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Preparation and *in vitro* characterization of slow release testosterone nanocapsules in alginates

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Slow release testosterone-loaded nanocapsules in alginate, biodegradable hydropolymer, were prepared by *in situ* nanoemulsion-polymer crosslinking approach. Different formulations varying in the drug loading solvent phase were prepared. Four different drug-loading solvents were assayed and the food grade hexane provided nanocapsules testosterone load of 30%. Testosterone loading was confirmed by FT-IR, DSC and quantitated by HPLC. Prepared nanocapsules appeared spherical with a dense drug core in transmission electron microscopy studies. Hydrodynamic diameter of nanocapsules was 34.5 ± 1.7 nm, with a Gaussian distribution and the zeta potential –5.0 meV. Sustained diffusive drug release was observed *in vitro*, following zero order kinetics releasing the drug payload over a period of 48 hours. Embedding testosterone in alginate provided sustained release. Different drug loading solvents have distinct influence on drug loading and nanocapsules size distribution. The nanocapsulation technique developed can be a good choice for the development of different sustained steroid hormonal drug carriers.

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Keywords: testosterone, nanocapsules, alginate, hexane

Testosterone is a steroidal male hormone that regulates cellular calcium transport (1). It is useful in preventing osteoporosis and coronary artery diseases (2), in suppressing diverse immune reactions (3) and in gerontology (4). Free testosterone is also emerging as a significant possibility in life quality improvements for HIV patients (5). Health implications of testosterone and also its large-scale use by athletes have propelled studies on different modified drug delivery systems for testosterone (6).

The drug testosterone, however, is practically insoluble in water, has a relatively short plasma half-life of about 10–20 minutes (7–9) and the drug as such undergoes extensive first pass metabolism in liver (10). Transdermal patches of testosterone are available but must be applied to scrotal skin to achieve some therapeutic levels (11). In view of several pharmacokinetic limitations of the natural hormone and its increasing multivarious thera-

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peutic requirements, a suitable slow release testosterone loaded nanocapsules (NCs) in biodegradable material was perceived.

NCs within a certain size range are known to be easily circulating, passing even through small capillaries without clogging (12). Selection of a suitable biodegradable and biocompatible polymeric coating material was found to provide protection of the bioactive hormone molecule within the polymeric coating, releasing the drug load in a definitive and sustained release manner.

Pursuing these objectives, this work was aimed at developing a suitable slow release testosterone NC formulation using hydrobased polymer material, alginate, following a nanoemulsion and polymer cross-linking approach. Different drug encapsulating solvents were used in order to evaluate the resultant drug payload, size distribution and to arrive at a suitable slow release testosterone NCs formulation.

EXPERIMENTAL

Materials

Sodium alginate (viscosity 250 cps, 2% m/V in water) was purchased from Sigma Chemicals (USA). Testosterone was obtained as a gift from Organon India Limited (India). All organic solvents and water used were of high performance liquid chromatography (HPLC) grade (Merck/Spectrochem, India). All other reagents of analytical grade were procured from Merck or Spectrochem and used as received.

Preparation of testosterone-loaded alginate nanocapsules

Testosterone was encapsulated in calcium alginate by the *in situ* nanoemulsification--polymer cross-linking method (Fig. 1). Thus, 5 mg of the drug, testosterone, was taken in 10 mL of different drug loading solvents and emulsified under sonication at 20 kHz in 30 mL of 0.1% m/V aqueous solution of sodium alginate, using polyoxyethylene sorbitan mono-oleate (Tween 80) as emulsifier. Stabilizer, glycerol (~30 mL), was then added dropwise to produce the nanoemulsion. Calcium chloride solution (2 mol L^{-1}, 1.5 mL) was added into the reaction mixture to effect cross-linking of the NCs produced. The reaction mixture was cured for 24 h at room temperature (25 °C). NCs were then separated by ultra centrifugation (Sorvall Ultra 80, Sorvall, USA) at 30,000 rpm, 0 °C, 30 min. NCs thus obtained were washed with 15 mL of water, recentrifuged and harvested in micro centrifuge tubes. These were then preserved in vacuum desiccators at 4 °C for further evaluations.

