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STABILITY STUDY OF NANOEMULSIONS OF DIOSGENIN[1]

ESTUDO DE ESTABILIDADE DE NANOEMULSÕES DE DIOSGENINA

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ABSTRACT

Nanotechnology is an interdisciplinary scientific field where it is possible to manipulate, alter and develop new materials at the nanoscale, changing their properties. In the pharmaceutical and cosmetic areas there is the production of nanocarriers for pharmaceuticals, which can protect, encapsulate and release pharmacologically active compounds. In our study we chose the nanoemulsion as carrier, since it is widely used as a vehicle for drugs, by the cosmetic and pharmaceutical industry. The drug used was Diosgenin, a steroidal sapogenin, found in roots of the wild yam (*Dioscorea villosa*). Diosgenin has a structure similar to progesterone and estradiol and is used as a chemical precursor in the industrial preparation of oral contraceptive pills and steroid hormones. The use of nanoemulsified diosgenin topically can bring a great number of benefits to the skin, considering its antioxidant properties and stimulating the production of collagen, thus being able to be incorporated into cosmetic anti-aging formulations. Thus, nanoemulsions can be a vehicle for diosgenin, encapsulating them, protecting them from possible degradation of the active substance and providing better permeation on the skin. The general objective was to produce and evaluate diosgenin containing nanoemulsions, aiming at the choice of a stable formulation. Nanoemulsions at concentrations 1mg.mL^{-1}, 3 mg.mL^{-1} and 5 mg.mL^{-1} were produced using the high energy emulsification method. All formulations were submitted to different forms of storage. The characterization of the formulations allowed to evaluate the formulations of diosgenin nanoemulsions in concentrations 1 mg.mL^{-1}, 3 mg.mL^{-1} and 5 mg.mL^{-1}. The best condition for storing 1mg.mL^{-1} nanoemulsions without losing their physical-chemical characteristics during the 90-day period was under refrigeration. With this, there is a guarantee of the quality and stability of the diosgenin nanoemulsion, compatibility for topical use and the possibility of reaching the deeper layers of the skin, enhancing the effects of diosgenin. It was also possible to co-validate an analytical method by spectrophotometry in the visible region to identify diosgenin in nanoemulsions, ensuring the presence of the active substance in the nanoemulsion.

Keywords: Nanocarriers, Steroid Hormone , Stability, Nanotechnology

RESUMO

A nanotecnologia é um campo científico interdisciplinar onde é possivel manipular, alterar e desenvolver novos materiais em escala nanométrica, ocorrendo alteração de suas propriedades. Nas áreas farmacêutica e de cosméticos ocorre a produção de nanocarreadores para fármacos, podendo proteger, encapsular e liberar compostos farmacologicamente ativos. Em nosso estudo escolhemos a nanoemulsão como carreador, pois é largamente utilizada como veículo para fámacos, pela indústria cosmética e farmacêutica. O fármaco utilizado foi a Diosgenina, uma sapogenina esteroidal, encontrada em raízes do inhame selvagem (Dioscorea villosa).

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A Diosgenina possui estrutura semelhante à progesterona e ao estradiol e é usada como um precursor químico na preparação industrial de pílulas anticoncepcionais orais e hormônios esteroides. a utilização da diosgenina nanoemulsionada de forma tópica pode trazer um grande número de benefícios para a pele, tendo em vista as suas propriedades antioxidantes e de estimulo da produção de colágeno, podendo assim ser incorporada às formulações cosméticas antiaging. Com isso, as nanoemulsões podem ser um veículo para a diosgenina, as encapsulando, protegendo de possível degradação da substância ativa e proporcionando melhor permeação sobre a pele. O objetivo geral foi produzir e avaliar nanoemulsões contendo diosgenina, visando a escolha de uma formulação estável. Foram produzidas nanoemulsões nas concentrações 1 mg.mL^{-1}, 3 mg.mL^{-1} e 5 mg.mL^{-1} utilizando o método de emulsificação sob alta energia. Todas as formulações foram submetidas a diferentes formas de armazenamento. A caracterização das formulações permitiu avaliar as formulações de nanoemulsões de diosgenina nas concentrações 1 mg.mL^{-1}, 3 mg.mL^{-1} e 5 mg.mL^{-1}. A melhor condição para o armazenamento das nanoemulsões de 1mg.mL^{-1} sem perder suas características físico-químicas durante o período de 90 dias foi sob refrigeração. Com isso, há a garantia da qualidade e estabilidade da nanoemulsão de diosgenina, compatibilidade para uso tópico e a possibilidade de alcance nas camadas mais profundas da pele, potencializando os efeitos da diosgenina. Foi possível, também, co-validar um método analítico por espectrofotometria na região do visível para identificar a diosgenina nas nanoemulsões, assegurando a presença do ativo na nanoemulsão.

Palavras-chave: *Nanocarreadores, Hormônio Esteroide, Estabilidade, Nanotecnologia*

INTRODUCTION

Nanotechnology is the property of manipulating, changing, and developing new nanometer-scale materials. It is an interdisciplinary scientific field that has advanced very rapidly in recent years, finding applications in the sectors of energy, electronics, medicine and cosmetology. With this, it has been exploited industrially with the manufacture of new cosmetics, medicines, paints, catalysts, coatings, fabrics, among others. (BERGMANN-ROSSI, 2008). The principle of this science is that the materials in this scale start to present chemical, physicochemical and behavioral properties different from those presented in larger scales (BARIL *et al*., 2012).

Studies carried out by several authors show that a greater emphasis has been placed on dermocosmetics, with a differentiated action, as in the case of nanocosmetics, where, for example, a more effective action on skin with different needs is expected, for the deeper penetration of particles in the epidermis without the risk of reaching the bloodstream. (FAPESP, 2008; NEVES, 2008; BONACIN, 2009; BARIL *et al*., 2012) This is because, when the molecules of the active ingredients of the creams have larger sizes they are only on the surface of the skin, protecting it from the loss of water, having a purely cosmetic effect (FAPESP, 2008, BONACIN, 2009)

The pharmaceutical industry is always looking for products with innovative properties, increasingly inserting nanotechnology into their formulations, for example using nanoemulsions as a vehicle. Nanoemulsions, with their small droplet size, have the following properties: better spreadability, hydration and skin penetration when compared to macroemulsions (BOUCHEMAL *et al*., 2004).

