Formulation Design and Optimization for the Improvement of Nystatin-Loaded Lipid Intravenous Emulsion

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ABSTRACT: Nystatin (NYS) is a polyene macrolide with broad antifungal spectrum restricted to topical use owing to its toxicity upon systemic administration. The aims of this work were the design, development, and optimization of NYS-loaded lipid emulsion for intravenous administration. A closed circuit system was designed to apply ultrasound during the elaboration of the lipid intravenous emulsions (LIEs). Additionally, a comparison with the commercially available Intralipid® 20% was also performed. Manufacturing conditions were optimized by factorial design. Formulations were evaluated in terms of physicochemical parameters, stability, release profile, and antimicrobial activity. The average droplet size, polydispersity index, zeta-potential, pH, and volume distribution values ranged between 192.5 and 143.0 nm, 0.170 and 0.135, −46 and −44 mV, 7.11 and 7.53, 580 and 670 nm, respectively. The selected NYS-loaded LIE (NYS-LIE54) consisted of soybean oil (30%), soybean lecithin (2%), solutol HS® 15 (4%), and glycerol (2.25%) was stable for at least 60 days. *In vitro* drug release studies of this formulation suggested a sustained-release profile. Equally, NYS-LIE54 showed the best antimicrobial activity being higher than the free drug. Thus, it could be a promising drug delivery system to treat systemic fungal infections. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: drug design; nystatin; lipids; emulsion; injectables; surfactants; *candida*; *aspergillus*; mathematical model

INTRODUCTION

Many patients under aggressive treatments (cancer, critically ill patients, major surgery, etc.), or severely immunocompromised (e.g., after organ or bone marrow transplantation), could develop opportunistic invasive fungal infections,[1] among them *Candida* and *Aspergillus sp* are the most common pathogens involved.[2] The management of these invasive infections is still problematic because most of the antifungals present resistances or high toxicity profiles, so renal and/or hepatic functions could be impaired[3] and therefore a likely increase in mortality rates.[4] Thus, user needs can be the starting point for developing, improving, and/or designing new more effective antifungal agents. In this way, the development of new formulations able to treat these infections reducing or avoiding the negative effects with affordable costs justifies that an increasingly area of pharmaceutical research is focused on this field.

Nystatin (NYS) is a polyene antifungal antibiotic, one of the oldest antifungal drugs, produced by *Streptomyces noursei* strains,[5] commonly used for the prophylaxis and treatment of candidiasis. It acts by interfering with the fungal cell membrane of the antibiotic-sensitive organism by binding to sterols, chiefly ergosterol, and the formation of barrel-like membrane-spanning channels.[6] NYS plays an essential role in antifungal chemotherapy.

Nystatin possesses a broad antifungal spectrum; it has been reported to be effective against azole-resistant strains of *Candida* and, in some cases, amphotericin B-resistant strains of *Candida albicans*.[7] NYS exhibits concentration-dependent activity, so at low concentration it shows fungistatic effect, whereas at high concentration, it exhibits rapid fungicidal activity. This fungicidal effect is a key factor because a rapid onset of action is quite important for a successful treatment. Moreover, NYS shows a long post-antifungal effect (PAFE), which allows the reduction in the frequency of administration.[8]

The structure of NYS reveals formulation challenges, because it is characterized by the presence of a large lactone ring containing several double bonds conferring an amphiphilic and amphoteric nature, which in turn contributes to its low solubility in aqueous media and poor bioavailability. For this reason, the controlled-release delivery of this drug is a complex task. To overcome this problem, several approaches have been reported. NYS has been formulated in micellar gels,[9] mucoadhesive devices for topical use,[10,11] liposomes,[12] nanoemulsions,[13,14] intralipids,[15] niosomes,[16] microparticles,[17] and pellets.[18] Liposomal NYS is a promising therapy,[19] but this formulation is expensive and not yet commercially available in all countries.

Lipid intravenous emulsions (LIEs) were first used for parenteral nutrition. Advantages such as reduction of irritation and/or toxicity of the drug loaded, high drug loading capacity, possibility of sustained release, targeting drug delivery to specific organs and industrial productivity make them appropriate drug carriers for poorly water soluble drugs.[20] The usefulness as drug delivery systems for drugs as propofol or lorazepam has been reported.[21,22]

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All these excellent properties of LIEs make them an appropriate carrier for the intravenous administration of NYS. Unfortunately, emulsions are intrinsically unstable formulations and normally experience changes in droplet size and distribution. For this reason, the main factors associated with the obtaining LIEs with adequate physical stability are appropriate elaboration method and parameters related to the emulsification process.

Among emulsification techniques used in the elaboration of LIEs, sonication enables not only complete dispersion of the active ingredient in the emulsion, homogenization, and stabilization, but also direct emulsification, in which ultrasounds act as single source of energy.[22] This method could be used in a hospital pharmacy, because it is easy to elaborate, fast, cheap and it does not require a large investment in facilities.

To our knowledge, only a formulation reported by Semis et al.[15] incorporated NYS in a commercial LIE at a final concentration of 0.8 mg/mL using dimethyl sulfoxide (DMSO) as organic solvent. In this work, we avoid the use of DMSO because of its well-known toxicity after intravenous administration, and the potential allergic reactions because of egg lecithin. The amount of NYS loaded was also increased (1 mg/mL) improving its antifungal activity.

On the basis of these considerations, the major aim of the present study was the development, optimization, characterization, and evaluation of a new NYS-loaded LIE (NYS-LIE) formulation without DMSO for the treatment of systemic fungal infections.

