ME was developed and the kinetics of the in vitro release of PB into ME was performed.

**MATERIAL AND METHODS**

**Chemicals**

PB raw material was used as standard (PharmaNostra, lot no. 10083132B, content 100.18%, Brazil). The Labrasol® (caprylocaproyl macrogol-8 glycerides) was purchased from Gattefosse (France). Ethanol (EtOH) 99.8% was purchased from Dinâmica (Brazil) and Vetec (Brazil). NaOH was acquired from Vetec (Brazil). The isopropyl myristate was purchased from Synth (Brazil). Purified water was obtained through reverse osmosis system (Milipore, Germany). Non-ionic self-emulsifying wax (PharmaNostra, Brazil), methylparaben, propylparaben and propylene glycol (Vetec, Brazil), were used. The dialysis membrane was acquired from Sigma-Aldrich (Brazil).

**Preparation of the ME and incorporation of the PB**

The ME was prepared as a cold mixture, under mild agitation, of Labrasol®, ethanol, isopropyl myristate and purified water. To design the pseudo-ternary phase diagram, proportions of surfactants and oil phase were fixed, followed by a drop-by-drop addition of purified water until the mixture became clear (microemulsion region). The PB was incorporated cold at concentrations of 50 mg/g (ME50) and 100 mg/g (ME100). In the preparation EM 50 mg/g (EM50) and 100 mg/g (EM100), non-ionic self-emulsifying wax, methylparaben, propylparaben, propylene glycol and purified water were used (Table I).

**Physicochemical characterization**

In all characterization tests performed, the mean±standard deviation of triplicate samples was used (also in the ME placebo, and with 5-10% of the incorporated drug).

**Solubilization Capacity**

In order to assess the maximum solubilization capacity of PB in ME, a solubility test was performed according to the adapted methodology of Üstündağ Okur et al. (2011). An excess of PB in a test tube containing 3 mL of ME (n=3) was added, then proceeding to the following conditions: stirring at 100 rpm for 72 hours at 37±2 °C. Subsequently, it was centrifuged for 30 min at 3,000 rpm. The sample was filtered; then, the filtered aliquots were transferred to a volumetric flask, supplementing it with a EtOH:NaOH mixture (pH 13, 0.1M) (50:50, v/v) (Boeris, Luco, Olsina, 2000). Readings were taken at UV λ = 242 nm.

**Resistance to centrifugation**

Samples of each formulation were subjected to centrifugation for 30 min at 3,000 rpm (HT, Brazil). Then, the occurrence or not of phase separation or any evidence of instability was observed.

**pH, conductivity and refractive index**

The pH of the ME was evaluated using a digital potentiometer (Bel Engineering, Italy) with a glass electrode and a temperature sensor previously calibrated with pH 4.0 and 7.0 buffer solutions. The glass electrode was inserted directly inside the formulation. The conductivity was determined in an electrical conductivity meter (Marte, Brazil). The refractive index was determined using a refractometer (Quimis, Brazil) calibrated with purified water. Readings were taken at 25±2 ºC.

**Viscosity**

The viscosity of the formulations was assessed in a digital rotary viscometer (Quimis, Brazil) through variations in the rotational speed between 6 and 60 rpm using nº 4 (ME) and nº 1 spindles (EM).

**Particle size and polydispersity index (PDI)**

The nanometric droplet size analysis was made using dynamic light scattering technique. It provides the hydrodynamic radius of colloidal particles (Malvern, England). The system temperature was kept at 25 °C and the droplet diameter (nm) and PDI (polydispersity index) (n = 3) were calculated.

**Preliminary stability**

The study of preliminary stability was conducted by analyzing the following parameters: organoleptic characteristics, pH, electrical conductivity and content. In the organoleptic analysis of the formulations, their appearance, color, homogeneity and instability processes as creaming and phase separation were observed.
(ANVISA, 2005). The parameters were analyzed at the beginning of the study and immediately after thermal stress tests, freeze-thaw cycle and 30 days at room temperature (25±2 °C). The drug content at 60 and 90 days after sample preparation was also evaluated.

