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Shivanand P. Puthli & Pradeep R. Vavia

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RESEARCH ARTICLE

Fabrication, characterization and in vivo studies of biodegradable gamma sterilized injectable microparticles for contraception

Shivanand P. Puthli, and Pradeep R. Vavia

Department of Pharmaceutical Sciences, University Institute of Chemical Technology, University of Mumbai, Nathalal Parikh Marg, Mumbai, Maharashtra, India

Abstract

A Levonorgestrel-loaded microparticulate system was developed with gelatin and bovine serum albumin using triple emulsion technique coupled with chemical cross-linking thermal rigidization method. The formulation was optimized for various formulation variables and process parameters. The microparticulate system was characterized by scanning electron microscopy, encapsulation efficiency, moisture content, IR, DSC, XRD, residual solvent content and evaluated for sterility, abnormal toxicity and absence of pyrogens. Microparticles were sterilized by gamma irradiation at 2.5 Mrad. The system was injected intramuscularly in rabbits and drug blood levels estimated using radioimmunoassay technique. An optimized drug to polymer ratio of 0.4:0.75 w/w gave drug encapsulation efficiency of about 40%. The in vitro drug release followed Higuchi square root kinetics. In in vivo studies the AUC0-t was found to be 12849.25 pg/mL.day^{-1} with mean residence time calculated to be about 16 days and Kel of 0.02 day^{-1}. Levonorgestrel (LNG) levels were maintained between 200 and 400 pg/mL. The pharmacokinetic results indicate that LNG is released from the injectable microparticles for a period of one-month duration.

Keywords: *Levonorgestrel; gelatin; bovine serum albumin; microparticle; contraceptive*

Introduction

Levonorgestrel (LNG), a levorotatory enantiomer derived from 19-nortestosterone, is used by medical practitioners for contraception. It is available as single drug product (progestin-only regimen) or in combination with other drugs like Estradiol, Estradiol valerate and Ethinyl estradiol. Additionally, these systems are available as implants, intrauterine devices and oral tablets. Implantable systems pose the problem of surgical manipulations during product insertion and retrieval. On the other hand, tablets which are taken orally on daily basis might lead to what is referred to as the 'missed-pill' period leading to contraceptive failure. Thus a product with long term beneficial effects would be user friendly.

Microparticles and microcapsules have been fabricated by various methods.[1–4] Many of the sustained release microparticulate systems are prepared using semi-synthetic or synthetic origin polymers.[5–7] Injectable contraceptives offer several advantages. A parenteral system would require lower dosage leading to fewer unwanted side-effects arising from the drug. This administration is highly effective with reliable reversibility. From the user's perspective, many women value injectable systems as they are long-acting and convenient. Miniaturized systems using biodegradable polymers bypasses the surgical complications associated with the implantable devices and are thus preferred over other non-biodegradable products.[8–12]

Gelatin (GEL) and bovine serum albumin (BSA) has been used in microparticle systems. Once-a-day formulation of alpha interferon has been reported using gelatin and bovine serum albumin which utilizes emulsion-solvent extraction method.[13] Further, it is reported that in vitro release studies showed that the release rate of FITC-dextran from the microparticles with BSA addition

Address for Correspondence: Pradeep R. Vavia, Department of Pharmaceutical Sciences, University Institute of Chemical Technology, Nathalal Parikh Marg, Matunga, Mumbai-400 019, Maharashtra, India. Email: drugdel@rediffmail.com

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to gelatin did not elucidate satisfactory sustained-release characteristics after subcutaneous injection. In another research article a combination of LNG and Estradiol in gelatin microparticles were formed using the phase-separation method.[14] Among the various methods of cross-linking, the chemical cross-linking technique is widely employed in the production of microparticles.[15–19] Thus, for the production of non-antigenic microparticles researchers have employed cross-linking agents either by modulating the type of cross-linker or by the specific preparative procedure employed. However, these studies have not demonstrated the effect of gamma radiation sterilization effects and safety of the systems in biological milieu. In our study we prepared the microparticles of gelatin and BSA by a different method of chemical cross-linking to evaluate the effect of BSA on the release characteristics of a steroid. In the preparation of soft gelatin capsules, cold conditions are employed to harden and cure the gelatin shell. We have experimented to incorporate this basic chilling principle in preparation of the microparticles. We have developed an innovative triple emulsion technique coupled with chemical cross-linking thermal rigidization method for preparation of microparticles.

The primary objective of this research work was to develop LNG microparticles as a monthly contraceptive system. Being a parenteral system the microparticles were subjected to gamma radiation sterilization before administration to animals. Pharmacokinetic studies were carried out in non-rodent species and blood levels monitored by sensitive radioimmunoassay technique. Here we report for the first time a biocompatible 'progestin-only' long-acting contraceptive microparticulate system prepared using cost-effective non-synthetic materials that can have a great potential to replace the synthetic systems. The in vivo studies have shown promising results. Both safety and efficacy has been demonstrated experimentally for this developed system.

Materials and methods

Materials

Levonorgestrel was gifted by Wyeth Laboratories Limited, India. Bovine serum albumin was purchased from Sigma Chemicals (USA). Gelatin was obtained from Hi Media Pvt. Ltd., India. Refined sunflower oil was gifted by Godrej Foods Ltd. (Mumbai, India). Glutaraldehyde (25% w/w), sodium dodecyl sulfate and sodium azide were purchased from S.D. Fine-chem Pvt. Ltd., India. Fluid thioglycollate medium was purchased from Hi Media Pvt. Ltd., India. Radioimmunoassay kit was gifted by the World Health Organization (Immunometrics, London). Double distilled water was employed in analytical methods. All other reagents used were of analytical grade and were purchased from Ranbaxy Chemicals, India.

Fabrication of LNG-BSA-GEL microparticles

Step 1: Triple emulsion method. LNG was dissolved in 3 mL of methylene chloride and was gradually dispersed in an aqueous solution of polymer mix. The polymer mixture consisted of 0.25 g of bovine serum albumin (BSA) and 0.5 g of gelatin (GEL) in 6 mL of deionized water. The mixture was emulsified in 20 mL of refined sunflower oil and an o/w/o emulsion was obtained using a high speed homogenizer probe (Homogenizer type L56-31, Remi Motors, India). This triple emulsion was then incorporated in a drop wise manner into the spherodizing bath comprising of 300 mL of refined sunflower oil.

Step 2: Chemical cross-linking. Chemical cross-linking was achieved by glutaraldehyde (GLU). The GLU was initially extracted into toluene and this toluene-extracted GLU was incorporated into the vegetable oil. Toluene-saturated GLU was prepared by taking GLU solution (25% w/w aqueous solution) and toluene (1:1 w/w ratio) in a separating funnel. The GLU was extracted into the organic phase with vigorous shaking for a period of 1 h. The two layers were allowed to separate and the toluene layer was collected.