The drug to polymer ratio (D:P) used in all cases was 1:6 (*m/m*) and a series of batches were prepared using four different drug loading solvents, *viz.*, hexane (food grade), ethyl acetate, dichloromethane and 1,2-dichloroethane to optimize drug loading.

Characterization of nanocapsules

Particle size distribution in PCS. – The particle size and size distribution of the prepared NCs were measured using Photon Correlation Spectroscopy, PCS (Zetasizer 1000HS,

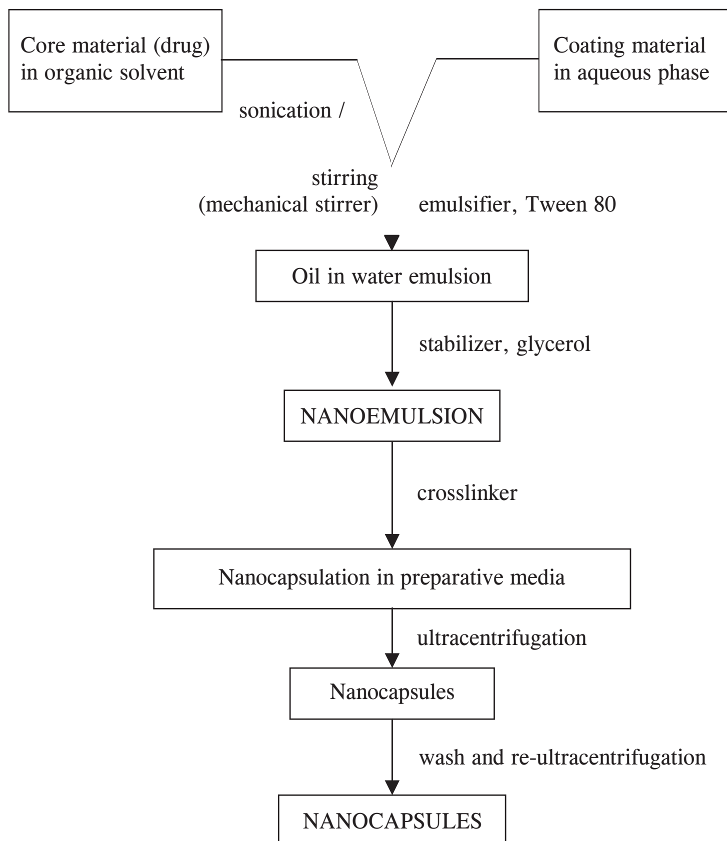

Fig. 1. Schematic representation of the preparation of nanocapsules (*in situ* nanoemulsion-polymer crosslinking method).

Malvern Instruments, UK) with a 4 mW He–Ne laser beam at a wavelength of 633 nm at 25 °C and at a scattering angle of 90°. Aliquots from each preparation batch were sampled in PCS cuvettes and NCs were then examined for equivalent diameters, size distribution and polydispersity.

Size distribution in TEM. – Transmission electron microscopy, TEM (FEI Technai 12 BioTwin, The Netherlands) with a CCD camera Megaview III soft imaging system was employed to visualize and record the nature and the size distribution of NCs. A generalized protocol was used for TEM studies. A drop of water suspension of the NCs was mounted on a carbon coated copper grid (CCG) and air-dried. NCs were stained with 2% uranyl acetate after washing with buffer solution (pH 4.0, phosphate) and micro graphed at 80–100 kV.

