HSOA Journal of Pharmaceutics, Drug Delivery & Safety

Research Article

Development of Liposomes-in-Hydrogel Formulations Containing Betamethasone for Topical Therapy

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Abstract

In this work, betamethasone was entrapped in unilamellar liposomes that were further dispersed in two different hydrogels to promote a localized effect of the drug with minimal systemic delivery in order to minimize the associated toxicity. The optimized betamethasone-loaded liposomes showed an average particle size of 155.1±4.9 nm, narrow size distribution (polydispersity index-PDI<0.1) and positive surface charge (mean zeta potential of +19.7±2.0 mV). The physico-chemical characterization of these vesicles also demonstrated that they presented spherical shape, good physical stability and drug entrapment efficiency values higher than 80%. Following characterization, the liposome dispersion was incorporated into 1% w/w poly(acrylic acid) gel base or in 2% w/w hydroxypropyl gum guar (Jaguar HP-8®) gel base. The betamethasone entrapment efficiency in liposomes and its incorporation in hydrogel were determined using a validated High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) method. The two hydrogel formulations demonstrated adequate properties as dermatological nanomedicines, namely pH, viscosity, physical stability and drug content.

Keywords: Betamethasone; Hydroxypropyl gum guar; Liposomes; Poly(acrylic acid); Topical effect

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Citation: Ferreira H, Gonçalves VMF, Silva R, Tiritan ME, Teixeira M, et al. (2017) Development of Liposomes-in-Hydrogel Formulations Containing Betamethasone for Topical Therapy. J Pharmaceut Drug Deliv Safety 1: 003.

Received: February 9, 2017; **Accepted:** April 20, 2017; **Published:** June 09, 2017

Introduction

Betamethasone is a highly effective corticosteroid widely used in the topical treatment of skin diseases, such as psoriasis and eczema. Indeed, this synthetic glucocorticoid presents immunosuppressive and anti-inflammatory activities. Unfortunately, due to the topic and systemic side effects that commonly occur, its use is frequently restricted [1]. Optimization of betamethasone-carrier formulations to modulate the release of this steroid over a long-lasting period and reduce its systemic absorption, improving, therefore, its therapeutic effectiveness are of growing interest. Liposomal formulations have been the focus of special attention, since their introduction in the early eighties as skin drug delivery systems. Mezei and Gulasekharam were the first to use liposomes for topical therapy [2]. *In vivo* studies have demonstrated that liposomes, when compared to other formulations, enhance steroid concentrations in the epidermis and dermis and reduce their systemic absorption [2,3]. These studies were criticized and contradictory results have been published [4-11]. However, it has become evident that liposomes without an edge activator (deformable liposomes), for example, did not present a significant skill for transdermal drug delivery [6,12,13]. Therefore, conventional liposomes can be used to obtain a localized skin effect.

In 1988, a liposomal formulation containing the antimycotic agent econazole was first introduced in the market [9]. Since then, other liposomal formulations have been marketed and many other may follow the same trend. For instance, promising clinical data was obtained by Agarwal et al., [14] with an aqueous gel-based containing dithranol-loaded liposome for the treatment of patients with stable plaque psoriasis. The developed formulation was effective and significantly reduced the adverse effects of dithranol, having, therefore, potential advantages over other available preparations of this drug. In another study, the efficacy of a liposomal formulation of betamethasone dipropionate was compared to that of a conventional gel in patients presenting atopic eczema and psoriasis vulgaris [15]. Although results reflected no improvements in the treatment of psoriasis, in eczema conditions the liposomal formulation led to a higher reduction of the erythema and skin scaling compared with the conventional gel.

Singh et al., reported that the entrapment of the non-steroidal anti-inflammatory drug Nimesulide in Multilamellar Liposomes (MLVs) improved its performance comparatively to the marketed gel and to a Carbopol® hydrogel base formulations [16]. Further studies demonstrated that Carbopol® hydrogel base containing liposomes was also successfully explored as a topical formulation to release an antifungal agent during a considerable period of time [17].

In the present study, betamethasone was entrapped into Large Unilamellar Liposomes (LUVs) to obtain a localized topical effect, without the undesired systemic side effects. LUVs were composed of Egg-yolk Phosphatidylcholine (EPC) and α-tocopherol, which was used to protect liposomes against oxidation. As liposomes were prepared from phospholipids, the main components of cells membranes, they act as non-irritating moisturizing agents and they are biocompatible, biodegradable and nontoxic [18,19]. It has also been reported that liposomes of EPC are biocompatible with human skin fibroblasts

