



Formulation and characterization of chitosan nanoparticles to enhance transdermal delivery of cinnarizine.

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ABSTRACT

Cinnarizine (CN) is an antihistaminic drug that is commonly used for the treatment of chronic conditions for the reduction of cerebral blood flow such as vertigo. However, CN is poorly water soluble with erratic absorption and low bioavailability. Chitosan is a biodegradable natural polymer with approved skin permeation enhancement properties; therefore, the formulation of CN-loaded chitosan nanoparticles could enhance the skin permeability of CN. In this work, for the first time, CN-loaded chitosan nanoparticles were prepared (using the nanoprecipitation method) and characterized. Additionally, the described nanoparticles were subjected to *in vitro* release and ex vivo permeation studies. Dynamic light scattering (DLS) characterization showed that the method was successful in the production of small (down to 12.4 nm), positive, and relatively homogenous or monodispersed nanoparticles. The highest entrapment efficiency (EE) of CN in the chitosan nanoparticles was $62 \pm 2.7\%$. Further, the release study showed that chitosan nanoparticles can effectively reduce the burst release and create a sustained release pattern of CN. The ex vivo permeation study, using rat skin as a model, showed that chitosan nanoparticles improved the CN permeability three times compared to the control. In conclusion, chitosan used as a nanocarrier enhances drug penetration in to the skin which may lead to the formulation of a transdermal formulation of CN

KEY WORDS: Cinnarizine, chitosan, nanoparticles, transdermal delivery, permeability

INTRODUCTION

Nanoparticles (NPs) have been developed as a promising approach to enhance skin penetration of drugs, allowing effective transdermal drug delivery (1,2). Different mechanisms are standing for the enhanced skin penetrating action of NPs, including enhanced drug solubility, protection the drug from degradation, controlled drug release, and tunable

surface properties that may allow surface adhesion of the NPs and drug permeation through the skin (3, 4). Recently, several reports have indicated that electrostatic interaction between NPs and the skin could have an impact on skin penetration of drugs. Skin has a negatively charged structure, therefore positively charged particles can penetrate deeper compared to negatively charged particles due to electrostatic interaction (5).

CN has histamine H1-receptor antagonist and calcium channel blocking activities. CN is known to enhance

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cerebral blood flow, therefore it is widely used in the treatment of cerebral atherosclerosis and posttraumatic cerebral symptoms. In addition, it is used for the treatment of vestibular vertigo and motion sickness. However, CN is poorly water soluble with a low and erratic oral bioavailability (6). Furthermore, CN has a short half-life (4-6 hours) and frequent dosing is required in short term use. Therefore, transdermal delivery of CN could have several advantages compared to oral administration, including sustained delivery, enhanced bioavailability, and reduce dosage frequency, leading to improve patient compliance.

Chitosan is a natural aminopolysaccharide that is derived from chitin (7). Chitosan has been used to design a wide range of polymeric nanoparticle-based drug delivery systems for its biodegradable, biocompatible and bioadhesive properties. In addition, chitosan nanoparticles have attracted many researchers to develop transdermal delivery systems due to their ideal permeation enhancing properties such as bioadhesion, positive charge, and tunable mechanical properties (1). In 2015, Abyoi *et. al.* used chitosan to prepare ibuprofen-chitosan nanocomposites which were successfully used to enhance skin penetration of ibuprofen (8). In another study, repaglinide-loaded nanoparticles were prepared using different polymers including chitosan, and their capacity to enhance the skin permeability of repaglinide was proven (9). Recently, Chitosan was also effectively used to prepare tacrolimus-loaded nanoparticles to enhance skin penetration and deposition of tacrolimus (10). Most of the chitosan nanoparticles were formulated using the ionic gelation method, however, nanoprecipitation method can produce smaller particle sizes and is less expensive.

The aim of this work was to prepare and characterize CN-loaded chitosan nanoparticles and investigate their capacity to enhance the skin permeation of CN. As far as is known, this is currently the first study investigating the permeation enhancing effect of chitosan nanoparticles on CN. The results showed an effective transdermal delivery system of CN with improved bioavailability and reduced side effects likely improving patient compliance.

MATERIALS AND METHODS

Materials

CN pure powder was generously received from Awa Medica company (Erbil, Iraq). Chitosan and Tween 80 were purchased from Hangzhou Hyper Chemicals, China. Absolute ethanol was from Scharlab S.L., Spain. All other ingredients used were of analytical grade.

Methods

Preparation of cinnarizine-loaded chitosan nanoparticles

Nanoparticles of CN were prepared by the nanoprecipitation method following the method described elsewhere with some modifications (11, 12). Briefly, the required quantity of CN was dissolved in ethanol and the required quantities of chitosan were dissolved in 1% (v/v) acetic acid. Thereafter, both solutions were mixed together to form the solvent phase (acidic phase, pH 3). The antisolvent phase was deionized using water containing 1% tween 80 (stabilizer) and its pH was adjusted to 8 using sodium hydroxide solution (basic phase). Thereafter, the solvent phase was dropped wise into the antisolvent phase under continuous stirring at 1000 RPM. The procedure included the investigation of different drug-to-polymer ratios, stabilizer concentrations, and solvent-to-antisolvent ratios and the exact components of each formula were detailed in Table 1.

Dynamic light scattering

Particles size and polydispersity index (PDI) of the freshly prepared nanoparticle formulae were measured using Dynamic Light Scattering (DLS) technique by particle size analyzer (lite sizer 500, Anton Paar, Austria) following a suitable dilution with distilled water, at 25°C (13).