Step 3: Rigidization of microparticles. The contents were chilled to 5–10°C (freezing mixture consisting of ice and sodium chloride was employed for this purpose) and stirred to effect rigidization of the coat.

Step 4: Harvesting and Freeze drying. The microparticles were then harvested and filtered using a sintered glass SG-4 filter. The product was washed with 15 mL of acetone (×2 washings) to remove any traces of oil and to make the product free flowing. To remove residual moisture, the microparticulate system was subjected to lyophilization at –0.004 mBar and –40°C using freeze dryer (Labconco Corporation, UK).

Optimization of LNG-BSA-GEL system

The preparative method of LNG loaded bovine serum albumin-gelatin microparticles was optimized by a battery of microparticle runs. The formulation variables and process parameters which were critically evaluated are described in subsequent sections.

Effect of drug to polymer ratio

Various trial batches were undertaken with different drug to polymer ratios. The drug to polymer ratios of 0.1:0.75, 0.2:0.75, 0.3:0.75 and 0.4:0.75 w/w were employed. The objective was to find the optimum ratio of the polymer

and the drug that would give the highest possible encapsulation efficiency.

Effect of speed of agitation

The effect of speed of agitation on the fractional (BSS sieve fraction 150/350) yield as also the drug loading was evaluated. Impeller agitation speeds of 880, 1260, 1650 and 1800 rpm were used in the study. The speed was regulated using a Chronometric hand tachometer, type H (Tedock Corporation, Japan). The influence of stirring speed on the sphericity of the final product was another important parameter that was investigated.

Effect of glutaraldehyde concentration

Experiments were conducted by varying the GLU concentration. For every batch consisting of 300 mL vegetable oil 0.5 mL, 1.0 mL, 3.0 mL, 5.0 mL and 10.0 mL of toluene extracted GLU was added and stirred for 2 h. The effect of the GLU on maintaining the sphericity as well as its influence on encapsulation efficiency was evaluated. The effect of the extent of cross-linking on drug release characteristics was also included in this study. Microparticles were formulated without the use of GLU and compared with the batches prepared using the cross linker.

Gamma radiation sterilization

The LNG-BSA-GEL system (batch code LBG10, the formulation with 5 mL toluene extracted GLU) was subjected to gamma radiation (source Cobalt-60). The product was subjected to dose of 2.5 Mrad. in a gamma chamber GC-900 (Bhabha Atomic Research Centre, Mumbai, India). The dose rate of 0.13 Mrad/h was employed.

The cumulative influence of the above parameters on the quality and characteristics of the product, namely, drug encapsulation efficiency, particle size, sphericity and in vitro drug release kinetics was investigated.

Characterization of LNG-BSA-GEL System

Particle size analysis

Apart from the process yields calculated for each formulation batch, the particle size analysis was also performed. The sieves Sieve No. 36 (420 microns), 60 (250 microns), 85 (180 microns), 100 (150 microns), 150 (105 microns), 200 (75 microns), 300 (53 microns), 350 (45 microns) and 400 (37 microns) were stacked one above another, with the coarsest sieve on the top. The weighed microparticles were carefully placed on the topmost sieve and the lid closed. The nest of microsieves were then loaded onto the Retsch (Germany) mechanical sieve shaker and subjected to agitation for 30 min. The microparticles retained on each of the sieves were carefully collected and each fraction was weighed. The percent fraction retained on each sieve was calculated. The particle size was determined from the plots of percent cumulative undersize versus mean particle size.

Drug encapsulation efficiency

Microparticles (BSS sieve fraction 150/350 fraction, 25 mg) were accurately weighed and powdered. The drug was extracted with 25 mL of chloroform (using sonication for 15 min for complete extraction) and filtered. After suitable dilution of the filtrate using the same solvent, the drug was estimated by HPTLC. The encapsulation efficiency was calculated by the actual and theoretical drug loading values.

Analytical method (HPTLC)

In this analysis, stationary phase was HPTLC precoated silica gel 60 F254 plates (Merck, Germany; size: 10 × 10 cm). The mobile phase consisted of benzene: methanol (9:1). Autosampler (Camag Linomat IV, Switzerland) was used for spotting with an application rate of 15 s/μl. Spectrodensitometric analysis was performed on a Camag TLC scanner II, (Switzerland) at a wavelength of 240 nm. Integration was done on Perkin Elmer integrator system LCI-100 (Switzerland). The blank was prepared by the same extraction process but using dummy microparticles. The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation 0.9913; slope 0.4421 and intercept 81.5992 (for peak height) and coefficient of correlation 0.9967; slope 10.9253 and intercept 1444.9893 (for peak area) over the concentration range of 200–700 ng.

Sphericity

The sphericity of the microparticle system was computed by the method of Lovgren and Lundberg.[20] Briefly, individual microparticle was viewed on a projection microscope (model MP3Nr 3725, Poland). The longest diameter (L) and that perpendicular to it at its mid-point (b) were measured for fifty particles. The ratio of L/b were put into class intervals and the percent sphericity calculated using the formula, $S = 1/(b^2 \times rf) \times 100$. Where b is the lower class interval limit and rf is the relative frequency.

Moisture content

Moisture content in the microparticulate system was determined by Karl Fischer/Autotitrator (model 831 KF Coulometer, Metrohm, UK). Dehydrated methanol (Merck, 20 mL) was titrated to the electrometric end point with the KF reagent (Merck). The microparticle sample was then carefully transferred to the titration

vessel and after stirring for 1 min titrated again using the KF reagent till the characteristic end-point.

In vitro release kinetics

In vitro drug release was studied for formulations prepared using different levels of GLU. Two conditions – static and shaking methods – were employed in the studies. In the shaking technique the samples were subjected to agitation using a constant temperature shaker water-bath (Shital Scientific Industries, Mumbai, India). Microparticulate LNG-BSA-GEL system (BSS sieve no. 150/350 fraction, 50 mg) was taken in 100 mL stoppered conical flask. The dissolution medium consisted of 20 mL solution that resembled saline,[21] and sodium dodecyl sulfate 0.5% w/v added to maintain sink conditions. To prevent microbial growth during the period of study, sodium azide 0.02% w/v were incorporated in the medium. The temperature of dissolution medium was maintained at 37 ± 0.5°C and the samples were subjected to shaking at the rate of 80 strokes/min. Microparticle-free samples (2 mL) were withdrawn at predetermined time intervals of 4, 8, 12, 16, 20, 24, 28, 32 days, replacing with 2 mL of fresh medium each time. The drug release was measured by HPLC method. Placebo microparticle system (50 mg) was also concomitantly subjected to dissolution studies to check excipient interference during analysis.

