CHAPTER – 2

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Preformulation studies were initiated with standardization of the drug. Polymers were also characterized for their important characters like molecular weights and glass transition temperatures. Stability of drug - polymer mixtures was evaluated at 0-4°C. Feasibility of sterilizing drug and polymers by gamma radiation was also evaluated.

2A.1 Standardization of Levonorgestrel

Materials :

Levonorgestrel B.P./U.S.P., chloroform, & phosphomolybdic acid reagent (Analytical grade), deutrated chloroform, methanol & potassium bromide (Spectroscopy grade) silica gel (TLC grade) and absolute alcohol I.P. were used for the standardization of LNG.

Experimental Methods :

The samples of LNG were standardized as per pharmacopoeial methods.[71,72] The various tests that were carried out included:

Identification :

a) The infra-red absorption spectrum of the drug (in the form of a pellets prepared by dispersing about 1mg of the drug in 50 mg of potassium bromide) was recorded on an infra-red spectrophotometer (Buck Scientific, Model 500) and compared with a standard spectrum of levonorgestrel.

b) The melting range of the drug was determined on a melting point apparatus (Veego Instruments).

c) 2% w/v solution of drug was prepared in chloroform and specific optical rotation of this solution was determined on a polarimeter (Jasco Polarimeter).

d) About 25 mg of drug, accurately weighed, was dissolved in 20 ml methanol, in a 25 ml volumetric flask. An aliquot (1 ml) from this solution was diluted to prepare 0.001% w/v solution in methanol. The light absorption (uv spectrum), in the range 220 to 350nm, of the diluted solution was recorded on a UV-Visible spectrophotometer (Jasco Model 7850).

Loss on drying :

About 1.0 gm of drug, accurately weighed, was taken in a tared weighing bottle, and dried in an oven at 105 ± 3°C for 5 hours. Percent loss on drying was calculated as :

$$\frac{(W_1 - W_2) * 100}{W_1}$$

W1 = Weight of sample before drying

W2 = Weight of sample after drying

Related substances or chromatographic purity :

On a 20 cm x 20 cm thin layer chromatographic plate, coated with chromatographic silica gel mixture, 5 µl of three solutions, containing (1) 2.0% w/v, (2) 0.01% w/v and (3) 0.004% w/v, of drug in chloroform, were applied separately. The plate was placed in a developing chamber containing mobile phase consisting of 96 volumes of chloroform and 4 volumes of absolute alcohol. The chromatogram was allowed to develop until the solvent front moved 15 cm above the line of application. The plate was dried in air and sprayed with phosphomolybdic acid solution. The colour intensity of the spots was noted visually.

Proton NMR spectrum :

The 300 MHz proton NMR spectrum, was obtained by dissolving levonorgestrel in deutrated chloroform.[73]

Assay :

About 100 mg of levonorgestrel was accurately weighed and dissolved in absolute alcohol and diluted quantitatively and stepwise with absolute alcohol to obtain a solution containing about 10 µg/ml. About 10 mg of U.S.P. Norgestrel RS was accurately weighed and dissolved in absolute alcohol to obtain a standard solution having a concentration of about 10 µg/ml. Absorbance of standard and test solutions were recorded at the wavelength of maximum absorbance (240nm), with double beam UV-visible spectrophotometer (Jasco Model

7850), using absolute alcohol as the blank. The quantity, in mg, of $C_{21}H_{28}O_2$ in the portion of LNG was calculated by the formula,

10C (Au/As), in which C is the concentration in µg/ml. of U.S.P. Norgestrel RS in the standard solution, and Au and As are the absorbances of the test and standard solutions respectively.

Results & Discussions :

Results of the various tests performed are given in Table-1. It was found that levonorgestrel samples complied with B.P./U.S.P. specifications, and hence were considered suitable for use in the study.

Table-1. Standardization of LNG sample

Sr. No.	TEST	SPECIFIED LIMIT	OBESERVED LIMIT
1	Identification		
a)	Infra-red spectrum	Principal peaks at 3350, 3270, 2940, 2865, 1655, 1448, 1420, 1335, 1360,1065, 700-670 cm^{-1}	Complied
b)	Melting Range (°C)	232-239	236-238
c)	Specific optical rotation	-30° to -35°	-31.5°
d)	UV spectrum of a methanolic solution	Maximum absorbance only at 240 nm. Extinction about 0.54	Complied
2	Loss on drying (% w/w)	0.5	0.16
3	Related substances	Any secondary spot in the chromatogram obtained with solution (1) is not more intense thsn the spot in the chromatogram obtained with solution (2) and (3)	Complied
4	Proton NMR spectrum	Triplet at δ1.02, singlet at δ2.62 and δ5.86	Complied
5	Assay (% w/w)	98.0-102.0	99.13

2A.2 Characterization of Poly-ϵ-caprolactone (PCL) and Poly (dl-lactide-co-glycolide) (PLGA) :

Biodegradable polymers have numerous physical and chemical characteristics, such as average molecular weight, molecular weight distribution, glass transition and/or melting temperatures and co-monomer ratios. All of these properties can influence the physical behaviour of raw polymers. Further, when these polymers are fabricated into controlled drug delivery systems, additional qualities such as surface area, surface morphology and particle size distribution are introduced and may affect both degradation and drug release from the polymeric system. Therefore, rigid characterization of the polymers and resultant polymeric drug delivery system was considered necessary.[216]

Numerous techniques (end-group analysis, ultra centrifugation, light scattering, electrospray mass spectroscopy) are available for the molecular weight determination of polymers but, the two most familiar methods are solution viscometry and GPC. Although, neither of these methods is a direct or absolute method of molecular weight determination they are being used frequently, as they give reliable relative results.[217]

The T_g of the biodegradable polymers influences the permeability and crystallinity of these polymers and hence alters the rate of drug release and rate of degradation, it was, therefore, necessary to characterize initial T_g and T_m values of the polymers used. DSC being the most widely used technique for measuring T_g and T_m of the polymers was adopted in this study.[218]

The infra-red spectra of the polymers were recorded for the identification of the polymers. Whereas, the moisture content of the polymers was determined because the stability of these polymers is greatly affected by moisture.

Materials :

Poly-ϵ-caprolactone (Sigma Lab. USA), Poly (dl-lactide-co-glycolide) [Courtesy Birmingham Polymers Inc., USA], Poly styrene standards (M_w 1320 to 156 000 daltons) [Polymers Lab. USA], Tetrahydrofuran (HPLC grade), chloroform (spectroscopy grade) and Karl Fischer reagent were used in these experiments.

Experimental Methods :

Determination of molecular weight :

Molecular weights of both the polymers were determined by gel permeation chromatography (GPC). The GPC system consisted of a solvent delivery device (spectraphysics P200, Germany), a refractive index detector (Shodex RI 71), an integrator installed with software capable of directly analyzing GPC molecular weight data, two PL gel columns 10µ, 500 Å for molecular weights of 600-10,000 Da and 5µ for molecular weights of 5,000-600,000 Da - connected in series and a rheodyne injector with 50 µL loop. HPLC grade tetrahydrofuran (THF) was used for solubilizing polymer samples as well as standards and also as the mobile phase. Narrow polystyrene standards with molecular weights ranging from 1320 to 156,000 Da were used for the calibration of GPC system and subsequent analysis of the polymer samples.