Diosgenin (DGN), a steroidal sapogenin, has been used by the pharmaceutical industry as a raw material for the production of steroid hormones. It has established hormone replacement therapy in menopausal women because it has similar structure to progesterone and estrogen (DIAS *et al.*, 2007, OKAWARA *et al.*, 2014). Through different mechanisms diosgenin has antifungal and antitumor activity (PATEL *et al.*, 2012). As a phytochemical is known to present anti-inflammatory action and also in the control of dyslipidemias (RAJU; MEHTA, 2009). It has antioxidant properties and stimulates the production of collagen (RAJU; MEHTA, 2009; CHIANG *et al.*, 2012), which are increasingly important in the pharmaceutical and cosmetic industries.

In this way, the use of topically-nano-emulsified diosgenin can bring a great number of benefits to the skin, considering its antioxidant properties and stimulating the production of collagen, so that it can be incorporated into cosmetic *antiaging* formulations. With this, nanoemulsions can be a vehicle for diosgenin, encapsulating, protecting of the active ingredient to prevent its degradation and distributing better on the skin. Bearing in mind that, increasingly, the pharmaceutical and cosmetics industries are looking for formulations with the most diversified purposes and that, allied to nanotechnology, these products have greater stability. The model drug for this research was diogenin, a substance that has lipophilia as characteristics, wich makes it difficult to permeat. For this, it is necessary to develop a methodology that enables such application on an industrial scale. The general objective was to produce and evaluate diosgenin containing nanoemulsions, aiming at the choice of a stable formulation.

MATERIAL AND METHODS

MATERIAL

Reference chemistry (SQR) diosgenin (96% purity) was obtained from Sigma-Aldrich (St. Louis, USA), ethanol PA was obtained from Synth (São Paulo, Brazil), methanol PA was obtained from Synth (São Paulo, Brazil), ultrapure water by Millipore (Guyancourt, France), p-anisaldehyde was obtained from Sigma-Aldrich (St. Louis, USA), ethyl acetate PA Synth (São Paulo, Brazil), sulfuric acid PA was obtained from Synth (São Paulo, Brazil), 0.45 μm pore membrane filter RC-45/25 by Macherey-Nagel (Düren, Germany). The medium chain triglyceride (TCM) mixture of caprylic/capric acid was obtained from the importing company Delaware Chemical (Porto Alegre, Brazil). Polysorbate 80 (Tween 80®) and Sorbitan monooleate (Span 80®) were obtained from LabSynth (São Paulo, Brazil). All chemicals and solvents were of pharmaceutical grade and were used as received.

METHODS

The activities developed during the research were carried out at the Nanotechnology Laboratory of the Franciscan University.

Production of Nanoemulsion Suspensions and Preformulation Studies

The nanoemulsions (NE) suspensions were prepared in triplicate using the emulsification method under high agitation using the Ultra Turrax® equipment, according to the methodology described in the literature by Fernandez *et al.*, (2004). Nanoemulsion formulations were composed of an organic phase containing: 2% sorbitan monooleate (Span 80®), diosgenin, 5% caprylic / capric triglyceride mixture (Crodamol®) and an aqueous phase composed of 2% polysorbate 80 (Tween 80®) and ultra pure water, according to the formulation described by Giongo *et al.*, (2016). Both phases were maintained under moderate magnetic stirring for 15 minutes in a water bath at 45 °C until complete dissolution of the active principle. The organic phase was then poured onto the aqueous phase with the aid of a funnel coupled to a 1000 μL automatic pipette tip under constant and moderate stirring maintained for 10 minutes on the Ultra Turrax® T25 with a rotation of 10,000 rpm then, rotation of the Ultra Turrax® T25 was increased to 17,000 rpm where the blend remained for 20 minutes. For this study, formulations of different concentrations of diosgenin (1, 3 and 5 $mg.mL^{-1}$) were prepared in triplicate for a total of 36 samples. The stability of these formulations was evaluated at the initial times, 15, 30, 45, 60 and 90 days after preparation with respect to the mean particle diameter, polydispersity index, zeta potential and pH. The samples were stored at room temperature (RT) (24 ± 2ºC), refrigerator (RE) (4 ± 2 ºC) and climatic chamber (CC) (40 ± 2 ºC) in amber glass bottles.

Physical-Chemical Characterization of Nanoemulsion Suspensions and Stability Assessment

The diosgenin nanoemulsions and blank nanoemulsion had their physico-chemical characteristics evaluated as described below for 90 days under different storage conditions. These conditions were: room temperature (RT) (25 ± 2 ºC), refrigerator (RE) (4 ± 2 ºC) and climatic chamber (CC) (40 ± 2 ºC)

Determination of pH

The pH determinations were performed using the Denver instrument® potentiometer previously calibrated with buffer solutions pH 4.0 and 7.0. The results were expressed from three different readings of the suspensions.

Distribution of the Mean Particle Size and Polydispersity Index

The mean particle diameter and polydispersity index of the nanoemulsions were determined by dynamic light scattering in the Zetasizer® Nano-ZS model ZEN 3600 (Malvern, England). The formulations were diluted 500-fold (v/v) in ultrafiltrated water filtered using the Millipore® 0.45 μm diameter porous membrane syringe. The results were expressed in nanometers (nm) and analyzed by the average reading of three replicates.

Zeta Potential

The zeta potential of the formulations was obtained by the electrophoretic mobility method using the Zetasizer® Nano-ZS model ZEN 3600 (Malvern, England). The formulations were diluted 500-fold (v/v) in 10 mM sodium chloride solution. The results were expressed in millivolts (mV) through the average reading of three replicates.

STATISTICAL ANALYSIS OF RESULTS

The statistical methodology of the data included a descriptive analysis of variables such as mean, standard deviation, coefficient of variation, correlation studies, simple linear regression, ANOVA and Dunett test, considering significance levels of $p<0.05$. Data were generated using GraphPad Prism® Version 5® software.