Analytical method (HPLC)

Jasco intelligent unit (Japan) equipped with Lichrospher RP-18 column (5 μ, 125 × 4.6 mm, Merck) was used for HPLC analysis. The mobile phase comprised of acetonitrile: water (6:4 v/v) with a flow rate of 1 mL/min. Jasco UV-975 UV/VIS detector coupled with Borwin V 1.21 chromatography software at a wavelength of 240 nm was used. The polynomial regression for the calibration plots showed good linear relationship over the concentration range of 10-80 μg/mL. Coefficient of correlation was 0.9998 with slope of 58381.76 and intercept –29375.17.

Surface topography

Scanning electron microscopy was done using a scanning electron microscope (model S-570, Hitachi, Japan). The microparticulate samples (with and without GLU rigidization) was carefully placed on a metal stud coated with adhesive label. It was then subjected to sputter coating with conductive gold palladium (Edwards sputter coater; model S 150B, Edwards High Vacuum International, UK).

Infrared spectroscopy

LNG, BSA, GEL, physical mixture of drug and polymers (1:1:1) and microparticles of LNG-BSA-GEL were subjected to Infrared (IR) spectroscopy using IR Spectrophotometer (Buck Scientific, Model 500, USA) using the KBr pellet technique. The possible interaction of the polymers with drug and the effect of the formulation process on the physical state of the drug were evaluated by infrared spectroscopy.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) scans were taken LNG, BSA, GEL, physical mixture of drug and polymers (1:1:1) and microparticles of LNG-BSA-GEL microparticles using Shimadzu Thermal Analyzer DT-40 apparatus (Japan). DSC thermograms were obtained at a heating rate of 10°C/min in nitrogen atmosphere, alumina being used as the reference substance.

X-ray diffraction studies

To establish the polymorphic form of the drug in the final microparticulate system, samples were subjected to x-ray diffractometric analysis. X-ray diffractograms were obtained for LNG, BSA, GEL, physical mixture of drug and polymers (1:1:1) and microparticles of LNG-BSA-GEL microparticles. An automatic x-ray diffractometer model (Siemens 5000, Germany) equipped with an x-ray generator was employed in the study. Nickel filtered Cu $K\alpha_1$ radiation having a wavelength of 1.5106 A°, operating at 35 KW and 20 mamps in the range (2θ) of 5–70° was used with scanning rate of 2°/min.

Glutaraldehyde content

The residual GLU in the microparticles was estimated by gas chromatography using Chemito 8510 HR Gas Chromatograph (Chemito Technologies Ltd, India) with Oracle 3 computing integrator. The microparticle sample (500 mg) was suspended in double distilled water and subjected to sonication for one hour. The volume was made up to 10 mL with distilled water and filtered, the filtrate being further subjected to gas chromatography. The same GLU solution (25% w/w) that was employed in the formulation of microparticles was used in the preparation of the standard. The GLU solution was suitably dilution with distilled water to a final concentration 0.1 μg/mL and injected in the gas chromatograph. A Glass U-tube column packed with 10% carbowax 20 M coated on chromosorb WHP, 80–100 mesh was employed. Carrier gas was nitrogen with a flow rate of 40 mL/min and injection volume was 1 μL. The temperatures of column, injection port and detector (FID 681 detector) were maintained at 125°C, 150°C and 200°C, respectively.

Residual solvent content

The microparticulate sample (200 mg) was initially extracted with 4 mL dimethyl formamide (DMF), sonicated for 5 min and then kept in the head space tube in the heating block. After heating to 80°C for 10 min, the head space was injected. A blank determination of DMF was also taken. Residual solvent in the product was estimated by head space gas chromatography on CHEMITO

8610 HT gas chromatograph (Chemito Technologies Ltd, India), BPX5 capillary column equipped with head space system and FID detector. The oven, injection port and detector temperatures were 40°C, 80°C and 240°C, respectively. Nitrogen with flow rate 5 mL/min was employed. The standard included 0.1 mL (0.79 g) of acetone (99.5% purity) diluted with DMF to get a final concentration of 47.4 μg/mL. Four mL of the above 47.4 ppm standard solution was taken in head space tube in the heating block and heated at 80°C for 10 min. The head space was then injected. Chromatograms of all the samples were recorded and the residual acetone content was determined by comparing the peak area of the test sample and that of the standard making necessary corrections for the blank.

Sterility testing

The formulation subjected to gamma radiation was subjected to the sterility testing. The test for sterility was conducted on the optimized batch according to the United States Pharmacopoeia method.[22] Briefly, the sample was added to fluid thyoglycollate medium and incubated at 32.5 ± 2.5°C for 14 days. Aseptic conditions were maintained during the study. The contents were observed for any microorganism growth.

Abnormal toxicity

The optimized formulation was subjected to abnormal toxicity studies as described in European Pharmacopoeia.[23] Briefly, five healthy mice (body weight 17–22 g) were injected with the gamma irradiated microparticles (25 mg) previously mixed with 0.5 mL of water for injection. The animals were observed for 24 h for any untoward reaction or death.

Test for pyrogens

Pharmacopoeial method was carried out to confirm the absence of pyrogens.[24] Three healthy, adult rabbits of either sex weighing not less than 1.5 kg were selected for the study. Basal body temperature was documented. Sterilized microparticles were diluted with pyrogens-free saline solution and injected slowly into the marginal ear vein of each rabbit. Rectal temperature of each rabbit was monitored every 30 min for 3 h.

Pharmacokinetic study

The study protocol was approved by Institutional Animal Ethics Committee (IAEC, India) prior to the study. Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) were followed during the study. To evaluate the performance of the formulations in biological milieu, in vivo studies were conducted in rabbits. Healthy female albino Belgium rabbits (from Institute for Research in Reproduction, Mumbai, India) selected from the same colony with body weight 1.5–2.0 kg were used in this study. The animals were quarantined in well ventilated room with defined room temperature, humidity and lighting conditions. The randomization technique was used in grouping the animals and each group contained six animals. For the purpose of comparison, a control group comprising of equal number of animals as that of the test group was taken. The animals did not receive any formulation. These rabbits were subjected to mating with male rabbits. This served as the positive control. All the animals received standard balanced diet and had free access to drinking water throughout the study. Formulation (batch LBG10) was selected for the in vivo studies. The LNG-loaded microparticles (previously sterilized by gamma radiation at 2.5 Mrad.) were reconstituted suspended in 1 mL sterile saline and was injected intramuscularly into proven-fertile female rabbits using sterile disposable hypodermic syringe equipped with a 22-gauge needle. Before injecting the product, blood samples were taken which served as the initial zero day reading. Blood samples were collected at predetermined time intervals of 1, 3, 4, 8, 12, 16, 20, 24 and 28 days from the marginal ear vein of the rabbits. The serum separated was collected and stored in stoppered vials at –20°C until analysis. The samples were analyzed by a sensitive RIA technique. Pharmacokinetic parameters – Cmax, AUC0-t, AUC0-inf and Kel – were calculated by the WINNONLIN® program (Pharsight). Mean residence time (MRT) was computed by the statistical moments theory method. In addition to the measurement of the drug in the body, the performance of the formulation as an effective contraceptive was assessed by pharmacodynamic study. The animals were kept with male rabbits and tested periodically for indication of conception/pregnancy.