Drug loading. – Reverse phase HPLC method was used to determine the total entrapment of testosterone. The HPLC set-up consisted of LC-6A, Shimadzu system (Japan) with a UV-Vis detector. Testosterone concentrations were determined using an ODS column (Zorbax-ODS, Agilent, USA) and methanol/water mobile phase in the ratio 80:20 at a flow rate of 0.5 mL min^{-1}. Eluates were monitored at a fixed λ_{max} of 239 nm. A 20 µL sample volume was injected each time through a Rheodyne injector. HPLC elution time for testosterone was 12 minutes.

A standard curve was first prepared using known concentrations of standard testosterone against the HPLC peak area and was used throughout for analysis. Standard curve equation was $y = 5.32 \times 10^6 \gamma + 686.03$, $R^2 = 0.9998$ (y = HPLC peak area, γ = concentration in mg mL^{-1}, R = correlation coefficient).

About 20 mg accurately weighed testosterone NCs were digested in 40 mL of 1% *m/V* sodium citrate solution and the final volume was made up to 50 mL with deionized water. The mixture was sonicated at 20 kHz for 30 minutes and the solution was filtered using centricon tubes (molecular mass cut off 50,000) in a cold centrifuge (Remi C30, India) at 0 °C, 10,000 rpm. 20 µL of filtrate was injected into HPLC. Six samples from each batch were analyzed.

Zeta potential. – Zeta potential was measured for each formulation batch using large bore capillary cells in the Zetasizer Nano-ZS (Malvern Instruments). One mL of NCs suspension from the preparation medium was sampled out and diluted to 8 mL with 0.9% (*m/V*) NaCl solution prepared in HPLC water for optimal signal intensity (13). The pH of the water used was 6.0 ± 0.1. Six samples of each batch were recorded to get the average zeta potential for different formulations.

Differential scanning calorimetry. – NC thermograms were studied using a differential scanning calorimeter (DSC-60, Shimadzu). Two mg samples of NCs were heated from 20 to 400 °C in crimped aluminum pans to produce thermograms at a heating rate of 10 °C min^{-1}. Alumina (2.0 mg) in crimped aluminum pans supplied by the instrument manufacturer was used as the standard reference material to calibrate the temperature and energy scales.

Fourier transformed-infrared spectra. – FT-IR spectra (FTIR 670, Jasco, Japan) of testosterone, testosterone loaded NCs and sodium alginate were recorded in potassium bromide pellets. Testosterone powder was mixed with dried and ground potassium bromide (E. Merck, FT-IR grade), pelletized at 10.3×10^4 Pa and the spectrum was recorded between 4000–400 cm^{-1} using a high-energy ceramic source and DLATGS detectors. Similarly, the NCs prepared and the sodium alginate powder were analyzed by FT-IR and compared using the Biorad knowItAll(tm) informatics system software (Biorad Laboratories, USA) for spectral analysis.

In-vitro *drug release.* – About 20.0 mg of accurately weighed NCs were taken in 30 mL of 100 mmol L^{-1} phosphate buffer (pH = 7.4) containing 5% *V/V* methanol. The release studies were carried out at 37 °C under continuous stirring at 50 rpm and cumulative drug release was measured under sink conditions. 0.1 mL aliquots were sampled out at regular time intervals, filtered in centricon tubes (molecular mass cut-off 50,000) and 20 µL was injected into the HPLC column. The samples withdrawn were replaced

each time with 1 mL of fresh buffer mixture. HPLC analysis conditions were as described earlier and the standard curve similarly prepared was used for data analysis with necessary corrections for the dilution factors.

RESULTS AND DISCUSSION

Testosterone loaded alginate NCs were prepared following oil in water nanoemulsion and *in situ* polymer crosslinking. Four different formulations were designed (Table I) using different drug loading solvents with differing dielectric constants, such as food grade hexane (ε, 1.89), ethyl acetate (ε, 6.02), dichloromethane (ε, 9.08) and 1,2-dichloroethane (ε, 10.36) (14). Different drug loading solvents have shown a distinct impact on the drug payloading and nanocapsular size distribution.

