UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Desenvolvimento de emulsões lipídicas de uso parenteral compostas de lecitinas de canola ou girassol produzidas por emulsificação espontânea e homogeneização a alta pressão

Roselena Silvestri Schuh

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Trabalho de Conclusão de Curso apresentado como requisito para a obtenção do título de Farmacêutica pelo curso de Farmácia da Universidade Federal do Rio Grande do Sul

Orientador: Prof. Dr. Helder Ferreira Teixeira

Co-orientadora: Profa. Dra. Fernanda Bruxel

Porto Alegre, junho de 2013

AGRADECIMENTOS

Agradeço ao Professor Dr. Helder Ferreira Teixeira pela orientação, pelo incentivo e pelos sábios conselhos. Agradeço à Professora Dra. Fernanda Bruxel pelos ensinamentos, e, em especial, pela sua paciência e dedicação. Sou muito grata pela oportunidade de trabalhar e aprender com vocês, que são professores maravilhosos, pelos quais tenho profunda admiração.

Agradeço a minha família pelo apoio, aos meus amigos e colegas pela companhia e amizade de todas as horas, e, em especial, ao João Paulo, pela injeção de autoestima e incentivo durante todo o curso de Farmácia.

Este trabalho foi escrito em formato de artigo científico para ser submetido ao periódico **LATIN AMERICAN JOURNAL OF PHARMACY**, cujas normas para submissão estão contidas em Anexo.

DEVELOPMENT OF PARENTERAL LIPID EMULSIONS COMPOSED BY RAPESEED OR SUNFLOWER LECITHINS BY SPONTANEOUS EMULSIFICATION AND HIGH-PRESSURE HOMOGENIZATION

Roselena S. SCHUH, Fernanda BRUXEL and Helder F. TEIXEIRA*

Laboratório de Desenvolvimento Galênico, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

* Correspondence:

Laboratório de Desenvolvimento Galênico - Faculdade de Farmácia - Universidade Federal do Rio Grande do Sul

Av. Ipiranga, 2752, Lab. 606, 90610-000, Porto Alegre, RS, Brazil

Tel.: (55) 51 3308 5231

E-mail: helder.teixeira@ufrgs.br

SUMMARY

In this study, we investigated the effect of two lecithins obtained from vegetal sources on the physicochemical properties of nanoemulsions intended for parenteral nutrition or drug delivery systems. Formulations composed by a medium chain triglycerides oil core stabilized by rapeseed or sunflower lecithins were obtained by means of spontaneous emulsification (SE) or high-pressure homogenization (HP) procedures. The main results were compared with formulations stabilized with egg lecithin. Formulations obtained by HP displayed larger droplet size (230-440nm) in comparison with those obtained by SE method (190-310nm), although the SE emulsions did not remain physically stable for more than one week. Zeta potential of the emulsions was negative and lower than -25 mV. Zeta potential inversion occurred at pH between 3.0 to 4.0 units, where droplet size showed an increase. The overall results demonstrated the feasibility of preparing injectable lipid emulsions composed by rapeseed or sunflower lecithins by SE and HP methods.

KEY WORDS

Lipid emulsion; lecithin; parenteral; rapeseed; sunflower.

INTRODUCTION

Since 1930 there are reports of the use of parenteral access for nutrients administration in patients that have feeding restrictions and need a supplementation. The parenteral nutrition regimen is basically composed by macronutrients (amino acids, carbohydrates and lipids) and micronutrients (vitamins, electrolytes and microelements), under prescription of a physician depending on the condition, age and weight of the patient [1].

Among the macronutrients, the lipids are included. They are administrated as lipid emulsions essentially as energy donors, essential fatty acids suppliers and fat soluble vitamins carriers. The fatty acids contained in those formulations have major metabolic importance, since they are cell membrane components and play specific roles in hormonal signalization and transportation. Moreover, they are precursors of prostaglandins, leukotrienes, thromboxanes and prostacyclins, which modulate inflammatory process, renal function and platelet aggregation [2,3]. An essential fatty acids deficiency in preterm infants during brain development results in learning problems and visual function impairment, which may be irreversible, even if an adequate fatty acids containing diet is provided later in child development. The clinical implication of the lack of essential fatty acids in the short term is that it interferes with the synthesis of pulmonary surfactant, damaging the lung function. Besides, it causes platelet function alterations, which could imply in clinical bleeding and susceptibility to infections. These occurrences can be prevented with the infusion of 0.5 to l g/kg/day of lipids, by starting early in the first day of life [3].

Parenteral lipid emulsions are heterogeneous systems, consisting of an oily phase homogeneously dispersed in an aqueous phase (dispersant), due to the presence of an emulsifier agent. Those formulations are characterized by a small droplet size, usually between 200 and 500 nm, due to the risk of embolism related to the use of larger particles. They must also present a physiologically compatible pH (around 7.0), isotonicity, low viscosity and a high zeta potential (module value), preventing the occurrence of instability phenomena [4].