MATERIALS AND METHODS

Materials

Nystatin was purchased from Fagron Iberica (Barcelona, Spain). Purified and ultrarefined soybean oil for intravenous use (AarhusKarlshamn®) was kindly supplied by IMCD (Madrid, Spain). Purified soybean lecithin (Phospholipon® 90) was also a kind gift from Phospholipid GMBH (Köln, Germany). Bi-distilled glycerin for intravenous use was provided by Acofarma (Barcelona, Spain). Polyoxyethylene-660-12-hydroxy stearate (solutol HS® 15) was obtained from BASF (Tarragona, Spain). Milli-Q water for injection was self-produced (Millipore Iberica S.A.U., Madrid, Spain). High-performance liquid chromatography (HPLC)-grade methanol, N-dimethylformamide (DMF), acetonitrile, and DMSO were obtained from Panreac (Barcelona, Spain). Cellulose membranes (MWCO 12 Kda) were purchased from Iberlabo (Madrid, Spain). Synthetic medium RPMI-1640, obtained from Invitrogen (Madrid, Spain), and buffer 3-(*N*-morpholino)-propanesulphonic acid, obtained from Sigma–Aldrich (Madrid, Spain), were also utilized.

Preparation of LIE

The soybean oil (oily phase) and the lecithin (surfactant) were heated under stirring (700 rpm) up to 90°C and the lecithin was dissolved. Independently, the aqueous phase, Milli-Q water, and glycerin were also heated up to 90°C. The aqueous phase was added to the oil phase, and after stabilizing at 90 ± 2°C, the mixture was submitted to successive cycles in a circuit system powered by a peristaltic pump (250 mL/min flow rate) and sonicated with a Branson sonifier 250 (Branson Ultrasonic Company, Danbury, Connecticut) at 75 ± 2°C. The mixture was exposed to different power levels and periods of time to evaluate the influence of these parameters.

Table 1. Independent Variables and Their Levels, the Lower (−1) and Upper (+1) Values for Each Variable

	Level	
Factors	Low Level (−1)	High Level (+1)
X_1: Sonication time (min)	30	50
X_2: Potency (W)	80	120
X_3: Neutralization	During production	After production

For NYS-LIEs manufacturing process, accurately weighed NYS at a concentration of 1 mg/mL was mixed with solutol® HS 15 at two different rates, 2% and 4%, and homogenized in an Ultra-Turrax® T 25 Basic (IKA Werke, Staufen, Germany) at 3000 rpm for 15 min at 25°C. Then, it was added to 100 mL of optimized LIE and maintained in Ultra-Turrax again for 15 min at the same speed. The resulting volume was added to the rest of the sample up to 250 mL in the reactor and sonicated for 1 h at 80 W. Formulations were then labeled and equilibrated for 24 h to detect possible precipitation risk of drug before their use in subsequent studies.

Optimization of LIE: Experimental Design

Three different factors and their influence on the LIE elaboration process were evaluated using two^3 full factorial design, composed of three variables set at two levels. The independent variables were: sonication time (X_1), potency of sonication (X_2), and neutralization (X_3) with NaOH 0.1 M (final pH 7.5 ± 0.2). Mean droplet size (Z-ave), polydispersity index (PI), zeta potential (ZP), and distribution parameter LD_{99} (laser diffraction volume diameter 99%) were the dependent variables. This design required a total of eight experiments performed in triplicate. For each factor, the lower and higher values of the lower and upper levels were represented by a (−1) and a (+1) sign, respectively, as summarized in Table 1. These were chosen on the basis of the tested lower and upper values for each variable according to preliminary experiments and literature research. A factorial design approach was applied to maximize the experimental efficiency requiring a minimum of experiments to optimize the LIE. To identify the significance of the effects and interactions among them, analysis of variance (M-ANOVA) was performed for each parameter. A value $p < 0.05$ was considered to be statistically significant. Data were analyzed using Statgraphics software version 5.1 (StatPoint Technologies Inc., Warrenton, Virginia).

Physicochemical Characterization

Physicochemical characterization of LIEs was carried out in terms of Z-ave and PI by photon correlation spectroscopy (PCS) with a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, United Kingdom) after dilution in ultrapurified water to reduce opalescence at 25°C. ZP was also determined with the same device. It allows determining the electrophoretic mobility to assess the surface electrical charge of droplets after dilution in ultrapurified water by adjusting electrical conductivity (50 μS/cm) with sodium chloride solution (0.9%, w/v) at 25°C. pH values were also tested (without dilution) by a pH-meter Micro pH 2000 (Crison Instruments S.A., Barcelona,

Spain) to check the suitability for intravenous administration and detect potential hydrolysis of phospholipids.

Additionally, droplet size analysis of LIE formulations was performed by laser diffractometry (LD) using a Coulter LS 13 320 (Beckman Coulter, Fullerton, California) yielding the volume distribution of the droplets. For the LD analysis, the diameter 99% was used. For example, a LD_{99} value indicates that 99% (volume distribution) of the measured droplets possess a diameter below the given value. All measurements were analyzed 24 h after preparation (t_0) and subsequently recorded again after 3, 6, and 9 months. Values are reported as the mean of at least three values ± standard deviation (SD).

Stability Under Storage

Lipid intravenous emulsions that exhibited the best physicochemical properties were selected for sterilization with a microclave P® Select (JP Selecta, Barcelona, Spain) 24 h after production, during 30 min at 121°C and 1.2 Kg/cm^2, to determine weather the process affected the stability of the emulsions. Then, selected LIEs and their sterilized fractions were submitted up to 12 months of storage period (three additional months) at 30 ± 1°C and 4 ± 1°C in other to discriminate the best of them. In parallel, Z-ave and PI measurements of the commercial standardized emulsion were carried out. Intralipid® 20% (Fresenius Kabi, Uppsala, Sweden) is an intravenous oil-in-water emulsion of soybean oil (20%) emulsified with egg phospholipids (1.2%). The emulsion contains also glycerol as osmotic agent (2.25%) and water for injection.

On the contrary, the physical stability of four NYS-LIEs was determined by measuring the variation of the physicochemical parameters of samples stored at 30 ± 1°C and at 4 ± 1°C, for 1, 15, 30, and 60 days after preparation.

Optical Characterization of the Stability

The stability of NYS-LIEs was assessed in triplicate by multiple light scattering using the Turbiscan® Lab (Formulaction Company, L'Union, France). Undiluted samples (35 mL) were placed into cylindrical glass measuring cells that were completely scanned by a reading head. A pattern of the light flux as a function of the sample height was obtained giving a macroscopic fingerprint of the sample at a predetermined time. Measurements were performed after 30 days of storage at room temperature.