Both the size and size distribution can strongly influence nanocapsular drug delivery (12). Particular drug carriers of an average diameter less than 10 nm in circulation are rapidly removed through extravation and renal clearance while particles ranging from 10–70 nm are the most effective systems for tissue distribution since they can circulate even in small capillaries and be taken up in tissues (15). Larger particles of diameter 200 nm are normally removed in phagocytosis and by the spleen as a result of mechanical filtration (16). Size distributions for all formulations were studied in PCS and the mean PCS diameters (Table II) were directly recorded as intensity-weighted.

PCS size distributions of formulation A provided a Gaussian size distribution with an average nanocapsular diameter of 34.5 nm (Fig. 2). Size distribution in formulation B was partially skewed, average diameter was 34.8 nm. Formulation C and formulation D produced larger particles of average PCS diameters of 585.2 nm and 819.8 nm, respectively. The polydispersity index (PI) was recorded as an index for particle size distribution in prepared formulations. PIs ranged from monodispersed, 0.000 to 1.000, where PIs greater than 0.500 indicated a relatively broader distribution. In injectable preparations, PIs near 0.250 are generally considered ideal (17). PIs of formulation A (0.22 ± 0.04) and formulation B (0.28 ± 0.04) were most suitable for circulating NCs (Table II).

Zeta potential is an important physicochemical parameter, which can influence factors like stability of a nano-drug carrier formulation. Extremely positive or negative zeta potential values cause larger repulsive forces, while electrostatic repulsion between parti-

Table I. Formulation design and drug payload for testosterone loaded alginate nanocapsules

Formulation	Solvent/oil phase (10.0 mL each)	Alginate sol. (0.1%, *m/V*, mL)	Tween 80 (mg)	$CaCl_2$ sol. (2 mol L^{-1}, mL)	Drug load (%)[a]
A	hexane	30.0	0.09	1.5	30.2 ± 1.9
B	ethyl acetate	30.0	0.09	1.5	12.9 ± 0.1
C	dichloromethane	30.0	0.09	1.5	5.1 ± 0.3
D	1,2-dichloroethane	30.0	0.09	1.5	2.6 ± 0.1

[a] Mean ± SE, $n = 6$.

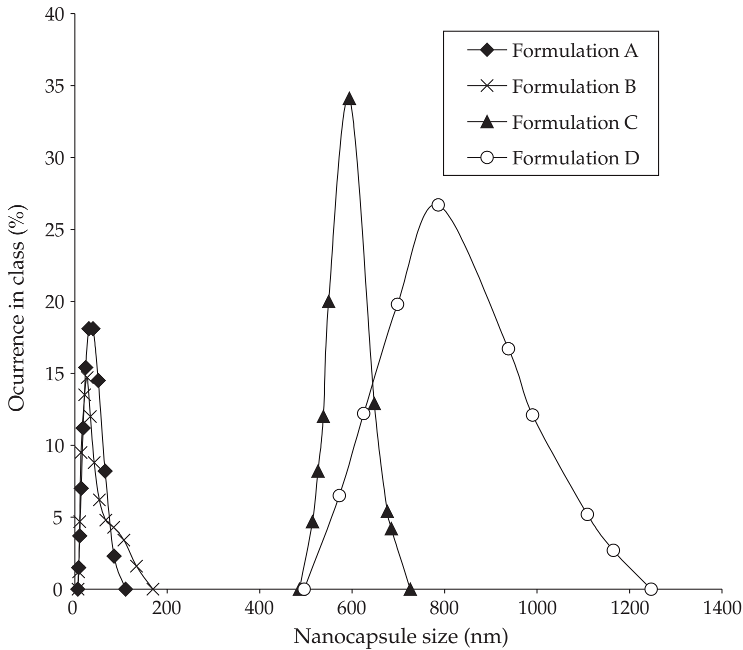

Fig. 2. PCS particle size distribution of testosterone nanocapsule formulations using: A (hexane), B (ethyl acetate), C (dichloromethane), D (1,2-dichloroethane).

cles with the same electric charge prevents aggregation of the spheres (18). Negative zeta potential values ranging from –3 to –5 meV were observed in four formulations (Table II).