Drug analysis (radioimmunoassay technique)

A sensitive RIA method was employed in estimating the drug in the serum samples. A standard curve was obtained at concentration levels of 1500, 750, 375, 188, 94, 47, and 23 fmol/tube. The serum samples were subjected to ether extraction. Buffer was added to each of the assay tube and vortex mixed. To each of the tube, antiserum and working tracer were added and mixed. Ether and serum blanks were also taken. The vials were then transferred into a beta counter (Wallac 1409 Liquid Scintillation Counter). An equilibration time of 24 h was allowed before the counting. Background counts of the vials were taken prior to use. The counting time was 180 cpm (beta spectrum). For the calibration curve, a dose response curve was plotted. The method consisted of plotting the logits on y-axis and log-dose on the x-axis. The concentration of drug in the serum samples was extrapolated from the calibration plot.

Table 1. Effect of drug to polymer ratio on the quality of LNG-BSA-GEL system.

Batch code	D/P ratio (w/w)	Process yield (%)	Sieve #150/350 yield (%) Avg ± SD	Average particle size (μ)Avg ± SD	% Sphericity	Drug EE (%) Avg. ± SD
LBG01	0.1/0.75	82.36	79.08 ± 4.5	105.55 ± 10.5	100	12.11 ± 1.35
LBG02	0.2/0.75	79.62	79.24 ± 5.2	104.62 ± 12.6	100	19.91 ± 1.89
LBG03	0.3/0.75	82.98	82.29 ± 4.8	103.70 ± 15.2	100	33.36 ± 1.12
LBG04	0.4/0.75	80.98	74.36 ± 4.4	108.33 ± 14.7	100	42.66 ± 1.09

D/P, Drug/polymer ratio (w/w); EE, Encapsulation efficiency.

Table 2. Influence of speed of agitation on the characteristics of LNG-BSA-GEL system.

Batch code	Speed of agitation (rpm)	Process yield (%)	Sieve #150/350 yield (%) Avg. ± SD	Average particle size (μm)Avg. ± SD	% Sphericity	Drug EE (%)Avg. ± SD
LBG05	880	75.98	35.10 ± 5.6	135.00 ± 15.5	87.69	47.44 ± 1.58
LBG06	1260	79.25	53.33 ± 7.2	122.50 ± 20.2	97.14	46.69 ± 1.33
LBG07	1650	78.44	56.09 ± 4.5	122.22 ± 14.6	100	46.09 ± 1.03
LBG08	1800	80.63	73.42 ± 8.5	104.88 ± 18.9	100	44.94 ± 1.22

EE, Encapsulation efficiency.

Table 3. Effect of glutaraldehyde levels on the final product (LNG-BSA-GEL system).

Batch code	GLU mL/300 mL oil	Process yield (%)	Sieve #150/350 yield (%) Avg. ± SD	Average particle size (μm)Avg. ± SD	% Sphericity	Drug EE (%)Avg. ± SD
LBG09	10	76.55	74.33 ± 6.5	100.78 ± 20.2	100	43.92 ± 1.09
LBG10	5	78.33	70.48 ± 8.2	110.22 ± 15.6	100	44.59 ± 1.89
LBG11	3	81.43	76.91 ± 7.6	104.98 ± 22.2	100	43.14 ± 1.03
LBG12	1	79.19	72.22 ± 5.5	107.25 ± 18.3	100	40.95 ± 1.33
LBG13	0.5	83.24	71.45 ± 4.9	109.77 ± 20.2	100	40.98 ± 1.20
LBG14	0	78.95	70.28 ± 9.2	100.53 ± 15.2	100	5.25 ± 1.07

GLU, Toluene-saturated GLU; EE, Encapsulation efficiency.

Results

Optimization and characterization of LNG-BSA-GEL system

Effect of drug to polymer ratio

The effect of drug to polymer ratio on the quality of microparticulate system is given in Table 1. All formulations with varying drug to polymer ratios gave good process yields of 79–82%. The fractional yield (sieve 150/350 fraction) was also about 74–82%. The average particle size was found to be in the range of 103–108 microns. The drug to polymer ratio had significant effect on the Encapsulation efficiency.

Effect of speed of agitation

The influence of the stirring speed on the characteristics of the microparticles is given in Table 2. The process yield was about 76–80% in all the cases and was not affected by agitation speed. The stirring speed played a major role in the fractional yield of microparticulate product, particle size and sphericity.

Effect of glutaraldehyde concentration

The effect of GLU levels on LNG-BSA-GEL microparticulate system is given in Table 3. The GLU had negligible effect on process yields as well as sieve 150/350 fractional yields. The average particle size was about 100–110 microns. However the GLU had a significant influence on drug loading. The sphericity of 87.69% was obtained for batch LBG05 and 97.14% for formulation LBG06. For all other formulations the sphericity was 100%. GLU had a significant influence on drug loading and encapsulation efficiency.

Moisture content

Since the LNG-BSA-GEL system contains gelatin as one of the component which is prone to mould growth in presence of moisture, it becomes increasingly important to measure moisture levels in the final product. The moisture content in all the batches was 0.12 ± 0.01% w/w for all the formulations.

In vitro release kinetics

The in vitro release profiles of formulations prepared using different concentrations of GLU are shown in Figure 1 (static condition) and Figure 2 (agitation method). The slopes and the corresponding coefficients of determination (r) were computed for zero-order, first order and Higuchi kinetics. There was no significant influence of gamma radiation on the in vitro drug release characteristics from the microparticle system.

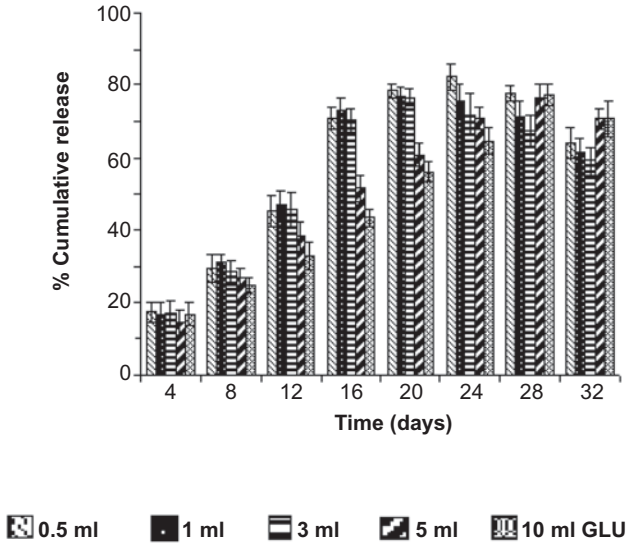

Figure 1. In vitro drug release from LNG-BSA-GEL microparticles: comparison of effect of glutaraldehyde and gamma radio-sterilization (static condition), n = 6 units.