The lipid nanoemulsions are commonly employed in total parenteral nutrition admixtures, known as 3 in 1 systems, in which all macronutrients and micronutrients are added to an ethylvinylacetate (EVA) bag. Such mixtures, however, are related to some physical instability problems, due to the presence of electrolytes and other components, which may precipitate or interact with the emulsion droplets. The precipitation of calcium and phosphate is well reported in literature [1,4-7]. Besides, bivalent ions (as calcium and magnesium) may interfere in emulsions zeta potential and induce aggregation/flocculation of lipid droplets, followed by coalescence. This phenomenon is a very serious event, since a droplet above 5 µm diameter that enters the bloodstream may cause fat embolism [8-10].

The physical characteristics and consequently the stability of lipid emulsions is strongly related to their production method and composition [4,11]. Their production methods are quite diverse and may require more than one step to produce an emulsion with reduced droplet size. Firstly, a coarse emulsion may be formed by a high speed homogenizer (Ultraturrax®), for example. Then, the droplet size reduction may be achieved by high-pressure homogenization, microfluidization or ultrasonication [12-16]. Among the methods that do not require a pre-treatment is the spontaneous emulsification, primarily used in studies of formulation and easily performed in a laboratory scale, since it is not necessary to use sophisticated equipments [17-19].

The composition of typical commercially available intravenous lipid emulsions is shown in Table 1. It must be mentioned that besides the components described, the formulations must meet the requirements for injectable products. The most commonly described isotonizing agents for those products are glycerol and sorbitol [11]. The pH may be adjusted either with diluted hydrochloric acid or diluted sodium hydroxide [4,20,21].

Table 1. Representative examples of composition (w/v) of emulsions currently marketed for parenteral nutrition.

Composition (%, m/v)	**Lipovenos® (Fresenius Kabi)**		**Intralipid® (Baxter)**		**Lipofundin® (B. Braun)**	
Soybean oil (LCT)	5.0	10.0	10.0	20.0	5.0	10.0
Medium chain triglycerides	5.0	10.0	-	-	5.0	10.0
Egg lecithin	0.6	1.2	1.2	1.2	1.2	1.2
Glycerol	2.5	2.5	2.25	2.25	2.5	2.5
Water to	100.0	100.0	100.0	100.0	100.0	100.0

The oil phase of parenteral emulsions is composed by long chain triglycerides (LCT), which may be combined with medium chain ones (MCT), as shown in Table 1. LCT comprises a wide variety of oils, as sunflower, castor, olive or more commonly soybean oil. All of them are composed by fatty acid chains longer than 12 carbons. MCT are obtained by esterification of coconut oil fatty acids. MCT containing emulsions have some clinical advantages in comparison to only LCT containing ones. Besides their improved solubility, the MCT are more easily hydrolyzed by lipases, rapidly removed from the bloodstream and absorbed by tissues, do not bind to unconjugated bilirubin, are less carnitine dependent, stabilize cell membranes, faster stimulate the immune reaction of the mononuclear phagocyte system (MPS), cause less immunogenic reactions and are a more efficient energy source [1-2,22]. Alternative marketed LCT containing higher amounts of omega-3 (fish oil) [23], showed less adverse effects [24], more significant health improvement and less accumulation of lipids in plasma [25,26], generating superior benefits especially for critical post-surgical patients. Another approach is the combination of different LCT in the same product, as the SMOFlipid®, which is composed by soybean oil (6%), olive oil (5%) and fish oil (3%), as well as MCT (6%).

The emulsifiers of choice for injectable emulsion stabilization are lecithins, since they are biocompatible and biodegradable. Lecithins are natural mixtures of polar and neutral phospholipids, obtained from animal or vegetable sources [27]. The phospholipid composition of lecithins from vegetable sources can be variable due to extraction, crop and other processing conditions [3,28].

Mainly, they contain amphoteric phospholipids as phosphatidylcholine and phosphatidylethanolamine, but anionic phospholipids may also be present [27].

Commercial available lipid emulsions for parenteral nutrition are most often composed by egg yolk lecithin, or rarely by soybean lecithin (Solipid® E&S). Despite the numerous benefits of fat supplementation, there are reports of adverse clinical effects related to long term supplementation, due to metabolic limitations and immune reactions in critic ill patients [29,30]. Adverse reactions to parenteral lipid emulsions were reported to be related to the presence of soybean and egg yolk lecithins [31-36]. Hofer et al [37] suggested that egg allergy was the cause of the anaphylactic reaction to propofol lipid emulsion, in a 14-month-old child. Lunn and Fausnight [38] reported diffuse pruritus caused by intravenous administration of total parenteral nutrition containing fat emulsion in a 2-year-old patient, who was also allergic to egg. Drug-food allergy interactions can lead to a range of adverse responses, from gastrointestinal upset to anaphylaxis [39].