showing the potentialities to be applied in the treatment of skin diseases [20]. The physicochemical characterization of the prepared liposomes included the determination of particle size and Poly Dispersity Index (PDI), zeta-potential, morphology, betamethasone entrapment efficiency and physical stability. To produce topical formulations, the liposomes were incorporated in 1% w/w poly (acrylic acid) gel base or in 2% w/w hydroxypropyl guar gum (Jaguar HP-8®) gel base. In contrast with marketed formulations where betamethasone is dispersed in gels or creams, in this work it was prepared liposomes-in-hydrogel formulations. Liposomes were incorporated in hydrogels to obtain a controlled release of betamethasone and to increase its concentration on the formulation, since that betamethasone has limited solubility in water. Poly (acrylic acid) and hydroxypropyl guar gum are able to retain large quantities of water even when subjected to some pressure [21,22]. These polymers provide, therefore, viscosity to aqueous solutions even at low concentrations and are also compatible with various drugs. Additionally, poly(acrylic acid) and hydroxypropyl guar gum have been shown to be biocompatible in several biomedical applications [23,24], making them a good candidate for the purposed work. Hydrogels are often used as skin formulations due to their technological feasibility of controlling their viscosity, providing suitable characteristics to be applied onto the skin [25]. Additionally, the use of liposomes-in-hydrogel as a delivery system can exhibit favorable texture properties for topical administration and can lead to a sustained drug release throughout their prolonged contact time with a tissue, contributing the high viscosity presented by some hydrogels to liposomes stabilization [26,27].

A High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) method was developed and validated for the determination of betamethasone entrapment efficiency in liposomes and of its concentration in the hydrogel. Formulation prepared. HPLC-DAD was also used to assess betamethasone stability to thermal, acidic and alkaline stress conditions.

The aim of this work is, therefore, the development and characterization of two novel liposomes-in-hydrogel formulations containing betamethasone with high potential application in the treatment of various topical disorders. Liposomes will allow a controlled release of the entrapped drug through the hydrogel into the skin, in order to achieve a prolonged and localized effect. Additionally, being EPC the main component of liposomes, it is expected a synergistic effect with betamethasone in counteracting inflammation, due to the phospholipid antioxidant activity [28,29].

Materials and Methods

Chemical reagents

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (Spain) and used as supplied. Acetonitrile HPLC gradient grade was purchased from Fisher Scientific (United Kingdom), ethanol HPLC gradient grade was acquired from Merck (Germany) and Jaguar HP-8® was generously supplied by Rhodia (France). Ultrapure water was provided by a SG Water System (Ultra Clear UV model).

Preparation of betamethasone-loaded liposomes

Liposomes were prepared according to the thin film hydration method [30] followed by extrusion. Basically, a known amount of EPC dissolved in ethanol was mixed with an ethanol solution of the anti-oxidant α-tocopherol (6:1; M/M). Then, the betamethasone ethanol solution (1:43 molar ratio of drug/EPC) was added. The use of ethanol, instead of chloroform or methanol, was to obtain a final product with no solvent-related toxicity. The formation of the lipid film was performed by evaporation of the ethanol in a rotary evaporator and to eliminate any traces of this organic solvent, the lipid film was left under high vacuum (Vaccuubr and GMBH+CO, VSP 3000- Germany) for at least 3h. The dried lipid film was hydrated with ultra-pure water and vortexed to originate MLVs. To produce LUVs, the MLVs suspensions were extruded five and ten times through Nucleopore® polycarbonate filters of 0.4 and 0.2 μm pore diameter, respectively. The non-entrapped drug was removed by size exclusion chromatography through a Sephadex G-25 M column (GE-Healthcare).

Phospholipid concentration determination

During liposomes preparation occurs some loss of the phospholipid content, and consequently, it was necessary to determine the final EPC concentration. The quantitative determination of lipid was assayed through an enzymatic method (Lab Assay™ Phospholipid, Wako, Osaka, Japan). In this assay, Phosphatidylcholine was hydrolyzed, by phosphalipase D, to choline. The oxidation of choline into betaine, mediated by choline oxidase, is accompanied by the simultaneous production of hydrogen peroxide. The reaction of the formed hydrogen peroxide with *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-Dimethoxyaniline Sodium Salt (DAOS) and 4-aminoantipyrine leads to the production of a blue pigment, which was quantified by measuring the absorbance at 600 nm in a multiplate reader (Synergy HT W/TRF from Bio-Tek).

Liposomes Characterization

Particle size, Polydispersity Index (PDI) and zeta-potential

The evaluation of size distribution and zeta-potential of the liposomes was performed in a Malvern zetasizer NS (Malvern Instruments) equipment, using Dynamic Light Scattering (DLS) and laser Doppler micro-electrophoresis, respectively. Before measurement, at 25.0±0.1 °C, lipid concentration was adjusted to approximately 500 μM by diluting the sample with ultra-pure water.