The samples to the thermal stress were subjected to the temperatures in the range of 40-80 °C, with elevation progression of 10 °C/30 minutes in thermostated water-bath (Nova ética, Brazil). The formulations were evaluated at the end of 80 °C, after returning to room temperature (25±2 °C) (Baby et al., 2008). For the freeze-thaw cycle, the samples were subjected at -5°C±2 °C in a refrigerator (Electrolux, Brazil) for 24 h and 50 °C±2 °C for 24 h in an oven (Icamo, Brazil), completing a cycle. At the end of 12 days (6 cycles) the formulations were analyzed after returning to room temperature (25 °C) (ANVISA, 2004). ME formulations with and without PB at 5 and 10% were evaluated, besides the comparative study with EM.

Validation of the analytical method to determine PB in ME

Sample preparation and analysis conditions

EtOH:NaOH (pH 13, 0.1 M) (50:50, v/v) was a solvent used to design the calibration curve PB. Aliquots of a 1 mg/mL solution were transferred to 10 mL volumetric flasks. In the preparation of the samples, an amount of ME containing 5 mg of PB was weighed in a 10 mL volumetric flask (Bioprecisa, Brazil). The sample was diluted with an EtOH:NaOH solution by stirring it manually for about 60 seconds. Then, an aliquot of this solution was transferred to another 10 mL volumetric flask containing the same solvent. All readings were made using spectrophotometry in the UV region at 242 nm using EtOH:NaOH as a blank solution (spectrophotometers Bioscience, England, and Shimadzu, Japan). The following analytical parameters were evaluated: specificity and selectivity, linearity and interval of the method, precision and accuracy, and robustness (ANVISA, 2003).

Specificity and selectivity

The following solutions were prepared: PB standard solution, solution of PB in ME, solution of ME without PB (placebo), and EtOH:NaOH solvent. A scan in the range of 200-400 nm was carried out and the absorption spectra shown by the curves were compared.

Linearity and interval of the method

A series of 7 dilutions in triplicate was conducted in the concentration range 1-24 µg/mL. The correlation coefficient was obtained by linear regression by the least squares method. Limits of detection (LOD) and limit of quantification (LOQ) were calculated according to RE no. 899/2003.

Precision and accuracy

The repeatability was evaluated by the mean of six sample determinations at a concentration of 12 µg/mL. In the intermediate precision assay, the between-run precision of sample determinations (n = 6) of a same concentration was evaluated within an interval of 48 hours of repeatability and performed by different analysts. The inter-laboratory precision (reproducibility) was performed on the same day under the same conditions of analysis in a laboratory B. Accuracy was assessed by adding PB in ME and its recovery percentage in three distinct concentration levels (n=3): 9.6, 12 and 14.4 µg/mL (80, 100 and 120% contents).

Robustness

The robustness was evaluated by the change in sample preparation conditions by the method of stirring during its preparation. Magnetic manual stirring and different manufacturers of the ethanol solvent were compared. In order to evaluate the stability of the analyte, ME samples were prepared for spectrophotometric determination according to the previous procedure and were subjected to the presence and absence of light. The reading was performed at 242 nm in intervals of 0 h to 3.5 h.

Kinetics of the in vitro release of PB in ME

The kinetics of the in vitro release of ME50 was evaluated and compared with EM50. The study was conducted using dialysis membranes in Franz type diffusion cells with a diffusional area of 1.15 cm² and a volume of ±14 mL. The receiver compartment was filled with borate buffer at a pH 9.6 associated with 10% of EtOH (previously defined) in a system composed of individual cells (37 ± 0.5 °C, 100 rpm). An amount of EM50 or ME50 (containing 20 mg of PB) was applied into the receptor compartment directly on the membrane. Samples of the receiving solution were collected at 2, 4, 6, 8, 12 and 24 h. Readings of the collected aliquots were performed at UV λ=242 nm. The total volume of the receptor phase was replaced at each sampling time for replacement of the medium maintaining sink conditions (Carvalho et al., 2012).

Statistical analysis

The results obtained in the validation of the analytical method and in the stability study were submitted to the
Student’s t test to evaluate possible statistical differences with a statistical significance of p<0.05. For data analysis, the software Microsoft Office Excel®, OriginLab® and Graphpad Prism® were used. The linearity of the method was subjected to analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Obtaining ME and physicochemical characterization

Figure 2 shows a pseudo-ternary phase diagram, which may show the ME region. The adopted criterion for considering ME was the observation of clear, single phase, isotropic and low viscous systems.

The formulations used in all tests of this work are described with their respective components on Table I.