In this context, the search continues for alternative raw materials in order to find hypoallergenic substitutes, which should be safer for parenteral administration in patients. This work has prioritized the search for different lecithins with the purpose of finding new alternatives to lipid emulsions intended for parenteral nutrition, or even for drug carrying, in order to provide the safest options for patients (especially preterm infants) with hypersensitivity to egg or soybean based emulsifiers. For this, we sought to develop parenteral lipid nanoemulsions stabilized by rapeseed or sunflower lecithins, comparing them to the egg lecithin containing ones. Besides a spontaneous emulsification method is compared to the high-pressure homogenization, commonly used for the industrial production of parenteral lipid emulsions.

MATERIALS AND METHODS

Chemicals and Reagents

Medium chain triglycerides (MCT), soybean oil, egg yolk (Lipoid E80®), rapeseed (Lipoid R20®)

and sunflower (Lipoid H100®) lecithins were obtained from Lipoid GmbH (Ludwigshafen, Germany), who kindly donated rapeseed and sunflower lecithins. Glycerol and ethanol were obtained from Merck (Brazil) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Ultrapure water was obtained from a Milli-Q® apparatus (Millipore, Billerica, USA).

Preparation of Nanoemulsions

Lipid emulsions were prepared in triplicate by the two following methods: spontaneous emulsification and high-pressure homogenization. The formulations obtained by means of spontaneous emulsification method were prepared according to the previously described procedure[17,40]. Briefly, soybean oil was mixed with MCT, lecithin and ethanol. Glycerol was dissolved in water, into which the ethanolic phase was then slowly added under moderate magnetic stirring for 30 min. The solvent was then removed by distillation under reduced pressure in a rotatory evaporator. The formulations obtained by high pressure homogenization were prepared as previously described [41]. Firstly, lecithin was dispersed in water containing glycerol and mixed under magnetic stirring at 40°C, until a homogeneous aqueous phase was obtained. The oil phase consisted of soybean oil and MCT. Both oil and water phases were mixed under magnetic stirring (15 min, at room temperature) to obtain a coarse emulsion. Afterwards, the coarse emulsions were mixed at 9,500 rpm for 2 min using an IKA® Ultra-Turrax T8 mixer (IKA® Works Inc., NC, USA) to form the crude pre-emulsions, that were individually subjected to high-pressure homogenization (EmulsiFlex-C3®, Avestin, Canada) at 750 bars (10,000 psi) for 10 cycles to get the final emulsion. The pH of all formulations was adjusted between 7.0 and 8.0 with a NaOH 0.01M solution and they were stored at 4 ± 2°C. The formulations and their constituents were named as in Table 2.

Table 2. Composition (%, m/v) of the nanoemulsions prepared by spontaneous emulsification and high-pressure homogenization.

Composition (%, m/v)	E-SE	R-SE	S-SE	E-HP	R-HP	S-HP
Egg yolk lecithin	1.2	-	-	1.2	-	-
Rapeseed lecithin	-	1.2	-	-	1.2	-
Sunflower lecithin	-	-	1.2	-	-	1.2
Soybean oil	5.0	5.0	5.0	5.0	5.0	5.0
MCT	5.0	5.0	5.0	5.0	5.0	5.0
Glycerol	2.5	2.5	2.5	2.5	2.5	2.5
Water to	100.0	100.0	100.0	100.0	100.0	100.0

SE: spontaneous emulsification
HP: high pressure homogenization

Physicochemical Characterization of Nanoemulsions

The pH values of the formulations were determined directly in the samples just after preparation, using a calibrated potentiometer (Digimed, São Paulo, Brazil), at room temperature. The mean droplet size and polydispersity index were measured by photon correlation spectroscopy (PCS) and zeta potential was determined by electrophoretic mobility, using a Malvern Zetasizer Nano ZS (Malvern Instrument, UK) at 25°C. For those measurements, nanoemulsions were adequate diluted in 1 mM NaCl solution. Zeta potential variation was measured as described, although the solutions had a pH range from 2.0 to 8.0 units. The viscosity was evaluated by capillary viscosimetry at 25°C (viscosimeter constant, k = 0.0212), at 25±0.1°C. The time was recorded, in seconds, for the liquid to flow from the upper to the lower mark in the capillary tube. All formulations were analyzed in triplicate.

Morphological Analysis

The morphologic examination was evaluated by transmission electron microscopy (TEM). One drop of the nanoemulsion was placed on a carbon-coated copper grid (200 mesh), negative stained with a 2.0% uranyl acetate solution and left to dry for 24 hours before the examination. A JEM-1200 EXII instrument (JEOL, Tokio, Japan) operating at 80 kV was used for those analyses.

