




Development of Sustained Release Formulations Based on Lipid-Liquid Crystal to Control the Release of Deoxycholate: *In Vitro* and *In Vivo* Assessment

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Received: 24 July 2023 / Accepted: 12 October 2023 / Published online: 10 November 2023
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Abstract

Subcutaneous injections of phosphatidylcholine (PC), sodium deoxycholate (NADC), and a mixture of them were found to be an effective option for treating cellulite. However, it is noteworthy that the injection of NADC may result in inflammation as well as necrosis in the injection area. The preparation of a sustained release formulation based on lipid-liquid crystal that controls the release of NADC could be a potential solution to address the issue of inflammation and necrosis at the site of injection. To present a practical and validated approach for accurately determining the concentration of NADC in LLC formulations, spectrofluorimetry was used based on the International Council for Harmonization (ICH) Q2 guidelines. Based on the validation results, the fluorometric technique has been confirmed as a reliable, efficient, and economical analytical method for quantifying NADC concentrations. The method demonstrated favorable attributes of linearity, precision, and accuracy, with an r^2 value of 0.999. Furthermore, it exhibited excellent interday and intraday repeatability, with RSD values below 4%. The recovery percentages ranged from 97 to 100%, indicating the method's ability to accurately measure NADC concentrations. The subcutaneous injection of the LLC-NADC demonstrated a reduction in inflammation and tissue necrosis in skin tissue, along with an increase in fat lysis within 30 days, when compared to the administration of only NADC solution. Moreover, the histopathological assessment confirmed that the use of the LLC formulation did not result in any detrimental side effects for kidney or heart tissue.

Keywords lipid-liquid crystal · sodium deoxycholate · spectrofluorometric method · sustained release · validation

Introduction

Recently, obesity and cellulite have become serious medical problems affecting both beauty and fitness, leading to

significant morbidity and mortality [1]. Cellulite is a condition that most women experience in their buttocks and posterior thighs after puberty and is caused by a change in the skin's topography. Cellulite is also referred to as gynoid lipodystrophy, adipose edematous, dermopanniculosis deformans, and nodular liposclerosis [2]. Despite its high prevalence (80–90%), little epidemiologic data have been published on its incidence, prevalence, and associated factors. In males, cellulite is rare and is caused by androgen deficiency secondary to castration, estrogen therapy for prostate cancer, hypogonadism, and Klinefelter's syndrome [2]. Several

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procedures have been claimed to be effective at reducing cellulite, including topical therapy, injectable medications (such as chemical septolysis with collagenase), sound wave therapy, light therapy, non-invasive external lasers, and radio frequency (RF) therapy [3, 4]. However, because of their unexpected effects and the possibility of short-term improvements even with multiple therapy sessions, the utilization of these treatments has not been as extensive as warranted.

One of the compounds proposed for the treatment of cellulite is the combination of phosphatidylcholine (PC) and sodium deoxycholate (NADC) [5]. NADC is frequently employed as a biological detergent to lyse cells and dissolve cellular and membrane components [6]. In April 2015, deoxycholic acid was authorized by the FDA for the treatment of submental fat to enhance the esthetic appearance and minimize facial fullness or convexity [7]. It is the first treatment available for submental fat removal, making it a less aggressive and safer alternative to surgical techniques [8].

Moreover, PC is the primary active substance of a commercially available injection lipolysis agent. Its subcutaneous injection is an off-label use to reduce local adiposities [9]. PC solubilized in deoxycholate has been suggested to help people lose weight by injecting it subcutaneously. Several studies have recently shown that this procedure may also be used to remove undesirable adipose tissue from various parts of the body, including the thighs, belly, neck, buttocks, and arms [10–12]. Deoxycholate increases the entry of PC into adipocytes; for this reason, deoxycholate is used in conjunction with PC [13, 14]. In a similar study, the clinical effectiveness of NADC along with PC was investigated. The final judgment was based on metric circumferential measurements and photographic and ultrasonographic evaluations throughout the study. In the presence of PC/NADC, the metric reduction value is 6.46% and the ultrasonographic reduction value is 36.87% [15].

Since the subcutaneous injection of NADC causes tissue necrosis and inflammation in the injection area, slow-release formulations can provide great help in solving this problem. Lipid-liquid crystal (LLC)-based drug delivery systems have received a lot of interest as drug carriers because of their distinctive microstructures, biocompatibility, thermodynamic stability, and effective sustained release profiles [16]. LLCs contain a variety of lipids, stabilizers, and solvents that transform into sustained release gels when contacted with aqueous media [17]. Several amphiphilic substances including monoolein, monovaccenin, and oleyl glycerate can absorb water from aqueous media or the injection site [18, 19]. Depending on the water amount entering LLC, lyotropic LLC structure is classified into reverse hexagonal (HII), lamellar ($L\alpha$), and bicontinuous cubic (Q2) [20]. LLC was used as a sustained release formulation of NADC due to the presence of PC, which is used both as a liquid crystal base and as a lytic agent for lipid tissues. Various analytical methods have been exploited to assess the amounts of NADC such as ultraviolet-visible (UV)

spectrophotometry, HPLC Refractive Index Detectors (HPLC-RI), and tandem mass spectrometry (HPLC-MS/MS) [21, 22]. While UV is a straightforward, quick, and simple-to-use technology, it has sensitivity and reliability limits [23]. HPLC methods are rapid and reproducible, and show high sensitivity but have disadvantages like high cost and time consumption, and require technical capability and skill to handle the system [24]. The fluorimetric method was designed to determine the quantity of NADC because this method is an effective, quick, repeatable, and low-cost method for analyzing substances that include fluorescent molecules [25]. Previous research has shown that this approach for determining the quantity of NADC is repeatable, quick, and sensitive [26].