In the investigation of the maximum solubility of PB in ME, the value 137.8±13.7 mg/mL was obtained, being 2.3 times higher than recently published results considering an oral PB loaded microemulsion (Monteagudo et al., 2013). The solubility of the drug in water is approximately 1.0 to 1.2 mg/mL. The ME was as effective in solubilizing PB as aqueous solutions, with a high amount of co-solvents such as propylene glycol at 50% (Shayanfar, Acree Jr, Jouyban, 2009) and ethanol at 80% (Attwood, Florence, Rothschild, 2003). Thus, it was possible to easily incorporate PB using mild stirring at concentrations of 5% and 10%, obtaining the formulations ME50 and ME100 that were evaluated in this work.

For the physicochemical characterization of ME placebo (ME) and formulations containing PB (ME50 and ME100) the results shown on Table II were obtained. It was observed that the tested formulations showed no phase separation. The approval in this test is a sign of the stability of evaluated preparations. The formation of precipitates or phase separation indicates the need of recasting them (ANVISA, 2004). Thus, by the spin-resistance test, a preliminary analysis of the presence of a possible physical instability was performed when PB was incorporated into ME.

The obtained formulations showed a pH compatible with the skin and without significant changes upon adding the drug (Table II). It is recommended that products with a prolonged permanence on the skin have a pH between 4.0 and 7.0 (Rebello, 2005). That is, the pH should be near the skin’s pH range, i.e., from 4.6 to 5.8 (Leonardi, Gaspar, Campos, 2002; Campos, Frasson, 2011).

The measurement of conductivity is an attempt to classify the type of formulation based on the distribution of the phases, allowing the determination of the conductivity of polar or apolar domains (Urban, 2004). The used
surfactant, Labrasol®, tends to form ME type O/A because its HLB value is 12 (Lawrence, Ress, 2000; Ash, Ash, 2004; Gatefossé, 2016), favoring the formation of micelles with an oily internal phase.

A small decrease in conductivity of ME was observed when the drug was incorporated into the system. This may characterize structural changes in ME by the incorporation of a hydrophobic molecule, since its conductivity is very sensitive to changes in the phase arrangement of a microemulsion system (Mo, Zhong, Zhong, 2000; Mehta, Kaur, 2011). Halle (1990) shows that the increase in the size of droplets in a ME O/A may influence the fluctuations of their charges, and thus reduce conductivity. A relation between the increase of droplets’ size and the reduction of the system conductivity (Table II) was observed.

The refractive index can be used to determine the purity of solutions or the proportions with which certain liquids are mixed. It is the ratio of the light propagation speed in air and its speed in the substance (Longo, 2006). The refractive index of the ME50 formulation achieved a significant reduction, being closer to the value of purified water (1.333) and more distant from the value of Labrasol® (1.450-1.470) (Ash, Ash, 2004). The formulation ME100 obtained a small but significant increase in its value, which may indicate the first signs of instability when increasing the drug content in the formulation.

The nanosize of ME droplets was affected with the incorporation of the drug. However, it remained within the ME range, i.e., 10-150 nm (Silva et al., 2009), or, according to Damasceno et al. (2011), 10-300 nm (Table II). The increase in droplet diameter might suggest the location of the drug in the interfacial layer of surfactants, which may decrease the curvature of the micelle and thus change the size of the droplets (Gomes, 2010). The PDI value lower than 0.5 indicates homogeneity of the droplet size (Üstündag Okur et al., 2011), which did not occur with the ME placebo. The incorporation of the drug may have helped to standardize the size of formed droplets and may thus stabilize the formulation.

In the evaluation of the viscosity with a rotation speed between 6 and 60 rpm, the viscosity curve was obtained for ME, ME50, ME100, EM, EM50 and EM100 (Figure 3). The incorporation of PB did not interfere significantly with viscosity values for any tested concentrations. The tested formulations showed a viscosity with a profile characteristic of non-Newtonian fluids, with a reduction in apparent viscosity with the increase in rotation speed.
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The viscosity of the ME proved to be approximately 200 times less than the viscosity of conventional EMs. This parameter may directly influence the drug release profile according to their formulations, because formulations with a higher viscosity may have a reduced \textit{in vitro} release drug profile (Chorilli \textit{et al.}, 2007).

**Preliminary stability**

In the preliminary evaluation of stability, the results were obtained according to Tables III and IV. In a stability study, important information can be obtained. Emulsified systems, when stored and submitted to several external factors, are subjected to the appearance of signs of destabilization, such as creaming, sedimentation, flocculation, coalescence, phase separation and loss of the content of active substances of the formulation (Baby \textit{et al.}, 2008).