Liposomes morphology

The examination of liposomes morphology was made using a scanning electronic microscope model NOVA Nano SEM 200 FEI. After deposition of the liposome suspension in copper grids with a 400 meshes and 3 mm diameter carbon film, Scanning Transmission Electron Microscopy (STEM) analyses were performed.

Betamethasone entrapment efficiency in liposomes

The betamethasone entrapment efficiency values were corrected to the concentration of lipids effectively present at the end of the LUVs preparation, by the following equation [6]:

$$\text{Entrapment efficiency } (\%) = \frac{[\text{Betamethas one}]_f / [\text{EPC}]_f}{[\text{Betamethas one}]_i / [\text{EPC}]_i} \times 100 \quad (1)$$

Where $[\text{Betamethasone}]_f$ and $[\text{EPC}]_f$ are, respectively, the betamethasone and lipid concentrations after liposomes preparation and $[\text{Betamethasone}]_i$ and $[\text{EPC}]_i$ are the respective instead of irrespective.

The corticosteroid concentrations were evaluated by a validated HPLC-DAD method described below. Analyses were performed with a La Chrom Merck Hitachi equipped with a L-7100 pump, a D-7000 interface, a L-7200 auto-sampler and a L-7455 Diode Array Detector.

For the chromatographic analyses, a HPLC System Manager HSMD-7000, version 3.0 and a Lichrocart® 250-4 LiChrospher® (100 RP-18, 5 µm; Merck) column preceded by a guard column LiChrospher® 100 RP-18 (5 µm) LiChrocart® (4x4 i.d.; Merck) were used. The system was operated at room temperature, in the isocratic mode at a flow rate of 0.8 mL/min, with a mixture of acetonitrile/water (50:50, v/v) as mobile phase. The injected volume was 20 µL and the monitorization of betamethasone was carried out at 240 nm.

Prior to HPLC quantification, samples were diluted 10x with ethanol.

HPLC-DAD method validation

The validation of the method was performed in accordance with the International Conference on Harmonization Guidelines [31-33] evaluating the following parameters: specificity, linearity and range, accuracy, recovery, precision, Detection Limit (DL) and Quantification Limit (QL).

A stock standard solution of betamethasone (1 mg/mL) in ethanol was diluted with water/ethanol (1:9, v/v) in order to obtain seven standard solutions of different concentrations (ranging between 5 and 130 µg/mL).

The specificity of the analytical method was evaluated by comparing the chromatograms of the standard solutions with samples of empty liposomes and samples obtained by the mixture of betamethasone and liposomes (i.e., spiked with empty liposomes).

Linearity was assessed through the construction of calibration curves with a set of seven concentration levels ranging from 5 to 130 µg/mL (5, 20, 40, 60, 80, 100 and 130 µg/mL), prepared in triplicate.

The accuracy of the method was evaluated as the percentage of agreement between the betamethasone concentration expected and the experimentally obtained value. For the determination of this parameter, three Quality Control (QC) standard solutions with concentrations of 10, 50 and 120 µg/mL were prepared and analyzed. The three replicates of QC standard solutions were prepared by diluting the stock standard solution of betamethasone (1 mg/mL) with a mixture of water/ethanol (1:9, v/v).

Recovery was assessed by adding known amounts of betamethasone to the suspension of empty liposomes. The corticosteroid concentrations used, in triplicate, were analogous to the three QC concentrations previously referred and recovery was calculated by comparing the peak area of the samples of liposomes spiked with betamethasone with those of equal drug concentration in a mixture of water/ethanol (1:9, v/v).

Intra- and inter-batch precisions were determined by the analyses of three replicates of the QC standard solutions obtained in the same day and in three different days, respectively. Precision was expressed as the Relative Standard Deviation (%RSD) of the determinations performed in triplicate. DL and QL were determined as detailed in equations 2 and 3:

$$DL = 3.3 \times \left(\frac{s}{S}\right) \quad (2)$$

$$QL = 10 \times \left(\frac{s}{S}\right) \quad (3)$$

where *s* is the standard deviation of the y-intercept of the regression line and *S* is the slope of the calibration curve [34].

The specificity of the analytical method was also evaluated in samples of betamethasone submitted to thermal, acidic and alkaline stress conditions. The resistance to thermal degradation was determined by placing a required amount (1 mg) of the compound in an oven at 120 °C during 72 h. At specified time points (2, 4, 10, 24, 48 and 72 h), an amount of sample was collected, dissolved in ethanol and analyzed by HPLC-DAD. To evaluate if the acidic and alkaline stress conditions provoked some corticosteroid degradation, 1 mg of the betamethasone was mixed with 25 mL of 1M HCl and with 1M NaOH, respectively. Following determined time points to a maximum of 72 h of stirring at room temperature, the pH values of aliquots of the acidic or alkaline solutions were adjusted to water pH with 6 M NaOH and HCl 98%, respectively. After that, samples were filtered and submitted to HPLC analysis.