Improving and validating the analytical approach are crucial in the discovery, development, and manufacturing of drugs. The primary objective of an analytical method is to obtain consistent, accurate, and reliable information. Validated methods play a vital role in achieving this objective, ensuring the integrity and quality of the obtained results [27]. These validation methods are also mandated by various regulations and quality standards that impact laboratories [28]. Therefore, validation is necessary for any new or modified method to ensure its capability to provide consistent and reliable results when utilized by different operators, using the same or different instruments in various laboratories. The aim of this study was to validate a spectrofluorometric technique in accordance with ICH guidelines for the determination of NADC quantity in a developed sustained release liquid crystals formulation. Additionally, the *in vitro* properties and *in vivo* performance in the lysis of lipid tissues in rabbits were evaluated following subcutaneous injection.

Materials and Methods

Materials

Phospholipid S100 was acquired from Lipoid (GmbH, Germany). Glycerol dioleate (GDO) was purchased from Dinfen Chemical Technology Company (Qingdao, China). NADC bulk powder was acquired from Sigma-Aldrich (St. Louis, MO). Absolute ethanol and other chemicals and reagents were acquired from Merck (Darmstadt, Germany).

Preparation of Standard Solution

A precise amount of NADC powder (1.5 mg) was weighed and dissolved in 10 mL of distilled water. The solution was then vortexed until the NADC was completely dissolved. For standard preparation, this solution was diluted with distilled water to attain a concentration in the range of 11.25 to 130 $\mu\text{g/mL}$.

Preparation of Formulation Based on Liquid Crystal Containing NADC

To prepare the LLC formulation, NADC (100 mg) was first precisely weighed and dissolved in 200 μL of ethanol. The LLC vehicle was then made by mixing PC (350 mg) and GDO (350 mg). The formulation was made by mixing the NADC solution into the LLC vehicle using a bath sonicator for 1 h at 60 $^{\circ}\text{C}$.

Fluorimetric Method

Fluorimetric measurements were conducted using an LS-45 fluorescence spectrophotometer (Perkin-Elmer, UK) with a 1-mm quartz cuvette at 25 $^{\circ}\text{C}$. Excitation and emission slits were both set to 10 nm. A sample scan was performed to validate the peak-emission wavelength of NADC, which was found to be between 400 and 700 nm. The excitation was set at 450 nm, based on NADC's highest absorbance wavelength.

Method Validation Studies

The analytical method was developed and confirmed based on the ICH Q2 (R1) guideline [29]. In the validation studies, linearity and range, specificity, precision, the limit of quantification (LOQ), the limit of detection (LOD), and accuracy were determined.

Linearity and Range

A wide range of concentrations was tested to determine an appropriate range, and seven points ranging from 11.25 to 130 $\mu\text{g}/\text{mL}$ were chosen for further evaluation. Three replicates of each sample were evaluated using a fluorescence spectrophotometer. The calibration curve was developed by plotting the fluorescence intensity of the standard solutions against their concentrations. Linearity was determined via linear regression and validated with the correlation coefficient.

Specificity

Specificity refers to the capability of an analytical method to differentiate between the analyte(s) and other components present in the sample matrix. In this study, the impact of components on the quantification of NADC was evaluated to assess the specificity of the method. This was achieved by calculating the fluorescence intensity of unloaded LLC (liquid crystals). The assessment of empty liquid crystals was performed in triplicate to ensure accurate and reliable measurements of specificity.

Precision

Precision was assessed by analyzing the repeatability of the procedure on 1 day (intraday) and three consecutive days (interday). The fluorescence intensity of the standard solutions was determined and precision was expressed as relative standard deviation (RSD %). Furthermore, six replicates of the highest concentration of a standard solution (130 $\mu\text{g}/\text{mL}$) were measured and the mean, SD, and RSD% were calculated.

Accuracy

The recovery percentage was determined by spiking three specific concentrations of NADC standard solution into empty liquid crystals. The fluorescence intensities of the samples ($n=3$) were then assessed, and concentrations were calculated using the equation provided by the calibration curve. The recovery percentage was calculated using Eq. 1:

$$R\% = (C_{sc}/C_{ss}) \times 100 \quad (1)$$

R is the percentage of recovery, C_{sc} is the NADC concentration ($\mu\text{g}/\text{mL}$) measured in liquid crystal by the fluorometric method, and C_{ss} denotes the concentration ($\mu\text{g}/\text{mL}$) of NADC spiked in the standard solution.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection is the lowest concentration of the sample, which could be identified with reasonable statistical certainty but not basically quantifiable. The lowest concentration of the sample that is measured with appropriate precision and accuracy is called the limit of quantification, which is determined directly from the calibration curve. LOD and LOQ were determined by Eqs. 2 and 3 respectively:

$$\text{LOD} = 3.3\delta/S \quad (2)$$

$$\text{LOQ} = 10\delta/S \quad (3)$$

where δ is the standard deviation (SD) of y -intercepts of the regression line and S is the calibration curve's slope [30].