Co-validation of analytical method by spectrophotometry in the visible region

The spectrophotometric method in the visible region was co-validated for the determination of diosgenin in nanoemulsions. This method was previously validated by Baldissera (2014) for analysis of diosgenin in lipid nanocarrier.

Equipment and conditions

For the quantitative determination of diosgenin, a Shimadzu UV-VIS 1650 PC dual beam spectrophotometer (Kyoto, Japan) was used as equipment. Absorption spectra were determined in the range of 350 to 750 nm using quartz cuvettes of 1 cm optical path.

Preparation of reagentes

Reagent A was prepared by measuring 0.5 mL of p-anisaldehyde and 99.5 mL of ethyl acetate and subsequently homogenized in beaker. Reagent B was prepared by measuring 50 mL of concentrated sulfuric acid and 50 mL of ethyl acetate and subsequently homogenized in beaker.

Co-validation of the method

The proposed method was co-validated according to Resolution RDC 166 (BRAZIL, 2017) and International Conference on Harmonization (ICH Q2A and IICH Q2B, 2005).

Specificity

In order to evaluate the specificity, a solution of the diosgenin standard, a nanoemulsion suspension containing 1 $mg.mL^{-1}$ diosgenin and a blank nanoemulsion suspension (without the active one) was prepared, and an analysis was then performed by superimposing the spectra in the range of 350 to 750 nm. To obtain the standard solution, 10 mg of the active substance diosgenin was weighed and transferred to a 25 mL volumetric flask containing ethanol as solvent. Then he was taken to the ultrasound for fifteen minutes. After dissolution, the volume was filled with the same solvent to give a 400 $\mu g.mL^{-1}$ solution. A 2.5 mL aliquot of this solution was removed and transferred to a 10 mL volumetric flask, the volume was quenched with ethyl acetate solvent to give a 100 $\mu g.mL^{-1}$ solution. From this solution aliquots of 200 µL were transferred into test tubes containing reagent A and reagent B to give the final concentration of 4 $\mu g.mL^{-1}$. The solution was brought to a water bath at 60 °C for 10 minutes and, after cooling for 10 minutes at room temperature, and the scan was performed between 350 and 750 nm. To obtain the nanoemulsion suspension, 1 mL was transferred to a 10 mL volumetric flask containing ethanol as solvent. The suspension at the concentration of 100 $\mu g.mL^{-1}$ was taken for 30 minutes. A 5 mL aliquot of this suspension was transferred to a 10 mL volumetric flask and the volume was rounded with the ethyl acetate solvent to give a 50 $\mu g.mL^{-1}$ suspension. An aliquot of 480 µL of the latter suspension was withdrawn and added to a mixture of 2.76 mL of reagent A and 2.76 mL of reagent B to make a 6 mL volume of suspension under analysis to give concentration of 4 $\mu g.mL^{-1}$ in the analysis tube. The suspension for the water bath at 60 °C was taken for 10 minutes and, after cooling for 10 minutes at room temperature, the scan was performed between 350 and 750 nm. To obtain the suspension of the blank nanoemulsion without the active ones the same procedure was repeated using 1 mL of the nanoparticle considered blank, that is, without the drug. The development of this technique was adapted from Chapagain and Wiesman (2005).

Linearity

The linearity of the method was evaluated from a standard curve analysis at concentrations of 1, 2, 3, 4, 5 and 6 μg.mL^{-1}. 10 mg of the active DGN was weighed and transferred to a 25 mL volumetric flask containing ethanol as solvent. Then he was taken to the ultrasound for fifteen minutes. After dissolution, the volume was filled with the same solvent to give a 400 μg.mL^{-1} solution. A 2.5 mL aliquot of this solution was removed and transferred to a 10 mL volumetric flask, the volume was quenched with ethyl acetate solvent to give a 100 μg.mL^{-1} solution. From this solution aliquots were transferred into test tubes containing reagent A and reagent B, obtaining final concentrations of 1 to 6 μg.mL^{-1} .The samples were taken to the water bath at 60 °C for 10 minutes and, after cooling for 10 minutes at room temperature, were analyzed by spectrophotometry in the visible region. DGN showed maximum wavelength absorbance at 434 nm wavelength. The analysis of variance (ANOVA) was performed (BALDISSERA, 2014).

Precision

Precision was assessed through a repeatability and intermediate accuracy study. Repeatability was determined from six test plugs at 100% of the test concentration carried out on the same day and by the same analyst. Intermediate precision was obtained by analyzing the sample on three different days by two different analysts, and performed in triplicate, at a concentration of 4 μg.mL^{-1}. From the results obtained, the mean and the relative standard deviation (DPR) were calculated. For the test, 1 mL of the nanoemulsion was transferred into a 10 mL volumetric flask and an amount of ethanol was added. Ultrasound was taken for 30 minutes and the volume was then quenched with the same solvent to give a 100 μg.mL^{-1} suspension. A 5 mL aliquot of this suspension was transferred to a 10 mL volumetric flask and the volume was rounded with the ethyl acetate solvent to give a 50 μg.mL^{-1} suspension. An aliquot of 480 μL of the latter suspension was withdrawn and added to a mixture of 2.76 mL of reagent A and 2.76 mL of reagent B to make a 6 mL volume of suspension under analysis to give concentration of 4 μg.mL^{-1} in the analysis tube. The suspension was taken to the water bath at 60 °C for ten minutes and, after cooling for ten minutes at ambient temperature, the analysis occurred at wavelength 434 nm (DGN). This procedure was repeated six times (BALDISSERA, 2014).

RESULTS AND DISCUSSION

PRE-FORMULATION STUDIES

The method used in the preparation of nanoemulsions (NE) was the emulsification method under high agitation using the Ultra Turrax® equipment, being frequently used due to its reproducibility and

easy handling. This method consists of the formation of the nanoemulsion obtained in the form of colloidal suspension by breaking the droplets of the internal phase, forming smaller globules (FERNANDEZ *et al.*, 2004; GUPTA 2010). One of the advantages of using high-emulsification methods is the better grain size control and the greater choice of formulation constituents (SONNEVILLE-ABRUN; SIMONNET; L'ALLORET, 2004).