System calibration was carried out using polystyrene standard solutions prepared in degassed, prefiltered THF at concentrations of 2 mg/ml. The system was allowed to equilibriate at a flow of 0.75 ml per minute. Polymer samples were prepared in THF at concentrations of 3 mg/ml stirred gently till clear solution was obtained. Solutions were filtered with a 0.2 micron PVDF filter. Duplicate injections were made for all samples.[219]

Determination of glass transition temperature (for PLGA) and melting point (for PCL) :

The glass transition temperature (Tg) or melting point of the polymer was determined by Perkin Elmer Differential Scanning Calorimeter (DSC4). Calibration of the system at a heating rate of 10°C per minute, was performed by temperature and area calibration of zinc and indium standard. Sample analysis was accomplished by accurately weighing 2-8 mg of the polymers samples and sealing them separately in two aluminium pans. An empty sealed aluminium pan was used as the reference. While under a nitrogen purge, the sample and reference were heated at a rate of 10°C per minute from 40°C to 150°C.[220]

Infra-red spectrum :

The infra-red absorption spectrum of the polymer (in the form of a solution prepared by dissolving 10 mg of the polymer in 25 ml of chloroform) was recorded on a infra-red spectrophotometer.[221,222]

Moisture-content :

The moisture content of both the polymers was determined by Karl Fischer method described in the USP.[72]

Results & Discussion :

Results of the study are summarized in Table-2, Fig.1 and Fig.2. GPC results indicate that the polymers have wide molecular weight distributions. The values of T_g and T_m were in good agreement with the reported ones.[178,188] The infra-red spectra of PLGA and PCL were concordant with the reported spectra.[178,223] Both the polymers were free from moisture.

Table-2. Charactrization of PCL and PLGA

Sr. No.	TEST	PCL	PLGA
1	GPC		
a)	M_w	155518 Da	89382 Da
b)	M_n	39346 Da	34818 Da
c)	PD = M_w/M_n	3.95	2.56
2	DSC	Endothermic peak at 63.38°C corresponding to its T_m	Endothermic peak at 57.95°C corresponding to its T_g
3	Infra-red spectrum	Principal peak at 1720 cm^{-1} corresponding to ester group	Principal peak at 1748 cm^{-1} corresponding to ester group
4	Moisture content	Nil	Nil

Fig - 1. IR spectrum of PLGA

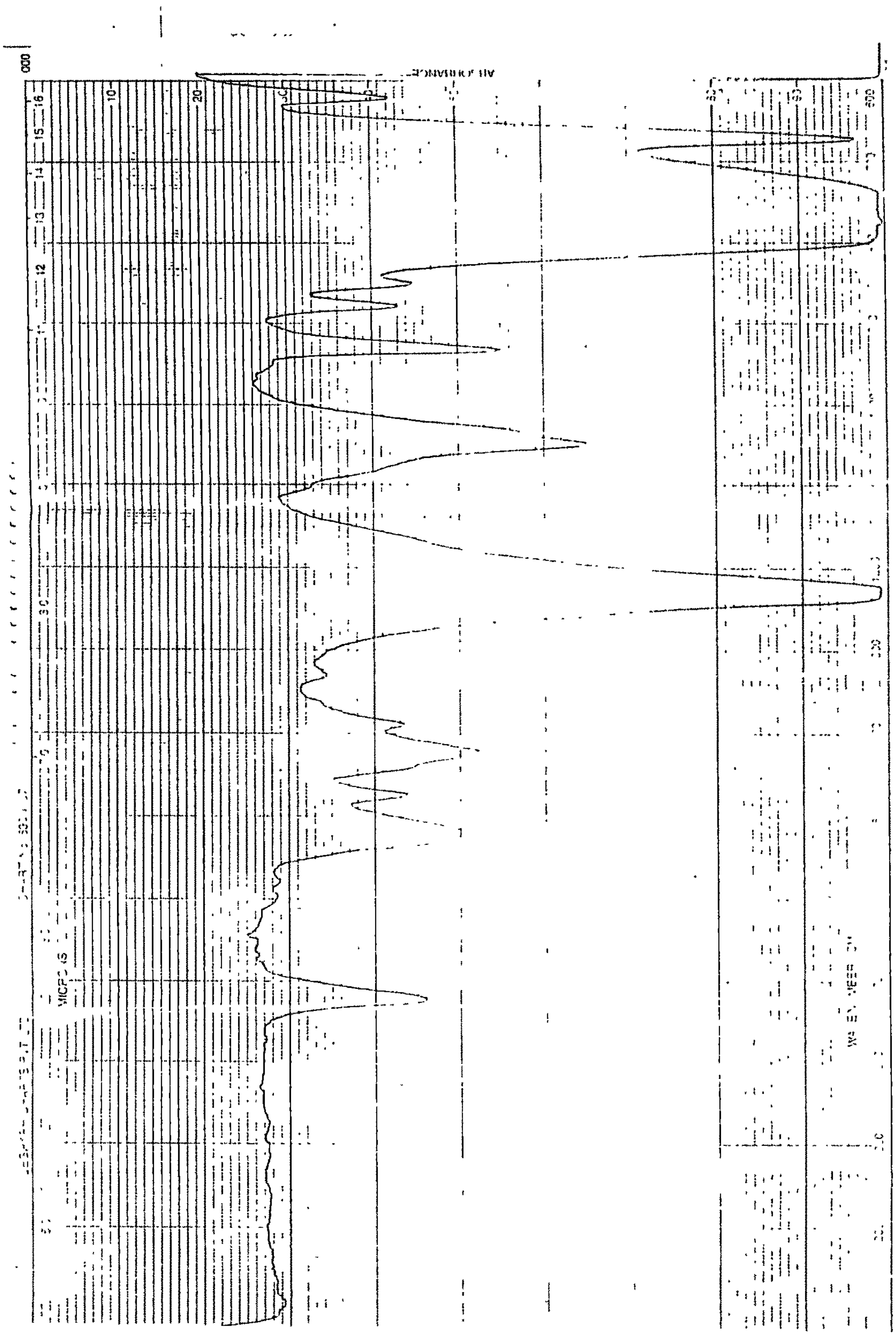
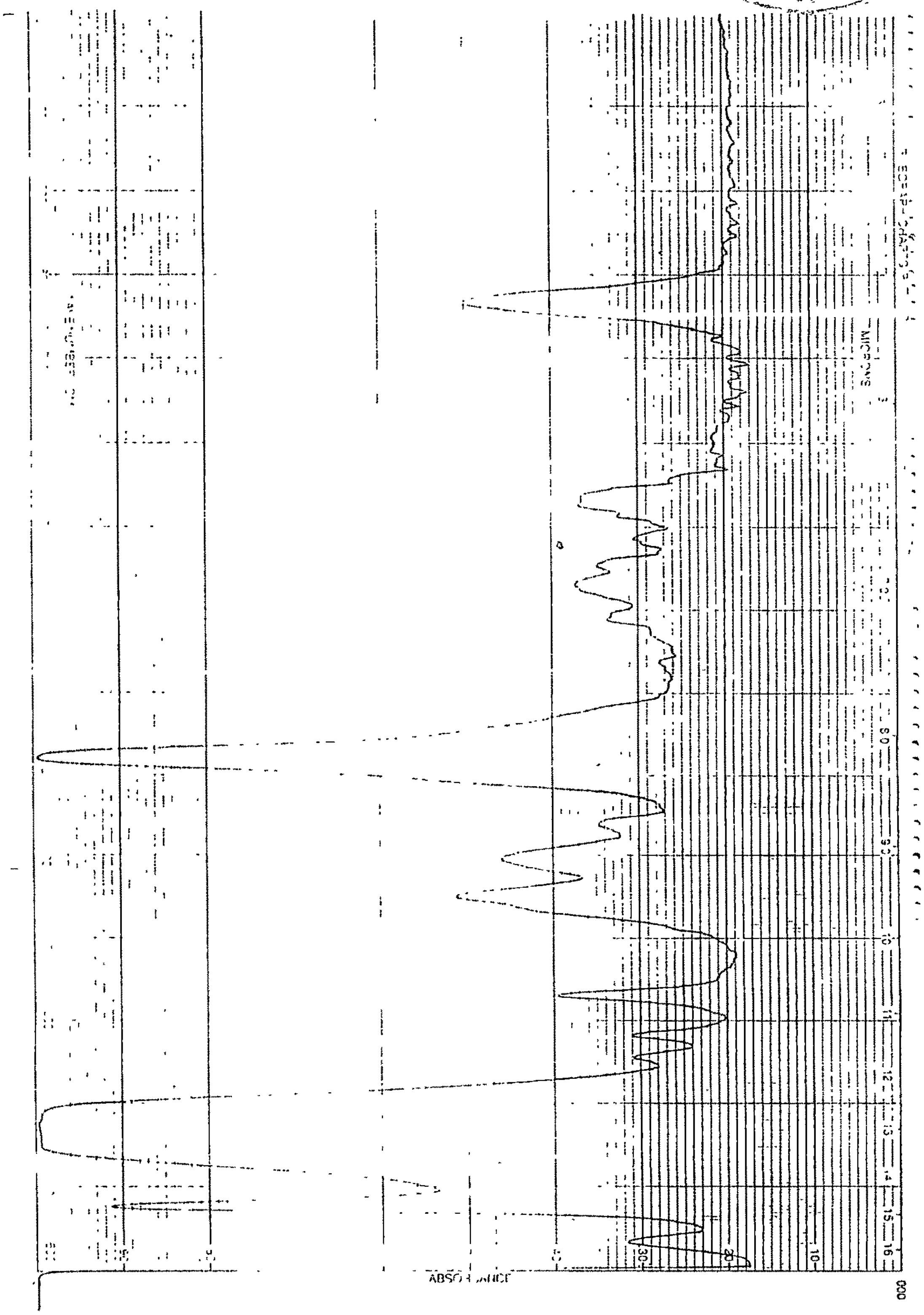