RESULTS AND DISCUSSION

In the present study, we developed lipid emulsions intended for parenteral nutrition or drug carrying, stabilized by two lecithins obtained from vegetable sources (rapeseed or sunflower), as alternatives to the egg yolk lecithin, a traditional stabilizer for parenteral emulsions. All other emulsion components were maintained in a concentration similar to those from the commercial lipid emulsion products, in order to compare the new formulations to the traditional ones. Besides, two different production methods were compared in this work: spontaneous emulsification and high-pressure homogenization.

The physicochemical properties of the resulted nanoemulsions are presented in Table 3. The formulations obtained by the spontaneous emulsification method displayed a mean droplet size from 220 to 300 nm, as determined by photon correlation spectroscopy technique. In theory, this is a range of high emulsion stability [21,42,43]. As the droplet size is reduced, the rate of self-diffusion increases to a point where very small droplets may be kept from creaming by diffusional mixing[12,43]. Nanoemulsions containing rapeseed or sunflower lecithin presented the smaller mean droplet size compared to the ones containing egg yolk lecithin. Similar results have been described for nanoemulsions obtained by the same method, composed solely by MCT as oil phase and stabilized by 2% (m/m) egg-lecithin [44]. Based on these data, one could conclude that 1.2% concentration would be sufficient to emulsify the mixture soybean oil, MCT and water. However, even if a small droplet size and low polydispersity index was obtained, all emulsions did not remain physically stable for more than one week after preparation. After that, a phase separation (coalescence) could be visually observed. The coalescence process is an irreversible instability phenomenon, since oil droplets lose their interfaces and fuse to larger droplets [6].

Table 3. Mean droplet size, polydispersity index, zeta potential and viscosity of nanoemulsions prepared by spontaneous emulsification (SE) or high-pressure homogenization (HP).

Sample	Droplet size (nm)	Polydispersity	Zeta potential (mV)	pH*	Viscosity (cP)
E-SE	292±18	0.20±0.05	-57.1±1.8	7.50±0.34	1.40±0.04
R-SE	221±23	0.26±0.05	-44.0±8.8	7.64±0.32	1.39±0.03
S-SE	243±24	0.26±0.07	-29.0±3.9	7.38±0.18	1.35±0.02
E-HP	243±12	0.08±0.04	-44.9±3.9	7.09±0.01	1.13±0.01
R-HP	296±8	0.28±0.08	-42.3±0.8	7.27±0.13	1.15±0.01
S-HP	417±25	0.44±0.14	-44.5±3.9	7.10±0.07	1.14±0.01

* Adjusted to about 8.0 just after measurement.

The qualitative and quantitative composition of nanoemulsions, in addition with the type of emulsifier and the method of emulsification can directly influence the droplet size [12,43]. Thus, a second method was tested for the nanoemulsions preparation. The high-pressure homogenization is commonly used in the pharmaceutical industry for the production of such formulations, although in an industrial scale. Among the various methods available for emulsification, it is preferred due to its efficiency for droplet disruption. It is a high energy method, where the size reduction may be achieved when a coarse emulsion is forced under high pressure through a homogenizing valve, deforming and reducing the droplets [45]. Otherwise, spontaneous emulsification is a low cost, easy and reliable method, and it is usually used in experimental studies instead of using the high-pressure homogenizer, that is a much more complex and expensive equipment [17,40].

As demonstrated in Table 3, high-pressure homogenization conducted to larger droplet size of nanoemulsions composed by rapeseed (296 nm ± 18) or sunflower lecithin (417 nm ± 25), in comparison to the previous method, or to the control egg-lecithin-emulsions (243 nm ± 12). Nevertheless, it must be pointed out that even if the high-pressure homogenization was less efficient in the droplet disruption, it conferred more stability for formulations. Contrary to emulsions obtained by spontaneous emulsification method, all those were visually stable for at least 30 days (Figure 1). The results confirm the importance of the preparation method in the emulsion stability.

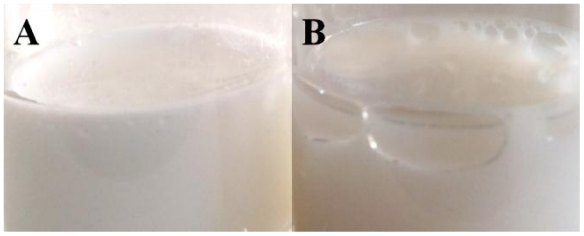

Figure 1. Visual aspect of E-HP (A) 30 days after preparation and E-SE (B) one week after preparation.