Zeta-potential

The charge of the CN-loaded chitosan nanoparticle was measured using Zetazizer 3000Hs. Samples from recently prepared formulations were diluted with

Table 1 Composition of the CN-loaded chitosan nanoparticles formulation

CODE	DRUG: POLYMER RATIO	CN (mg)	CHITOSAN (mg)	ACETIC ACID 1%: ETHANOL (1: 1) (mL)	TWEEN 80 SOLUTION (pH 8)	SOLVENT: ANTISOLVENT
F1	1:0.2	10	2	5	1%	1:4
F2	1:0.4	10	4	5	1%	1:4
F3	1:0.6	10	6	5	1%	1:4
F4	1:1	10	10	5	1%	1:4
F5	1:0.4	10	4	5	1%	1:8
F6	1:0.4	10	4	5	1%	1:16
F7	1:0.4	10	4	5	2%	1:4
F8	1:0.4	10	4	5	0.5%	1:4

distilled water and placed into an electrophoretic cell, the potential of 150 mV was set.

Entrapment efficiency

The amount of CN entrapped in the chitosan nanoparticles, the entrapment efficiency (EE), was quantified using the indirect method. The nanoparticle dispersion (1.5 mL) was placed in an Eppendorf tube and centrifuged twice at 16000 RPM for 30 minutes each time. The supernatant was collected and the amount of the unbound CN was quantified spectrophotometrically at λ_{max} 252 nm. The calibration curve of CN in distilled water was established with a correlation coefficient (R2) of 0.992. The EE was measured using Equation 1.

$$\text{EE (\%)} = \frac{\text{Total Amount of Drug} - \text{Amount of Drug in Supernatant}}{\text{Total Amount of Drug}} \times 100 \quad \text{Eq. 1}$$

Scanning electron microscope

The morphology of the CN-loaded chitosan nanoparticles was observed using a Scanning Electron Microscope (SEM). A small quantity of the lyophilized sample was mounted on a metal stub, the stub was then coated with gold and the SEM microscope (EVO 10 ZEISS GERMANY) was used to investigate the nanoparticles.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR)

spectrums of pure CN, chitosan, and tween 80 and their physical mixture were observed using FTIR spectroscopy (Bruker, Germany) in the range of 4000-400 cm^{-1} . The samples were prepared as KBr pellets by compressing at 6 tons/ nm^2 . The wavelength range was selected between 400 and 4000 cm^{-1} .

In vitro release study

To investigate the release profile of CN from the chitosan, three formulations (F2, F6 and F7) were selected and an aqueous drug solution was used as a control. The experiment was carried out using a USP type II dissolution apparatus using the inverted tube approach. Wherein, an aliquot (5 mL) of the samples or the control was placed in a glass test tube and the tube was then sealed using a dialysis membrane (MWCO 8000-14000 Daltons) and fixed to the paddle of the dissolution apparatus so that the membrane surface was immersed in the release medium (inverted tube). The release medium was 200 mL of phosphate saline buffer (pH 7.4). At different time points (0.15, 0.5, 1, 2, 3, 4, 5, 8, 24 and 48 hours), an aliquot of 5 mL of the release medium was withdrawn and replaced with fresh buffer as soon as possible (14). The CN concentration of the samples and the control was measured spectrophotometrically at 252 nm using an established calibration curve. The control was an aqueous solution of CN with an equivalent concentration to that of the formulations. The control was prepared by dissolving 10 mg of the pure CN powder in 5 mL of solvent

phase (1:1 acetic acid 1%: ethanol) which was then diluted with distilled water at a ratio of 1:4, so that the final concentration of CN in the control was 0.4 mg / mL. The experiment was performed in three replicates.

Ex vivo permeation study

The *ex vivo* permeation study was performed using an abdominal skin of a rat. Animal handling was licensed by the Institutional Animal Care and Use Committee at the College of Veterinary Medicine, University of Mosul (Ref: UM.VET.2022.028). Male Albino rats weighing 160-200 grams were obtained from the animal house of the College of Veterinary Medicine, University of Mosul. The abdominal area of the rat was carefully shaved using an electrical clipper removing all hair. Thereafter, the animals were sacrificed and the skin was excised under general anaesthesia. The skin was either used fresh or stored at -20°C to be used the next day (15). The skin was then fixed on a 10 mL plastic tube (cross-sectional area was 3 cm²) and sealed tightly using rubber bandage, wherein the epidermis layer (outer surface of the skin) faced the donor compartment and the dermis layer faced the receptor compartment. The experiments were performed in the dissolution apparatus using the inverted tube procedure. The tubes were fixed in the paddle of the dissolution apparatus (type II) in an inverted way so that the tube end which is covered by the skin is immersed in the dissolution medium. The temperature was set at 37°C, rotation was 100 RPM and 200 mL of phosphate saline buffer (pH 7.4) was used as dissolution medium. Samples (5 mL) of each formula and control was loaded into the tube (donor compartment). Aliquots (5 mL) were drawn at different time points 0.15, 0.5, 1, 2, 3, 4, 5, 8, 12 and 24 hours and replaced with fresh buffer as soon as possible. The samples were filtered using a 0.45 µm pore filter (16) and analysed using a spectrophotometer at 252 nm to measure the concentration of CN. An aqueous drug solution having an equivalent concentration of CN to the formula was used as a control (17).