Fig - 2. IR spectrum of PCL



2A.3 Stability of the Drug - Polymer(s) Mixtures :

It has been reported that, biodegradable polymers, such as poly(dl-lactide-co-glycolide), when stored at a temperature above their glass transition temperature and at high humidity conditions, show changes in their physicochemical properties.[224-226] Therefore, the drug delivery systems prepared from such polymers have to be stored at low temperatures, preferably below their glass transition temperature. However, this is not possible in cases where the glass transition temperature of the polymer is very low eg. PCL T_g : - 65°C. The objective of this study was to evaluate the feasibility of storing the dosage forms prepared with PCL and PLGA at 0-4°C.

<u>Materials</u> :

The materials used for this study were; Levonorgestrel B.P./U.S.P., Poly-ϵ-caprolactone (M_w 155000), Poly (dl-lactide -co-glycolide) (M_w 89000) and absolute alcohol I.P.

<u>Experimental Methods</u> :

Levonorgestrel 50 mg, was accurately weighed and mixed with either PCL alone (50 mg) or PCL and PLGA (each polymer 25 mg). The mixtures were separately sealed in glass vials (U.S.P. Type 1) and stored at 0-4°C for one month. The DSC thermograms of these mixtures, before and after storage at 0-4°C, were recorded by the method described under the characterization of polymers (Section 2A.2).

The drug content of these mixtures, before and after storage at 0-4°C, was determined by UV-spectrophotometry. An accurately weighed sample of the drug-polymer mixture, equivalent to 5 mg of levonorgestrel, was transferred to a 100 ml round bottom flask, containing 50 ml of absolute alcohol. The contents were refluxed on a water bath for about 15 min., cooled to ambient temperature (30 ± 3°C) and transferred to a 100 ml volumetric flask. The round bottom flask was rinsed thrice with 10 ml of absolute alcohol. All the rinsings were mixed with the contents of 100 ml volumetric flask. The volume was made up to 100 ml with absolute alcohol. About 10 ml of this solution was filtered through a 0.45 micron filter attached to a syringe and collected in a stoppered test tube. An aliquot (2 ml) from the filtered solution was diluted to 10 ml, with absolute alcohol. A standard solution of levonorgestrel, in absolute alcohol, having concentration of about 10 μg/ml was prepared. Absorbances of standard solution and test solutions were recorded at 240 nm, on a double beam UV-visible spectrophotometer, using absolute alcohol as a blank. The quantity, in mg, of levonorgestrel in the portion of drug-polymer mixture was calculated by the formula 0.5C (Au/As) in which C is the concentrations, in μg per ml, of the levonorgestrel in standard solution and Au and As are the absorbances of the sample and standard solutions respectively.

<u>Results & Discussions</u> :

DSC thermograms (Fig.3 and Fig.4) of the drug-polymer mixtures, before and after storage at 0-4°C for one month, were found to be superimposable. The levonorgestrel content of these mixtures, before and after storage, was found to be same. These results indicated that the dosage forms prepared using these polymers can be stored at 0-4°C.