After the production, the formulations of nanoemulsions presented good visual stability, without observation of phase separation, that is, macroscopically homogeneous, with slightly milky-white appearance, bluish and opalescent appearance, due to the *tyndall* effect. The *tyndall* effect is due to the Brownian motion of suspended nanoparticles, characteristic of suspensions with submicron particles (RAFFIN *et al.*, 2003).

Several factors influence the stability of a system, such as physical (temperature, agitation, freezing and thawing) or chemical factors (pH, presence of electrolytes, lipid peroxidation), and the composition of the system is a factor that may contribute to this problem (DRISCOLL, 2006).

Preformulation studies were conducted to verify the viability of the production of diosgenin-containing nanoemulsions. Nanoemulsions of 1, 3 and 5 $mg.mL^{-1}$ were prepared and analyzed for their physico-chemical characteristics at the initial, 15, 30, 45, 60 and 90 days storage conditions at room temperature (RT), refrigerator (RE) and climatic chamber (CC) after its preparation as described in table 1, 2 and 3.

Table 1 - Results of the physico-chemical characterization of diosgenin nanoemulsions at concentrations of 1, 3 and 5 $mg.mL^{-1}$ over time conditioned at room temperature (RT) (n = 3).

Time	Formulation	Size (nm)	PDI	Zeta Potential (mV)	pH
Initial	1 $mg.mL^{-1}$	101 ± 0.87	0.23 ± 0.00	-6.79 ± 0.55	5.52 ± 0.12
	3 $mg.mL^{-1}$	102 ± 1.07	0.24 ± 0.01	-6.66 ± 0.24	5.52 ± 0.13
	5 $mg.mL^{-1}$	105 ± 2.45	0.24 ± 0.01	-7.55 ± 1.02	5.53 ± 0.11
15 days	1 $mg.mL^{-1}$	101 ± 1.24	0.24 ± 0.00	-6.53 ± 1.10	5.58 ± 0.25
	3 $mg.mL^{-1}$	104 ± 3.52	0.25 ± 0.00	-7.63 ± 1.10	5.58 ± 0.15
	5 $mg.mL^{-1}$	106 ± 5.95	0.24 ± 0.01	-7.68 ± 0.96	5.53 ± 0.21
30 days	1 $mg.mL^{-1}$	102 ± 1.63	0.24 ± 0.00	-7.60 ± 0.63	5.43 ± 0.16
	3 $mg.mL^{-1}$	103 ± 5.52	0.24 ± 0.00	-8.08 ± 0.96	5.01 ± 0.17
	5 $mg.mL^{-1}$	107 ± 0.71	0.24 ± 0.01	-8.15 ± 1.20	5.15 ± 0.14
45 days	1 $mg.mL^{-1}$	103 ± 2.45	0.24 ± 0.00	-7.68 ± 0.48	4.98 ± 0.08
	3 $mg.mL^{-1}$	105 ± 0.39	0.24 ± 0.01	-8.06 ± 1.00	4.65** ± 0.40
	5 $mg.mL^{-1}$	106 ± 0.93	0.25 ± 0.00	-8.07 ± 0.49	4.74* ± 0.17
60 days	1 $mg.mL^{-1}$	102 ± 2.05	0.25 ± 0.01	-7.86 ± 0.44	4.61* ± 0.07
	3 $mg.mL^{-1}$	105 ± 0.32	0.24 ± 0.01	-8.57 ± 0.31	4.33*** ± 0.38
	5 $mg.mL^{-1}$	106 ± 0.36	0.24 ± 0.00	-8.41 ± 1.07	4.45*** ± 0.17
90 days	1 $mg.mL^{-1}$	102 ± 2.49	0.23 ± 0.00	-7.86 ± 0.76	4.61** ± 0.07
	3 $mg.mL^{-1}$	105 ± 0.47	0.23 ± 0.02	-8.05 ± 0.18	4.12*** ± 0.50
	5 $mg.mL^{-1}$	108* ± 0.20	0.23 ± 0.00	-8.09 ± 0.27	4.23*** ± 0.12

**P<0,05 **P<0,001 ***P<0,0001.*

Table 2 - Results of the physicochemical characterization of diosgenin nanoemulsions at the concentrations of 1, 3 and 5 mg.mL^{-1} over the time conditioned to the refrigerator (RE) (n = 3).

Time	Formulation	Tamanho (nm)	PDI	Zeta Potential (mV)	pH
Initial	1 mg.mL^{-1}	101 ± 0.87	0.23 ± 0.00	-6.79 ± 0.55	5.52 ± 0.12
	3 mg.mL^{-1}	102 ± 1.07	0.24 ± 0.01	-6.66 ± 0.24	5.52 ± 0.13
	5 mg.mL^{-1}	105 ± 2.45	0.24 ± 0.01	-7.55 ± 1.02	5.53 ± 0.11
15 days	1 mg.mL^{-1}	103 ± 1.33	0.24 ± 0.00	-7.11 ± 0.59	5.58 ± 0.00
	3 mg.mL^{-1}	103 ± 0.89	0.23 ± 0.00	-6.46 ± 0.43	5.55 ± 0.00
	5 mg.mL^{-1}	106 ± 0.87	0.24 ± 0.00	-7.19 ± 0.12	5.67 ± 0.09
30 days	1 mg.mL^{-1}	103 ± 0.21	0.23 ± 0.01	-7.19 ± 0.16	5.82 ± 0.04
	3 mg.mL^{-1}	102 ± 0.22	0.24 ± 0.00	-8.22 ± 0.37	5.73 ± 0.01
	5 mg.mL^{-1}	106 ± 0.87	0.24 ± 0.00	-8.31 ± 0.48	5.80 ± 0.02
45 days	1 mg.mL^{-1}	101 ± 0.43	0.24 ± 0.01	-7.33 ± 1.55	5.62 ± 0.24
	3 mg.mL^{-1}	105 ± 1.97	0.23 ± 0.00	-6.50 ± 0.33	5.56 ± 0.04
	5 mg.mL^{-1}	108*** ± 0.73	0.24 ± 0.00	-7.74 ± 0.86	5.72 ± 0.10
60 days	1 mg.mL^{-1}	103 ± 0.41	0.24 ± 0.00	-7.83 ± 0.30	5.56 ± 0.02
	3 mg.mL^{-1}	103 ± 1.39	0.23 ± 0.00	-7.57 ± 0.31	5.67 ± 0.00
	5 mg.mL^{-1}	110*** ± 0.21	0.24 ± 0.00	-7.48 ± 0.45	5.72 ± 0.00
90 days	1 mg.mL^{-1}	103 ± 0.63	0.25 ± 0.00	-7.91 ± 0.76	5.93 ± 0.00
	3 mg.mL^{-1}	103 ± 0.64	0.23 ± 0.00	-7.13 ± 0.82	5.73 ± 0.01
	5 mg.mL^{-1}	109*** ± 0.31	0.24 ± 0.00	-8.02 ± 0.38	5.72 ± 0.01