Characterization of Formulation

Polarized Optical Microscopic (POM)

Polarized optical microscopy (POM) was used to identify the structure of the liquid crystal and the transformation of the oil phase to a gel-like mesophase (liquid crystal) using a Motic BA300 device in BC, Canada. A small amount of a formulation containing GDO/PC/NADC was placed on a glass slide. When a liquid crystalline phase formed, it was

put in a Petri dish containing 10 mL of double-distilled water for 15 min. To prevent air bubbles from forming, the cover glass was carefully put on the glass slide, and silicone grease was used to seal the glass and prevent water from evaporating at room temperature. Each image was captured using cross-polarized conditions [31].

Viscosity

Rheological measurements were conducted at 25°C using a Brookfield viscometer [32]. In brief, 0.3 mL of the LLC formulation was loaded into the Brookfield viscometer, which was equipped with a concentric cylinder spindle (CC3-14). The shear stress was incrementally increased from 0 to 100 Pa, and the corresponding viscosity values were determined. To ensure the reliability of the obtained data, all measurements were conducted in triplicate.

Determination of Water Content

The water content of the LLC was quantified using Karl-Fischer (Metrohm 870 KF Titrino plus, Swiss) titration. Five hundred microliters of the LLC precursor was transferred to 5 mL of PBS at room temperature (pH=7.4), and the amount of absorbed water was determined using a Karl Fischer device (Metrohm 870 KF Titrino plus, Switzerland) and the method described by Miao-Yao *et al.* [31].

In Vitro Release Evaluation

To determine the % cumulative release of NADC from the liquid crystal system, a formulation containing 10.0 mg of NADC was placed in a microtube, and 4.0 mL of PBS buffer was added. Over a period of 1 month, 1 mL of the release medium was collected at predetermined intervals (2, 4, 8, and 24 h and 2, 4, 7, 15, 18, 22, 26, 30, and 35 days) followed by replacing 1 mL of fresh buffer. The amount of NADC in each collected sample was determined using a validated fluorescence spectrophotometer method (Ex: 450, Em: 400–700). The cumulative percentage of NADC released was calculated using the amount of NADC loaded in the LC formulations (Eq. 4).

$$M_n = C_n V_t + \sum C_{(n-1)} V_s \quad (4)$$

M_n cumulative value of sample n ($\mu\text{g/mL}$)

C_n apparent concentration of sample n ($\mu\text{g/mL}$)

V_s volume removed (mL)

V_t total release medium (mL)

$\sum C_{(n-1)}$ total apparent concentrations of samples ($\mu\text{g/mL}$)

In Vitro Liquid Crystal Degradation

The *in vitro* degradation of the lipid-liquid crystal was assessed by measuring the weight loss of the formulation over time. For this purpose, 10 optimal formulations were prepared in deionized water (DW) and incubated at 37°C. On days 1, 2, 3, 5, 7, 14, 21, 28, and 35, the water was discarded from the samples, and the residual gels were kept at -20°C and freeze-dried for 48 h. The degradation percentage was determined using Eq. 5, where W_i is the initial weight before adding the water, and W_t is the final weight of the gel [33].

$$\text{Degradation \%} = \frac{W_i - W_t}{W_i} \times 100 \quad (5)$$

W_i the initial weight

W_t the final weight.

Pharmacodynamics and Histopathology of NADC-LLC in Rabbits

Twelve New Zealand rabbits with an average weight of 2 ± 0.1 kg were obtained from the Pasteur Institute in Tehran, Iran, and were housed under a 12-h dark/light cycle with free access to food and water. All animal experiments were performed in accordance with the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences (MUMS) (Ethical Number: IR.MUMS.PHARMACY.REC.1397.047).

To interpret research findings, the conversion of drug doses from human studies to animal studies involved the application of body surface area (BSA, m^2) [33, 34]. The correlation between drug dosage in humans and animals can be accurately assessed by employing Eq. 6 in the following manner.

$$\text{Animal drug dose (mg/kg)} \times \text{animal } K_m = \text{human drug dose (mg/kg)} \times \text{human } K_m \quad (6)$$

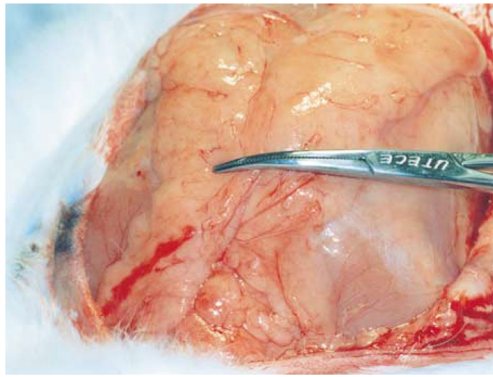


Fig. 1 Rabbit back with the fat location

where K_m is $Weight (kg)/BSA (m^2)$, human K_m for the weight of 60 kg is $37 \text{ kg}/m^2$, and rabbit K_m for weight of 2 kg is $13.3 \text{ kg}/m^2$.