Liposomes stability

The stability of the liposomes, stored at 4 °C over 2 months, was evaluated by the weekly determination of the particle size and zeta-potential, as described in the Particle size, PDI and zeta-potential section.

Preparation of hydrogels containing liposomes entrapping betamethasone

The produced liposomes were incorporated in 1% w/w poly(acrylic acid) (number average molecular weight equal to 130,000) gel base or in 2% w/w Jaguar HP 8® gel base in order to obtain a betamethasone concentration of 0.5 mg/g. This concentration was based on the typical levels found in marketed betamethasone topical medications.

Hydroxypropyl guar gum was dispersed in the liposomes suspension and to obtain an appropriate viscosity of the gel, the pH of the polymeric dispersion was adjusted to ≈ 6 with lactic acid. In the case of poly (acrylic acid), it was necessary to neutralize the polymeric dispersion to a pH near to 6 with triethanolamine before addition of liposomes, in order to avoid a production of a heterogeneous formulation. Placebo hydrogels using empty liposomes or deionized water were also prepared.

Hydrogels characterization

The physicochemical characterization of hydrogels consisted in the determination of pH, viscosity, centrifugation stability, drug content and physical stability. The pH was measured with a calibrated Metrohm 691 pH Meter. The rheological characteristics of the prepared formulations were determined, at 25 ± 0.1 °C using a rotational viscometer (Digital viscometer, Brookfield DV-E) and a spindle nº 1 for poly (acrylic acid) gel and a spindle nº 6 for Jaguar HP-8® gel. The centrifugation stability was evaluated at 3500 rpm for 30 min. For drug quantification in hydrogels containing liposomes entrapping betamethasone, a HPLC-DAD method was also developed and validated for its specificity, linearity and range, accuracy, recovery and precision. Specificity was verified in samples containing betamethasone and hydrogel excipients. Linearity of the method was examined in a concentration range of 3-10 µg/mL. Accuracy, recovery and intra- and inter-batch precision were estimated by performing three replicates of three QC standard solutions with concentrations equal to 0.4, 0.5 and 0.6 mg/g (corresponding to 80%, 100% and 120% of betamethasone in hydrogels). The quantification of betamethasone in the hydrogels was performed after its extraction with ethanol, as follows: after accurately weighted 0.1 g of each formulation, 10 mL of ethanol was added and the resulting mixture was subjected to ultrasound for 30 min, at room temperature. The sample was then filtered through a nylon membrane (Acrodisc 13 mm, 0.45 µm pore diameter, PALL) and analyzed by HPLC-DAD.

The stability of the hydrogels, stored at 4°C, over 1 month, was measured in terms of pH, viscosity and centrifugation stability by the previously described procedure.

Statistical analysis

Data was expressed as mean ± standard deviation of three independent experiments. The significance of the differences was determined by the Kruskal-Wallis One Way Analysis of Variance on Ranks (Sigma Plot 12.3).

Results

Size distribution, zeta-potential and morphology of liposomes

The size of the liposomes entrapping betamethasone presented a mean value of 155.1±4.9 nm, and the PDI was always lower than 0.1 (0.066±0.009). The liposomes produced exhibited a zeta-potential of approximately +19.7±2.0 mV, indicating the presence of a positive charge on the vesicles surface.

Figure 1 illustrates that the obtained liposomes are spherical. In addition, the size distribution obtained with STEM was identical to that reported using DLS analyses.

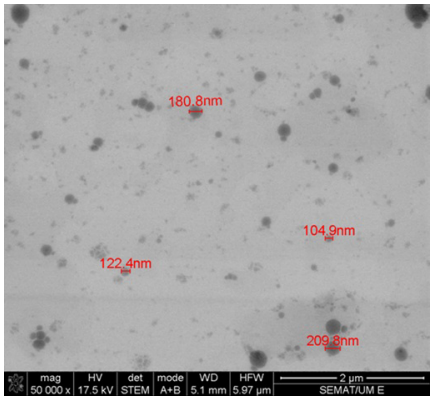

Figure 1: STEM microphotographs of liposomes entrapping betamethasone.

The microphotographs with a magnification of x50000 present the shape and size of the liposomes population.

Betamethasone entrapment efficiency

To determine the entrapment efficiency of the glucocorticoid by HPLC-DAD, increasing ratios of EPC/betamethasone were tested. The determination of this parameter considered the initial and final lipid concentration, once that about 6-7% of EPC was lost during the liposomes preparation. With an initial molar ratio of 1:43 of drug/EPC the entrapment efficiency of betamethasone into LUVs presented a value of 85.7±4.5%.