*P<0,05 **P<0,001 ***P<0,0001.

The values of particle diameter obtained by dynamic light scattering, which evaluates the fluctuation of particles in Brownian motion (MUSTAFA *et al.*, 2009) during the evaluation period of all samples stored in the room temperature and refrigerator conditions were between 101 and 110 nm (Table 1 and 2). There was a significant effect of the concentration of the drug related to this parameter, whereas no significant differences between the average values of polydispersion index, pH and zeta potential were observed for the storage conditions in the refrigerator. According to Mcclements (2012) and Gutiérrez *et al.* (2008), nanoemulsions have an average size of 50 nm to 200 nm, corroborating the present study and the study by Guttoff *et al.* (2015).

Nemen; Lemos-Senna (2011) states that the polydispersity index provides information regarding the homogeneity of the system's droplet size distribution. Values below 0.3, as found in our study for all formulations, indicate that the system is monodispersed, with good homogeneity in the particle size distribution (IZQUIERDO *et al.*, 2005). Guttoff *et al.*, (2015) state that the polydispersion index depends strongly on the type of surfactant used, with the smallest droplets and the lowest polydispersion indices being those produced with Tween 80®.

The zeta potential values obtained in the nanoemulsions are in the range of -6.0 to -8.0 mV. For oil-in-water type nanoemulsions, where the oil droplets have organic acids in their constitution and the organic and aqueous phases are stabilized with the addition of two nonionic surfactants, this is the expected result. The interfacial region between the droplet (dispersed phase) and water (continuous phase) can be described as an interfacial region with excess dipoles with some orientation which results in a small potential drop. The total net charge density in the region is small, which explains the

small potential drop. This result implies that the droplet repulsion intensity is small, facilitating the instability processes characterized by coalescence or Ostwald ripenig, depending on the interfacial contact of the droplets (CAPEK, 2004).

The zeta potential plays an important role in the stability of nanoemulsions as it reflects the surface potential of the droplets. This potential depends on the degree of ionization of the surfactant and, therefore, depends on the pH values, and also emphasizes that the pH value provides information on the stabilization of nanoemulsions, since its reduction may indicate the presence of free fatty acids in the formulation, (KLANG and BENITA, 1998). In the present work, it is possible to determine the relationship between the hydrolysis of the surfactant system (phospholipids) and the triglycerides of the oily nucleus. The zeta potential of formulations 1 $mg.mL^{-1}$, 3 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$ was negative, due to the nonionic surfactants used in the formulation.

A slightly acidic pH, around 5.0 was observed for all formulations stored in the refrigerator, not showing significant differences in terms of pH values for the different concentrations of diosgenin. It can be said that the formulations remained stable throughout the period elapsed (90 days). The pH value is an important parameter for monitoring the stability of nanoemulsions, as changes in their value indicate the occurrence of chemical reactions that may compromise the quality of the final product. The decrease in pH may be due to the hydrolysis of the esters of fatty acids, which produce free fatty acids. The free fatty acids reduce the pH value of the formulations and are used as indicators of nanoemulsion stability under different storage conditions (MARTINI, 2005; MASMOUDI *et al.*, 2005).

Nanoemulsions containing 3 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$ demonstrated increased diameter and polydispersity index in the climatic chamber storage condition, as well as decreased pH values (Table 3).

Table 3 - Results of the physico-chemical characterization of diosgenin nanoemulsions at concentrations of 1, 3 and 5 $mg.mL^{-1}$ over time conditioned in the climatic chamber (CC) (n = 3).

Time	Formulation	Size (nm)	PDI	Zeta Potential (mV)	pH
Initial	1 $mg.mL^{-1}$	101 ± 0.87	0.23 ± 0.00	-6.79 ± 0.55	5.52 ± 0.12
	3 $mg.mL^{-1}$	102 ± 1.07	0.24 ± 0.01	-6.66 ± 0.24	5.52 ± 0.13
	5 $mg.mL^{-1}$	105 ± 2.45	0.24 ± 0.01	-7.55 ± 1.02	5.53 ± 0.11
15 days	1 $mg.mL^{-1}$	102 ± 1.79	0.21 ± 0.00	-7.30 ± 0.35	5.04 ± 0.04
	3 $mg.mL^{-1}$	103 ± 0.29	0.20 ± 0.00	-6.91 ± 0.50	4.58*** ± 0.04
	5 $mg.mL^{-1}$	107 ± 1.69	0.23 ± 0.00	-6.61 ± 0.05	4.43*** ± 0.01
30 days	1 $mg.mL^{-1}$	111 ± 1.70	0.21 ± 0.01	-6.32 ± 0.16	4.53*** ± 0.01
	3 $mg.mL^{-1}$	276*** ± 4.78	0.23 ± 0.00	-8.27 ± 0.48	4.04*** ± 0.02
	5 $mg.mL^{-1}$	318*** ± 2.69	0.31*** ± 0.03	-7.28 ± 0.77	3.64*** ± 0.01
45 days	1 $mg.mL^{-1}$	124 ± 0.45	0.19 ± 0.01	-8.17 ± 0.62	4.05*** ± 0.03
	3 $mg.mL^{-1}$	412*** ± 3.31	0.23 ± 0.01	-8.53 ± 0.44	3.79*** ± 0.01
	5 $mg.mL^{-1}$	682*** ± 4.08	0.31*** ± 0.03	-8.46 ± 0.21	3.29*** ± 0.04
60 days	1 $mg.mL^{-1}$	135 ± 0.31	0,17** ± 0.01	-8.37 ± 0.79	3.95*** ± 0.01
	3 $mg.mL^{-1}$	ND	ND	ND	ND
	5 $mg.mL^{-1}$	ND	ND	ND	ND
90 days	1 $mg.mL^{-1}$	168*** ± 4.03	0.18* ± 0.01	-8.20 ± 0.14	3.91*** ± 0.02
	3 $mg.mL^{-1}$	ND	ND	ND	ND
	5 $mg.mL^{-1}$	ND	ND	ND	ND