Scanning Electron Microscopy

A JSM-840 Jeol scanning electron microscope with a ZT 1500 Cryo transfer system (Oxford Instruments, Tubney Woods Abingdon, Oxfordshire, UK) was used to characterize the interfacial structure of NYS-LIE 54 formulation. The emulsion samples were cryofixed by immersion in solid nitrogen slush (−210°C) and quickly transferred to a cryo-transfer system (−150°C) under vacuum pressure (1 kPa). The sample was fractured using a small punch precooled at −150°C. The fractured surface of the emulsion was introduced into the chamber of the microscope to be sublimated at −90°C in order to remove excess frost accumulation. Finally, the sample was coated with Au (0.2 kPa and 40 mA). After metallization, it was introduced into the microscope to be observed at 13 kV and working distance of 11 mm.

Quantification of NYS (HPLC Analysis)

Nystatin was quantified using a HPLC validated previously[14] according international guidelines. Samples were taken from the top, middle, and the bottom of the tubes containing formulations and diluted (1:100) in methanol–DMF–water (55:15:30). Measures were performed in triplicate.

High-performance liquid chromatography assay was performed isocratically at room temperature. The system consisted of a Waters 515 pump (Waters, Milford, Massachusetts) with UV–Vis 2487 detector (Waters) set at 305 nm. A reverse-phase column (Kromasil 100, 5 μm, 15 × 0.46 cm^2) with a flow rate of 0.8 mL/min was used. The mobile phase consisted of a mixture of 1% glacial acetic acid in acetonitrile–water (40:60, v/v). The injection volume was 50 μL and total run time was 8 min.

Drug Release Studies

The release studies were performed using vertical Franz diffusion cells (FDC 400; Crown Glass, Somerville, New Jersey) with cellulose membranes (MWCO of 12 KDa) between the donor and the receptor compartment (12 mL), with an effective diffusional area of 2.54 cm^2. The receptor chamber was filled with methanol–DMF–water (55:15:30, v/v/v), according to previous studies.[13,14] This receptor medium allowed keeping sink conditions in the whole experiment. Amounts of formulations equivalent to 1 mg of NYS were added to the donor compartment. The system was kept at 37 ± 0.5°C to mimic *in vivo* conditions and stirred continuously. Samples (200 μL) were withdrawn at selected time intervals for 76 h and replaced with the same volume of fresh receptor medium.

The concentration of the released drug was measured as described in section *Scanning Electron Microscopy*. Values are reported as the mean ± SD of the six replicates.

Model Parameters.

Three different kinetic models (first order, Weibull, and korsmeyer-Peppas) were used to fit the experimental data obtained from the release assay.

$$\%R_t/\%R_\infty = 1 - e^{-K\times t} \quad \text{First order} \tag{1}$$

$$\%R_t/\%R_\infty = 1 - e^{-(t/td)ß} \quad \text{Weibull's equation} \tag{2}$$

$$\%R_t/\%R_\infty = k \times t^n \quad \text{Korsmeyer} - \text{Peppas's equation} \tag{3}$$

where $\%R_t$ is the percentage of drug released at time t, $\%R_\infty$ is the total percentage of drug released, $\%R_t/\%R_\infty$ is the fraction of drug released at time t, K is the release rate constant, and n is the diffusion release exponent. It could be used to characterize the different release mechanisms, $n \leq 0.43$ (Fickian diffusion), $0.43 < n < 0.85$ (anomalous transport), and ≥ 0.85 (case II transport; i.e., zero-order release); td is the time in which the 63.2% of the drug is released and β is the shape parameter.[23]

A nonlinear least-squares regression was performed using the WinNonLin® software (WinNonlin® Professional edition version 3.3; Pharsight Corporation, Sunnyvale, California), and the model parameters calculated. Also the Akaike's information criterion (AIC) was determined for each model as it is an indicator of the model's suitability for a given dataset.[24] The smaller the AIC value, the better the model adjusts the data. For the

model selection, the parameter accuracy, expressed as percentage coefficient of variation (%CV), and the residual distribution plots were also taken into account.

Antimicrobiological Efficacy

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of NYS-LIE and free NYS for yeasts and moulds according to international guidelines.[25,26] *C. albicans* ATCC 10231 was first cultured on Sabouraud's dextrose agar (SDA) plates at 35°C for 24 h before testing. The inoculums were prepared by suspending colonies in sterile distilled water to achieve the desired density equivalent to the 0.5 McFarland standard and counted in a Neubauer chamber ($1 - 5 \times 10^6$ Colony Forming Unit, CFU/mL). Working suspension was prepared diluting the standardized suspension in sterile distilled water (1:10) to transfer $1 - 5 \times 10^5$ CFU/mL.

Aspergillus fumigatus ATCC 9197 was cultured on SDA at 25°C for 5 days before testing. The spores and mycelium were suspended in 1 mL of sterile 0.85% saline buffer. The resulting suspension was withdrawn, and the heavy particles were allowed to settle for 3 –5 min. The upper homogeneous suspension was mixed for 15 s with a vortex mixer. The conidial suspension was adjusted to a concentration of 2×10^6 CFU/mL.

A synthetic medium RPMI-1640 2% G (RPMI-1640 with glutamine, pH indicator, without bicarbonate and glucose 2%; w/v) was used as working culture. pH was adjusted to 7.0 with 1 M sodium hydroxide and the final solution filtered through a 0.22-μm filter. Because the samples were not sterile, chloramphenicol was incorporated to RPMI (500 μg/mL). This antibiotic does not inhibit the yeast or moulds growth.[27] Serial dilutions of RPMI-1640 double strength from 500 to 0.00762 μg/mL were prepared for NYS-LIE. Equally, serial LIE without NYS was prepared to verify that the carrier did not interfere with the test. Free NYS solution in DMSO was used as reference antifungal. The final concentration of DMSO comprised less than 1% (v/v) of the total solution concentration used in the experiment.[7] Finally, 100 μL of inoculum suspension was added to each well. MIC was determined after 48 h of incubation at 30°C. Readings were performed at $\lambda = 595$ nm by a microdilution plate reader model 680 (Bio-Rad, Madrid, Spain).