HPLC-DAD method validation

Selectivity is highlighted in figure 2, in which the chromatograms obtained for a sample of empty liposomes, for a standard solution of betamethasone (10 µg/mL) and for a sample of betamethasone (10 µg/mL) spiked with empty liposomes are shown. It is possible to verify that no interference is present in the betamethasone retention time (approximately 4.7 min), thus substantiating the selectivity of the method concerning liposome composition. In fact, the UV spectra of the standard solution and of the sample spiked with liposomes were identical. Furthermore, the peak purity obtained for the two peaks was 99.99%.

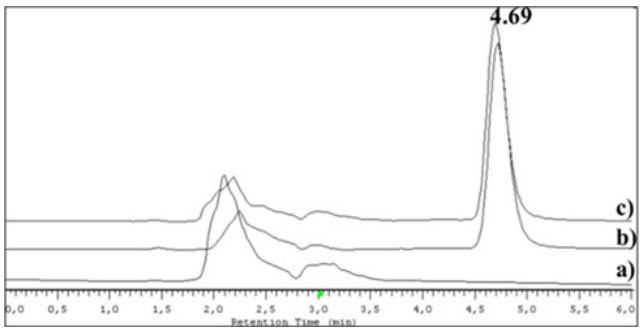

Figure 2: HPLC-DAD chromatograms to demonstrate the selectivity of the method.

In a) is presented the chromatogram obtained for empty liposomes; b) is the chromatogram obtained for betamethasone standard solution (10 µg/mL) and c) represents the chromatogram of a betamethasone standard solution (10 µg/mL) spiked with empty liposomes.

The method was linear in the concentration range considered (5-130 µg/mL). The calibration curve (equation: y=21173x-15133) showed a correlation coefficient of 0.9998 and the %RSD ranged between 0.8-2.1%. The values calculated for DL and QL were 0.20 μg/mL and 0.60 μg/mL, respectively.

Table 1 contains the results obtained for accuracy and recovery assays and, as observable, they ranged between 100.1-105.4% and 99.7-102.6%, respectively. The intra and inter-day assays, expressed by %RSD, were performed to estimate the precision of the chromatographic method. The %RSD calculated for both parameters presented a maximum value of 2.96% (Table 1), which indicates that the method presents an acceptable precision [34].

Nominal concentration (µg/mL)	Accuracy (%)	Recovery (%)	%RSD (inter-day)	%RSD (intra-day)
10	105.4	100.5	1.92	2.96
50	103.4	99.7	1.35	0.83
120	100.1	102.6	1.49	2.82

Table 1: Accuracy, recovery and precision (intra and inter-day) obtained for the HPLC-DAD method under validation.

Chromatograms of the samples containing the drug submitted to thermal, acidic and alkaline stress conditions are shown in figure 3. Comparison of the representative chromatogram of betamethasone (Figure 2) with those in figure 3 allows concluding that betamethasone did not suffer any quantifiable degradation under the tested temperature and time conditions (Figure 3A). Additionally, betamethasone presents a higher resistance to acidic degradation than to the considered alkaline conditions. The samples subjected to acidic conditions only showed a small additional peak (retention time approximately at 8.0 min), after 72h of incubation (Figure 3B). On the other hand, in the samples submitted to alkaline stress conditions, after 72h of incubation, almost all betamethasone was degraded (Figure 3E). In fact, after 20 min (Figure 3C) of incubation, additional peaks were found and after 4h (Figure 3D) betamethasone concentration was below the QL. The betamethasone UV-spectrum was the same after submitting the samples to thermal, acidic and alkaline (considering only 20 min of incubation; Figure 3C) conditions and the peak purity tests performed by the HPLC-DAD software revealed that the peaks had a purity of 100%. These results highlight that there were no degradation products eluting simultaneously with betamethasone.

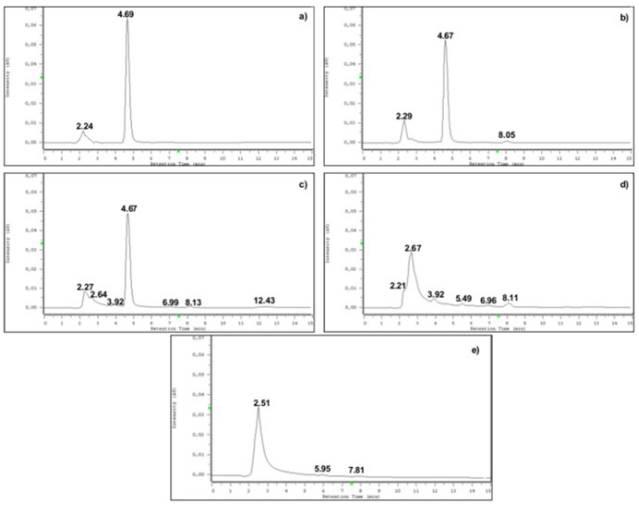

Figure 3: HPLC-DAD chromatograms of betamethasone subjected to stress conditions.

a) Chromatogram obtained for betamethasone samples subjected to thermal degradation (120 °C, 72 h) and b) to acid degradation (1M HCl, 72 h). c), d) and e) represents the chromatograms obtained under alkaline degradation conditions (1M NaOH) at 20 min, 4 h and 72 h, respectively.