Calculations of the permeability parameters

Permeability parameters, including steady state flux

(J_{ss}), permeability coefficient (K_p), enhancement ratio (ER) and the total permeated amount (Q), were calculated. J_{ss} was calculated from the slope of the straight line of the graph between the cumulated permeated amount of per surface area (cm²) and time in hours. J_{ss} and the permeability coefficient (K_p) were calculated by using Equations 2 and 3 (18).

$$J_{ss} = \frac{\text{Slope of Curve}}{\text{Surface area of membrane}} \quad \text{Eq. 2}$$

$$K_p = \frac{J_{ss}}{C_d} \quad \text{Eq. 3}$$

Where, C_d is the drug's initial concentration in the donor compartment.

The enhancement ratio (ER) can further be calculated using Equation 4 (19, 20).

$$ER = \frac{J_{ss \text{ of test}}}{J_{ss \text{ of control}}} \quad \text{Eq. 4}$$

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was carried out using GraphPad Prism 9 software. Data sets were tested for statistical significance using one-way ANOVA with Tukey's multiple comparisons test when it is applicable. Results were considered statistically significant if P < 0.05 and the significance levels were *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

RESULTS

Hydrodynamic diameter of cinnarizine-loaded chitosan nanoparticles

To characterize the CN-loaded chitosan nanoparticle particle size, polydispersity index and surface charge DLS was used. The detailed measurement values are shown in Table 2. The results show a statistically significant increase in particle size with increasing polymer concentration, where the hydrodynamic diameter increased from 14.5±0.3 nm (F1) to 24.6±1.5 nm and 21±3.42 nm (F2 and F3, respectively). However, increasing the chitosan concentration further (F4) the

particle size significantly decreased again to 15.8 ± 0.7 nm, suggesting a chitosan concentration-dependent increase in NPs diameter with a threshold.

To further optimize the nanoparticles, the solvent:antisolvent ratio was modified. When this ratio was changed from 1:4 in F2 to 1:8 and 1:16 (F5 and F6, respectively), the particle size decreased to 14.8 ± 0.66 nm (F5) and 12.8 ± 0.33 nm (F6) compared to 24.6 ± 1.5 nm (F2), suggesting a reduction in particle size with the increase in the antisolvent volume. Moreover, the particle size of the chitosan nanoparticles was slightly reduced when reducing the concentration of tween 80 (stabiliser). The hydrodynamic diameter of the particles in F8 (0.5% tween 80) is 29 ± 4.7 nm compared to F2 (1% Tween 80) and F7 (2% tween 80) where the diameters of the particles are 24.6 ± 1.5 and 18.4 ± 1.6 nm, respectively. Regarding the PDI values, the results show that, except for F7 where PDI is high, all other formulas have PDI value in the range between 0.14 ± 0.03 and 0.5 ± 0.05 , suggesting a monodispersed nanoparticle size.

Zeta-potential

Table 2 shows the zeta-potential values of the CN-loaded chitosan nanoparticles. The average zeta-potential of the described nanoparticles was ranging between 18.5 ± 2.4 mV and 29.5 ± 2.4 mV. The current data showed that increasing the concentration of chitosan caused a statistically significant increase in

the zeta-potential values. However, increasing the antisolvent volume and stabilizer concentration slightly reduced the zeta-potential values as shown in Table 2. A high zeta-potential value creates an electrical repulsion between particles and stabilises the nanodispersions.

Entrapment efficiency

The entrapment efficiency (EE) of CN within the chitosan nanoparticles was characterized using the indirect method. In general, EE values of the described formulations were within the range of 36.6 ± 3 % (F7) and 62 ± 2.7 % (F6) (Figure 1). The results show no statistically significant change in EE in response to increase chitosan concentration (Figure 1A). However, the EE significantly increased with increasing the antisolvent volume (F6) and decreased with increasing the tween 80 concentration (F7), as shown in Figure 1 (A) and 1 (B), respectively, suggesting an important influence of solvent:antisolvent ratio and stabilizer concentration on the EE of chitosan nanoparticles.

Fourier Transform Infrared Spectroscopy

The Fourier Transform Infrared Spectroscopy (FTIR) spectrum of the pure CN shows characteristic peaks at 2949.96 cm^{-1} (C-H aromatic, alkene, mono-substituted), 2869.99 cm^{-1} (C-H stretch, aliphatic, alkane), 1593.7 cm^{-1} (C=C aromatic stretching), 1489.32 cm^{-1} and 1446.68 cm^{-1} (CH₂, alkane), 1133.46 cm^{-1} (C-N stretching) and 995.61 cm^{-1} and 964.86 cm^{-1} (C-H aromatic, alkene), as shown in Figure 2 (A). Figure 2

Table 2 DLS and zeta-potential characterization of the CN-loaded chitosan nanoparticles

CODE	HYDRODYNAMIC DIAMETER (nm)	ZETA-POTENTIAL (mV)	POLYDISPERSITY INDEX (PDI)
F1	14.5 ± 0.3	18.5 ± 2.4	0.166 ± 0.02
F2	$24.6 \pm 1.5^*$	26 ± 1.8	0.39 ± 0.04
F3	$21 \pm 3.4^*$	27.4 ± 1.4	0.48 ± 0.18
F4	$15.8 \pm 0.7^\circ$	29.5 ± 2.4	0.5 ± 0.05
F5	$14.8 \pm 0.66^\circ$	23.5 ± 4.4	0.24 ± 0.06
F6	$12.8 \pm 0.33^\circ$	22.7 ± 2.4	0.14 ± 0.03
F7	$18.4 \pm 1.6^\circ$	22.2 ± 3.1	0.68 ± 0.17
F8	$29 \pm 4.7^\circ$	26.5 ± 0.05	0.32 ± 0.09

Values are mean $\pm$ SD. N=3. * = Statistically significant from F1, ° = Statistically significant from F2, P<0.05.