Considering the intravenous route, size distribution of lipid emulsion droplets may be even a more important information than the average droplet size. A small population of oil droplets presenting huge size may be sufficient to cause fat embolism in patients [8,9]. The droplet size distribution of the prepared formulations is presented in Figure 2.

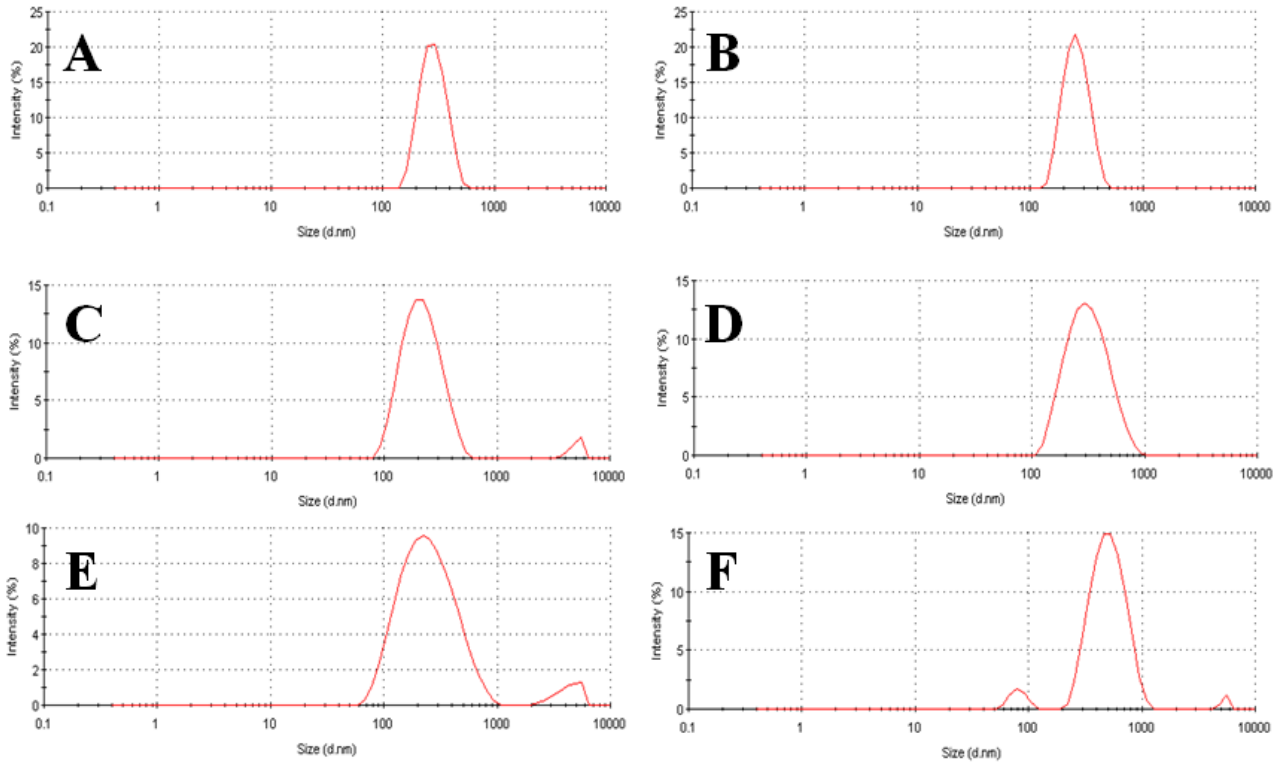

Figure 2. Comparison of the graphics of polydispersity of the formulations; (A) E-SE, (B) E-HP, (C) R-SE, (D) R-HP, (E) S-SE, (F) S-HP.

In Figure 2, at least two populations can be observed in formulations composed by rapeseed or sunflower lecithins, obtained either by spontaneous emulsification (Figures 2C and 2E), or by high-pressure homogenization (Figure 2F). As a result, a polydispersity index higher than 0.20 was

obtained for those formulations.

The stability of emulsions may be correlated to the composition and properties of their interfacial film (lecithin), since it determines the zeta potential of formulations and the repulsion between droplets, which is one of the mechanisms for emulsion stabilization [46]. Lecithin is a heterogeneous mixture of phospholipids, and its heterogeneity is extremely beneficial because of the fluidity of the interfacial film, when compared to a pure phospholipid [47,48]. The major phospholipids of lecithin mixtures are phosphatidylcholine and phosphatidylethanolamine, which are uncharged at physiological pH (7.4). Smaller quantities of acidic lipids as phosphatidylinositol, phosphatidylserine and phosphatidylglycerol may also be present. These lipids are ionized at pH 7.0 and promote a negative surface charge on emulsion droplets, which contributes to their stability. Any added substance which interferes with this charge is likely to alter the stability of the system [7]. Even if the parenteral grade lecithin is highly purified, it still contains a small amount of other phospholipids, as shown in Table 4. Table 4 describes the composition of the three lecithin raw materials used in this study, obtained from Lipoid.