Stability of liposomes

Figure 4 shows that size and zeta-potential present similar values before and during two months of storage (no statistical significant differences were found), at 4˚C, which emphasizes the stability of the formulation developed. Furthermore, the PDI remained under 0.1, which also indicates the absence of liposomes aggregation.

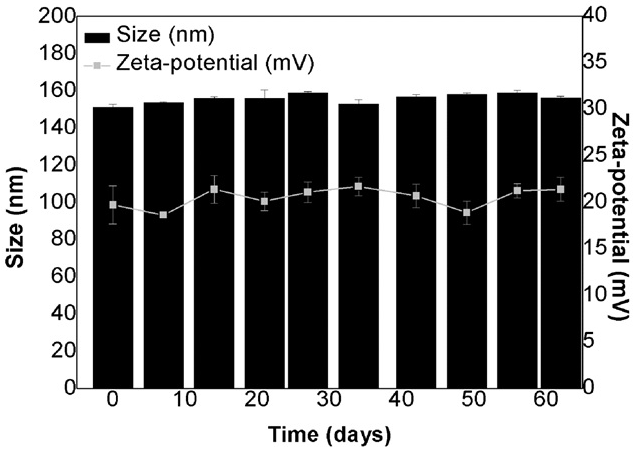

Figure 4: Evaluation of the liposomes stability. Size (nm; ■) and zeta-potential (mV; ■) obtained for LUVs entrapping betamethasone over a period of two months.

Hydrogels characterization

The macroscopic aspect of hydrogels based on poly (acrylic acid) or hydroxypropyl gum guar are shown in figure 5.

The pH values of the hydrogels developed were ≈ 6.3, which is compatible with skin application [25]. The rheological behavior of the formulations developed is shown in figure 6. The hydrogels prepared with hydroxypropyl gum guar revealed a non-Newtonian behavior, more specifically, a pseudoplastic flow, as viscosity decreased with increasing shear rate [35]. In non-Newtonian systems, this rheological behavior is intended, as the application of medium to high shear

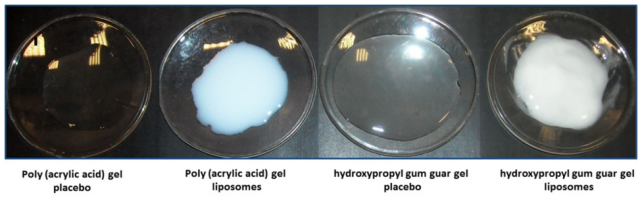

Figure 5: Physical appearance of the hydrogels. Hydrogels based on poly(acrylic acid) or hydroxypropyl gum guar without (placebo) or with liposomes presents, respectively, a translucent or opalescent aspect.

conditions leads to a low flow resistance of the formulation [36]. On the other hand, for the poly(acrylic acid) gel base, viscosity did not change significantly with shear rate, suggestive of a Newtonian behavior. However, the viscosity values were higher when the gel base was enriched with the liposomes suspension (Figure 6). As illustrated in figure 7, no signs of phase separation or sedimentation were observed after centrifugation, thus proving that the developed formulations presented a good physical stability.

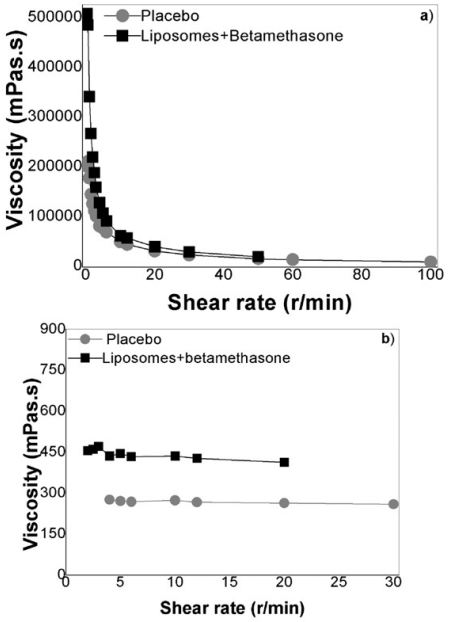

Figure 6: Viscosity curve of hydrogels.

Viscosity values variation with shear rate for hydroxypropyl gum guar (a) and poly(acrylic acid) (b) hydrogels prepared with water (●) or liposomes suspension (■), at 25±0.1 °C.