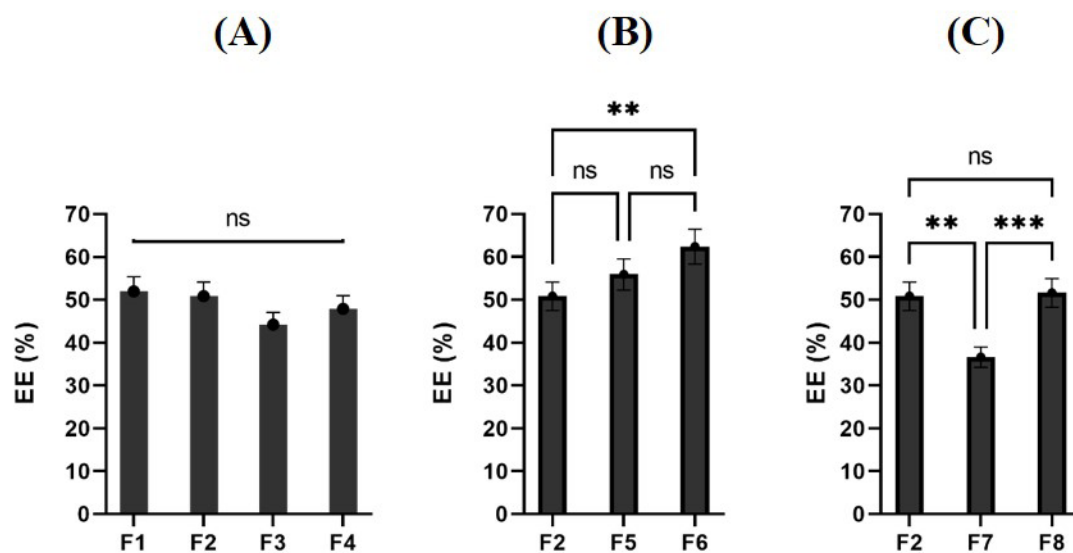


Figure 1 EE of the CN-loaded chitosan nanoparticles. Values are mean $\pm$ SD, N=3. *Statistically significant at $P < 0.05$, **statistically significant at $P < 0.01$, ***statistically significant at $P < 0.001$, **** statistically significant at $P < 0.0001$.

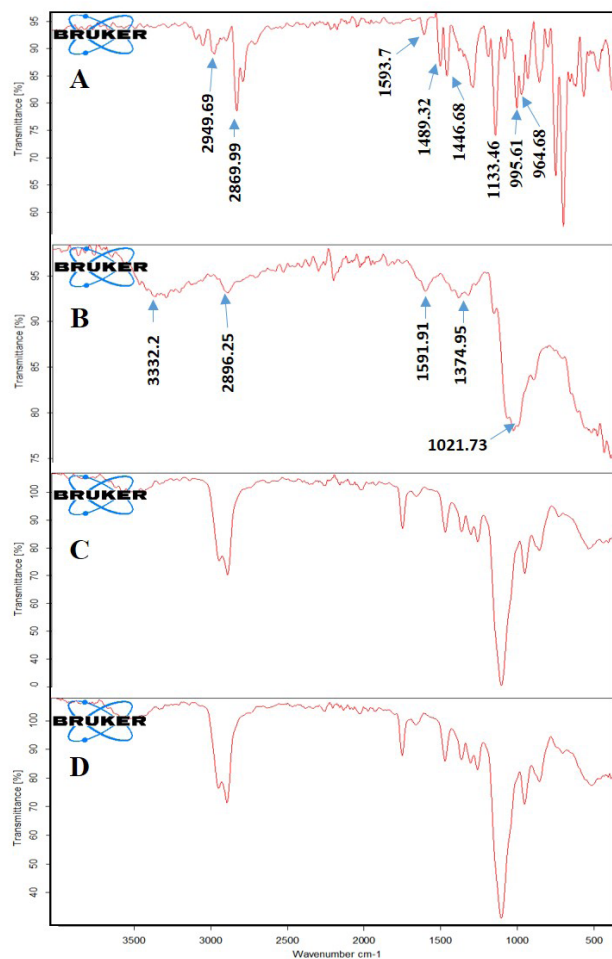


Figure 2 FTIR spectrum of the pure CN (A), chitosan (B), tween 80 (C) and physical mixture of CN, chitosan and tween 80 (D).

(B) shows the characteristic peaks of chitosan, 3332.2 cm^{-1} corresponds to the N-H and OH stretching, 2896.25 cm^{-1} indicates C-H symmetric and asymmetric stretching, 1591.91 cm^{-1} indicates C=O stretching of amide I and 1374.94 cm^{-1} indicates C-N stretching of amide III and 1021.73 cm^{-1} . The spectra of physical mixtures of CN with chitosan and tween 80 shows simple shifting in position and the intensity of the characteristic peaks of CN/chitosan which could be due to H-bond formation (Figure 2), suggesting good compatibility of the drug with the polymers used in the CN/chitosan nanoparticles.

Scanning electron microscope

Figure 3 shows the scanning electron microscope (SEM) photographs of the selected CN-loaded chitosan nanoparticles (F6). The photographs show homogeneously distributed, spherical shape nanoparticles with few particle aggregates, confirming the formation of the nanoparticles with a particle size comparable to that of the DLS results.