RESULTS AND DISCUSSION

LIE Optimization

Lipid intravenous emulsions were optimized varying elaboration parameters based on dependent variables closely related to the stability and safety after intravenous administration (Z-ave, PI, LD_{99}, and ZP). Table 2 shows the observed responses. Three LIE replicates were prepared for each factor level.

Analysis of variance of the effect of factors on Z-ave values showed that neutralization was the only factor showing significant affectance on the droplet size ($p < 0.0001$). The range of Z-ave values was 237.5 ± 2.67−258.5 ± 2.98 nm when neutralization was performed during preparation and 225.7 ± 3.29−266.1 ± 2.53 nm when carried out after processing. However, this variation was not relevant from a technological point of view because all measures are within an appropriate range for intravenous administration.

Polydispersity index is an important parameter in emulsion characterization showing the real difference in droplet size distribution as an evidence of the system stability. As regards to the effect of the assayed factors on PI, only neutralization had a significant effect ($p < 0.05$). The lowest PI values were achieved when neutralization was carried out during preparation and ranged from 0.110 to 0.148.

Laser diffraction volume diameter 99% parameter expresses the droplet's volume distribution, which means that 99% of the measured particles are smaller than the values listed. It is a critical factor when the formulation is administrated intravenously. LD_{99} parameter was used for the determination of particle aggregates that could not be detected by PCS. Results of the analysis showed that neutralization ($p < 0.0001$) and time of exposure to ultrasound ($p < 0.0095$) were found to be significant factors on this dependent variable. The lowest LD_{99} values were achieved when neutralization was carried out during preparation (586−629 nm) and the exposure time was 50 min (780−825 nm).

The surface charge of the droplet is a parameter that determines the stability, when this charge is sufficiently large, the droplets are prevented from aggregation because of the repulsive electrical forces between approaching droplets. When the ZP is relatively high (25 mV or more, absolute value), the repulsive forces exceed the attractive London forces leading to enhanced stability. Neutralization process had also a significant effect on ZP. LIEs neutralized during the preparation process showed higher ZP values and ranged from −50.70 to −45.18 mV; in this way, neutralization would void free fatty acids and their decrease in pH, thus a potential decrease in ZP and ultimately instability.[28]

Finally, it was observed that ultrasound potency was not a determining factor on dependent variables.

Regarding the stability of LIEs, samples that were macroscopically altered in the course of the 9-month study

Table 2. Coded Values and Measured Responses of the Three Factors Depicted in Table 1 for the Eight Experiment LIE Runs

Run	ST (min)	Potency (W)	N	Z-ave (nm) ± SD	PI ± SD	LD_{99} (nm) ± SD	ZP (mV) ± SD
LIE1	30	80	NDE	257.3 ± 2.95	0.112 ± 0.04	565.3 ± 5.27	−50.70 ± 1.42
LIE2	30	80	NAE	245.1 ± 3.13	0.206 ± 0.01	857.3 ± 7.54	−46.45 ± 1.82
LIE3	30	120	NDE	258.5 ± 2.98	0.176 ± 0.04	623.4 ± 1.86	−45.18 ± 2.02
LIE4	30	120	NAE	246.7 ± 2.98	0.189 ± 0.03	685.1 ± 1.87	−40.89 ± 0.92
LIE5	50	80	NDE	237.5 ± 2.67	0.110 ± 0.05	567.8 ± 4.09	−46.77 ± 1.71
LIE6	50	80	NAE	225.7 ± 3.29	0.265 ± 0.02	734.2 ± 3.91	−41.39 ± 3.32
LIE7	50	120	NDE	245.6 ± 1.86	0.148 ± 0.01	562.5 ± 2.01	−46.44 ± 1.20
LIE8	50	120	NAE	266.1 ± 2.53	0.255 ± 0.05	786.8 ± 1.98	−44.76 ± 3.51

Values are reported as the mean ($n = 3$) ± SD.

ST, sonication time; N, neutralization; NDE, neutralization during elaboration; NAE, neutralization after elaboration; Z-ave, mean droplet size; PI, polidispersity index; LD_{99}, laser diffraction volume diameter 99%; and ZP, zeta potential.

Table 3. Physicochemical Results of the LIEs Stored for 12 Months at 4 ± 1°C and 30 ± 1°C

Run	*T* (°C)	AC	Z-ave (nm)	PI	LD_{99} (nm)	pH	ZP (mV)
LIE3	4	No	230.0 ± 2.32	0.213 ± 0.03	898.2 ± 1.09	6.57 ± 0.11	−39.57 ± 3.32
LIE3	4	Yes	229.2 ± 1.86	0.206 ± 0.01	877.5 ± 3.91	6.46 ± 0.14	−37.98 ± 1.24
LIE5	4	No	223.3 ± 1.87	0.173 ± 0.01	651.2 ± 1.86	7.50 ± 0.15	−45.19 ± 1.20
LIE5	4	Yes	231.2 ± 3.90	0.176 ± 0.02	642.8 ± 2.01	7.41 ± 0.23	−44.24 ± 3.51
LIE3	30	No	237.9 ± 3.29	0.199 ± 0.01	738.5 ± 1.86	6.62 ± 0.13	−39.65 ± 2.71
LIE3	30	Yes	239.6 ± 1.66	0.205 ± 0.03	695.5 ± 2.01	6.40 ± 0.24	−38.46 ± 1.36
LIE5	30	No	229.0 ± 2.72	0.175 ± 0.11	654.3 ± 1.98	7.37 ± 0.05	−45.18 ± 1.80
LIE5	30	Yes	234.6 ± 1.45	0.179 ± 0.02	639.1 ± 2.98	7.34 ± 0.1	−44.37 ± 2.75

Values are reported as the mean ($n = 3$) ± SD.
AC, autoclaved; Z-ave, mean droplet size; PI, polidispersity index; LD_{99}, laser diffraction volume diameter 99%; and ZP, zeta potential.