Table 4. Composition (%) of lecithins obtained from Lipoid and used in nanoemulsion production.

Composition* (%)	**Egg yolk**	**Rapeseed**	**Sunflower**
Phosphatidylcholine	82.7	95.5	94.3
Phosphatidylethanolamine	8.1	0.1	0.1
Other phospholipids	9.2	4.4	5.6

* According to Analytical Data from Lipoid.

As demonstrated in Table 3, a smaller zeta potential (module value) was observed for nanoemulsions composed by rapeseed or sunflower lecithin when obtained by spontaneous emulsification method when compared to the egg-lecithin based nanoemulsion. However, no differences were observed in zeta potential of nanoemulsions produced by high-pressure homogenization. The results indicate that the main factor affecting zeta potential is the preparation method of nanoemulsions. Although both methods have been optimized

by our group [49,50], usually the experimental conditions must be adjusted taking into account the composition of formulations. The parameters such as the number of cycles and pressure may be modified in order to obtain the desired physicochemical properties of the final formulations.

Zeta potential of nanoemulsions depends also on the ionization of the emulsifier [51]. The occurrence of a reduction in the resulting charge (module value) from 40 mV to less than 25 mV can promote an increase in the flocculation and coagulation rates [52]. The zeta potential and mean droplet size of nanoemulsions composed by different lecithins and produced by different emulsification methods were evaluated at a pH range from 2.0 to 8.0. The results are presented in Figure 3.