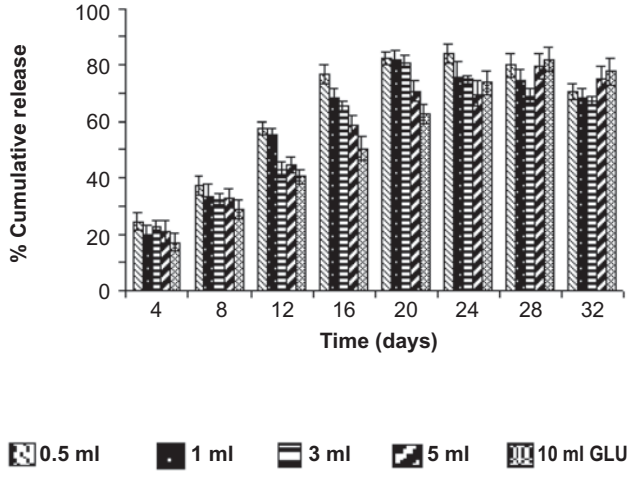

Figure 2. In vitro drug release from LNG-BSA-GEL microparticles: comparison of effect of glutaraldehyde and gamma radio-sterilization (agitation method).

Surface topography

The scanning electron photomicrographs for the glutaraldehyde-treated and non-glutaraldehyde-treated microparticles are shown in Figure 3. The spherical shape of microparticle was retained even after gamma radiation at 2.5 Mrad. The microparticle had a smooth surface without any invagination. There were no pores on surface of microparticles prepared using GLU as the cross-linking agent. It should be noted that miscellaneous matter seen on the surface of microparticle may be due to polymer remaining in medium during the coat hardening process. Adhesive polymer sundries could be a result of ruptured fractions of polymer or the remaining gelatin-bovine serum albumin fractions that were cross-linked with GLU upon hardening.

Table 4. Infrared spectrum of LNG, physical mixture of drug + polymer and microparticulate system.

Group assignment	Wavenumber (cm^{-1})		
	LNG	PM	MS
Conjugated C = O s	1655.4	1654.6	1654.2
C = C s	1617.8	1612.5	1615.7
Methyl C-H asym. b	1444.2	1449.8	1450.6
Methyl C-H sym. b	1365.8	1368.2	1362.5
Alcoholic C-O s	1067.3	1067.3	1067.3
Acetylene C-H b	691.2	691.9	691.2

PM, Physical mixture of drug + polymer; MS, Microparticulate system.

Infrared spectroscopy

In the IR study, characteristic IR absorption peaks of drug were present in the spectra obtained for the physical mixture and the microparticle system. The results suggest that there is no interaction of LNG with polymers employed in the formulation. The characteristic absorption peaks in the IR spectrum of the drug, physical mixture of drug with polymer and microparticulate system are listed in Table 4.

Differential scanning calorimetry

The DSC thermogram (as seen in Figure 4) of pure LNG shows a characteristic endotherm at 241.9°C. The same was shifted slightly to the left (237.9°C) in case of physical mixture of drug and polymers. Plot E depicts DSC pattern of the LNG-BSA-GEL system. The endothermic peak was absent in case of microparticulate system. The above results suggest that LNG is uniformly distributed in the polymer matrix.

X-ray diffraction studies

The x-ray pattern of both the polymers is typically amorphous. The x-ray diffraction spectrum of drug loaded microparticle system shows a lowering of diffraction peak intensity as compared to that of pure LNG. The weakened peaks may be a result of the rapid solvent evaporation during formation of microparticles, converting the drug to amorphous form or due to low concentration of the drug in the sample. Thus the results were not conclusive.

Gamma radiation sterilization

Physical characteristics of the microparticles were not altered after gamma radiation. A free-flowing product was obtained. There was no clumping or aggregation behavior observed. The color of the product also did not change after exposure to cobalt-60 irradiation. The mean particle size was 109.25 microns for the irradiated sample and 102.5 microns for non-radiated. The drug content in microparticles was 98.29 ± 1.28% post-irradiation as compared to initial value of non radiated

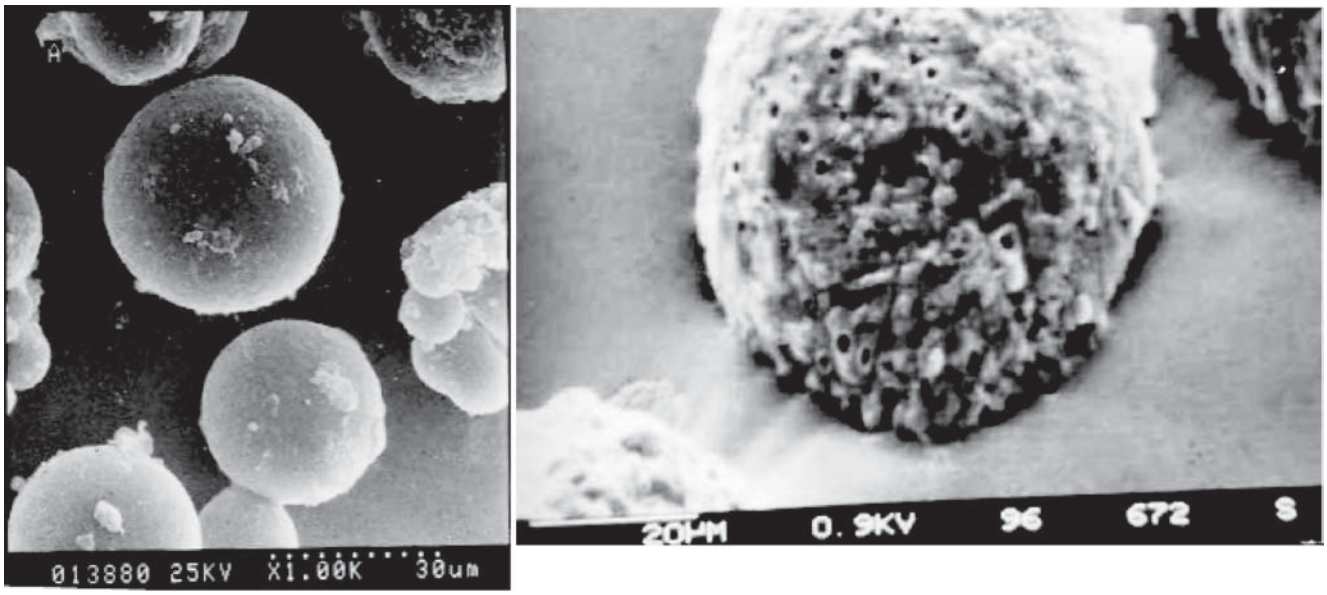

Figure 3. Scanning electron photomicrographs (SEM) of microspheres. (A) Gamma irradiated microspheres. (B) Microspheres without glutaraldehyde.

sample of 99.56 ± 1.56%. The microparticle system also did not show any decrease in drug content.