Since the drug dose of the human was considered $2 \text{ mg}/\text{cm}^2$, the rabbit drug dose will be $5.56 \text{ mg}/\text{cm}^2$. Moreover, a volume of 1 mL (drug samples) was injected subcutaneously in the back of the rabbit. The rabbits were divided into four groups ($n=3$) as follows: group I received the LLC formulation containing 100 mg of NADC subcutaneously into the back of the rabbit at a predetermined fat location. Group II received the LLC formulation without NADC. Group III received the NADC solution in ethanol (containing 100 mg of NADC). Group IV served as the control group (no treatment). The injection site and incision on the rabbit's back are shown in Fig. 1 [35]. Finally, the process of fat mass reduction (pharmacodynamics) and any pathological changes at the injection site and in other important tissues were evaluated [36]. After a period of 35 days, during which histopathological examinations and pharmacodynamics studies were conducted, the rabbits were subjected to a 12-h fast. Subsequently, they were euthanized using CO_2 at the conclusion of the evaluations. The injection site was shaved, and the skin tissue at this site was removed ($10 \times 10 \text{ cm}$). Tissue samples from the heart, liver, and kidneys were also obtained. After washing the isolated tissues with normal saline, they were fixed in 10% formalin, paraffinized, and cut into $5\text{-}\mu\text{m}$ -thick sections. Finally, the tissues were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope in the Pathology Department of Ghaem Hospital in Mashhad, Iran.

Results and Discussion

Results of Method Validation

Fluorimetry is a simple, cost-effective, rapid, and reproducible method for estimating a wide range of chemicals. In

this study, the fluorimetry method was validated for NADC determination according to the ICH guideline Q2 [37, 38]. The precision, accuracy, specificity, linearity, LOD, and LOQ of NADC in the LLC formulation were studied for method validation.

Linearity and Range

The calibration curve (Fig. 2) was constructed by plotting fluorescence intensity in arbitrary units (au.) versus NADC standard concentrations. The correlation coefficient was 0.999 over the range of 11.25 to $130 \mu\text{g}/\text{mL}$.

Precision

Precision was quantified by assessing intraday and interday repeatability. The results (Table I) indicated that intraday and interday variability were appropriate, with RSD values of less than 4%, which is within acceptable limits. The repeatability of six $130 \mu\text{g}/\text{mL}$ samples was also tested, with a mean fluorescence intensity of 98.6 ± 0.4 and an RSD value of 0.4% (Table II).

Accuracy

The drug recovery percentage for the standard solutions and LLC formulations was found to be between 97 and 100% ($\text{RSD} < 1\%$), confirming the accuracy of the proposed technique for assessing NADC (Table III).

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) are commonly used parameters to assess the sensitivity of analytical methods. The computed LOD and LOQ

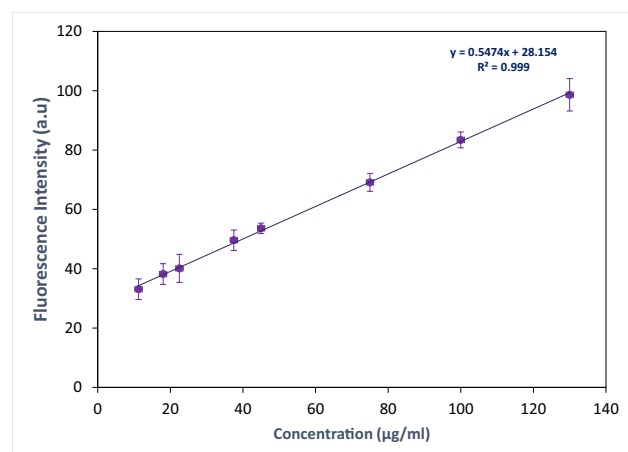


Fig. 2 Calibration curve of NADC fluorescence intensity (au.) at excitation 450 nm and emission 400–700 nm ($n=3$, mean \pm SD)

Table I Interday and Intraday Repeatability for Precision Determination

Amount of NADC $\mu\text{g/mL}$ (theoretical)	Day	Time	Fluorescence intensity \pm SD ^a	RSD ^b %	
11.25	Intraday ($n=3$)	9:00	33.2 \pm 0.8	2.4	
		12:00	31.3 \pm 0.9	2.9	
		15:00	33.6 \pm 0.8	2.5	
	Interday ($n=9$)		32.6 \pm 0.9	3.0	
	18	Intraday ($n=3$)	9:00	38.9 \pm 0.6	1.7
			12:00	37.4 \pm 0.7	1.8
15:00			36.9 \pm 0.6	1.6	
Interday ($n=9$)			38.3 \pm 0.7	2.5	
22.5		Intraday ($n=3$)	9:00	40.1 \pm 0.2	0.6
			12:00	37.9 \pm 0.3	0.6
	15:00		39.5 \pm 0.3	0.9	
	Interday ($n=9$)		40.1 \pm 0.4	0.9	
	37.5	Intraday ($n=3$)	9:00	50.3 \pm 0.6	1.2
			12:00	50.5 \pm 0.8	1.6
15:00			52.2 \pm 0.7	1.3	
Interday ($n=9$)			50.8 \pm 0.7	1.5	
45		Intraday ($n=3$)	9:00	54.3 \pm 0.8	1.6
			12:00	54.6 \pm 0.7	1.3
	15:00		55.3 \pm 0.7	1.4	
	Interday ($n=9$)		54.2 \pm 0.8	1.5	
	75	Intraday ($n=3$)	9:00	69.2 \pm 0.8	1.1
			12:00	70.8 \pm 0.7	0.9
15:00			73.5 \pm 1.1	1.6	
Interday ($n=9$)			71.9 \pm 1.3	1.9	
100		Intraday ($n=3$)	9:00	83.6 \pm 1.2	1.5
			12:00	84.1 \pm 0.7	0.9
	15:00		80.5 \pm 0.9	1.29	
	Interday ($n=9$)		82.9 \pm 0.9	1.1	
	130	Intraday ($n=3$)	9:00	98.0 \pm 0.9	0.91
			12:00	98.6 \pm 1.0	1.0
15:00			99.0 \pm 0.8	0.8	
Interday ($n=9$)			98.2 \pm 1.0	0.9	