(e.g., broken or cremated emulsions) were discarded. In this way, samples that were neutralized during elaboration process showed in general better stability properties. Statistically significant differences were found for PI values throughout the study, increasing as time progressed among samples neutralized after elaboration (LIE2, LIE4, LIE6, and LIE8). On the contrary, samples neutralized during elaboration showed PI values in acceptable limits for parenteral use ≤ 0.250.[29] As results demonstrated, neutralization methodology is a critical factor affecting LIE stability.

Furthermore, statistically significant differences were observed in LD_{99} values among assayed LIEs, but none of the samples at the end of 9 months exceeded the given limit value and ranged between 729 and 938 nm. The maximum LD_{99} value was set at 1 μm; this guaranteed that the volume of the droplets was even small enough to prevent any potential vascular injury.

Lipid intravenous emulsion 3 and LIE5 were the formulations that showed the best physicochemical results and thus were selected for further stability studies. In this way, as it is shown in Table 3, both formulations (sterilized and nonsterilized) were submitted to a 12-month stability study at two temperatures, from which LIE5 was finally selected as the best formulation for further stages, based on its stability results scarcely but better than LIE 3.

Intralipid® 20% emulsion is normally used for parenteral nutrition and its composition is very similar to LIE5 (20% soybean oil, 2% soybean phospholipids, 2.25% glycerin, and water for injection). Both vehicles were characterized in terms of Z-ave and PI for 12-month storage at two temperatures. Table 4 shows the results of the paired *t*-test used to compare those parameters. No significant differences were found between commercial and novel vehicle in terms of time or temperature. Hence, it can be observed that the replacement of egg lecithin for soy lecithin did not modify the physical stability of the formulations. This substitution could be interesting as lecithin derived from egg yolk could cause allergic reactions in some patients.

NYS-Loaded LIEs

Four different formulations were prepared by adding 1 mg/mL of NYS to LIE5. Compositions of these NYS-LIEs are reported in Table 5. To facilitate the incorporation of the drug, a predispersion of NYS in solutol HS® 15 was performed at final concentration of 2% and 4%. Solutol HS® 15 is an amphiphilic, nonionic surfactant, with potent emulsifying ability and was chosen because of its physiological compatibility with intravenous application. According to the procedure reported by Semis et al.,[15] the NYS-loading process into the emulsion required DMSO and a prolonged agitation period. We have managed to overcome these drawbacks by incorporating solutol HS® 15, allowing a rapid NYS inclusion at higher concentration (1 mg/mL). Moreover and because of the poor water solubility of NYS, LIE5 was prepared at 20% and 30% of oily phase (soybean oil).

After 24 h and 60 days after preparation, pH values of the unloaded LIEs and NYS-LIE formulations ranged from 7.11 to 7.53; ZP values from −46 to − 44 mV; and LD_{99} values between 580 and 670 nm. These parameters remained almost constant for 60 days (data not shown). M-ANOVA demonstrated

Table 4. Changes in Z-ave and Mean PI of the Developed LIE and the Commercially Available Intralipid® 20%

		LIE5		Intralipid® 20%			
Time	*T* (°C)	Z-ave (nm)	PI	Z-ave (nm)	PI	PI *p* Value	Z-ave *p* Value
24 h	30	237.5 ± 2.11	0.108 ± 0.08	213.3 ± 2.98	0.105 ± 0.02	0.561	0.557
3 Months	30	220.2 ± 3.30	0.116 ± 0.04	229.6 ± 2.53	0.124 ± 0.03	0.476	0.554
6 Months	30	236.0 ± 3.77	0.136 ± 0.01	222.2 ± 1.92	0.153 ± 0.08	0.256	0.604
9 Months	30	215.4 ± 4.00	0.162 ± 0.04	243.7 ± 5.27	0.157 ± 0.02	0.414	0.390
12 Months	30	234.4 ± 3.29	0.169 ± 0.05	240.3 ± 7.54	0.171 ± 0.04	0.748	0.579
3 Months	4	228.5 ± 2.35	0.116 ± 0.01	237.9 ± 3.90	0.119 ± 0.01	0.561	0.527
6 Months	4	239.1 ± 3.54	0.139 ± 0.02	241.0 ± 4.09	0.143 ± 0.04	0.369	0.478
9 Months	4	229.4 ± 2.32	0.157 ± 0.01	247.5 ± 3.91	0.164 ± 0.02	0.144	0.375
12 Months	4	226.0 ± 1.86	0.170 ± 0.02	239.6 ± 1.86	0.169 ± 0.07	0.711	0.698
p Values		0.735	0.410	0.168	0.533		

Column *p* value indicates the significance of the PI and Z-ave, mean droplet size differences for each time point, respectively. File *p* value indicates the significance of the PI and Z-ave differences within all time points and temperatures.

Table 5. Nystatin-Loaded Lipid Intravenous Emulsions Composition (%, w/v)

Formulation	Nystatin	Solutol HS® 15	Soybean Oil	Soybean Lecithin	Glycerol	Water
NYS-LIE51	0.1	2	20	2	2.25	73.65
NYS-LIE52	0.1	4	20	2	2.25	71.65
NYS-LIE53	0.1	2	30	2	2.25	63.65
NYS-LIE54	0.1	4	30	2	2.25	61.65