Glutaraldehyde content

In the estimation of residual glutaraldehyde, the retention time of GLU was 3.58 ± 0.2 min. There was no peak observed in the injected samples of microparticulate system (batch LBG10). The residual level of the free GLU in the product is less than the lowest detectable limit of 100 ng. Furthermore, the extractable amount of the potentially toxic residual GLU in microparticles was very low and should therefore not limit the therapeutic applicability of the formulation.

Residual solvent content

In the estimation of residual solvents, acetone exhibited a retention time of 251 s, dimethyl formamide was observed at 253 s. The residual acetone content was about 12.28 ppm in the LNG-BSA-GEL system (batch LBG10) and is well below the International Conference on Harmonization (ICH) guideline limits for residual solvents.

Sterility testing

The product (at all the experimental doses of gamma radiation) complied with the test for sterility.

Abnormal toxicity

In the abnormal toxicity study, there was no immediate reaction observed in animals in both control as well as the test group. During the test period there was no weight reduction and convulsions observed in any of the test animals. All the animals survived till the end of the study. The product complied with the test for abnormal toxicity.

Test for pyrogens

Product passed the test for pyrogens since the summed response in the body temperature of rabbits did not exceed 1.15°C.

Pharmacokinetic study

The serum concentration-time profile is depicted in Figure 5. The final formulation and process parameters is given in Table 5. It was observed that there was increase in the blood levels of LNG to about 300 pg/mL which indicated that there was a burst effect from the system. The AUC0-t was 12849.25 ± 3083.82 pg/mL.day^{-1}, AUC0-inf was 43476.59 ± 9564.8 pg/mL.day^{-1} with Cmax of 586.3 ± 140.71 pg/mL and Kel 0.02 ± 0.004 day^{-1}. The MRT was calculated to be about 16 days. The control group exhibited signs of conception (checked by palpation method). No pregnancies occurred in all test animals during the one month study period. However, there was reversibility of fertility after 5–6 months time. There was no tissue inflammation or erythemia observed at the site of injection of LNG-BSA-GEL microparticles which indicates that this dosage form is biocompatible.

Discussion

Injectable microparticulate drug delivery system offers various advantages such as long duration of action,

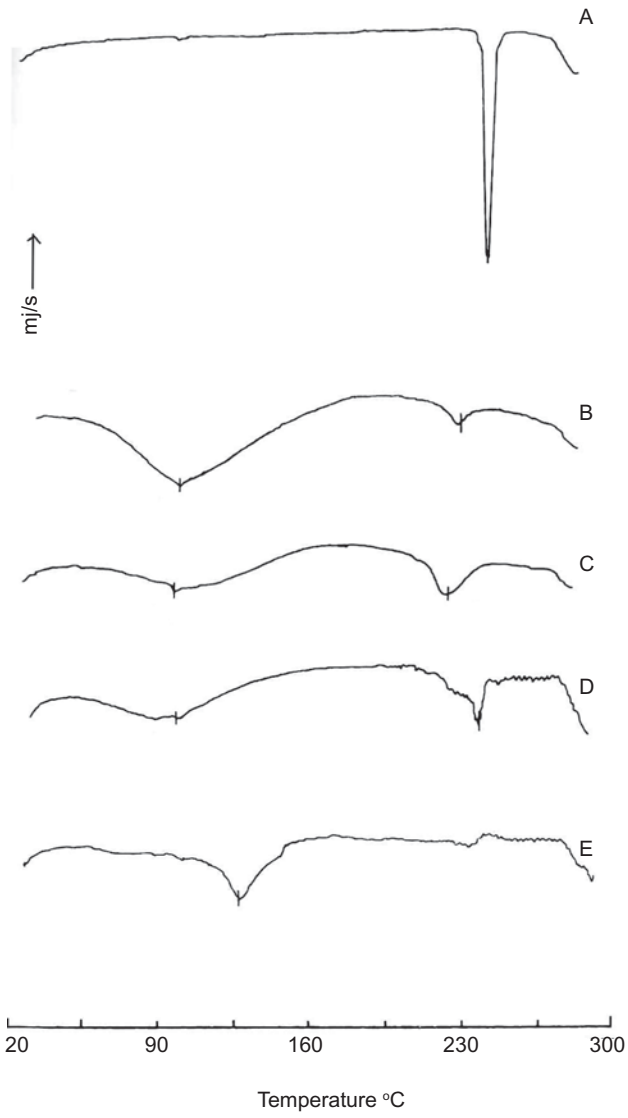

Figure 4. Differential scanning thermogram (DSC) of (A) LNG, (B) GEL, (C) BSA, (D) LNG+BSA+GEL (Physical mixture) and (E) LNG-BSA-GEL microparticles.

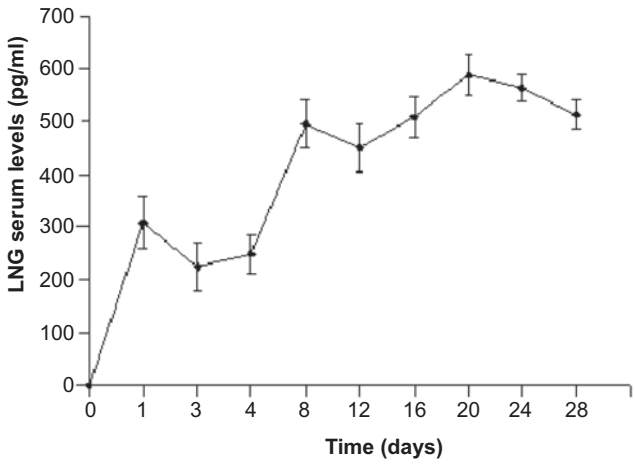

Figure 5. Serum drug concentration versus time profile after intramuscular administration of LNG-BSA-GEL microparticles to rabbits (n = 6). Dose administered 4 mg/kg.

Table 5. Optimized formulation of LNG-BSA-GEL microparticle system.

Ingredient/parameter	Quantity/Process conditions
Levonorgestrel	0.4 g
Methylene chloride	3 mL
Bovine serum albumin	0.25 g
Gelatin	0.5 g
Deionized water	6 mL
Refined sunflower oil (for emulsion)	20 mL
Refined sunflower oil (spherodizing bath)	300 mL
Homogenization time for emulsification	20 min
Toluene-saturated GLU	5.0 mL
Speed of agitation	1800 rpm
Rigidization time period	2 h
Temperature during coat hardening	5–10 °C
Acetone (quantity per washing)	15 mL
Lyophilization conditions	−0.004 mBar, −40°C
Gamma radiation (Cobalt-60)	2.5 Mrad.

ease of administration by intramuscular or subcutaneous injection and biodegradability. Another aspect is that they can be a valuable tool to improve drug efficacy and in reducing unwanted side-effects. LNG and Norethisterone have been incorporated in long acting contraceptive microspheres, implants and intrauterine devices. However, most of these works deal with the use of synthetic polymeric materials. Here we present a novel system utilizing non-synthetic excipients in fabrication of contraceptive delivery system. The advantages of LNG microparticles will be longer duration than Norethisterone system and a much smaller injectable dose. This would increase the convenience and acceptability of such a contraceptive preparation.