^aStandard deviation, ^brelative standard deviation

Table II Repeatability for Precision Evaluation

No	Amount of NADC $\mu\text{g/mL}$	Fluorescence intensity	Mean \pm SD ^a	RSD ^b %
1	130	97.9	98.5 \pm 0.4	0.4
2		98.1		
3		98.6		
4		99		
5		98.3		
6		98.9		

^aStandard deviation, ^brelative standard deviation

Table III The Recovery Percentage of NADC Standard Solution Added to Liquid Crystal for Accuracy Evaluation

Amount of drugs added to empty liquid crystal ($\mu\text{g/mL}$)	Amount of measured ($\mu\text{g/mL}$)	Recovery%	Mean	RSD%
130	126.6	97.3	98.3	1.0
	129.2	99.4		
	127.8	98.3		
75	74.5	99.3	99.2	0.7
	73.9	98.5		
	74.9	99.9		
37.5	37.5	100.0	99.7	0.2
	37.3	99.5		
	37.4	99.7		

^aRelative standard deviation

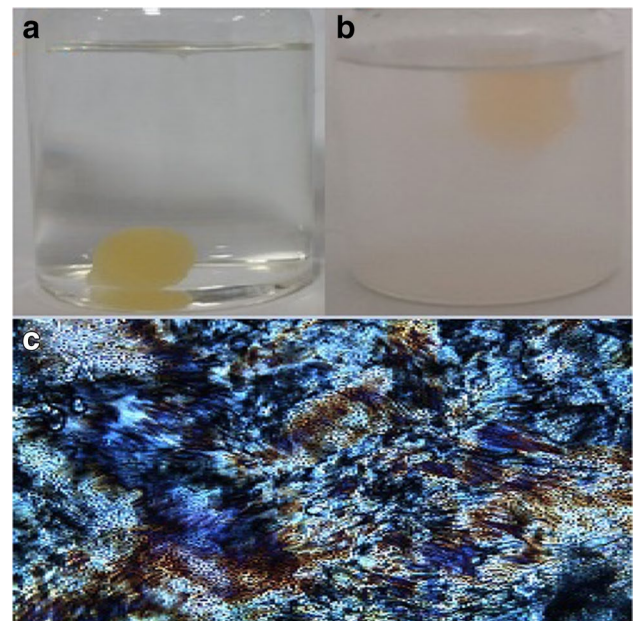


Fig. 3 Gelation of the liquid crystal formulation in phosphate buffer solution immediately after injection (a), after 35 days (b), and polarized optical microscopy of the lipid-liquid crystal gel (c)

were 4.87 $\mu\text{g/mL}$ and 14.78 $\mu\text{g/mL}$, respectively, which demonstrates the selected method is sensitive.

NADC-LLC Formulation

Characterization of Formulation

As shown in Fig. 3a, the formulation immediately turned into a gel-like opaque mesophase upon contact with the aqueous medium of PBS (pH 7.4) at room temperature, forming a spherical and uniform structure. Figure 3b shows the changes in the LLC structure after 35 days. POM was

used to assess the texture structure of LLC and identify the phase, as shown in Fig. 3c. The formula was observed under the supervision of POM and showed the formation of the HII phase. The hexagonal phase is the most common non-lamellar phase formed by amphiphilic molecules mixed with water and has great potential for use as a delivery vehicle for a wide range of materials, from low molecular weight drugs to proteins, peptides, and nucleic acids [39, 40]. Furthermore, this structure is very suitable for the formation of slow-release formulations [41]. The viscosity of the formulation was 530 centipoise (cPs), which is lower than 650 cPs, indicating that the resulting formulation can be injected under the skin [41].