Fig - 3. DSC scans of LNG and PCL mixtures

——— INITIAL, ------- AFTER STORAGE AT 0-4°C

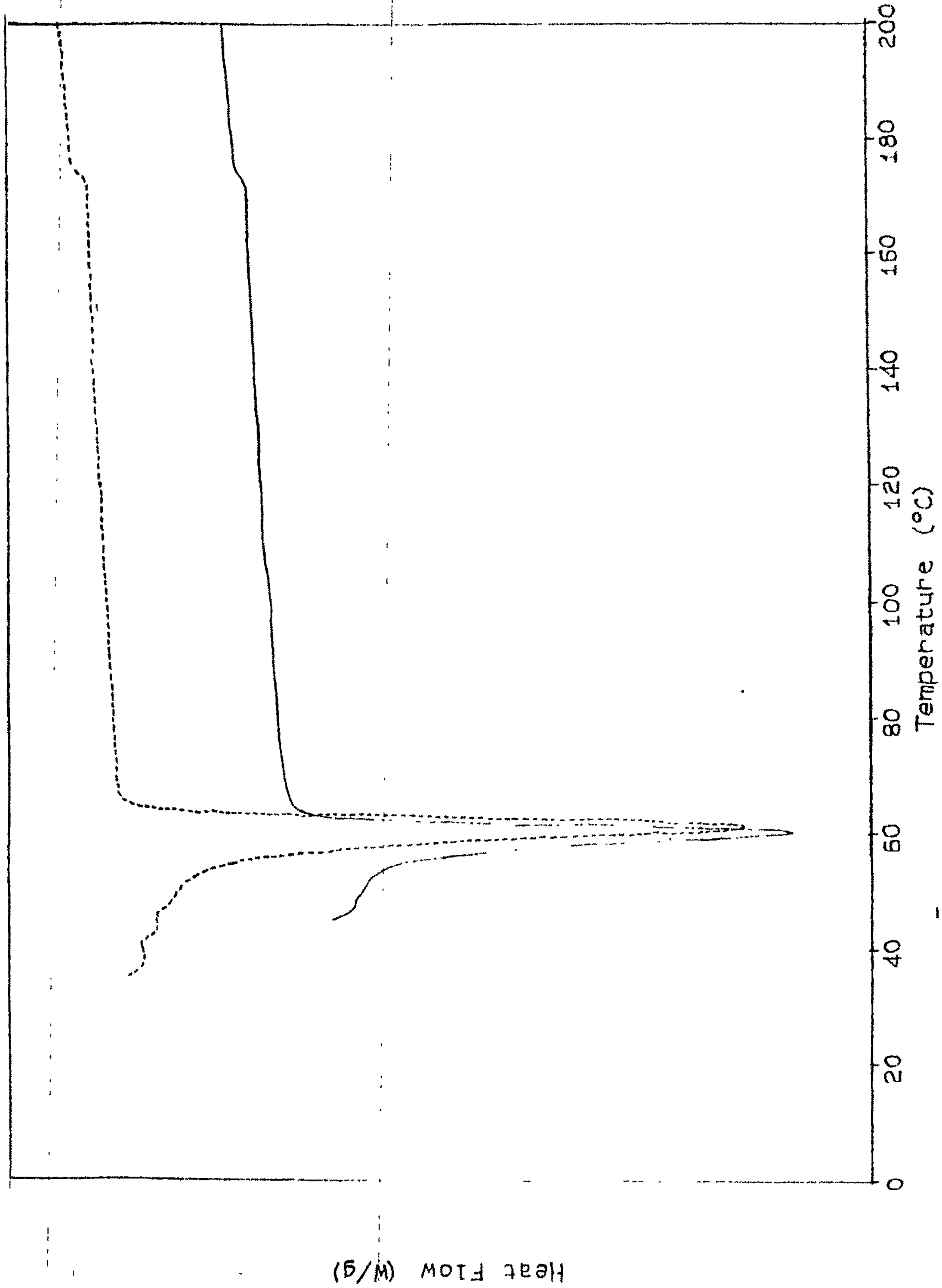
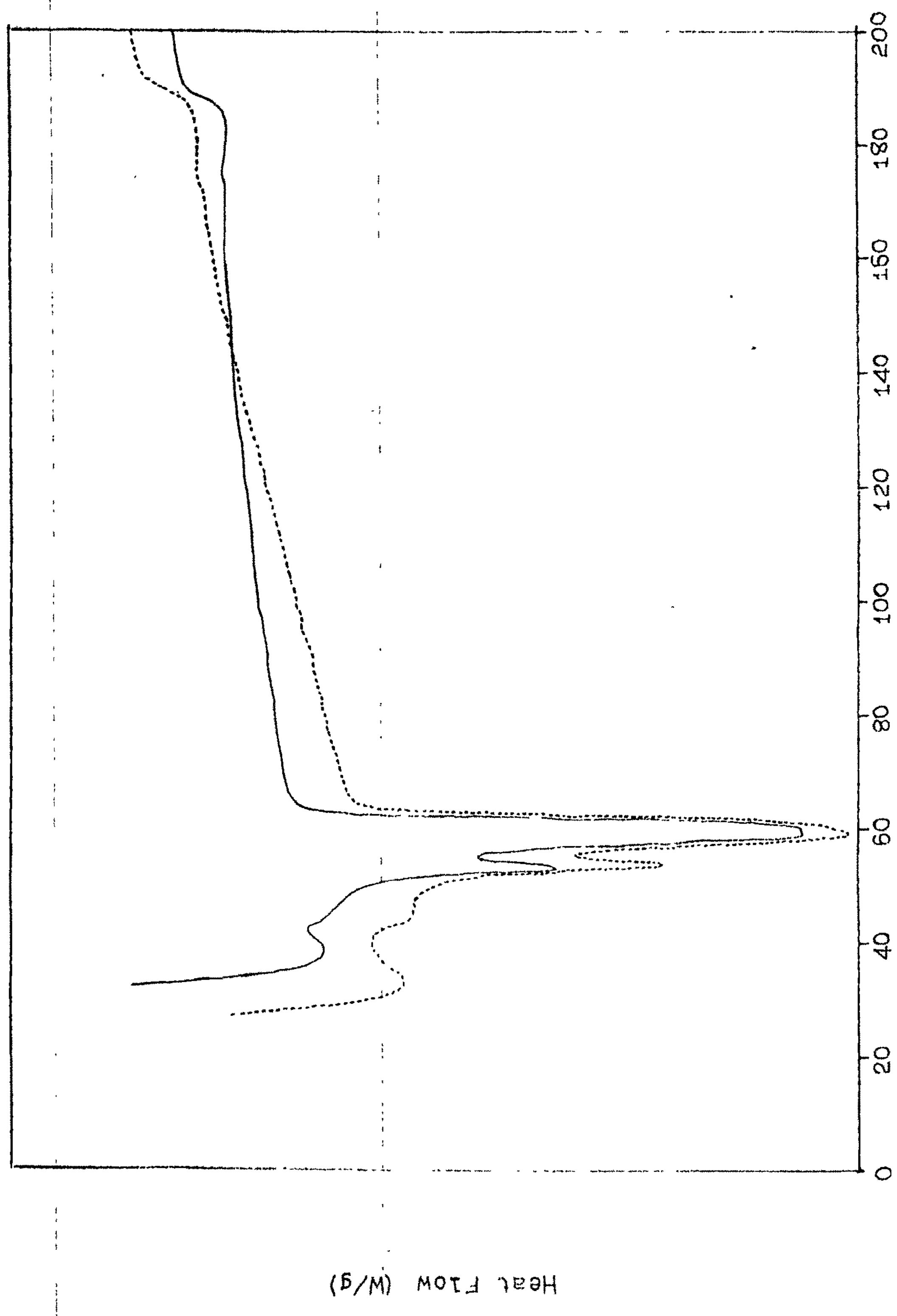


Fig - 4. DSC scans of LNG, PCL and PLGA mixtures

——— INITIAL, ------- AFTER STORAGE AT 0-4°C



2A.4 Feasibility studies on Radiation Sterilization of Drug and Polymers :

Sterility is an important criterion required of parenteral products. Hence all parenteral delivery systems have to be subjected to a suitable sterilization process. However, for biodegradable polymers or drug delivery systems made from these polymers, the choice of sterilization process is restricted. Parenteral drug delivery system based on biodegradable polymers need to be prepared by aseptic processing or they have to be terminally sterilized by gamma irradiation.