Formulation A provided the best loading for testosterone of 30% while in formulation B, significantly lower testosterone loading of 13% was observed (Table I). Formulation C and formulation D, however, carry very low drug payloads of 5 and 3%, respectively. When compared to formulations B–D, the solvent dielectric constant of hexane in formulation A was much lower, which might have contributed to higher drug loading. In the case of fat-soluble drugs like testosterone, the solvent dielectric constant possibly pro-

Table II. Particle size, polydispersity and zeta potential studies of different formulations in PCS

Formulation	Particle size (nm)[a]	Polydispersity[b]	Zeta (meV)[b] potential
A	34.5 ± 1.7	0.22 ± 0.04	–5.0 ± 0.0
B	34.8 ± 2.6	0.28 ± 0.04	–4.8 ± 0.0
C	585.2 ± 4.9	1.00 ± 0.75	–3.5 ± 0.0
D	819.8 ± 13.6	1.00 ± 0.75	–3.0 ± 0.0

Mean ± SE: [a] $n = 4$, [b] $n = 6$.

vides for a reasonable degree of predictability; selection of a suitable encapsulating solvent might, in general, improve molecular payloading of lipophylic drugs in hydro-polymer nanocapsules.

In view of the drug payload and PCS size distribution, formulation A was chosen for further physicochemical studies and necessary evaluations in order to arrive at a desired natural androgenic hormonal drug delivery system.

The DSC and the FT-IR studies were carried out to determine whether testosterone was incorporated in formulation A NCs in crystalline, amorphous or bound form. DSC

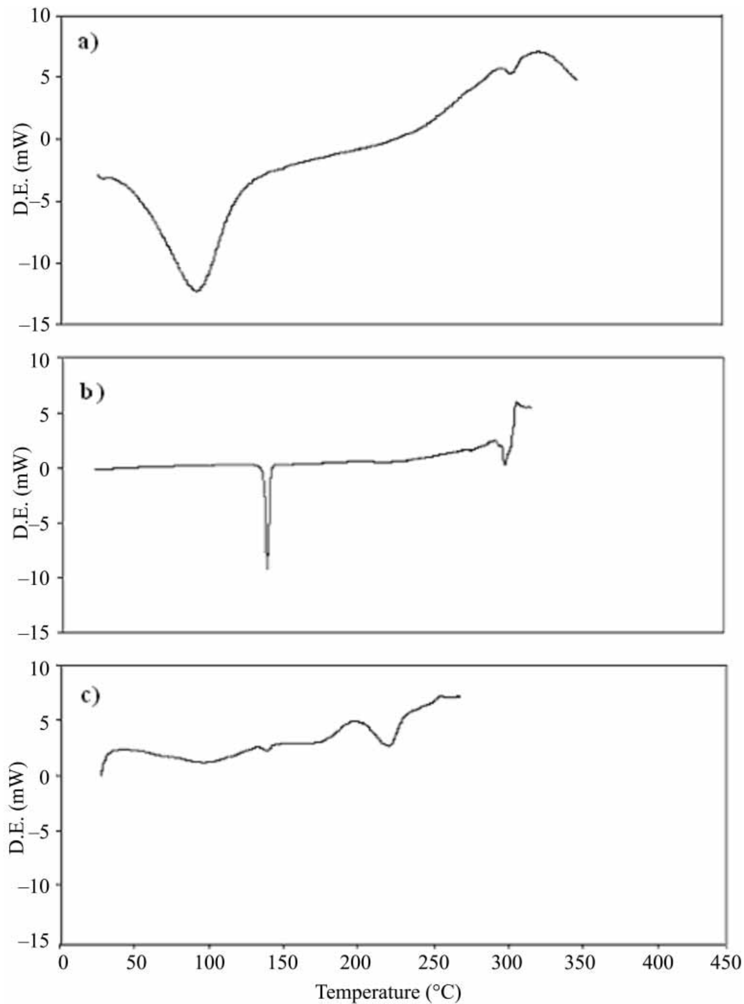

Fig. 3. DSC thermograms for: a) alginate, b) testosterone, c) formulation A (using hexane) nanocapsules.