**P<0,05 **P<0,001 ***P<0,0001*. ND= Not determined.

Nanoemulsions showed low pH values, indicating instability of the formulation at 3 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$. After 60 days it was possible to observe the phase separation at these concentrations, possibly due to the high concentration of drug, which led to the destabilization of the formulation (Figure 1).

Figure 1 - Nanoemulsion suspensions containing 1 $mg.mL^{-1}$ and 5 $mg.mL^{-1}$ DGN after 60 days of production

(Source: The author herself)

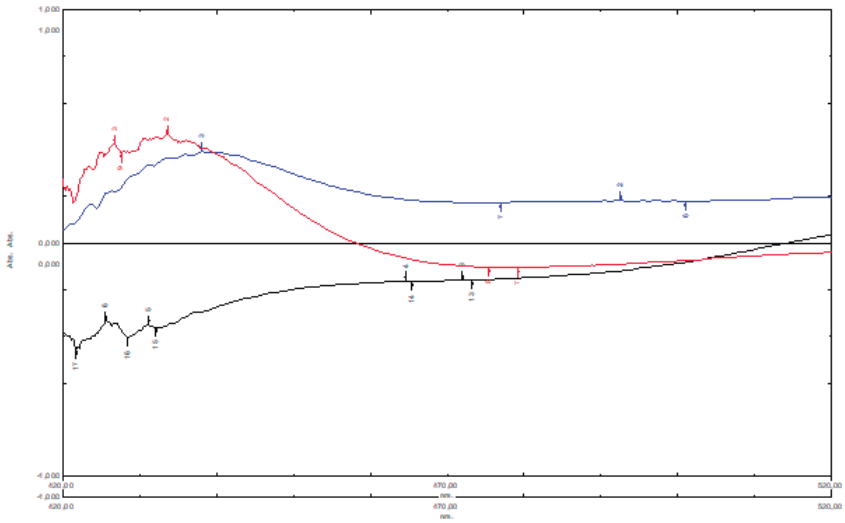
With the choice of the best formulation, aiming at stability and cost, nanoemulsions suspensions were prepared at the final concentration of diosgenin 1 $mg.mL^{-1}$ to proceed with the characterization and stability studies. Suspensions of blank nanoemulsions were prepared in the same way, discarding the presence of diosgenin.

CO-VALIDATION OF ANALYTICAL METHOD BY SPECTROPHOTOMETRY IN THE VISIBLE REGION

Specificity

The determination of diosgenin occurred at the wavelength of 434 nm, as shown in Figure 2. It is possible to determine the diosgenin present in the nanoemulsion, without interfering with the constituents present in the formulation.

Figure 2 - Overlap of spectra for suspension of blank nanoemulsion (black), nanoemulsion containing diosgenin (blue) and standard solution containing diosgenin (red)



(Source: The author herself)

Linearity

Analysis of the linearity results by ANOVA showed P <0.0001 for diosgenin (DGN), demonstrating that the reading difference between the analyzed concentrations was significant. From the standard curve found for the active DGN (y = 0.0584 x + 0.26652), the Pearson linear correlation coefficient was calculated, obtaining a value of 0.9918, being within the range recommended by ANVISA, which establishes a correlation coefficient greater than or equal to 0.99 (BRASIL, 2017).

Precision

The results obtained with the parameters, repeatability and intermediate accuracy for diosgenin are shown in Table 4. The method was found to have repeatability and appropriate accuracy, since all relative standard deviations (RSD) were less than 5%, the limit established by ANVISA (BRASIL, 2017).

Table 4 - Intermediate accuracy and repeatability results for the diosgenin active in the visible region.

	Diosgenin (%)			Intermediate Accuracy	
Samples	**Day 1[a]**	**Day 2[a]**	**Day 3[b]**	**Mean (%)**	**RSD (%)**
1	98.20	97.98	99.70	98.31	0.89
2	96.27	98.63	98.63		
3	97.56	98.20	98.20		
4	99.49	97.34	99.91		
5	98.63	98.20	98.20		
6	96.27	99.06	99.27		
Mean (%)	97.73	98.23	98.99		
RSD	1.33	0.59	0.76		