In vitro release study

The *in vitro* release profile of CN from the chitosan nanoparticles was investigated using three selected formulations (F2, F6, and F7) and an aqueous solution

of CN (as control). Figure 4 shows the release profile of the CN-loaded chitosan nanoparticles as a function of time for up to 48 hours. The release profile of the CN-loaded chitosan nanoparticles (from all formulations) shows a statistically significant reduction in the burst release phase compared to the control (CN solution). After 15 minutes, the cumulative release was $8.7 \pm 0.3\%$, $26.3 \pm 1\%$ and $8.3 \pm 0.21\%$ for F2, F6 and F7 compared to about $63 \pm 2.5\%$ of control, suggesting a sustained release effect of the chitosan nanoparticles. The burst release that can be observed in the formulas is most probably due to the unbound or free drug in the nanodispersions. After 24 hours, F2, F6 and F7 had reached approximately 80% accumulative release compared to 95% in the control.

Release kinetics of cinnarizine

In order to investigate the release kinetics of CN from the chitosan nanoparticles, the *in vitro* release data were fitted into zero-order, first-order, Higuchi, and Korsmeyer-Peppas models. Table 3 shows the regression coefficients (R^2) of the fitted release data of formulations (F2, F6 and F7) and the drug solution (control). The release data of the formulas were perfectly fitted with the first-order kinetic model ($R^2 = 0.95\text{--}0.97$) and Higuchi model ($R = 0.96\text{--}0.99$) followed by zero-order kinetic ($R^2 = 0.87\text{--}0.88$). Although the

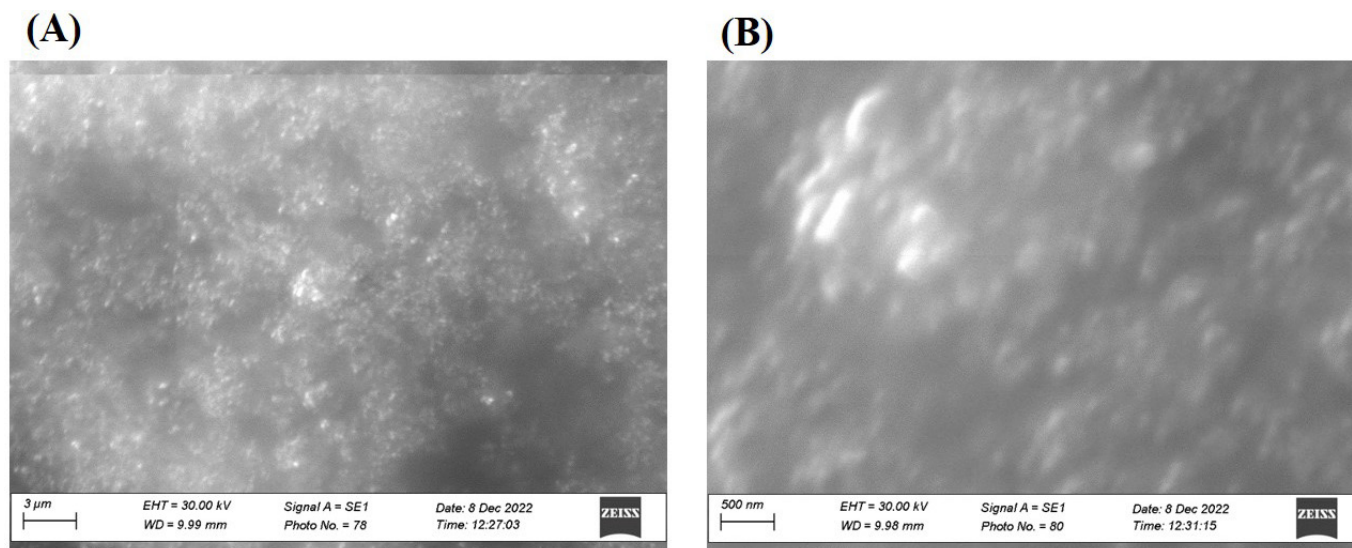


Figure 3 SEM images of the CN-loaded chitosan nanoparticles (F6). Low magnification (A) and high magnification (B).

Table 3 Release kinetics of the CN-loaded chitosan nanoparticles. The values are the correlation coefficients (R^2) of the fitted kinetic models

FORMULATION	ZERO-ORDER KINETICS	FIRST-ORDER KINETICS	HIGUCHI	KORSMEYER-PEPPAS	
				R^2	n
CONTROL	0.21	0.69	0.4	0.61	0.96
F2	0.88	0.97	0.99	0.96	1.1
F6	0.87	0.95	0.96	0.8	0.97
F7	0.88	0.95	0.99	0.97	0.73

*n is the diffusional exponent of Korsmeyer-Peppas model

drug solution was moderately correlated with first-order kinetic ($R^2 = 0.69$) there was no correlation with the Higuchi and KorsmeyerPeppas models which is due to the absence of the polymeric matrix of the control. The chitosan nanoparticles containing formulations also exhibited perfect fitting in KorsmeyerPeppas model with $R^2 = 0.8-0.97$ and diffusional coefficient (n) = 0.98-1.1. Fitting with Higuchi and korsmeyerPeppas models with diffusional exponent (n) value above 1 (Table 3), suggest that the release mechanism is controlled by both drug diffusion and polymer erosion with a super-case (II) transport.