Table 6. Changes in Z-ave and PI of Unloaded LIE and NYS-LIE

Formulation	Storage (Days)	T (°C)	Z-ave (nm)	PI
Unloaded LIE	0	30	236.0 ± 1.25	0.108 ± 0.06
	15	30	220.5 ± 1.92	0.105 ± 0.08
		4	209.8 ± 2.32	0.115 ± 0.03
	30	30	226.0 ± 5.27	0.130 ± 0.02
		4	220.5 ± 1.86	0.124 ± 0.03
	60	30	225.4 ± 7.54	0.128 ± 0.04
		4	242.1 ± 1.87	0.153 ± 0.05
NYS-LIE51	0	30	192.5 ± 2.32	0.170 ± 0.03
	15	30	203.5 ± 3.64	0.186 ± 0.05
		4	187.7 ± 3.90	0.136 ± 0.06
	30	30	201.9 ± 2.95	0.178 ± 0.02
		4	177.1 ± 4.09	0.215 ± 0.01
	60	30	212.0 ± 3.13	0.215 ± 0.07
		4	251.7 ± 3.91	0.120 ± 0.04
NYS-LIE52	0	30	181.0 ± 1.47	0.158 ± 0.03
	15	30	183.5 ± 1.86	0.149 ± 0.04
		4	182.3 ± 2.43	0.143 ± 0.04
	30	30	179.7 ± 2.01	0.140 ± 0.05
		4	177.3 ± 2.98	0.134 ± 0.05
	60	30	201.7 ± 1.98	0.193 ± 0.02
		4	201.5 ± 2.98	0.172 ± 0.02
NYS-LIE53	0	30	158.2 ± 1.62	0.149 ± 0.05
	15	30	160.8 ± 2.60	0.134 ± 0.02
		4	162.7 ± 3.29	0.148 ± 0.01
	30	30	215.7 ± 2.67	0.204 ± 0.02
		4	164.0 ± 1.86	0.151 ± 0.04
	60	30	161.1 ± 3.29	0.203 ± 0.07
		4	156.8 ± 2.53	0.127 ± 0.07
NYS-LIE54	0	30	143.0 ± 1.50	0.135 ± 0.02
	15	30	163.4 ± 2.98	0.150 ± 0.03
		4	144.6 ± 2.11	0.147 ± 0.04
	30	30	169.4 ± 3.65	0.147 ± 0.02
		4	145.0 ± 2.35	0.144 ± 0.02
	60	30	133.7 ± 5.86	0.158 ± 0.05
		4	141.1 ± 3.30	0.158 ± 0.08

Values are reported as the mean ($n = 3$) ± SD.

that formulation composition ($p < 0.01$) and the storage time (p = 0.0152) had significant effect on Z-ave. Despite these significant differences between 24 h and 60 days, these were small among NYS-LIEs, as can be seen in Table 6. The incorporation of NYS in solutol HS® 15 decreased Z-ave significantly. It has been hypothesized that apart from the emulsion structure and composition, the incorporated drug participates in the microstructure of the system and may influence it because of molecular interactions, especially if the drug possesses amphiphilic and/or mesogenic properties.[30]

But NYS was incorporated by means of the cosurfactant solutol HS® 15; the effect of this addition on Z-ave is clear. When NYS-LIEs was compared with the same percentage of oil phase (e.g., NYS-LIE51 and NYS-LIE52 or NYS-LIE53 and NYS-LIE54), the increase of solutol HS® 15 content implied a decrease of Z-ave.

Polydispersity index values remained between 0.105 and 0.215 in the course of the study. NYS-LIE54 and unloaded-LIE exhibited lower data dispersion than the rest of formulations, and thus more uniform droplet size distribution, highly recommended for emulsions development indicating good stability. Although the storage temperature was found to be a significant factor affecting the PI ($p = 0.0117$), differences were small, ranging from 0.14 to 0.16, for 4°C and 30°C, respectively. Finally, significant differences of PI were also observed when storage time was compared ($p = 0.0026$), a slight increase in PI was observed over time. As in the case of storage temperature, differences found in different days were within the preestablished threshold for PI ($\leq$0.25).

Optical Stability Assays for NYS-LIE

Turbiscan® Lab is considered as a device that predicts the stability, being able to detect the sample destabilization faster than other conventional techniques (microscopy, spectroscopy, turbidity, and particle size analysis). Moreover, it provides real-time information on the destabilization process. When sedimentation process is produced, a backscattering increase versus time at the bottom of the sample is observed. When the sample suffers a creaming process, an increase of backscattering versus time on the top of the vial is observed. If the destabilization phenomenon occurs because of particle aggregation, a backscattering increase versus time can be observed over the whole height of the sample.[31] If backscattering profiles have a deviation of at least ±2%, it can be considered that there are no significant variations on particle size. Variations up to ±10% indicate instable formulations.

Figure 1 shows the backscattering profiles of the four NYS-LIEs, corresponding to the measurements on different days: 1, 7, 15, 21, and 26 after production. The left side of the curve corresponds to the bottom of the vial, whereas the right side corresponds to the sample behavior on the top of the vial. As time passed, a decrease in backscattering at the bottom of the vial and an increase at the top of the vial could be observed, corresponding to an incipient creaming process. Moreover, a little backscattering peak appeared at the bottom. When the vial was observed at naked eye after 24 h, it could be seen a little yellow sediment easy to disperse, which could correspond to NYS sedimentation. This sediment was observed in NYS-LIE51, NYS-LIE 52, and NYS-LIE53. It was inexistent in NYS-LIE54. In our study, a predispersion of solutol HS® 15 and NYS was performed, obtaining a nistatin suspension, which was resistant to drug precipitation. When the predispersion was incorporated to the final emulsion (NYS-LIE54), it allowed NYS to be dissolved in the resulted emulsion. The precipitate was separated and analyzed for NYS quantification, Z-ave, PI, and LD_{99}. Results showed that NYS percentages were 35.48 ± 3.95%, 31.25 ± 3.73%, and 14.61 ± 4.02%,