The main difference in the two approaches is the sterility assurance level (SAL) of the final product. Typically aseptic processes result in an SAL of 10^{-3}, or that there is chance that as many as 1 unit per 1000 units of final product is contaminated. Whereas, terminal sterilization processes generally result in an SAL of 10^{-6} or the chance that upto 1 unit per one million units of product is non-sterile. Therefore, terminal sterilization process is preferred over aseptic processing.[205]

The objective of this study was to find out the minimum dose of gamma radiation required for the sterilization of drug and polymer and also to evaluate the stability of the samples thus sterilized. The irradiation was carried out at the radiation sterilization plant, ISOMED, B.A.R.C., Mumbai.

2A.41 Radiation sterilization of levonorgestrel

Materials :

Levonorgestrel B.P./U.S.P., chloroform, & phosphomolybdic acid reagent (Analytical grade), deutrated chloroform, methanol & potassium bromide (Spectroscopy grade) silica gel (TLC grade), absolute alcohol I.P., Fluid thioglycolate medium & Soyabean casein digest medium (Hi media) were employed in this study.

Experimental Methods :

Levonorgestrel, sealed in glass vials under nitrogen atmosphere, was exposed to four doses of gamma radiation viz. 1.0, 1.5, 2.0 and 2.5 Mrads. The irradiated samples were subjected to sterility test.

Test for sterility :

The samples of LNG exposed to different doses of gamma radiation were evaluated for sterility by direct innoculation technique.[227] Fluid thioglycolate medium was used for detection of aerobic and anaerobic bacteria and soyabean casein digest medium was used for detection of fungi.

The media were sterilized by autocloaving at 15 psi and 121°C for 30 minutes. About 50 mg of the samples in triplicate were aseptically added to tubes of the two media. The innoculated tubes were gently shaken, and incubated as follows :

- Fluid thioglycolate medium tubes : 37°C ± 1°C

- Soyabean casein digest medium tubes : 25°C ± 0.5°C

Controls :

Uninnoculated tubes of both media, as negative control and tubes of media innoculated with test cultures (B.Subtilis, S.aureus and A.niger) as positive control, were also incubated, similarly. Also sets of tubes were innoculated with samples of the substance under test along with the test cultures and incubated to check whether the sample had any growth inhibiting properties on bacteria and fungi. All the tubes were incubated and observed for a period of 15 days for signs of growth.

The various other tests which were performed to detect any change in the physicochemical properties of the radiated drug samples included :

- Visual appearance
- Melting range
- Specific optical rotation
- Infra-red spectrum
- UV spectrum
- Proton NMR spectrum
- Related substances and
- Assay

The procedures used for these tests were the same as described in standardization of LNG (Section 2A.1).

Results & Discussions :

The characteristics of the samples of LNG exposed to different doses of Co-60 gamma irradiation, are given in Table-3. LNG did not show any marked changes in physicochemical properties at the studied doses of gamma radiation. The IR and proton NMR spectra of the samples subjected to gamma radiation dose of 1.0 and 2.5 Mrads, showed similar principal peaks as the unsterilized sample (Fig.5 to Fig.8), the UV spectra of all samples were superimposable. The TLC showed absence of additional spots, and the Rf value, of the principal spot, in all the samples was 0.75. The LNG samples subjected to radiation dose of 1.5 Mrads, 2.0 Mrads, and 2.5 Mrads were found to be sterile and those exposed to lower radiation dose were found to be non-sterile.

2A.42 Radiation sterilization of PCL and PLGA

Materials :

Poly-ϵ-caprolactone, Poly (dl-lactide - co -glycolide), Poly styrene standards (M_w 1320 to 156 000 daltons) [Polymers Lab. USA], Tetrahydrofuran (HPLC grade), Fluid thioglycolate medium & Soyabean casein digest medium (Hi media) were required for this study.

Experimental Methods :

Since the minimum dose for sterilization of LNG was found to be 1.5 Mrads, all the polymer samples were exposed to only two different doses of gamma radiation i.e. 1.5 Mrads and 2.5 Mrads. The latter is a normally recommended dose of

Table-3. Physicochemical characterization of LNG samples exposed to different doses of gamma radiation

Sr. No.	Characteristic	0.0 Mrads	1.0 Mrads	1.5 Mrads	2.0 Mrads	2.5 Mrads
1	Visual Appearance	-------- White crystalline odourless powder ---------				
2	Melting Range (°C)	236-238	235-237	235-237	235-237	235-237
3	Specific Optical Rotation	-31.5°	-34.1°	-32.0°	-32.5°	-32.5°
4	Related Substances	-- Complied with the pharmacopoeial specifications -- (no additional spots seen)				
5	Assay (% w/w)	99.13	98.82	98.71	98.93	98.98