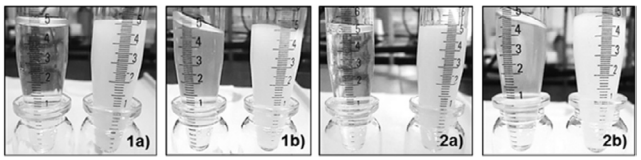

Figure 7: Physical stability of the hydrogels.

Visual aspect of poly(acrylic acid) (1) and hydroxypropyl gum guar (2) hydrogels prepared with water (translucent aspect) or liposomes suspension (opalescent aspect), before (a) and after (b) centrifugation (3500 rpm, 30 min).

The percentage of drug content, assayed by HPLC-DAD, was 98.8±1.8% and 99.6±2.2% for hydrogels of poly(acrylic acid) and hydroxypropyl gum guar, respectively.

Finally, the determination of pH, viscosity and centrifugation stability, over 1 month, demonstrated that the hydrogels prepared

showed good physical stability, once that the data obtained presented approximately the same behavior throughout the time considered.

HPLC-DAD method validation

The quantification of the betamethasone entrapped in liposomes and incorporated in a poly (acrylic acid) gel base or in a hydroxypropyl gum guar gel base was performed with the same chromatographic conditions previously described for the betamethasone entrapment efficiency. However, the method was also validated, in a range between 3-10 µg/mL, taking into account the specificity, linearity and range, accuracy, recovery and precision in this matrix.

Figure 8 allows the comparison between the chromatograms obtained for a betamethasone standard solution and for the placebo hydrogels (with or without empty liposomes). As none of the chromatograms exhibits interfering peaks in the betamethasone retention time, the selectivity of the method was verified. A calibration curve with standard solutions that were submitted to the extraction method was also prepared, in order to demonstrate that this process did not induce any modification on the drug. In fact, liposome preparation method and their incorporation into hydrogels did not induce any degradation of the betamethasone. As expected, the assay was linear in the concentration range of 3 to 10 µg/mL, with a correlation coefficient higher than 0.9993 and a %RSD ranging between 0.6-2.5%.

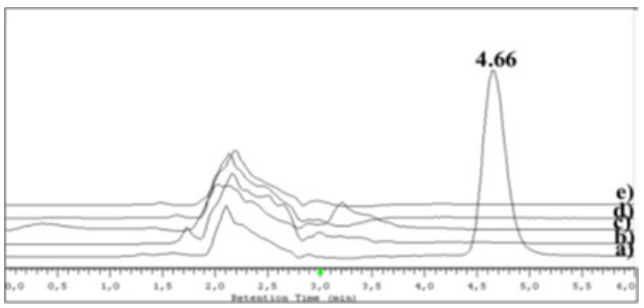

Figure 8: HPLC-DAD chromatograms to demonstrate the selectivity of the method in the presence of hydrogels.

a) Chromatogram obtained for a betamethasone standard solution (10 µg/mL); b) and c) presents the chromatograms obtained for poly(acrylic acid) gel without and with empty liposomes, respectively and in d) and e) are the chromatograms obtained for hydroxypropyl gum guar gel without and with empty liposomes, respectively.

Three QC standard solutions, corresponding to 80%, 100% and 120% of betamethasone in hydrogels, were used to assess accuracy, recovery and precision of the method. In table 2, it is possible to note that accuracy ranged between 98.9-101.0% and 98.8-101.7% and recovery was between 97.7-101.7% and 97.1-99.3% for hydrogels of poly (acrylic acid) and hydroxypropyl gum guar, respectively. Additionally, table 2 shows that the method was precise, taking into account the values of %RSD calculated for intra- and inter day assays.

Hydrogels	Nominal concentration (mg/g)	Accuracy (%)	Recovery (%)	%RSD (inter-day)	%RSD (intra-day)
Poly(acrylic acid)	0.4	98.9	97.7	1.42	1.58
	0.5	99.7	97.8	1.26	1.35
	0.6	101	101.7	1.18	2.49
Hydroxypropyl gum guar	0.4	98.8	97.9	1.58	2.11
	0.5	101.7	99.3	1.25	1.41
	0.6	99.9	97.1	1.45	1.97

Table 2: Results of accuracy, recovery and precision (intra and inter-day) obtained for the HPLC-DAD determinations in hydrogels of poly(acrylic acid) and hydroxypropyl gum guar.

Discussion

The main purpose of this work was the development of topical formulations based in hydrogels presenting in their composition bethametasone-loaded liposomes to avoid the systemic absorption of this corticosteroid, thus contributing to the improvement of betamethasone therapeutic index.