studies revealed that the pure testosterone powder was crystalline with sharp melting at 149 °C (Fig. 3b) and the alginate polymer was amorphous showing a decomposition endotherm at around 292 °C (Fig. 3a). Testosterone endotherm devoid of powder melting was clearly observable at 149 °C in formulation A NCs (Fig. 3c) with the nanocapsule polymer decomposition at 220 °C. The drug was therefore considered encapsulated in its amorphous form (19) and was presumably unbound in the polymer coat.

FT-IR studies at 280 scans were recorded for the pure powdered testosterone (Fig. 4b), formulation A NCs (Fig. 4c) and the alginate powder (Fig. 4a). In FT-IR studies, the characteristic tautomeric testosterone C=O stretching at around 1660 cm^{-1} was clearly

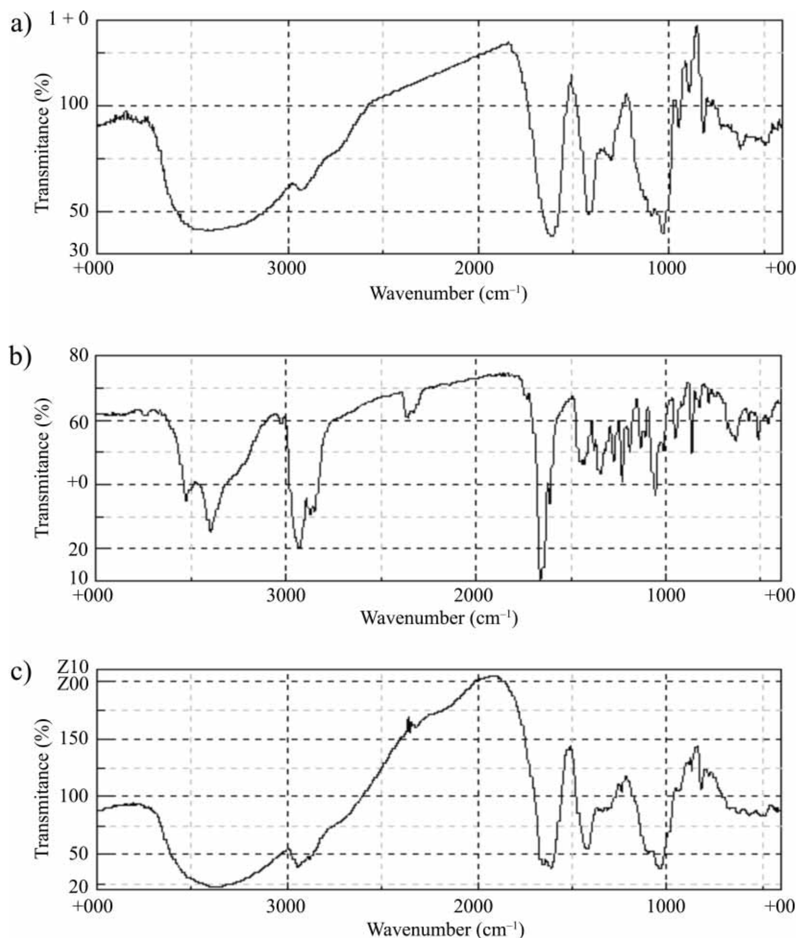

Fig. 4. FT-IR spectra for: a) alginate (COOH at 1611cm^{-1}), b) pure testosterone powder (C=O stretching at 1660 cm^{-1}), c) formulation A (using hexane) nanocapsules with both testosterone (C=O stretching at 1660 cm^{-1}) and alginate (COOH stretching at 1611 cm^{-1}).