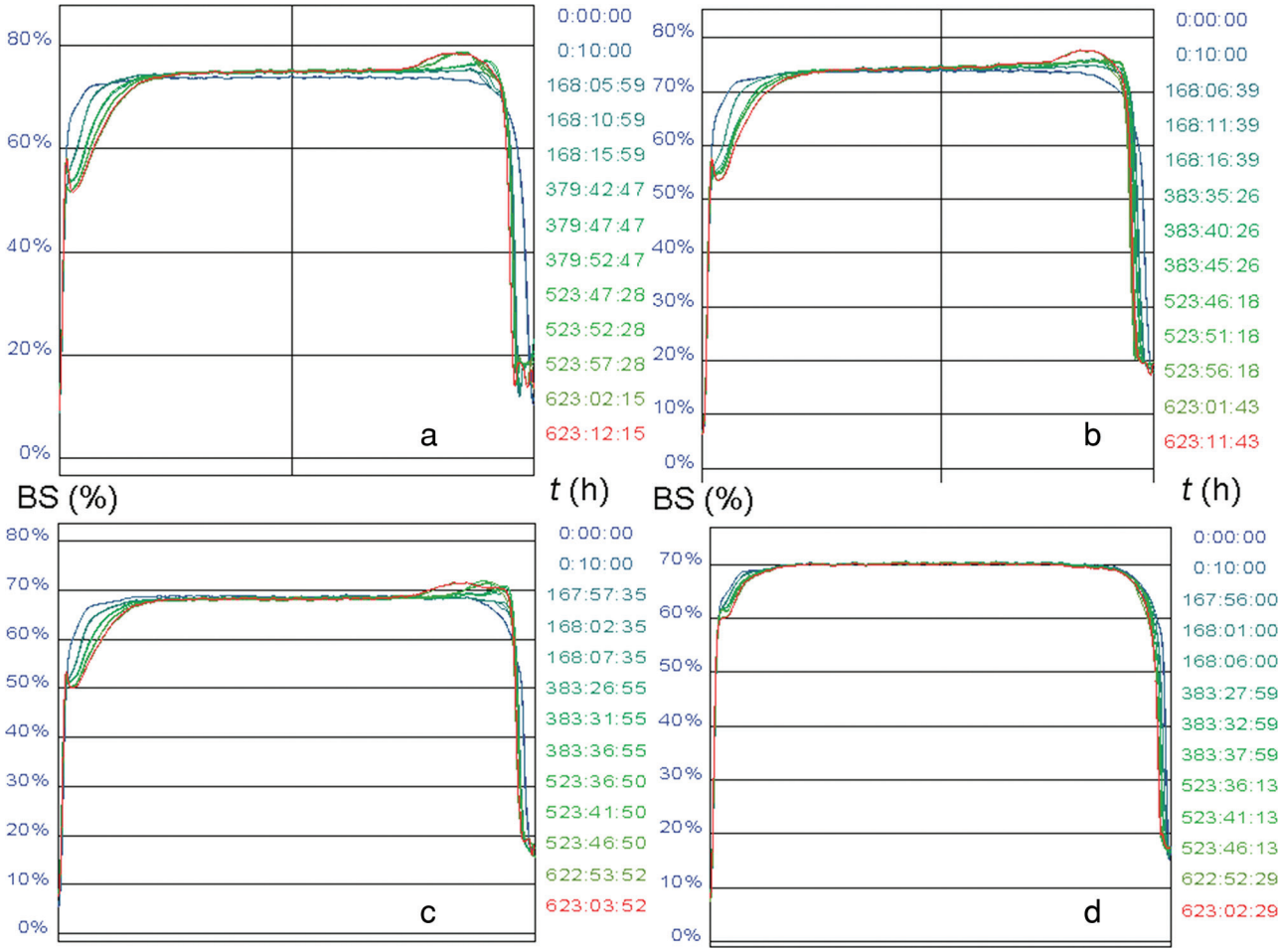

Figure 1. Turbiscan analysis of nystatin-loaded lipid intravenous emulsions (NYS-LIEs) at room temperature. Backscattering (BS), %, versus time (*t*), h. Panel a: NYS-LIE51; panel b: NYS-LIE52; panel c: NYS-LIE53; and panel d: NYS-LIE54.

respectively. These values decreased as the oily phase increased. Z-ave values ranged from 104.7 ± 2.63 to 115.18 ± 5.78 nm, PI ranged from 0.107 ± 0.01 to 0.118 ± 0.04, and LD_{99} ranged from 325 ± 6.29 to 376 ± 2.43 nm, indicating smaller drop sizes and a decrease of size distribution.

On the contrary, NYS-LIE54 (30% oil phase and 4% cosurfactant) was proposed to be the most stable confirming the results obtained in section *NYS-loaded LIEs*. Its Z-ave and PI remained almost constant along the study and no evidence of creaming or NYS precipitation were detected.

Morphological Analysis

After 30 days of storage, the freeze fracture–scanning electron microscopy images of NYS-LIE 54 revealed almost spherical-shaped droplets in the submicron size range, with the narrowest droplet size distribution. The macroscopic appearance and consistency suggested that emulsion remained stable. Microphotograph is available in the Supporting Information.

Quantification of NYS Within LIE

In all cases, the drug content, 24 h after preparation, was at least 98 ± 1.5%. One month after NYS-LIE51–53 elaboration, the drug content at the top of the container decreased (range 68%–81%). On the contrary, the drug content at the bottom increased (range 123%–142%), confirming the results observed in optical analysis. Drug content in emulsion NYS-LIE 54 remained almost constant along the vial up to the end of the study.

Release Studies

In the designing of colloidal drug-carrier formulations, the study of the rate at which the drug is released from the vehicle is particularly important, because it can be used as quality control data to predict *in vivo* behavior, or to study the structure and release mechanism of the system. Previous studies[13,14] demonstrated the ability of the mixture methanol–DMF–water (55:15:30, v/v/v) to maintain sink conditions in NYS release experiments and excellent compatibility with cellulose membranes. Figure 2 shows NYS release profiles from their respective emulsions ($n = 6$) at 37°C for 76 h. The maximum amount of NYS release at the end of the experiment ($\%R_\infty$) is relatively small; NYS-LIE51 and 53 exhibited similar release rates, followed by NYS-LIE52, and finally NYS-LIE54.