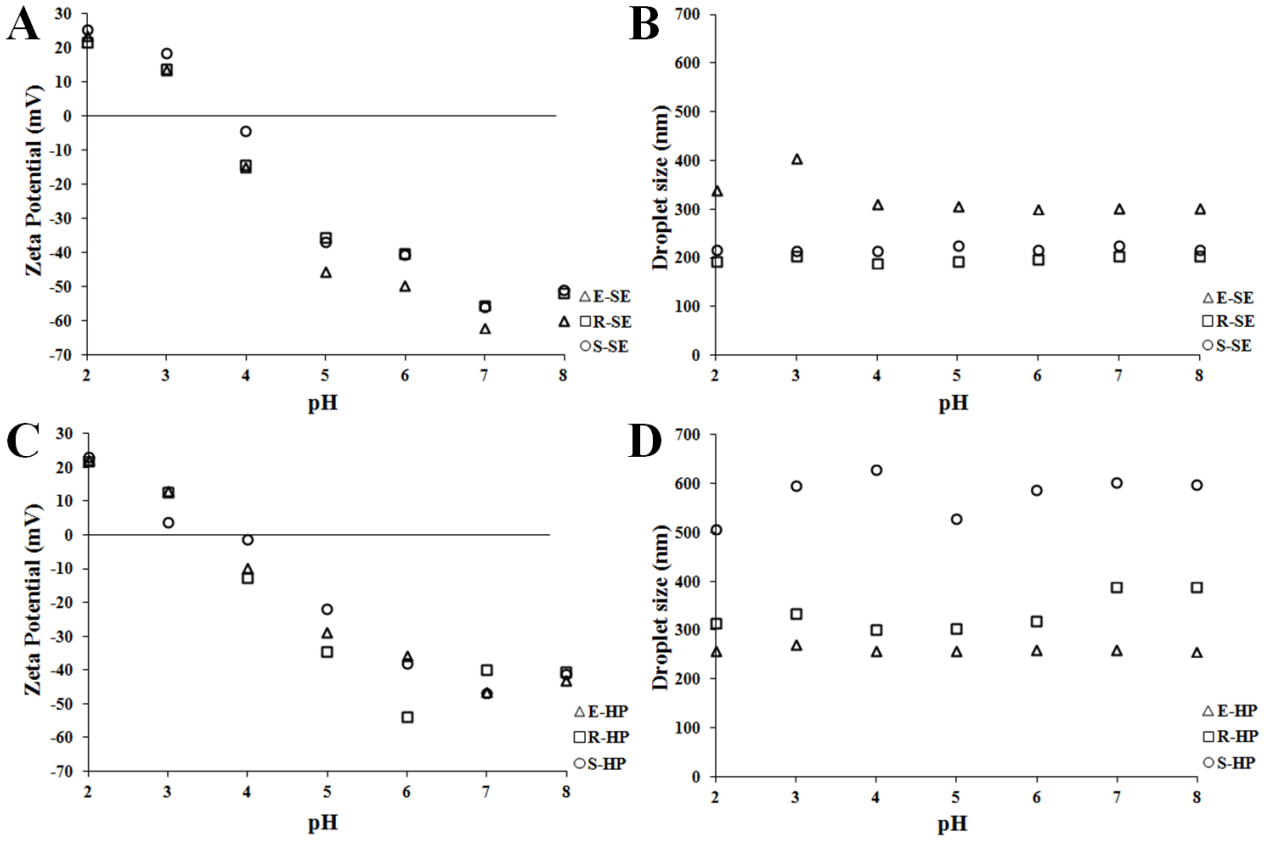

Figure 3. Zeta potential values (A, C) and mean droplet size (B, D) of formulations obtained by spontaneous emulsification (A, B) or high-pressure homogenization (C, D) in 1 mM NaCl solution at various pH. Key: E-SE and E-HP (triangle), R-SE and R-HP (square), S-SE and S-HP (circle).

It can be observed that the surface charge of all the studied formulations declines to zero at pH between 3.0 and 4.0 units, as previously observed for Intralipid®, an egg-lecithin stabilized triglyceride emulsion [6]. Zeta potential depends on the pH, since H^+ is a potential-determining ion

on phospholipid surfaces, with an isoelectric pH of 3.1 [53]. A reduction of pH results in a decreased (less negative) zeta potential and a more rapid rate of flocculation [54]. Mean droplet size showed a small increase at the pH of zeta potential inversion. According to Figure 3 one can conclude that the pH of nanoemulsions should be preferably higher than 7.0, since a plateau is achieved at that pH value, where a maximum repulsion between oil droplets is observed.

Finally, the morphology of the oil droplets of the nanoemulsions prepared by high-pressure homogenization was examined by TEM (Figure 4). The Figure reveals homogeneous and spherical particles, showing that emulsion droplets present a mean droplet size in the nanometer range, and these results corroborate to the droplet size analysis showed previously.

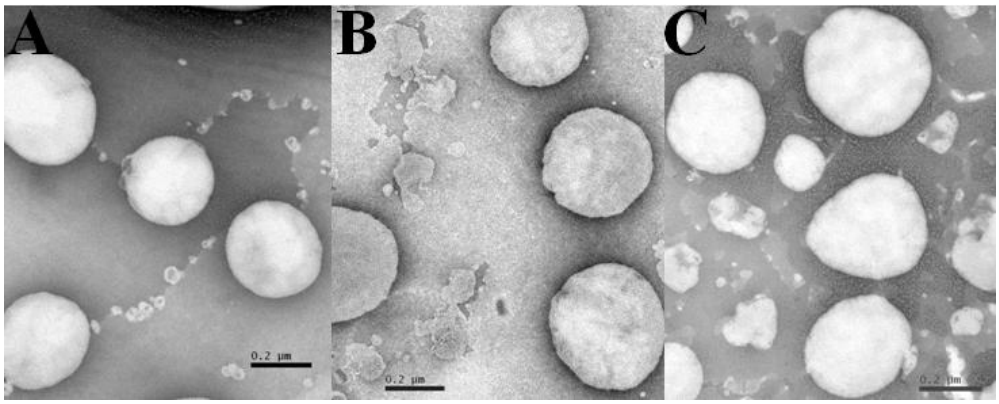

Figure 4. Morphology of the oil droplets examined by TEM at 80 kV. Key: (A) E-HP, (B) R-HP and (C) S-HP nanoemulsions.

Nanoemulsions are low viscosity systems, with a Newtonian behavior. The evaluation of the emulsion viscosity is very crucial, since the intravenous administration of high viscosity emulsions can be very painful to the patient [43,52]. Nanoemulsions composed by different lecithins showed similar viscosities. As expected, no relation between mean droplet size and viscosity of nanoemulsions was observed, since all formulations contained only 10% oil [55]. In contrast, some differences in viscosity values could be observed for formulations obtained by different methods. Slightly more viscous emulsions were produced by spontaneous emulsification method.

It is worth mentioning that the composition of nanoemulsions studied in this work was totally based on the commercial nanoemulsions composed by egg-lecithin as emulsifier. The use of a different

emulsifier may require an optimization of its concentration or the emulsification conditions.

Commercial injectable nanoemulsions composed by soybean lecithin (Solipid®) require a concentration of 1.5% of the emulsifier, for example. Sometimes additional co-emulsifiers are used as an artifice to stabilize the emulsions and promote less polydispersity and smaller droplets. However, their application is restricted to lipid emulsions as drug carriers, since small quantities of the formulations are administrated for that purpose. In fact, co-emulsifiers are not frequently used in emulsions of parenteral nutrition, due to the volume of administration of those formulations and safety problems, especially in the case of preterm infants [56]. Sodium oleate is commonly used in formulations of injectable lipid emulsions with a stabilizing purpose [57], acting as an anionic surfactant and solubilizing agent [46].