Water Content in Mesophases Using the Karl Fischer

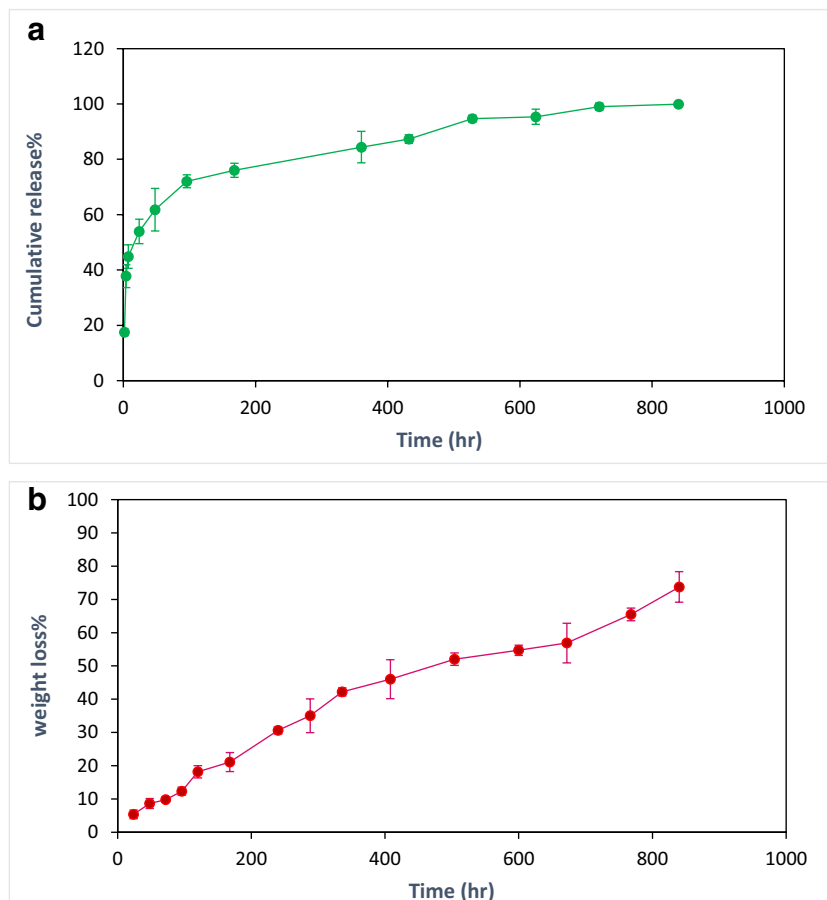
The Karl Fischer test was employed to evaluate the amount of water absorption in the formulation. For the formulation containing 10 mg of NADC, 350 mg of PC, 350 mg of glycerol dioleate, and 20 μL of alcohol, the amount of water absorption was found to be $1.5 \pm 0.2\%$ w/w. In gel form, the water absorption was $23 \pm 2.1\%$ w/w. Ki *et al.* demonstrated a maximum water content of 21% in the sorbitan monooleate (SMO)-LLC, which is necessary for the formation of a water

channel inside the liquid crystal [41]. In another study involving colloidal carriers comprising poloxamers and sorbitol, the amount of water required to develop a hexagonal phase was found to be $29.3 \pm 0.7\%$ w/w [42]. Generally, the water content in different formulations may be attributed to the varying percentages of the ingredients [31].

In Vitro Release and Degradation

The concentrations of NADC released at each time point were determined by using a standard curve, and the percentage of cumulative release was calculated. The cumulative percentage release profile of NADC is shown in Fig. 4a. It was observed that the cumulative release of NADC from the liquid crystals increased from 18.9% on day 1 to 97.8% on day 26, and 100% of drug release was achieved after 35 days. Other studies have reported similar release results for LLC. For example, a 100% release of naltrexone from LLC containing GDO was obtained after 35 days [43], and LLC-GDO exhibited a sustained release pattern for risperidone [19]. The LLC phase structure strongly influences the drug release profile. The sustained release pattern observed was consistent with the POM results, which confirmed the formation of an HII structure for the LLC formulation. This

Fig. 4 Cumulative release curve of NADC from LLC (a) and the degradation profile LLC containing NADC in deionized water (b)



structure provides sustained drug delivery systems [44]. Wadsater *et al.* demonstrated that the release of sodium fluorescein from GDO-PC-water strongly depended on the phase, and release from hexagonal and cubic phases was much slower than from a lamellar phase [45].

The degradation data of LLC are presented in Fig. 4b. The LLC formulation exhibited 70% degradation after 35 days, and slow degradation led to sustained drug release. Moreover, release data fitted with different kinetic models (zero order, first order, Higuchi, and Korsmeyer-Peppas models). The results showed good fitness with Korsmeyer-Peppas with an n value < 0.43 , indicating that the main release mechanism is diffusion [46]. Hence, the release pattern demonstrates a concentration-dependent phenomenon, wherein the active diffusion of the drug occurs due to a significant concentration gradient between the LLC and the surrounding environment. This leads to an initial release of nearly 80% within a short period, followed by a slower release as the concentration gradient decreases.