Fig - 5. IR spectra of un-irradiated & irradiated LNG samples

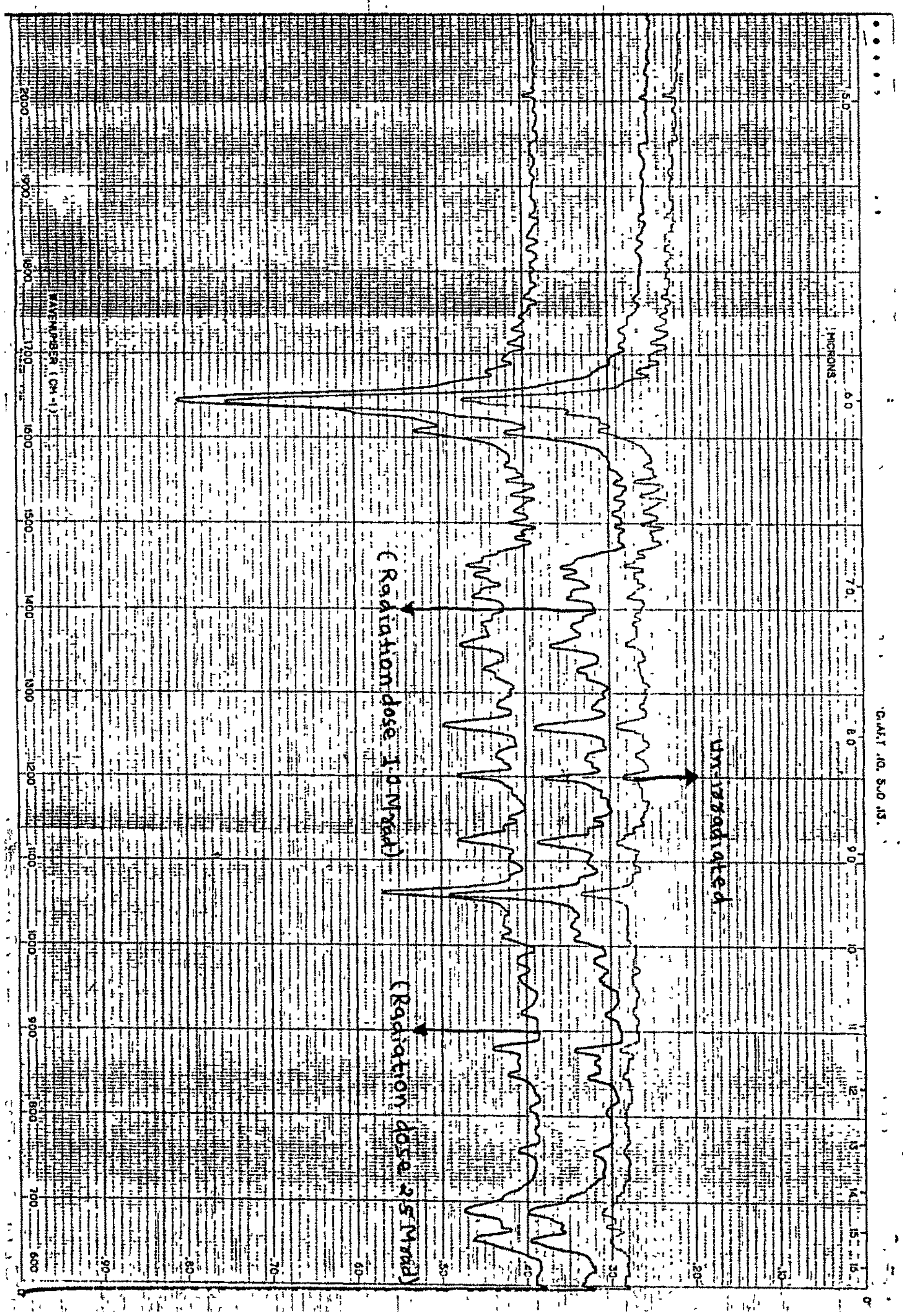


Fig - 6. ^1H NMR of un-irradiated LNG

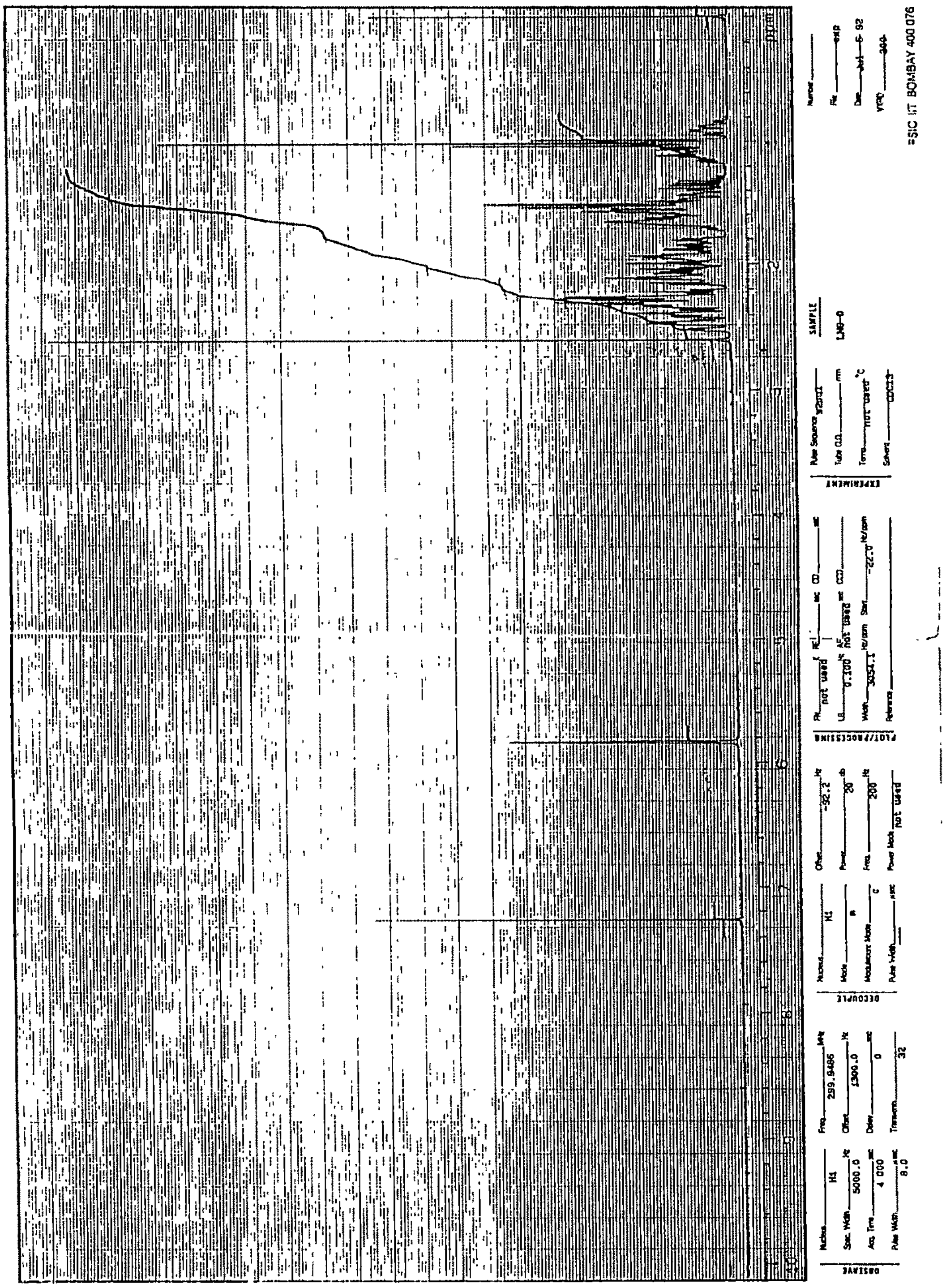


Fig - 7. ^{1}H NMR of LNG exposed to gamma radiation
(Dose 1.0 Mrads)