CONCLUSIONS

The overall results demonstrated the feasibility of preparing injectable lipid emulsions composed by rapeseed or sunflower lecithins by spontaneous emulsification and high-pressure homogenization, as an alternative to the traditional egg-lecithin nanoemulsions for patients that are sensitive to egg derivatives. Further studies should be conducted to optimize the conditions of the emulsification methods in order to improve the long term stability of the formulations.

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ANEXO

NORMAS PARA SUBMISSÃO DE TRABALHOS
LATIN AMERICAN JOURNAL OF PHARMACY

Manuscripts submitted to *Latin American Journal of Pharmacy* are only accepted on the understanding that they are subject to editorial review and that they have not been, and will not be, published in whole or in part in any other journal.

Papers must be written in English. If English is not authors' native language, the manuscript should be checked by someone proficient in the language before submission. Manuscripts in which English is difficult to understand may be returned to the author for revision before scientific review.

Types of Contribution

Original articles should contain material that has not been previously published elsewhere, except in a preliminary form. These papers should not exceed 5000 words including tables, references and legends of tables and figures. *Short Communications* are research papers constituting a concise but complete description of a limited investigation, which will not be included in a later paper. They should be as completely documented as a regular paper and should not occupy more than 2,500 words including tables, references and legends of tables and figures. *Reviews and mini-reviews* will be exceptionally accepted in areas of topical interest and will normally emphasize literature published over the previous five years. *Letters to the Editor* are published from time to time on subjects of topical interest.

Manuscript Preparation

Manuscripts must be neatly typed (size page A4), double-spaced throughout, including figures and tables, with at least 2 cm margins on all sides. The Editor reserves the right to adjust style to certain standards of uniformity. Every page of the manuscript must be numbered at the right top, preceded by the name of the author to whom the correspondence should be sent. The usage of italics should be limited to scientific names of organisms. A cover letter is not required, but if included it should be placed at the beginning of the manuscript.

Manuscripts in general should be organized in the following order:

- *Title:* should be clear, concise, and unambiguously reflect the paper's contents.
- *Name(s) of author(s):* first name, initial(s) of the middle name(s), and family name of each author. The corresponding author should be identified with an asterisk (*).
- *Affiliations:* include the name of department (if any), institution, city and state or country where the work was done, indicating which authors are associated with which affiliation.

- *E-mail address of the corresponding author*, as all correspondence, including proofs, should be sent only to him.
- *Summary*: not exceeding 150 words, reporting concisely on the major findings. Many abstracting services use abstracts without modification, so this section should be comprehensible in its own right.
- *Key Words:* at least three and not more than six in alphabetical order will be listed.
- *Introduction*: briefly review important prior publications and state the reasons for the investigation being reported.
- *Materials and methods*: description of methods, equipment and techniques (including statistical treatments used in the research).
- *Results*: efforts should be made to avoid jargon, to spell out all non-standard abbreviations the first time they are mentioned and to present the contents of the study as clearly and concisely as possible.
- *Discussion* (may be combined with the Results section).
- *Conclusions (at the author's discretion):* must not reiterate any discussion or introductory comments, they must be genuine conclusions drawn from the results of the study.
- Acknowledgements and any additional information concerning research grants, etc.
- *References:* will be numerated correlatively as they are cited in the text and listed separately under the title "References" (please use a hanging indent: second and subsequent lines indented). The style used for citation of articles in journals (1), monographs (2), chapters in books (3), and internet references (4) which must be strictly observed, is given in the following examples:

  (1) Medeiros, R., G.F. Passos, C.E. Vitor J. Koepp, T.L. Mazzuco, L.F. Pianowski, M.M . Campos & J.B. Calixto (2007) *Brit. J. Pharmacol*. **151**: 618-27.Journal names should be abbreviated according to ISI style (you are invited to consult the sites http://www.efm.leeds.ac.uk/~mark/ISIabbr/A_abrvjt.html or http://images.isiknowledge.com/WOK46/help/WOS/L_abrvjt.html)

  (2) Vogel, W.H., B.A. Scholkens, J. Sandow & G. Muller (2002). "*Drug discovery and evaluation, Pharmacological assay*", Second Edition, Spinger-Verlag, Berlin Heidelberg, New York, pp. 906-44.

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For 2-6 authors all authors are to be listed, with "&" separating the last two authors; for more than six authors, use the first six authors followed by et al. For three or more authors use et al. in the text.

- *Tables and Figures:* will be numbered using Arabic numerals in the order they appear in the text. *Letters and symbols included into the figures should be made in a suitable size, since figures are usually reduced to half a column wide size (7.5 cm)*. Photographs, charts and diagrams are all to be referred to as "Figures". They should accompany the manuscript. All illustrations should be clearly marked with the figure number. All figures are to have a self-explanatory caption.