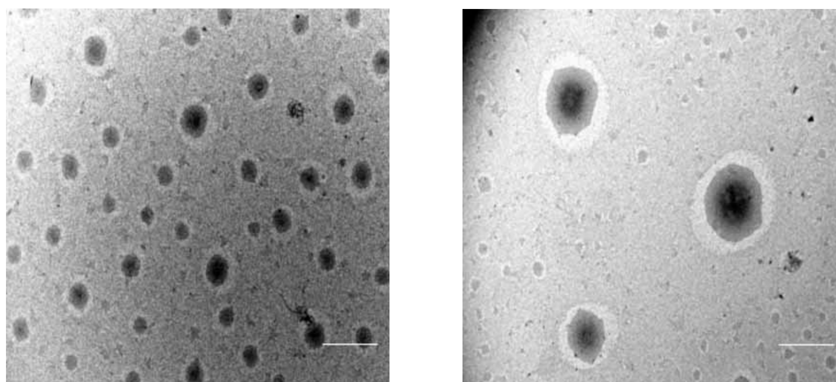

Fig. 5. TEM micrograph of formulation A (using hexane) nanocapsules.

distinguishable in the testosterone loaded formulation A (Fig. 4c). Additionally, O=C—H stretching of testosterone at 2871 cm^{-1} was also observed unchanged in formulation A, suggesting no drug-polymer chemical interactions in loaded NCs.

TEM is a 2D image of a 3 dimensional nanocapsule while PCS provides NCs hydrodyanamic diameter in terms of equivalent sphere. Transmission electron microscopy of uranyl acetate stained formulation A NCs were spherical with a dense central core for encapsulated testosterone (Fig. 5). Average TEM diameter for formulation A was observed to be 150 nm from 200 counts in four observation plates. TEM diameters though appeared relatively larger they were within the higher ranges of PCS observations. Both PCS and TEM are independent techniques of observation, but were sufficiently informative and complementary to each other (20).

The time dependent cumulative percentage release profile of formulation A was studied (Fig. 6). A sustained release profile for testosterone payload was observed over a period of 48 h. Both the size and the amount of the drug loading were known to influence the nanocapsular drug release profile (21). Smaller particles were evidently supporting faster the initial drug release equilibrium (22). Goodness of fit in different release kinetic models was evaluated in order to understand the mechanism of drug release (Table III). In general, the drug release mechanism of formulation A followed an overall zero-order kinetics dependent only on time. Formulation A drug release rate did not, however, fit into the concentration and time dependent first-order release mechanism model, suggesting that the overall *in vitro* testosterone release mechanism was of payload independent zero-order type, as expected for particular fat soluble drug carriers. As the drug loading in formulation A was near 30%, the surface area dependent Higuchi's dissolution model was evaluated and was found valid. The power law model (23) is a very suggestive tool to study the drug release mechanism dependent on the n' value. When n' is 0.43, drug diffusion is proportional to the concentration gradient indicating Fickian diffusion, while, n' value reaching 0.85 is indicative of the drug concentration gradient independent, non-Fickian, drug release mechanism. The power law was generally evaluated up to 60% of the total drug release (24, 25). Testosterone release mechanism from formulation A was evaluated and the n' value observed to be 0.36. The *in vitro* release