In the optimization trials we find that a manipulation of the drug to polymer ratio did not have any significant influence on the process yields, fractional yields as well as the average particle size. However, it had a marked effect on the drug encapsulation efficiency. It was observed that as the drug to polymer ratio increased from 0.1:0.75 to 0.4:0.75, the drug encapsulation efficiency also increased. An optimum drug loading of 42.66% was achieved with the drug/polymer ratio of 0.4:0.75. This ratio was the highest possible ratio that could be taken for formulation due to the fact that an increase in ratio caused the polymer-drug mixture to be too viscous with difficulty in processing of the emulsion. It was observed that as the speed of agitation increased

from 880 rpm to 1800 rpm, the fractional yield increased from about 35–73%. This is probably due to the 'slicing' effect of the impeller blade on the drug-polymer mix while its addition into the spherodizing bath. The average particle size varied according to stirring speed. The encapsulation efficiency remained unaltered and was found to be about 45%. A significant parameter that was affected by the agitation speed was sphericity. At low speeds, the microparticles deviated from sphericity (e.g. a speed of 880 rpm yielded particles having a sphericity of 87.67%). Speeds higher than 1650 rpm gave microparticles with perfect sphericity of 100%. This may be attributed to the fact that at low speeds of agitation the nascent microparticles (the particles in the oil bath before complete rigidization of the coat) probably tend to collide with each other. This would result in distortion of final shape of the microparticles. High speed of agitation keeps individual particles in motion and sphericity is thus maintained.

Although there was a marginal difference in drug loading in the formulations that contained GLU, there was a marked influence in the same when the cross-linker was not employed during formulation. The low drug loading may be ascribed to the fact that when GLU is not used, the polymer coat is not rigidized. Hence, in acetone wash step in the formulation protocol, the drug probably leaches out resulting in a decrease in encapsulation efficiency from about 43% to about 5%. This assumption was further confirmed by scanning electron microscopic studies. The scanning electron photomicrograph taken for batch prepared without the GLU (batch LBG14) reveals microscopic pores on the surface of microparticles. It is interesting to note here that sphericity was not altered by the presence of GLU. The formulations that were devoid of the cross-linking agent also exhibited excellent sphericity (100%). This brings us to a conclusion that the GLU does not have a role in maintaining the sphericity of LNG-BSA-GEL microparticulate system. Probably the chilled conditions employed in formulation retain the spherical geometry of microparticles.

The drug release data was fitted to various kinetic models – zero-order, first-order and Higuchi kinetics.[25] The release data was also fitted to Baker-Lonsdale kinetic model.[26] Zero order kinetics is obtained when the drug release from a system is independent of the concentration of the drug.[27] When the percent drug release was plotted as a function of time, a linear relationship was obtained with the '*r*' value closet to unity. The fit of different kinetic models and comparison of coefficients of determination for LNG-BSA-GEL system (effect of GLU levels and gamma sterilized microparticles) indicated that the release data fitted adequately to Higuchi kinetics. Drug release could be effectively modulated by the amount of GLU. It was observed that an increase in concentration of the cross-linking agent decreased drug release. In case of formulations containing 0.5, 1.0 and 3.0 mL of the cross-linking agent, most of the drug was released in 20 day time period. Since our aim was to fabricate a system that would release the drug over a period of one month, these formulations were not of much concern. It is interesting to examine the release profiles of microparticles prepared with 5 mL and 10 mL of GLU. Both the systems exhibited a controlled release of LNG for one month. From toxicological point of view, GLU level in the final product is important. Thus, batch prepared with 5 mL of the cross-linker (batch LBG10) was preferred and hence selected for gamma radiation studies. Comparable release profiles were obtained for both the methods, namely, static and agitation employed in the study. The gamma radiation dose did not have any significant impact on release kinetics. In all the cases, $t^{1/2}$ kinetics was observed. Comparing the results of static and agitation methods, not much change was observed in the release data. Thus, the cobalt-60 irradiation at 2.5 Mrad dose can be used safely for terminal sterilization of this product.

In the scanning electron microscopy studies, an interesting phenomenon was observed with the microparticles prepared without GLU. The scanning electron photomicrograph of this formulation that eliminated the GLU shows microscopic pores on its surface. This confirms our proposition that the drug loss might have occurred during the solvent wash step through micropore formation.

In the Pharmacokinetic study, a dose of 4 mg/kg was employed. LNG levels which were raised initially subsequently reduced on day 3 and day 4. A subsequent increase in the concentrations was seen till day 20. However, this tendency of rapid initial release followed by slower release was not observed in in vitro dissolution studies. This may be attributed to the presence of proteolytic enzymes that might digest the gelatin-albumin matrix leading to the development of the rapid release fraction. It has been reported that LNG concentration of 500–800 pg/mL is sufficient for inhibition of ovulation.[28] The serum LNG levels during the 12 month of use were 500–800 pg/mL in the polydimethylsiloxane (silastic) rod users while in the capsule users they were about 150–300 pg/mL.[29] Suppression of ovulation may not be critical for contraceptive efficacy since progestin-only contraceptive systems have achieved acceptable rates of contraception without inhibition of ovulation. In fact, their mechanism of action has been by rendering the endometrium inhospitable to nidation or cervical mucus impermeable to sperm migration.[30] A randomized trial provides evidence that the minimum LNG concentration necessary to protect against pregnancy is below 200 pg/mL, and possibly is below 175 pg/mL. This research also indicates that drug concentration

in upper part of the range 151–200 pg/mL has protective action against pregnancy.[31] One can reasonably expect similar release from the LNG-BSA-GEL system in humans as there are reports that there exits a good correlation of in vivo drug release from microparticles in rabbits and humans.[32,33]

Conclusion

A biodegradable microparticle system was successfully developed for long-term delivery of contraceptive agent. The in vivo results indicate that the drug is released for a period of one month. The safety and efficacy of the developed system in biological milieu was successfully demonstrated by experiments. Salient features of the system include ease of administration, longer duration of action, user compliance and no need of system retrieval after use due to its biodegradable nature. An injectable dosage form would also mitigate any surgery as in the case of implantable dosage forms. Unwanted side-effects of the molecule can be eliminated using lower dose of the drug with better tolerance. The proposed system can be a very potential alternative to the expensive synthetic polymer-based products, thus reducing the overall cost of the product and therapy.