When stored at room temperature for 30 days, the formulations with ME did not show significant changes in the evaluated parameters. However, conventional EMs showed a significant loss of chemical stability, with a reduction in drug content after 30, 60 and 90 days. The ME50 and ME100 content was kept for 90 days after preparation (Table IV).

**Validation of the analytical method to determine PB in ME**

In order to verify the specificity and selectivity of the method, spectrophotometric scans were performed at the UV regions 200-400 nm. These analyzed parameters

<table>
<thead>
<tr>
<th>Test</th>
<th>ME</th>
<th>ME50</th>
<th>ME100</th>
<th>EM</th>
<th>EM50</th>
<th>EM100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organoleptic characterization</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>pH</td>
<td>5.1±0.1</td>
<td>5.1±0.2</td>
<td>5.1±0.1</td>
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<td>4.9±0.2</td>
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<td>29.7±1.3</td>
<td>22.4±2.2</td>
<td>35.2±3.7</td>
<td>43.9±1.6</td>
<td>47.5±2.4</td>
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<tr>
<td>Organoleptic characterization</td>
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<td>Normal</td>
<td>Normal</td>
<td>Phase separ., creaming</td>
<td>Phase separ., creaming</td>
<td>Phase separ., creaming</td>
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<tr>
<td>pH</td>
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<td>4.9±0.1</td>
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<tr>
<td>Conductivity (μS.cm(^{-1}))</td>
<td>35.5±0.4</td>
<td>32.1±1.6</td>
<td>26.8±2.8</td>
<td>33.9±2.5</td>
<td>42.5±3.8</td>
<td>43.1±1.7</td>
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<td><strong>Thermal stress</strong></td>
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<tr>
<td>Organoleptic characterization</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Phase separ., creaming</td>
<td>Phase separ., creaming</td>
<td>Phase separ., creaming</td>
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<tr>
<td>pH</td>
<td>5.3±0.1*</td>
<td>5.0±0.0</td>
<td>4.9±0.0*</td>
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<td>5.1±0.1*</td>
<td>5.2±0.1***</td>
</tr>
<tr>
<td>Conductivity (μS.cm(^{-1}))</td>
<td>30.4±1.0*</td>
<td>27.8±0.7</td>
<td>24.5±1.3</td>
<td>43.6±3.1*</td>
<td>101.1±6.0***</td>
<td>140.6±6.7***</td>
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<tr>
<td><strong>Freeze-thaw cycle</strong></td>
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<td></td>
</tr>
<tr>
<td>Organoleptic characterization</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>pH</td>
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<tr>
<td>Conductivity (μS.cm(^{-1}))</td>
<td>38.5±2.2</td>
<td>34.2±0.2*</td>
<td>28.2±0.8</td>
<td>40.2±2.2</td>
<td>45.6±3.5</td>
<td>54.6±2.9*</td>
</tr>
</tbody>
</table>

T0 – time: 0 days; T30 – time: 30 days. *p<0.05; **p<0.01; ***p<0.001 in relation to T0. Student’s t test. Results were expressed as mean ± standard deviation.
are important in the validation of analytical methods to discard interference from impurities, excipients and degradation products by determining the amount of a drug in the formulation under study (Streck et al., 2011). The method was able to perform the determination of the drug without a significant interference from ME constituents in the region UV \( \lambda = 242 \text{ nm} \), where there was no significant absorbance of the placebo sample compared to the used solvent (data not shown).

On Table V, the results of the study of linearity, precision, accuracy and robustness of the developed analytical method are observed. Linearity data were subjected to analysis of variance (ANOVA) with statistically significant linear regression and without lack of adjustments \((p < 0.05)\). The values of limit of detection (LOD) and limit of quantification (LOQ) are shown on Table V, demonstrating the good sensitivity of the method.