The characterization of the LUVs demonstrated that they are homogeneous in terms of size (PDI < 0.1), presenting a mean diameter around 155 nm. These data were corroborated by STEM analyses, which also allowed concluding that the liposomes were morphologically spherical. To confirm this assumption, it was calculated the sphericity factor [37]. As the values of the sphericity factors were less than 0.15, it is possible to conclude that LUVs are spherical. Besides other factors, this shape presents advantages in the field of drug controlled release [38]. Additionally, LUVs showed a significant positive surface charge (+19.7±2.0 mV), related to the protonation of EPC phosphate group at an acidic medium (water pH ≈ 5.5) [39]. The zeta-potential values gives an indication of the surface charge of liposomes, allowing the prediction of the physical stability of the colloidal dispersion [40-42]. In general, particle aggregation of charged particles is less likely to occur, due to electric repulsion. Taking into account the zeta-potential results, it is possible to conclude that the liposomal suspension is relatively stable, once that its value is in the range of ±10-20 mV [43]. This was corroborated with the stability assays. Indeed, liposomes can suffer destabilization and aggregation over time, particularly in aqueous dispersions. Therefore, particle size was monitored for two months, after LUVs preparation, in order to evaluate their physical stability. The zeta-potential was also determined, since this parameter can change over time, due to chemical and/or physical structural alterations. The stability studies demonstrated that LUVs maintained their properties in terms of size, PDI and surface charge during storage. As previously referred, the positive zeta-potential value obtained for this drug-carrier can produce repulsive interactions between the lipid vesicles dispersed in the water, leading to long-lasting stability [44].

The increase of phospholipid concentration promoted an increment of anti-inflammatory entrapment, since betamethasone, due to its hydrophobic nature, is mainly located between phospholipids in the lipid bilayer. In fact, betamethasone is associated with the lipid bilayer, as demonstrated resorting to differential scanning calorimetry analysis [5]. On the other hand, the inclusion of the betamethasone into the lipid bilayer versus the aqueous compartment through, for example, the use of cyclodextrin complexes, seems to be the best choice as it gives rise to higher entrapment efficiencies [6]. The developed and validated adequate HPLC-DAD method presented specificity, linearity, accuracy, recovery and precision values that allowed performing the determination of the betamethasone concentrations when entrapped or not into the liposomes. Indeed, the method validation was performed to demonstrate that the HPLC-DAD method used in this work accomplishes all the requirements of the analytical applications, ensuring the reliability of the results.

The characterization of the hydrogels based on poly(acrylic acid) or hydroxypropyl gum guar showed that they present adequate pH values for topical formulations, since that the skin pH is ideally slightly acidic [25]. Additionally, the determination of the rheological behavior of the two hydrogels demonstrated that the poly(acrylic acid)

gel base is more fluid than the hydroxypropyl gum guar hydrogel, which, consequently, can be suited to the treatment of more extensive areas. Moreover, the addition of liposomes increased the hydrogels viscosity, particularly of the poly(acrylic acid) gel. However, the significant difference of viscosity of the two hydrogels was maintained at the end. The assessment of stability and drug content (quantified by the validated HPLC-DAD method) demonstrated that these formulations preserved their properties and presented a drug concentration similar to the marketed betamethasone topical medications. In this sense, these formulations have potential to promote a prolonged and localized therapeutic effect, allowed and coadjuvated by the presence of liposomes of EPC, which presents antioxidant properties [28,29].

Conclusion

This work exploited the potential of liposomes as carriers for topical delivery of betamethasone. The use of liposomes may represent a promising approach in the treatment of inflammatory skin diseases, by increasing betamethasone benefit-risk ratio, which is crucial for this drug presenting severest side effects. In this sense, two hydrogel formulations containing betamethasone-loaded liposomes were developed and characterized, which demonstrated compatible properties with topical application, regarding pH values, physical stability, drug content and rheological behavior. Additionally, the higher fluidity displayed by the poly (acrylic acid) gel can be useful to treat extensive areas. By contrast, the hydroxypropyl gum guar gel, more viscous, may be used in the treatment of smaller areas without considerable risk to drain to healthy skin.

As conclusion, it is possible to state that the hydrogels formulations developed may have potential to control and heal topical inflammatory conditions, as psoriasis and eczema.

Acknowledgment

Helena Ferreira acknowledges Human Potential Operational Programme/European Social Fund (POPH/FSE) for co-financing and Portuguese Foundation for Science and Technology (FCT) for the fellowship SFRH/BPD/38939/2007. The authors acknowledge Cooperativa de Ensino Superior Politécnico Universitário (CESPU) for financial support (04-GCQF-CICS-2011N). This work was also partially supported through national funds provided by FCT/MCTES - Foundation for Science and Technology from the Minister of Science, Technology and Higher Education (PIDDAC) and European Regional Development Fund (ERDF) through the COMPETE - Programa Operacional Factores de Competitividade-(POFC) program, under the Strategic Funding UID/Multi/04423/2013, in the framework of the program PT2020.

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