Ex-vivo permeability

To investigate the effect of the chitosan nanoparticles on the skin permeation of CN, an *ex vivo* permeability experiment using rat skin was performed. Figure 5 shows the cumulative permeated amount ($\mu\text{g}/\text{cm}^2$) as a function of the time of the formulas (F2, F6, and F7) and the control (pure drug solution). The result shows that F6 has the highest permeated amount of CN compared to the control. However, F2 and F7 offered lower and comparable drug permeation, respectively,

compared to the control. Permeation parameters were also calculated to further investigate the influence of the chitosan nanoparticles. Table 4 shows that formulation F6 had the highest Jss which was $14 \mu\text{g}/\text{cm}^2/\text{h}$ compared to the control ($4.7 \mu\text{g}/\text{cm}^2/\text{h}$). This difference is considered statistically significant. The permeation was remarkably enhanced in F6 (3-fold) compared to F2 and F7. The formulation F2 showed a reduction in the permeability of CN through rat skin as indicated by its Jss and EE values compared to the respective control values (Table 4). F2 showed statistically lower permeation parameters compared to those of the control, suggesting retention of the CN-loaded chitosan nanoparticles within the superficial skin layers.

DISCUSSION

The data presented in this study describes the formulation and evaluation of CN-loaded chitosan nanoparticles. Moreover, the study investigated the feasibility of using chitosan nanoparticles as a dermal/transdermal delivery system of CN. CN is a well-known drug commonly used in cerebral conditions with reduced

Table 4 *Ex vivo* permeation parameters of the CN-loaded chitosan nanoparticles

	STEADY STATE FLUX (JSS) ($\mu\text{g}/\text{cm}^2/\text{h}$)	PERMEABILITY COEFFICIENT (cm/h)	ENHANCEMENT RATIO	CUMULATIVE AMOUNT (Q) ($\mu\text{g}/\text{cm}^2$)
F2	$2.1 \pm 0.11^{***}$	5.25 ± 0.3	$0.5 \pm 0.03^*$	$119 \pm 6.7^{**}$
F6	$14 \pm 0.7^{****}$	$116.6 \pm 6^{****}$	$3 \pm 0.5^{****}$	$414 \pm 29^{****}$
F7	5.7 ± 0.3	14.25 ± 0.7	1.2 ± 0.06	220 ± 10.9
Control	4.7 ± 0.2	11.75 ± 0.6	1 ± 0.05	180 ± 9

Values are mean $\pm$ SD, N=3. *Statistically significant from the control at $P < 0.05$, **statistically significant from the control at $P < 0.01$, ***statistically significant from the control at $P < 0.001$, **** statistically significant from the control at $P < 0.0001$.

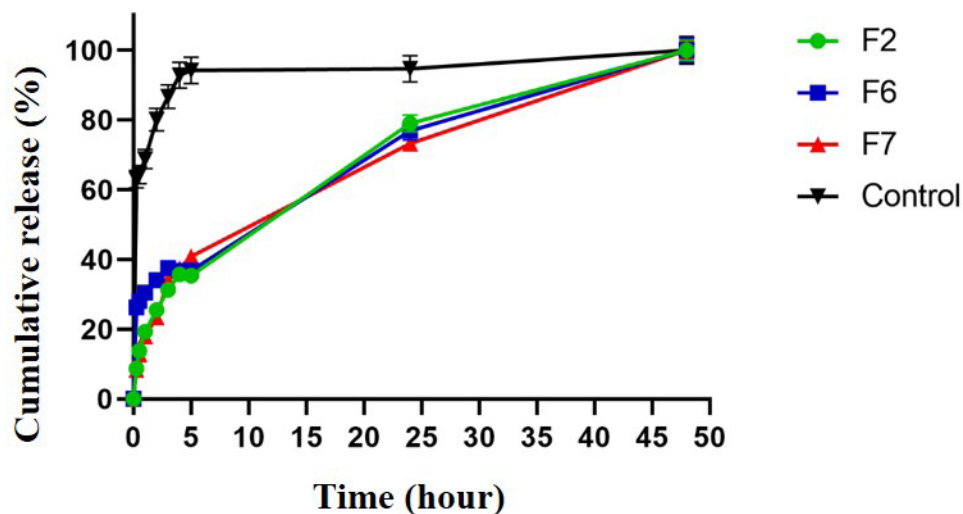


Figure 4 *In vitro* release profile of the CN-loaded chitosan nanoparticles. Values are mean $\pm$ SD, N=3.

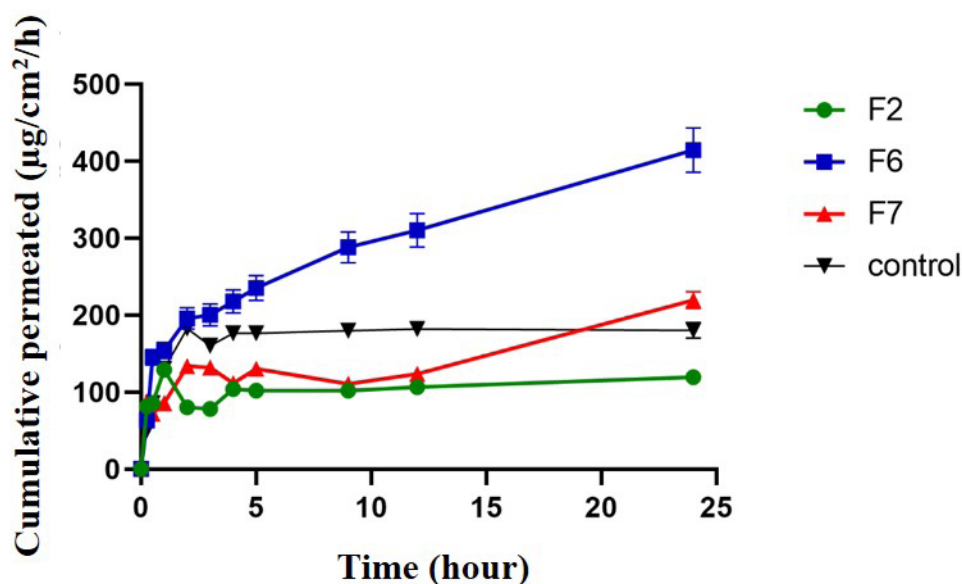


Figure 5 *Ex vivo* permeability profile of CN-loaded chitosan nanoparticles using rat skin.