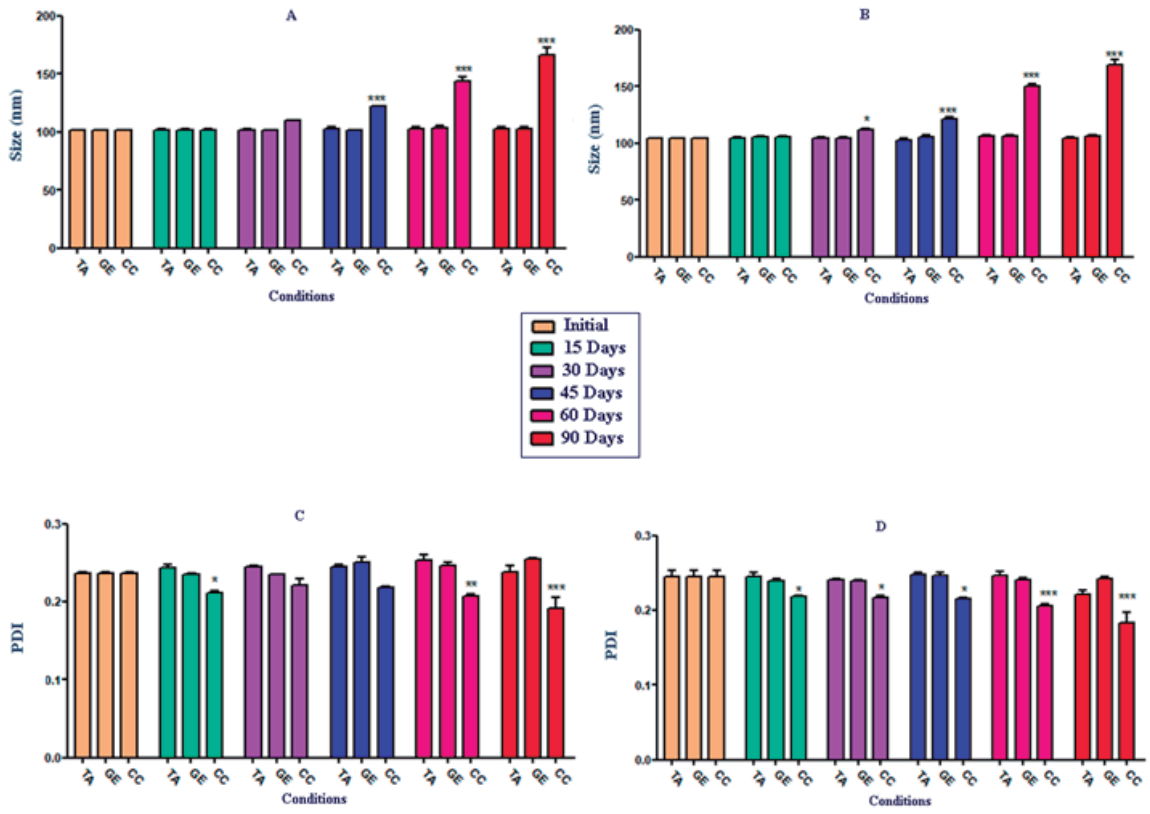
[a]: analyst 1, [b]: analyst 2.

STABILITY STUDY OF DIOSGENINE NANOEMULSIONS

With the choice of the best formulation, aiming at stability and cost, suspensions of nanoemulsions were prepared in the final concentration of diosgenin 1 mg.mL^{-1} to continue the characterization and stability studies. Suspensions of blank nanoemulsions were prepared in the same way, discarding the presence of diosgenin.

It was possible to evaluate the physicochemical characteristics during the whole analyzed period and in all storage conditions without phase separation, as shown in the figures below. All formulations presented homogeneous, milky and *tyndall* effect after preparation, characteristics found in colloidal systems (RAFFIN *et al.*, 2003).

Figure 3 - Mean particle diameter (nm) and polydispersity index for nanoemulsions containing diogenin at a concentration of 1 mg.mL^{-1} and blank nanoemulsions, stored at different temperatures



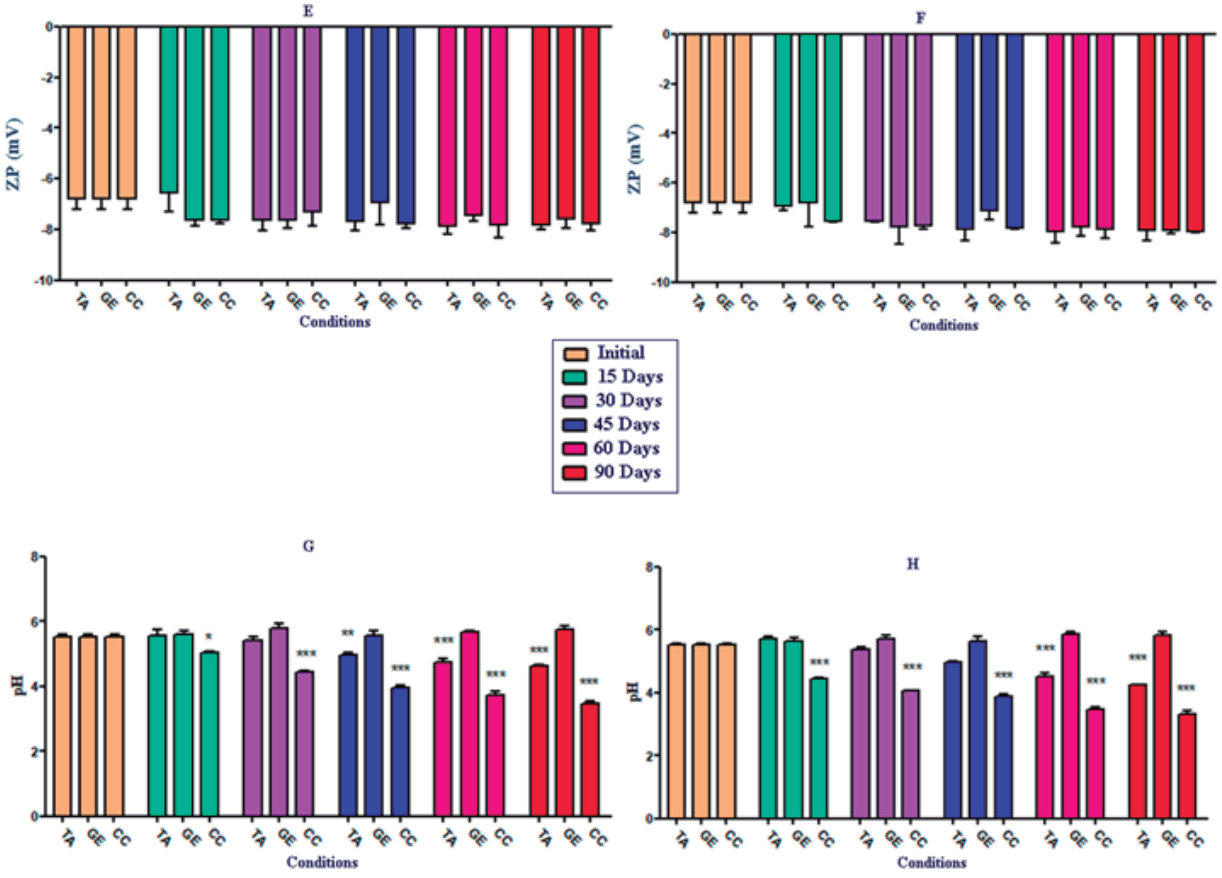
A: Size (NE-DGN), B: Sizer (NE-Blank), C: PDI (NE-DGN), D: PDI (N-Blank) under different storage conditions. * P <0.05 ** P <0.001 *** P<0.0001. TA = Room temperature; RE = refrigerator; CC = climatic chamber.