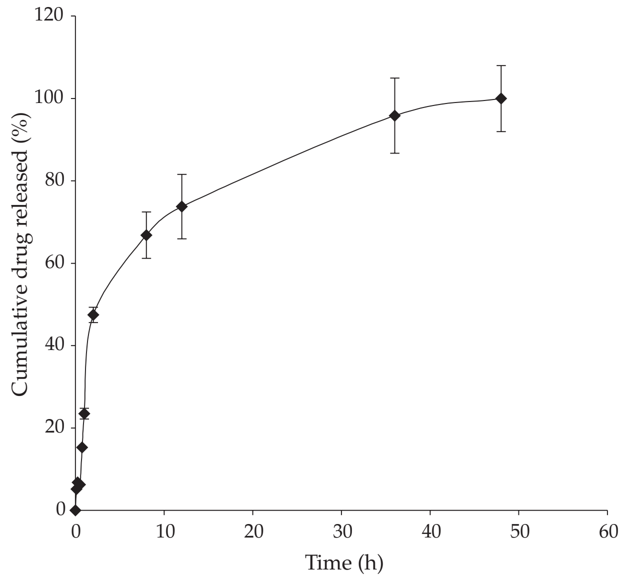

Fig. 6. Testosterone release profile for formulation A nanocapsules (using hexane). Each point represents mean ± SE, $n = 4$.

mechanism in formulation A was therefore taken to be following a Fickian diffusion mechanism. Hence the preparative technique followed has a predictive ability for androgenic hormonal drug payloading and release.

Table III. Testosterone release mechanism from formulation A nanocapsules

Release kinetic model	Zero-order[a]	Higuchi's model[a]	Power law[a]
Equation	$D_t = k_0\, t$	$Q = k_H\, t^{1/2}$	$M_t = M_\alpha\, k_P\, t^{n'}$
k value	$k_0 = 0.0503 \pm 0.0075$	$k_H = (6.9 \pm 0.4) \times 10^{-5}$	$k_P = 0.2479 \pm 0.0044$ $n' = 0.3615 \pm 0.0730$
R^2	1.000	0.98155	0.93378

k_0 – zero-order release rate constant, D_t – drug release mass at time t, Q – mass of drug release per unit surface area at time t, k_H – Higuchi's release rate constant, M_t – mass of drug released over time t, M_α – mass of drug released over infinite time, k_P – kinetic constant of power law, n' – release component, R – correlation coefficient
[a] Mean ± S.E, $n = 6$.

CONCLUSIONS

Natural androgenic nanocarriers in biodegradable polymer material, following a controlled release profile, have been formulated. Significant testosterone payload in alginate NCs was achieved using the food grade hexane as a drug loading solvent. The *in vitro* release of testosterone from alginate nanocapsules followed a Fickian diffusion mechanism. This nanocapsulation technique can be further tested for the development of different steroid hormonal drug carrier therapeutics.

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S A Ž E T A K

Priprava i *in vitro* karakterizacija alginatnih nanokapsula za polagano oslobađanje testosterona

BENOY BRATA BHOWMIK, BISWANATH SA i ARUP MUKHERJEE

U radu je opisana priprava nanokapsula za polagano oslobađanje testosterona. Nanokapsule su pripravljene iz biorazgradljivog hidrofilnog polimera alginata umrežavanjem *in situ*. Varirajući udio lijeka u tekućoj fazi pripravljeno je nekoliko različitih nanokapsula. Upotreba heksana kao otapala omogućila je visoki sadržaj testosterona u nanokapsulama (30%). Prisutnost testosterona potvrđena je pomoću FT-IR i DSC, a za kvantitativno određivanje upotrebljena je HPLC metoda. Pomoću transmisijskog elektronskog mikroskopa utvrđeno je da su pripravljene nanokapsule sferno simetrične i imaju gustu ovojnicu s ljekovitom supstancijom. Hidrodinamički promjer nanokapsula bio je 34,5 ± 1,7 nm (Gaussova raspodjela), a zeta potencijal –5,0 meV. Oslobađanje testosterona *in vitro* bilo je polagano tijekom 48 sati, a slijedilo je kinetiku nultog reda. Otapalo koje je

upotrebljeno za punjenje nanokapsula ljekovitom tvari utječe na količinu ljekovite tvari i raspodjelu veličine nanokapsula. Opisana metoda nanokapsuliranja može se primijeniti i na druge steroidne hormone.

Ključne riječi: testosteron, nanokapsule, alginat, heksan

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