blood flow (21) is approved for nausea, vomiting, motion sickness, inner ear disorders and is considered as first-line pharmacotherapy for management of vertigo. It acts as an anti-vasoconstrictor activity,

reducing blood viscosity and reducing nystagmus in labyrinth. Lack of adequate literature on clinical evidence of CN and its combination (dimenhydrinate). However, CN exhibited very low solubility and erratic

oral bioavailability making the transdermal route of administration a good alternative to oral administration especially in chronic treatments to reduce drug frequency and improve patient compliance (22). Drug penetration into the skin is a very crucial issue in both dermal and transdermal drug administration. Recently nanoparticles made of biodegradable polymers such as chitosan have attracted many researchers to develop nanocarrier-based formulations to enhance drug penetration (23). Moreover, following the penetration of a drug into the skin, drug permeability and/or retention will determine whether this system is suitable for transdermal or dermal applications (24).

CN-loaded chitosan nanoparticles were formulated using the nanoprecipitation method. The concept was based on using the pH-dependent solubility of both CN and chitosan to create solvent and antisolvent phases. Where the drug and the polymer are soluble in acidic pH (2-3) and their solubilities are remarkably reduced at alkaline pH (8-9). The method is rapid, simple, and inexpensive and was effective in generating small size, uniform nanoparticles with acceptable EE, this finding was in agreement with previous studies using chitosan to formulate drug-loaded nanoparticles by the nanoprecipitation method (12, 25). Preparation of the chitosan nanoparticles was started with several preliminary experiments to determine the solvent and antisolvent pH, the concentration of the drug and the concentration of chitosan that resulted in the formation of a clear formulation and avoided phase separation due to gelling of chitosan.

DLS characterization of the particle size showed an increase in the particle size with an increase in chitosan concentration. However, further increase in chitosan concentration resulted in decreasing particle diameter. Both effects have been observed in previous studies using chitosan to prepare nanoparticles (8,9). In preparation of polymeric nanoparticles by nanoprecipitation increase polymer concentration usually causes increase in the viscosity of the solvent phase leading to the slower diffusion of the solvent phase and formation of larger particles (16, 26, 27). However, sometimes an increase in a polymer concentration may lead to more effective precipitation

of the polymer/drug mixture forming smaller nanoparticles (8, 28). The formulation of the chitosan nanoparticles also showed a statistically significant reduction in particle size in response to the reduction of solvent to the nonsolvent volume ratio (i.e., an increase of the nonsolvent volume compared with the solvent volume). A similar finding was shown in a previous study that investigated the effect of various preparation parameters on the diameter of chitosan nanoparticles prepared by the nanoprecipitation method. In that study, the particle size was significantly reduced when the non-solvent-to-solvent volume ratio was increased from 10:1 to 40:1 (12). A clear reduction in the mean particle diameter was observed in response to increasing in tween 80 concentration which most portably due to the more effective prevention of particle aggregation and formation of larger particles (12, 29). A positive zeta-potential of the prepared CN-loaded nanoparticles with values increased gradually from 18 mV to 30 mV in response to increasing chitosan concentration can enhance the repulsion between particles, indicating the formation of stable nanoparticle dispersions. In addition, the positive charge can enhance drug penetration into the skin via electrostatic interaction with the negatively charged skin cells (1). PDI is an indicator of the uniformity of the size distribution of the nanoparticles. All formulas, except F7, showed a PDI of less than 0.5, suggesting a monodispersed nanoparticle (30).

The EE (%) of CN in chitosan nanoparticles was also determined. The results showed low (36% for F7) to moderate EE (62% for F6) but this result was expected due to the high drug: polymer ratio used in the formulation too compensate for the solubility limits of chitosan in the solvent (acetic acid:ethanol) phase. In general EE of drugs in chitosan nanogel prepared by the nanogelation method was founded to be significantly depending on the solubility of the drug in the solvent phase and on drug and chitosan concentrations (27), suggesting that multi-factors could affect EE in chitosan nanoparticles. A better EE of citral in chitosan nanoparticles prepared by nanoprecipitation was achieved in a previous study but at a much lower drug: polymer ratio, compared to the current study (12). In another study, the EE

of repaglinide in chitosan nanoparticles prepared by nanoprecipitation was up to 84% but at a drug:chitosan ratio of 1:20 (9), whereas the maximum drug:chitosan ratio used in the current work was 1:1. SEM characterization was used to further confirm the formation of the nanoparticles and visualize their morphology. The small spherical shape of the chitosan nanoparticles was in agreement with previous reports (31, 32). Compatibility of the CN with chitosan and other excipients was also confirmed by the FTIR spectroscopy.