Precision is the assessment of the results proximity obtained in a series of measurements of a multiple sampling of the same sample (ANVISA, 2003). Table 5 shows the result of six determinations at a 100% concentration of the within-run (repeatability) and between-run (intermediate precision) precision tests. There were no statistically significant differences between the means of these determinations. Moreover, the coefficients of variation (CV) were lower than 5% (ICH, 1996). Regarding the

### TABLE IV - Preliminary stability of the formulations tested at 30, 60 and 90 days at room temperature, thermal stress and freeze-thaw cycle to PB content (%)

<table>
<thead>
<tr>
<th>Test</th>
<th>ME50</th>
<th>ME100</th>
<th>EM50</th>
<th>EM100</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>96.9±1.3</td>
<td>98.5±2.6</td>
<td>93.5±4.4</td>
<td>103.3±5.5</td>
</tr>
<tr>
<td>T30</td>
<td>96.2±1.4</td>
<td>103.9±5.1</td>
<td>83.5±2.3*</td>
<td>93.2±1.5*</td>
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<tr>
<td>T60</td>
<td>100.5±3.5</td>
<td>104.7±3.5</td>
<td>64.9±4.6'</td>
<td>95.5±1.9</td>
</tr>
<tr>
<td>T90</td>
<td>97.1±2.0</td>
<td>100.1±1.3</td>
<td>54.2±3.7'</td>
<td>79.6±3.8**</td>
</tr>
<tr>
<td>Thermal stress</td>
<td>95.5±5.5</td>
<td>98.2±2.5</td>
<td>32.7±3.4***</td>
<td>17.5±3.5***</td>
</tr>
<tr>
<td>Freeze-thaw cycle</td>
<td>97.5±1.7</td>
<td>101.9±4.0</td>
<td>87.8±4.9</td>
<td>97.5±4.7</td>
</tr>
</tbody>
</table>

T0 – time: 0 days; T30 – time: 30 days; T60 – time: 60 days; T90 – time: 90 days. * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \) in relation to T0. Student’s t test. Results were expressed as mean ± standard deviation.

### TABLE V - Parameters obtained in validation of the analytical method for determining PB-loaded microemulsion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental results</th>
<th>CV (%)</th>
<th>Value ( p' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>1 – 24 ( \mu \text{g/mL} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Straight-line equation</td>
<td>( y = 0.037x - 0.007 )</td>
<td>3.94</td>
<td>0.88261</td>
</tr>
<tr>
<td>Linear correlation coefficient</td>
<td>( r = 0.9996 )</td>
<td>4.47</td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>0.409 ( \mu \text{g/mL} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOQ</td>
<td>0.619 ( \mu \text{g/mL} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>12.10±0.48 ( \mu \text{g/mL} )</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>12.05±0.54 ( \mu \text{g/mL} )</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Laborat. A: 11.96±0.16 ( \mu \text{g/mL} )</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laborat. B: 12.11±0.16 ( \mu \text{g/mL} )</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Accuracy (%recuperation)</td>
<td>9.6 ( \mu \text{g/mL} )</td>
<td>100.87±2.12%</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>12.0 ( \mu \text{g/mL} )</td>
<td>100.29±0.9%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.4 ( \mu \text{g/mL} )</td>
<td>101.18±0.83%</td>
<td>0.82</td>
</tr>
<tr>
<td>Robustness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stirring method</td>
<td>Manual: 11.85±0.23 ( \mu \text{g/mL} )</td>
<td>1.97</td>
<td>0.9601</td>
</tr>
<tr>
<td></td>
<td>Magnetic: 11.84±0.17 ( \mu \text{g/mL} )</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>Manufacturer solvent</td>
<td>Manufact. A: 11.89±0.19</td>
<td>1.58</td>
<td>0.28184</td>
</tr>
<tr>
<td></td>
<td>Manufact. B: 12.07±0.16</td>
<td>1.34</td>
<td></td>
</tr>
</tbody>
</table>

LOD – limit of detection; LOQ – limit of quantification; CV – coefficient of variation. * Statistical significance at \( p < 0.05 \), Student’s t test.
assessments of inter-laboratory precision (reproducibility), the method also demonstrated a concordance between the results obtained in different laboratories when compared to measurements with different equipment and the same analyst, with p>0.05 (Table V).

The accuracy of the method was evaluated by the mean of three determinations at 80% (9.6 µg/mL), 100% (12 µg/mL) and 120% (14.4 µg/mL) of the PB concentration (Table V). Recovery measures the efficiency of the extraction procedure of an analytical method within a limited variation. Desirable percentages are close to 100% (ANVISA, 2003). In all three tested concentrations (low, medium and high), the developed method demonstrated a percentage of recovery very close to 100, with CV lesser than 5%.