The results indicated that the technique used allowed the formation of nanometric particles in all formulations, regardless of the storage condition. However, in the storage conditions, the droplets remained with an average diameter of around 102 nm (Figure 3A and 3B**)**. significant difference in the climatic chamber storage condition with an increase of this diameter. The polydispersity index remained below 0.3 for all storage conditions, characterizing a monodisperse system.

As for the polydispersion index (PDI) for diosgenin nanoemulsions and blank nanoemulsions (figure 3C and 3D), there was a significant difference in the climatic chamber storage condition in the 15, 45, 60 and 90 day intervals for diosgenin nanoemulsions and 15, 30, 45, 60 and 90 for nanoemulsions blank In the 90-day refrigerant storage condition for diosgenin nanoemulsions, the PDI was 0.255 ± 0.001, the highest value found, but still a very low value and proved adequate to maintain the homogeneity of the formulation. For blank nanoemulsions, the highest polydispersity index value found was 0.248 ± 0.003 at 45 days of storage analysis at room temperature.

In the case of the zeta potential (Figure 4E e 4F), the results found for diosgenin nanoemulsions (NE-DGN) and blank nanoemulsions (NE-Blank) demonstrate anionic charge, as expected due to the addition of two nonionic surfactants (CAPEK, 2004).

Figure 4 - Zeta potential and pH in nanoemulsions containing diogenin at a concentration of 1 mg.mL^{-1} and blank nanoemulsions, stored at different temperatures.



E: Zeta Potential (NE-DGN), F: Zeta Potential (NE-Blank), G: pH (NE-DGN), H: pH (N-Blank) under different storage conditions. *P<0,05 **P<0,001 ***P<0,0001. TA = Room temperature; RE = refrigerator; CC = climatic chamber.

The results found for blank nanoemulsions remained between -6.77 ± 1.40 and -7.97 ± 0.62 and there was no significant variation between any of the times and storage conditions analyzed. The zeta potential values found for diosgenin-containing nanoemulsions were also not significant between any of the analyzed periods and conditions. The results found for diosgenin-containing nanoemulsions remained between -6.53 ± 0.55 and -7.86 ± 0.48 (Figure 4E e 4F).

According to Jaiswal *et al.* (2015), the zeta potential is used to know what the charge on the surface of the drop of the nanoemulsion is determined by specific electrophoretic techniques.

The results obtained for the nanoemulsions containing diosgenin showed significant differences in the pH values, in the period of 90 days, under storage conditions at room temperature and climatic chamber. The same can be noted for the values of the blank nanoemulsions.

Only in the refrigerator storage condition there was no significant difference in pH values during the whole period. PH remained between 5.78 ± 0.24 and 5.56 ± 0.19 for diosgenin-containing nanoemulsions and 5.85 ± 0.14 and 5.64 ± 0.17 for blank nanoemulsions (Figure 4G e 4H).

The pH results showed a gradual decrease over the analysis period and there was a significant difference for all the formulations conditioned in the climatic chamber during the 90 days, with the exception of the initial time analysis. Driscoll (2006) points out that the pH value provides information about the stability of the nanoemulsion, and the use of heat in this system can cause its reduction, as observed in nanoemulsions.

Masmoudi *et al.* (2005) point out that nanoemulsions may present a decrease in pH due to hydrolysis of the fatty acid esters in free fatty acids which is the degradation product.

However, these decreases do not represent visual changes for the formulations. Taking into account that the pH of the human skin is slightly acidic, around 5.5, it is desirable that topical formulations maintain pH between 3 and 10, so as not to cause changes in the pH of the skin (LEONARDI; GASPAR; MAIA CAMPOS, 2002).

With the conclusion of the stability study, it was possible to verify the refrigerator condition (RE) as the best storage form of nanoemulsion suspensions containing 1mg.mL^{-1} of diosgenin. This condition ensured that the physico-chemical characteristics were preserved during the 90 days analyzed.

CONCLUSION

In this study it was possible to produce nanoemulsions containing the active substance diosgenin at concentrations 1, 3 and 5 mg.mL^{-1} by the emulsification method under high agitation. After the production, the formulations of nanoemulsions presented good visual stability, without observation of phase separation, that is, macroscopically homogeneous, with slightly milky-white appearance, bluish and opalescent appearance, due to the *tyndall* effect. The physicochemical characterization of the formulations over the course of 30 days was shown to be adequate, but after 60 days concentrations of 3 and 5 mg.mL^{-1} began to show too much increase in diameter and phase separation.

The stability study at different temperatures allowed to evaluate formulations of diosgenin nanoemulsions at concentration 1 mg.mL^{-1} and blank nanoemulsions for 90 days. The characterization of the diosgenin-containing nanoemulsions showed a nanometric and homogeneous particle diameter with negative zeta potential, low polydispersity index and slightly acidic pH, which guarantees the quality and

stability of diosgenin nanoemulsion, compatibility for topical use and the possibility reaching the deepest layers of the skin, potentializing the effects of diosgenin. The best condition for storage of nanoemulsions without losing their physico-chemical characteristics during the 90-day period was under refrigeration.

It was also possible to co-validate an analytical method by spectrophotometry in the visible region to identify diosgenin in nanoemulsions, assuring the presence of the active in the nanoemulsion.

PERSPECTIVES

With the results obtained in the present research, the prospects are to continue the study in order to verify the safety of the formulations regarding their toxicity, evaluate the pharmacological profile in vitro, as well as the release profile, as well as the study of quantification of diogenin in the nanoemulsion at different times of analysis and temperature conditions to prove the stability of 90 days.

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