This pattern could be explained by the formulation ingredients. Solutol HS® 15 was at concentration of 2% in NYS-LIE51 and 53, and 4% in NYS-LIE52 and 54. Higher surfactant concentration would prolong the NYS residence time in the emulsion droplets, decreasing the drug release rate. According to results reported above, it has been demonstrated that an increase of the oil phase up to 30% in NYS-LIE54 enhanced its

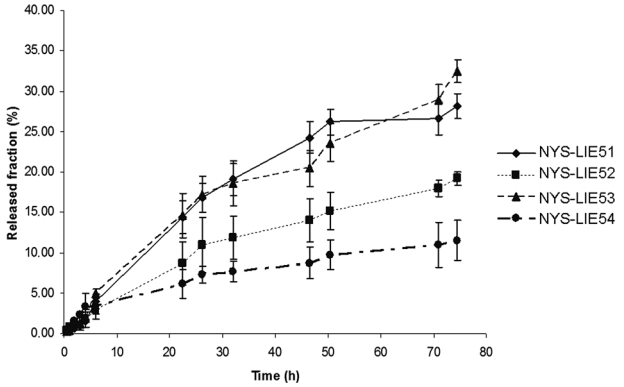

Figure 2. Release profiles of NYS-LIEs ($n = 6$) at 37°C for 76 h.

stability and could have a synergic effect with the augmentation of surfactant.

In order to ascertain the drug release mechanism from the elaborated emulsions, three kinetic models were fitted to experimental data: first-order equation (Eq. 1, Weibull's equation (Eq. 2, and Korsmeyer-Peppas equation (Eq. 3. The best model was selected based on the lowest AIC value. The precision of the biopharmaceutical parameters was expressed as %CV and the distribution of the residuals were also taken into account. The release parameters of the prepared emulsions are depicted in the Supporting Information. NYS release from LIE51 followed the Weibull's equation; it has been typically considered as an empirical equation, in which the value of the exponent β has been proposed as indicator of the mechanism of diffusion release.[32] The value 1.36 corresponds to a sigmoid curve-like indicative of a complex release mechanism. This fact was confirmed by the release exponent n of the Korsmeyer-Peppas's equation with a value of 0.67, being indicative of anomalous transport. NYS-LIE52 and 53 exhibited a first-order kinetic release with slow release constants, 0.021 and 0.018 h^{-1}, respectively; but this difference did not result as significant ($p > 0.05$). First-order model means that drug release from emulsion followed a concentration gradient pattern, based on the first Fick's law, where the released amounts are directly proportional to the amounts remaining in the dosage form offering sustained release of drug. As in the case of NYS-LIE51, when release exponent n of Korsmeyer-Peppas's equation was calculated, the anomalous transport could be responsible of NYS release in both cases. Anomalous transport has been classically attributed to a combination of diffusion and erosion in solid matrixes,[33] but little is known in emulsions. Most authors have attributed the anomalous transport to surfactant phase transitions, such as the formation of lamellar or cubic phases.[34] Finally, NYS-LIE54 exhibited a release exponent n equal to 0.5. At this point, the Korsmeyer-Peppas's equation is equivalent to Higuchi's square root of time equation and is indicative of a diffusion-controlled release.[33] Higuchi release model assumes a steady-state diffusion of nondegradable systems when the drug is uniformly dispersed throughout the matrix. Therefore, a controlled release was only achieved by NYS-LIE54, which was also the formulation that exhibited the best stability.

Microbiological Studies

The broth microdilution method was used to assess the *in vitro* antifungal activity of NYS-LIEs against strain of yeast *C. albicans* ATCC 10231 and *A. fumigatus* ATCC 9197. Lipid emulsion excipients showed no inhibitory effect on the yeast grown, proving that the excipients did not interfere the test. MIC values of NYS-LIEs were lower than free NYS (0.3906 μg/mL); these values suggested that lipid emulsion enhanced the antimicrobial activity of NYS and its efficacy. In the case of *C. albicans*, NYS-LIE51 and 53 showed slightly lower MIC values (0.3882 μg/mL, both) but were not statistically significant compared with free NYS. On the contrary, NYS-LIE52 and 54 showed approximately two-fold lower MIC values than free NYS (0.2441 μg/mL, both). These emulsions contained greater proportion of solutol HS® 15 (4%) in contrast to other two formulations (containing 2%). As stated previously, solutol HS® 15 is a cotensoactive, which apart from the stability improvement of the formulation might allow the system to provide better interaction with the fungi membrane, and thus decrease the MIC value and increase the potency of the drug. The two-fold MIC reduction is in agreement with the study reported by Semis et al.[15]

When *A. fumigatus* was tested, a strong efficacy increase was observed. According to results, the MIC value for *Aspergillus* was approximately two times lower when NYS was introduced within the emulsion (3.906 μg/mL, all) compared with free NYS (6.250 μg/mL). In this case, there were no significant differences among different emulsions, and thus the proportion of solutol HS® 15 does not seem to have a direct relationship with the LIE efficacy.

A comparison between MIC values obtained in this study with those reported by other authors was difficult, because of the variance of protocols, strains, and culture medium used, which could lead to MIC values modification.

The pronounced MIC reduction found in *Aspergillus*, in contrast to *Candida*, could be because of the different ergosterol content in these microorganisms. According to NYS antifungal mechanism (by binding to membrane ergosterol), the results obtained in this study could be attributed to the higher ergosterol content in *Aspergillus* membranes.

The enhancement of antifungal activity of NYS, when vehiculized in a lipid-based formulation, compared with free NYS, was previously reported,[13–15] confirming the necessity to formulate NYS in lipid-based system to improve its efficacy.

CONCLUSIONS

A stable LIE was successfully developed. This formulation exhibited a small Z-ave and PI when neutralized during the emulsification process. LD_{99} values showed no aggregation, being appropriate for intravenous administration. The in-house low-scale production gave equivalent results to the commercially available emulsion Intralipid® 20%. The incorporation of NYS in the emulsion was successfully achieved by the addition of Solutol HS® 15 at 4% and the increase of the oil phase to 30%, resulting in a stable formulation with Z-ave and PI values lower than unloaded emulsion. The drug release followed a controlled-diffusion mechanism according to Higuchi's equation. The incorporation of NYS in the lipid-based formulation improved the antifungal efficacy by the reduction of MIC value of *C. albicans* and *A. fumigatus*. This vehicle had an adequate technological and biopharmaceutical profile, being a promising, low-cost NYS delivery system for invasive fungal infection treatments.

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