Changes in Fat Tissue Under the Skin of Rabbits

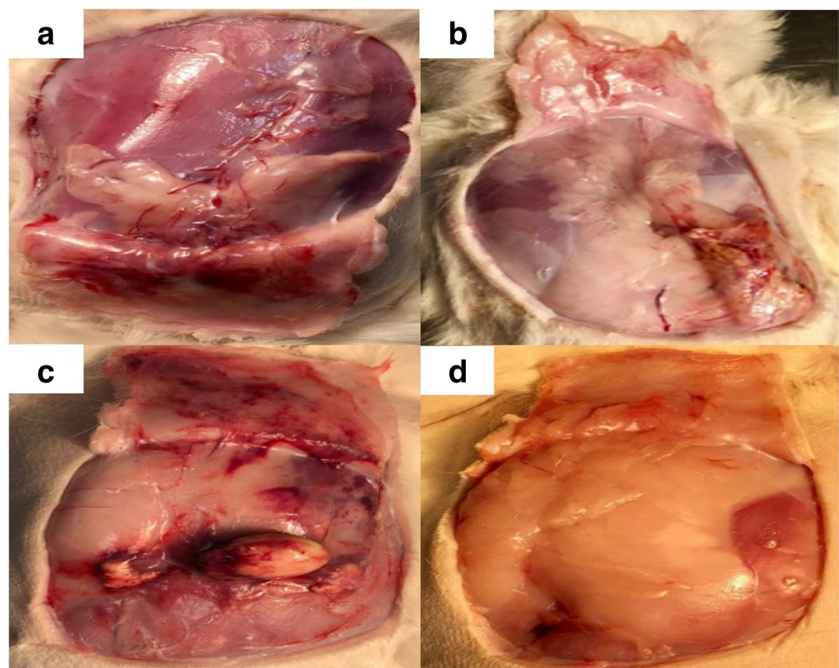
Figure 5 depicts the injection sites of the various treated groups, and it is evident that subcutaneous fat lysis is more prevalent in the formulation containing the drug group than in the control group. A comparison of images from the drug formulation group and the drug alone group revealed that the drug formulation group had more lysis than the drug alone group (Fig. 5). However, in comparison to the control group, there was more subcutaneous fat lysis at the injection site in the drug alone group. When comparing the formulation

group alone to the formulation group with the drug, it was observed that there was more lysis in the formulation group with the NADC than in the formulation group without the NADC. These results indicate that the LLC formulation containing NADC can effectively lyse fat tissue compared to the other groups. This is because NADC helps break down fat cells, while PC emulsifies and transports fats. Therefore, the combination of these compounds promotes the breakdown of more fat and its elimination from the body. Another study also demonstrated that a formulation containing PC and deoxycholate can effectively destroy fat cells at the microscopic level [47].

Pathology

Figure 6 displays the histopathological findings in the skin, heart, kidney, and liver for the four groups studied. The skin tissue in the control group (group IV) was entirely healthy and normal, but slight inflammation and subcutaneous fat necrosis were observed in the LLC formulation without drugs (group II) and the NADC-LLC formulation (group I). These results were expected due to the injection of the needle tip and mechanical damage to the fat tissue, as well as the fact that PC and deoxycholate are known to lyse fat tissue [48]. Furthermore, the solution of NADC in ethanol (group III) exhibited more inflammation in the injection area than the NADC-LLC formulation (group I) since the LLC formulation can help slow down the release of the NADC, reducing the risk of inflammation. In another study conducted by Ki *et al.*, histopathological results indicated that

Fig. 5 Images showing the injection sites of **a** the LLC formulation containing NADC, **b** the LLC formulation group without the NADC, **c** a solution of NADC in ethanol, and **d** the control group