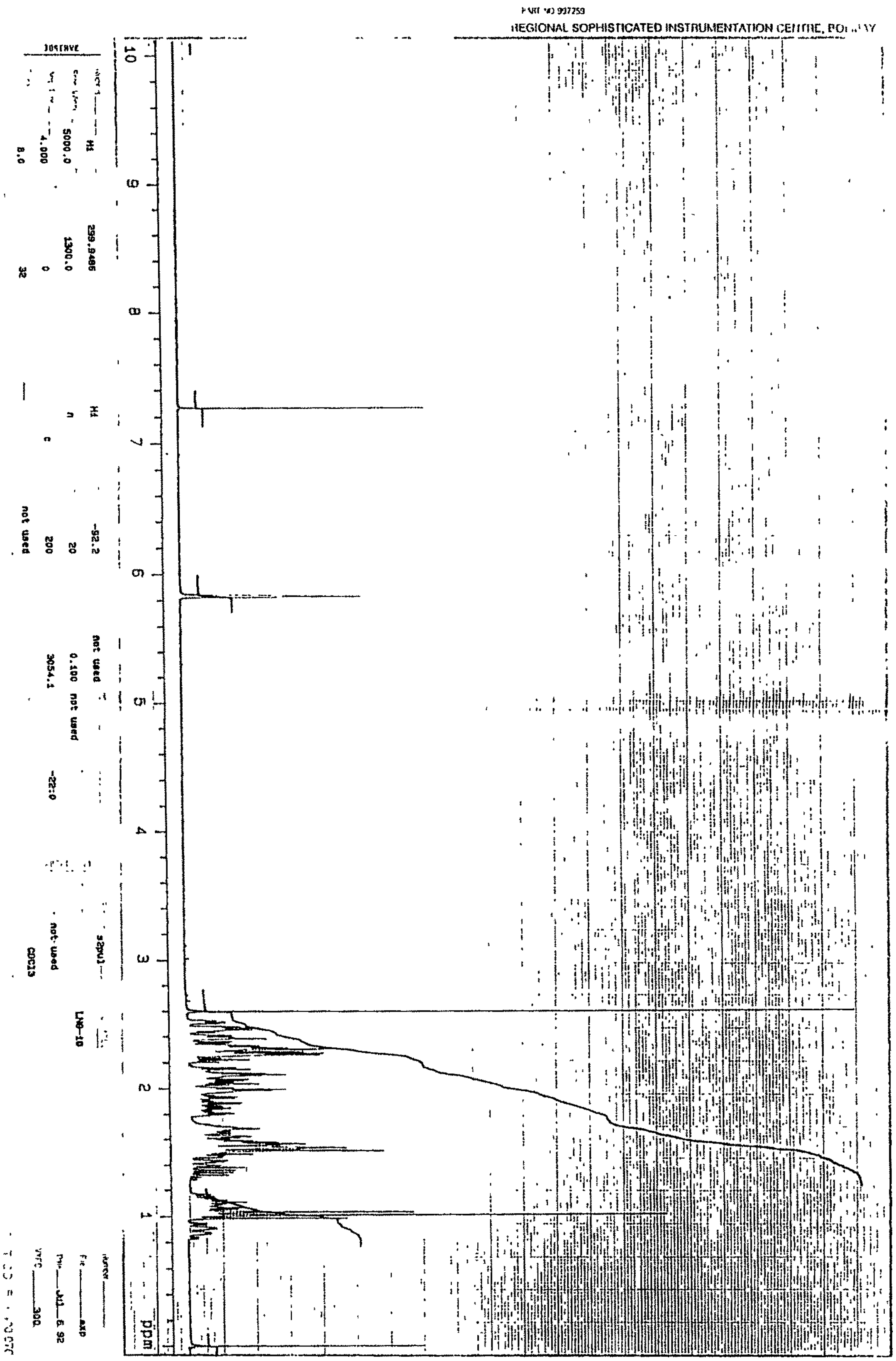
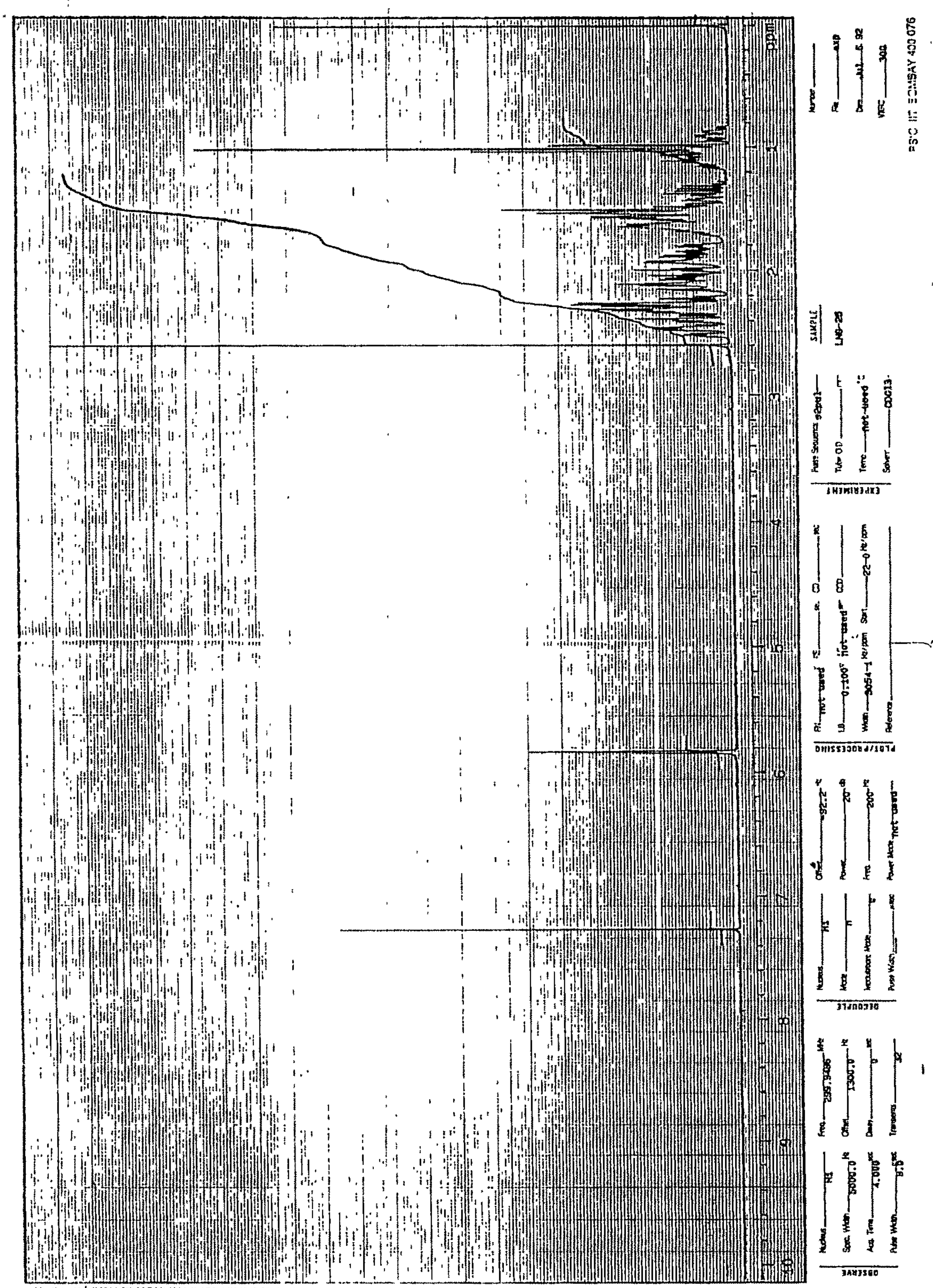


Fig - 8. ^{1}H NMR of LNG exposed to gamma radiation
(Dose 2.5 Mrads)



gamma radiation for the sterilization of pharmaceutical products.

Polymers were separately sealed in glass vials, under nitrogen atmosphere. These vials were then exposed to the different doses of gamma radiation. The irradiated polymers were evaluated for sterility by the method described earlier. The samples were also characterized by GPC and DSC to evaluate the effect of radiation dose on each polymer. The procedures used for these tests were same as those described in characterization of polymers (Section A.2).

Results & Discussion :

Average molecular weights and T_g or T_m of the samples of PCL and PLGA exposed to 1.5 and 2.5 Mrads dose are given in Table-4 and Table-5. Molecular weight distribution and DSC thermograms are shown in Fig.9, 10, 11 and 12. Both the polymers when exposed to radiation dose of 1.5 Mrads and 2.5 Mards were found to be sterile.