For the validation of an analytical method to determine a drug in a pharmaceutical formulation, the ability of this method to resist small and deliberate variations of an analytical parameters should be evaluated. Different values of pH, temperature and different manufacturers of the solvent used can be evaluated, as well as changes in sample preparation (ANVISA, 2003). The developed method was considered to be robust regarding the variation of the evaluated parameters, comparing manual stirring with magnetic stirring and the use of ethanol from different manufacturers. No significant differences were observed during the preparation of the samples, with p values of 0.9601 and 0.28184, respectively (Table V). In addition, as a parameter for evaluating the robustness of the method, the stability of a PB sample in ME was demonstrated at 100% test concentration (12 µg/mL) in up to 2.5 hours (Figure 4). There was a significant loss of approximately 3.11% of drug content in the presence (p=0.0286) and 3.08% in the absence of light (p = 0.0260) when comparing 0 and 3.5 h times. There was no influence of light on sample stability loss (p = 0.9189), comparing the 3.5 h time in both groups. Therefore, the use of samples prepared within 2 h is recommended considering that, although it does not exceed 10%, the loss is significant. Furthermore, the solution EtOH:NaOH used tends to interfere with the method 2 hours after preparation (data not shown).

The literature reports several spectrophotometric methods to determine drugs in MEs, such as benznidazole (Streck et al., 2011), nortriptyline (Moreno et al., 2000), diazepam (Dastidar, Sa, 2009) and nifedipine (Castro, Moreno, Alastres, 2001), as being as effective in determining drugs in MEs as HPLC methods (Moreno et al., 2000, Silva et al., 2014). From the results obtained, it can be shown that the reported analytical method was rightfully determined and validated. It proved easy to be performed, inexpensive, using few toxic solvents and applicable as a quality control method to laboratories lacking chromatographic methods.

Kinetics of the in vitro release of PB in ME

In order to determine the solubility condition for the establishment of sink conditions (Klose, Delplace, Siepmann, 2011) for the kinetic of the in vitro release of PB in ME, a prior study of drug solubility was performed using different buffer media and different pH values. The borate buffer solution at a pH 9.6 with 10% of EtOH was used, for it has a better solubility and does not interfere with the wavelength 242 nm (data not shown). A basic pH facilitates the solubility of PB (pKa = 7.3) due to its conversion into an ionized tautomeric form, in addition to increasing its molar absorptivity compared to more acidic pHs (Romer, Donaruma, Zuman, 1977; Jelveghari, Nokhodchi, 2008).

Factors such as surfactants/co-surfactants, microemulsion type, viscosity, drug solubility, particle size, among others, directly influence the behavior of drug release (Mortazavi, Pishrochi, Azar, 2013). In assessing the kinetics of the in vitro release of PB, ME50 obtained an amount of PB 4.8 times greater in 24 h (17.39 ± 3.13 mg.cm⁻¹) than EM50 (3.84 ± 1.12 mg.cm⁻¹), with p=0.0003 for the point 24 h (Figure 5). This result was in accordance with the literature, considering that the ME is capable of increasing the drug release system (Hashem et al., 2011; Kreilgaard, 2002). Thus, the ME provides benefits, including increased solubility and absorption of drugs, and the possibility of modifying pharmacokinetic parameters, decreasing toxicity and increasing its clinical efficacy (Formariz et al., 2005; Klein, 2007).
Besides the influence of droplet size, the presence of the co-surfactant used (ethanol) may have contributed to the differences observed in the release of PB of the two systems. In the obtained ME, ethanol may further reduce the surface tension of the surfactant, making the surface of droplets more flexible and dynamic. The energy of this system facilitates the flow of drugs through the interface between the inner and outer layers. This facilitates their release and diffusion across membranes (Yi-Hung et al., 2011). Thus, the study on kinetics of the in vitro release showed promising results for MEs, which may provide, in the future, a product with transdermal application for the treatment of epilepsy.

CONCLUSION

The PB loaded microemulsion developed in this work has a great potential for a transdermal delivery of the drug. The incorporation of the drug was easily accomplished through mild stirring, besides providing a great solubilization capacity and little change in its physicochemical characteristics with the incorporation of the drug. Thus, it demonstrated a physicochemical stability greater than EM. The analytical method of determining PB in ME can be used as a quality control method, and proved easy to be performed and inexpensive. It uses few toxic solvents and is applicable to laboratories lacking chromatographic methods. Moreover, the ME obtained an in vitro release profile significantly greater than EM containing PB. Thus, the obtained ME has a potential for future transdermal applications, being able to compose a drug delivery system for the treatment of epilepsy.

REFERENCES


Phenobarbital loaded microemulsion: development, kinetic release and quality control


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Received for publication on 15th January 2015
Accepted for publication on 02nd May 2016