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References

1. Reis CP, Neufeld RJ, Vilela S, Ribeiro A.J, Veiga F. Review and current status of emulsion/dispersion technology using an internal gelation process for the design of alginate particles. J Microencapsul. 2006;23:245–257.
2. Shi Y, Li LC.Current advances in sustained-release systems for parenteral drug delivery. Expert Opin Drug Deliv. 2005;2:1039–1058.
3. Freitas S, Merkle HP, Gander B. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microparticle preparation process technology. J Control Release. 2005;102:313–332.
4. Okada H, Toguchi H. Biodegradable microparticles in drug delivery. Crit Rev Therap Drug Carrier Syst. 1995;2:1–99.
5. Drieu K, Devissaguet JP, Duboistesselin R, Dray F, Ezan E. Pharmacokinetics of D-Trp6 LHRH in man: sustained release polymer microparticle study (I.M. route). Prog Clin Biol Res. 1987;243A:435–437.
6. Dhanaraju MD, Rajkannan R, Selvaraj D, Jayakumar R, Vamsadhara C. Biodegradation and biocompatibility of contraceptive-steroid-loaded poly (DL-lactide-co-glycolide) injectable microparticles: in vitro and in vivo study. Contraception. 2006;74:148–156.
7. Dhanaraju MD, Gopinath D, Ahmed MR, Jayakumar R, Vamsadhara CJ. Characterization of polymeric poly(epsilon-caprolactone) injectable implant delivery system for the controlled delivery of contraceptive steroids. Biomed Mater Res. 2006;76:63–72.
8. Urata T, Arimori K, Nakano M. Modification of release rates of cyclosporin A from polyl(L-lactic acid) microparticles by fatty acid esters and in vivo evaluation of the microparticles. J Control Release. 1999;58:133–141.
9. Viswanathan NB, Thomas PA, Pandit JK, Kulkarni MG, Mashelkar RA. Preparation of non-porous microparticles with high entrapment efficiency of proteins by a (water-in-oil)-in-oil emulsion technique. J Control Release. 1999;58:9–20.
10. Sah HK, Chien YW.Evaluation of a microreservoir-type biodegradable microcapsule for controlled release of proteins. Drug Dev Ind Pharm. 1993;19:1243–1263.
11. Heya T, Okada H, Ogawa Y, Toguchi H. Factors influencing the profiles of TRH release from copoly(d,l-lactic/glycolic acid) microparticles. Int J Pharm. 1991;72:199–205.
12. Chattaraj SC, Rathinavelu A, Das SK. Biodegradable microparticles of influenza viral vaccine: comparison of the effects of routes of administration on the in vivo immune response in mice. J Control Release. 1999;58:223–232.
13. Yoshikawa Y, Komuta Y, Nishihara T, Itoh Y, Yoshikawa H, Takada K. Preparation and evaluation of once-a-day injectable microparticles of interferon alpha in rats. J Drug Target. 1999;6:449–461.
14. Guo R, Lu B. Study on compound levonorgestrel microparticles. Hua Xi Yi Ke Da Xue Xue Bao. 1993;24:376–380.
15. Chen Y, Willmott N, Anderson J, Florence AT. Comparison of albumin and casein microparticles as a carrier for doxorubicin. J Pharm Pharmacol. 1987;39:978–985.
16. Jones C, Burton MA, Gray BN. Albumin microparticles as vehicles for the sustained and controlled release of doxorubicin. J Pharm Pharmacol. 1989;41:813–816.
17. Orienti I, Zecchi V. Progesterone-loaded albumin microparticles. J Control Release. 1993;27:1–7.
18. Egbaria K, Friedman M. Sustained release albumin microparticles containing antibacterial drugs: effects of preparative conditions on kinetics of drug release. J Control Release. 1990;14:79–94.
19. Luftenstelner CP, Schwendenwein I, Paul B, Eichler HG, Viernstein H. Evaluation of mitoxantrone-loaded albumin microparticles following intraperitoneal administration to rats. J Control Release. 1999;57:35–44.
20. Lovgren K, Lundberg P.Determination of sphericity of pellets prepared by extrusion/spheronization and the impact of some process parameters. Drug Dev Ind Pharm. 1989;15:2375–2392.
21. Farmacopea Ufficiale Italiana, IX. Vol. 1; 1985, pp. 744.
22. United States Pharmacopoeia, Microbiological tests: Sterility Tests, USP 29-NF 24, The United States Pharmacopoeial Convention, Inc., Rockville; 2006.
23. European Pharmacopoeia, Test for abnormal toxicity, Ph. Eur. method 2.6.9, Council of Europe; 2005, Appendix XIVE.
24. European Pharmacopoeia, Test for pyrogens, Ph. Eur. method 2.6.8, Council of Europe; 2005, Appendix XIVD.
25. Higuchi T. Mechanism of sustained action medication: theoretical analysis of rate of release of solid drugs dispersed in solid matrices. J Pharm Sci. 1963;52:1145–1149.
26. Doshi CC, Bhalla HL. In vitro release studies of levonorgestrel loaded biodegradable microspheres. Ind J Pharm Sci. 1999;61:39–43.
27. Ranga Rao KV, Padmalatha DK, Buri PK. Cellulose matrices for zero-order release of soluble drugs. Drug Dev Ind Pharm. 1988;14:2299–2320.
28. Roy S, Stanczyk FZ, Mishell DR, Lumkin M, Gentzschein E. Clinical and endocrinologic study of continuous levonorgestrel

administration from subcutaneous solid polydimethylsiloxane rods. Contraception. 1980;21:595–615.
29. Moore DE, Roy S, Stanczyk FZ, Mishell DR. Bleeding and serum d-norgestrel, estradiol and progesterone patterns in women using d-norgestrel subdermal polysiloxane capsules for contraception. Contraception. 1978;17:315–328.
30. Ory SJ, Hammond CB, Yancy SG, Hendren RW, Pitt CG. The effect of a biodegradable contraceptive capsule (Capronor) containing levonorgestrel on gonadotropin, estrogen, and progesterone levels. Am J Obstet Gynecol. 1983;145:600–605.
31. Sivin I, Lähteenmäki P, Ranta S, Darney P, Klaisle C, Wan L, Mishell DR, Lacarra M, Viegas OA, Bilhareus P, Koetsawang S, Piya-Anant M, Diaz S, Pavez M, Alvarez F, Brache V, LaGuardia K, Nash H, Stern J. Levonorgestrel concentrations during use of levonorgestrel rod (LNG ROD) implants. Contraception. 1997;55:81–85.
32. Lancranjan I, Bruns C, Grass P, Jaquet P, Jervell J, Kendall-Taylor P, Lamberts SW, Marbach P, Orskov H, Pagani G, Sheppard M, Simionescu L ,Sandostatin LAR: a promising therapeutic tool in the management of acromegalic patients. Metabolism. 1996;45:67–71.
33. Comets E, Mentre F, Kawai R, Nimmerfall F, Marbach P, Vonderscher J. Modeling the kinetics of release of octreotide from long-acting formulations injected intramuscularly in rabbits. J Pharm Sci. 2000;89:1123–1133.