Three formulations (F2, F6, and F7) were selected based on their charge, EE, and smallest particle size surfactant concentration to study the release profile of CN from chitosan nanoparticles and skin permeability. The *in vitro* release profile of CN-loaded chitosan nanoparticles showed a marked reduction in the burst release within the first 15 min and a marked increase in the time to 80% release of the CN, suggesting a sustained release profile. Comparable findings were obtained in previous studies using chitosan to prepare nanoparticles or nanogels of ibuprofen (33), tacrolimus (10), and myricetin (34). The burst release could be due to the free or the adsorbed drug on the nanoparticles surface. The burst release was significantly lower in F2 and F7 than F6 which is most probably due to larger particle size and less EE of F2 and F7 compared to F6. Reduction of burst release may lead to zero-order kinetic release allowing more effective treatment and fewer side effects (35, 36). The release kinetics of all chitosan formulae showed a combined first-order and zero-order kinetic, suggesting initial rapid release and subsequent slow-release profile compared to the control. The fitting of the release with a Higuchi and Korsmeyer-Peppas indicated that the release mechanism is a combination of drug diffusion through the matrix and polymer erosion. The release kinetics were in agreement with the results of previous studies using chitosan nanoparticles (33).

Rat skin was used as a model to investigate the effect of chitosan nanoparticles on the skin permeability of CN (37). Chitosan and its derivatives are widely accepted as a skin penetration enhancers and used as a form of a nanocarrier for this purpose. Different

mechanisms have been suggested for its penetration enhancing action including changing the *stratum corneum* and tight junction zones, interaction with intracellular lipid, and increasing stratum corneum water content (2). In addition, the cationic nature of chitosan enhance the electrostatic interaction with the cellular membrane allowing deeper penetration of the drug into skin layers (38). On the other hand, several other reports have suggested that the penetration of the nanoparticles can result in an accumulation of the nanoparticles within the superficial layers of the skin and cause drug retention in the skin rather than permeation into the systemic circulation (38, 39). The latter action will be useful for dermal targeting and reducing systemic side effects and can be achieved via optimization of particle size, charge or other functionalities (40). The current *ex vivo* permeability results suggested that chitosan nanoparticles could enhance permeability (F6), no significant effect on permeability (F7) or reduce permeability (F2) and such effect could be explained on the basis of the particle size of each formula, wherein the particle size decreases from high to low size in the following order: F2>F7>F6. Recent studies strongly support the relation between the nanoparticle size and its capacity to penetrate the skin, and most of these studies have showed that only nanoparticles with a diameter <20 nm can penetrate the intact skin while those with a diameter <45 nm can only penetrate damaged skin (41, 42). These findings were in agreement with a previous study using chitosan nanoparticles to enhance ibuprofen permeability through porcine skin. Where the skin permeability was increased from comparable to the control to enhanced by factor 4 in response to an increase in particle size (8). In another study, chitosan nanogel of glibenclamide and quercetin was used to investigate the skin permeability of these drug. The result showed that a reduced skin permeability of both drugs, however in this study the particle size of the chitosan nanogel was much higher than size of the nanoparticles of the current study (up to 400 nm) (43). In 2017, chitosan-loaded 5-fluorouracil nanoparticles were successfully prepared by spray drying and investigated for skin permeation and retention effects. Chitosan nanoparticles significantly enhanced skin permeation and skin retention extent of 5-fluorouracil

compared to the control (44).

In a different study, positively charged polymeric nanoparticles were used to enhance the transdermal delivery of ondansetron via electrostatic interaction with skin (45). Tacrolimus-loaded chitosan nanoparticles were also used for targeted treatment of psoriasis (dermal targeting). In this study, chitosan nanoparticles with a particle size of 140 nm and zeta-potential 22mV enhanced skin deposition of the tacrolimus and reduced drug permeation through the skin, suggesting the usefulness of the chitosan nanoparticles for local targeted therapy and reduction of systemic side effects (10). Our finding of the *ex vivo* permeation suggests that chitosan nanoparticles could either drug permeation or deposition in the skin following a careful optimization of the particle size. However, CN assay in different layer of the skin are necessary in the futur to confirm skin retention of chitosan nanoparticles and the relation between other physical properties such charge and skin penetration.

CONCLUSIONS

The nanoprecipitation method using pH-dependent solubility of drug/polymers offered a simple, rapid, and reproducible method to prepare CN-loaded chitosan nanoparticles. The method exhibited a good capacity to generate small and uniform polymeric nanoparticles, suggesting its feasibility to enhance drug permeability through biological tissues. The nanoparticles exhibited a sustained release of CN with a marked reduction in the burst release suggesting lower side effects. The described nanodispersion system has also enhanced skin permeation of the CN, suggesting the feasibility of chitosan nanoparticles to develop a transdermal delivery system of CN following its incorporation in a topically applied formula such as a patch. However, further investigations are required in the future to explore the relation between chitosan particle size/charge and its ability to enhance transdermal permeation or retention of drugs.

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ADHERENCE TO ETHICAL STANDARDS

Not applicable.

CONFLICT OF INTEREST

The Authors declare that there are no conflicts of interest regarding the publication of this manuscript

AUTHORS' CONTRIBUTIONS

All the authors contributed to the study conception and design. Concept, experimental design, data collection and analysis and writing were performed by Omar A Hamid. Fadia Yassir AL-bazzaz contributed in material preparation and experimental design and conduction. Rasha Khalid Dhahir contributed in material preparation, experiments conduction and reviewing. All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

An approval letter from the Institutional Animal Care and Use Committee at College of Veterinary Medicine, University of Mosul (Ref: UM.VET.2022.028) was issued before starting the *ex-vivo* permeation study using rat skin.

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