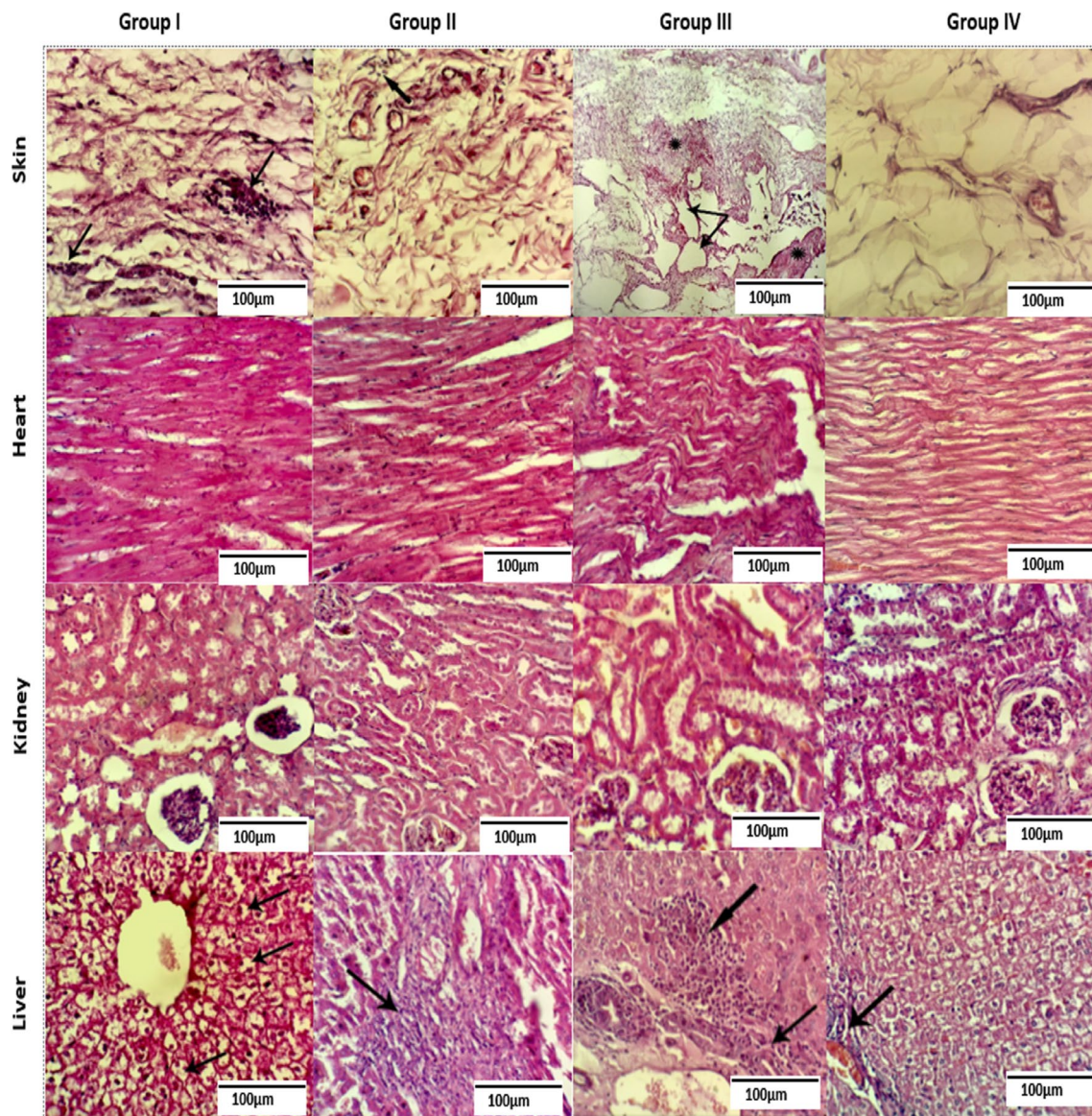


Fig. 6 Histopathology of skin, heart, kidney, and liver. Group I received the slow-release formulation of NADC, group II received the slow-release formulation without drugs, group III received NADC in

ethanol, and group IV was the control. H&E staining was performed at a magnification of $\times 400$

sustained release of drug-loaded LLC prepared by sorbitan monooleate had no considerable inflammation or tissue abnormalities [41].

The cardiac tissue myocytes showed no necrosis, hemorrhage, or infiltration of inflammatory cells in the LLC formulation without drugs (group II) and the NADC-LLC formulation (group I). The glomeruli and renal tubules of the kidneys appeared healthy, with no degeneration, inflammation, bleeding, or necrosis observed in both the LLC formulation without drugs (group II) and the NADC-LLC formulation (group I). This study confirmed similar results from previous studies demonstrating that animals treated with

sustained release of drug-loaded LLC had no side effects on tissue pathology [19, 43]. Another study performed by Liang *et al.* showed that the group receiving drug-loaded LLC exhibited similar tissue pathology to the control group, and no tissue damage was observed [49].

The liver structure for NADC in ethanol (group III) and NADC-LLC (group I) showed slightly mononuclear inflammatory cells (mainly lymphocytes) infiltrating the portal triad associated with periportal hepatocellular necrosis. The reason for this response was found in the NADC structure, which indicated that increasing deoxycholic acid levels increased enterohepatic circulation. These events resulted in

DNA damage and cellular senescence in liver stellate cells, which secreted various tumor-promoting and inflammatory factors [50].

Conclusion

Cellulite, a harmless skin condition that causes the skin to look dimpled, is a common concern, especially among women. One of the effective cellulite treatments is the injection of a mixture of PC and NADC into the subcutaneous fat layer, as this combination has been shown to effectively reduce localized fat deposits. However, the need for an efficient, quick, repeatable, and cost-effective method to quantify NADC remains apparent. To address this requirement, the present study successfully developed and validated a spectrofluorometric technique in accordance with the ICH guidelines. The spectrofluorometric technique demonstrated desirable linearity ($r^2 = 0.999$) and exhibited low limits of detection (LOD) and quantification (LOQ), indicating the method's appropriate sensitivity. Furthermore, the developed method proved to be accurate, specific, and precise with high interday and intraday repeatability. For formulation development, the use of an LLC can offer several advantages, such as sustained release of the drug, biocompatibility, non-toxicity, and stability. Consequently, the LLC formulation of NADC causes a more controlled release of NADC over time, which can reduce the risk of tissue damage, inflammation, and other adverse events. Furthermore, subcutaneous injection of LLC-NADC provides more fat tissue lysis and lower tissue inflammation compared to NADC injection because of the *in vitro* and *in vivo* slow release of NADC for 30 days. Therefore, the use of LLC could represent a promising approach to reduce localized fat deposits with minimal side effects.

Author Contribution Maryam Kouhjani: conceptualization, methodology, original draft, writing and critical revision; Arezoo Saberi: methodology, original draft, writing and critical revision; Farzin Hadizadeh: writing and revision. Elham Khodaverdi: revised the manuscript. Malihe Karimi: original draft, writing, data analysis. Elaheh Gholizadeh: data analysis. Hossein Kamali: supervision, design of study, validation, writing and critical revision. Ali Nokhodchi: supervision, design of study, validation, writing and critical revision. All authors read and approved the final manuscript.

Funding This study was financially supported by a grant from Mashhad University of Medical Sciences (grant no. 970090).

Data Availability Data are available on request.

Declarations

Ethics Approval All of the experiments were ethically approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences and carried out according to

their recommendations under registration number (IR.MUMS.PHARMACY.REC.1397.047). The study was also performed in compliance with the ARRIVE guidelines [51].

Conflict of Interest The authors declare no competing interests.

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