The poly-ϵ-caprolactone sample exposed to radiation dose of 1.5 Mrads showed increase in the M_w, M_n, T_m and decrease in the PD, values as compared to the un-irradiated sample. On the other hand, sample exposed to radiation dose of 2.5 Mrads showed decrease in M_w & PD and increase in M_n values as compared to un-irradiated samples. However, these changes were not signficant. T_m of the un-irradiated sample and the sample sterilized at 2.5 Mrad was same. These results showed that at lower doses (1.5 Mrads) of gamma irradiation PCL has tendency to undergo polymerization, whereas, at higher dose (2.5 Mrads) depolymerization occurs. Such results have been reported for poly lactic acid which is often used in drug delivery systems.[205]

The PLGA samples exposed to radiation dose of 1.5 Mrads and 2.5 Mrads showed decrease in M_w, M_n, T_g and increase in PD values as compared to those of un-irradiated PLGA, indicating significant depolymerization of the polymer at the studied doses of gamma irradiation. Deluca et al also observed similar effects of gamma irradiation on the molecular weights of poly (dl-lactide-co-glycolide) microparticles.[228,229] However, Aso et al have reported that the *in vitro* release pattern, and hence the release rates, of progesterone from the PLGA microspheres altered significantly after subjecting them to different doses of gamma radiation. Further, it has been reporetd that the desired *in vitro* release profile could be obtained by careful selection of the radiation dose.[230]

It was therefore, decided to prepare drug delivery systems either with only PCL which has higher permeability for steroids and lower biodegradation rate as compared to PLGA or using combination of PCL and PLGA so that the rate of biodegradation of the dosage form as well as the rate of drug release can be altered beneficially.

Further, since the radiation dose of 2.5 Mrads did not show any adverse effects on the properties of LNG or PCL, it was considered as suitable dose of gamma radiation for the sterilization of drug delivery systems.

Table-4. Characteristics of PCL samples exposed to different doses of gamma radiation

Sr. No.	Test	0.0 Mrads	1.5 Mrads	2.5 Mrads
1	GPC (Fig.9)			
a)	M_w (Daltons)	155518	157231	147867
b)	M_n (Daltons)	39346	47461	45601
c)	PD	3.95	3.31	3.24
2	DSC (Fig.10) T_m (°C)	63.38	64.04	63.44
3	Test for Sterility	Fails	Passes	Passes

Table-5. Characteristics of PLGA samples exposed to different doses of gamma radiation

Sr. No.	Test	0.0 Mrads	1.5 Mrads	2.5 Mrads
1	GPC (Fig.11)			
a)	M_w (Daltons)	89392	82128	73254
b)	M_n (Daltons)	34818	31820	26573
c)	PD	2.56	2.58	2.75
2	DSC (Fig.12) T_g (°C)	57.95	57.72	51.98
3	Test for Sterility	Fails	Passes	Passes

Fig - 9. M_w V/S % Area plots of PCL before and after subjecting to different doses of gamma radiation

——— 0.0 Mrads; ...... 1.5 Mrads; ----- 2.5 Mrads

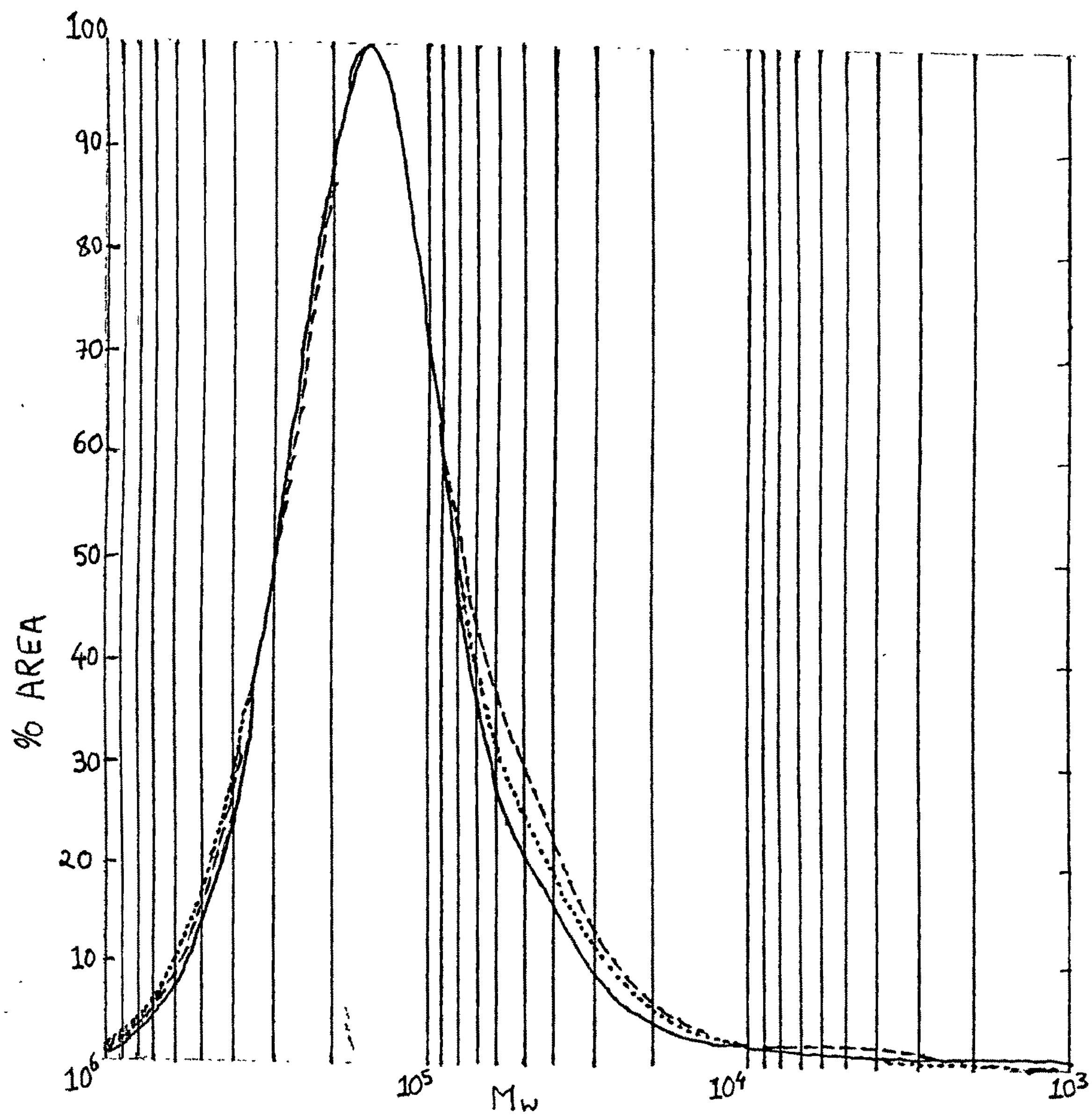


Fig - 10. DSC scans of PCL before and after subjecting to different doses of gamma radiation

——— 0.0 Mrads; ...... 1.5 Mrads; ----- 2.5 Mrads

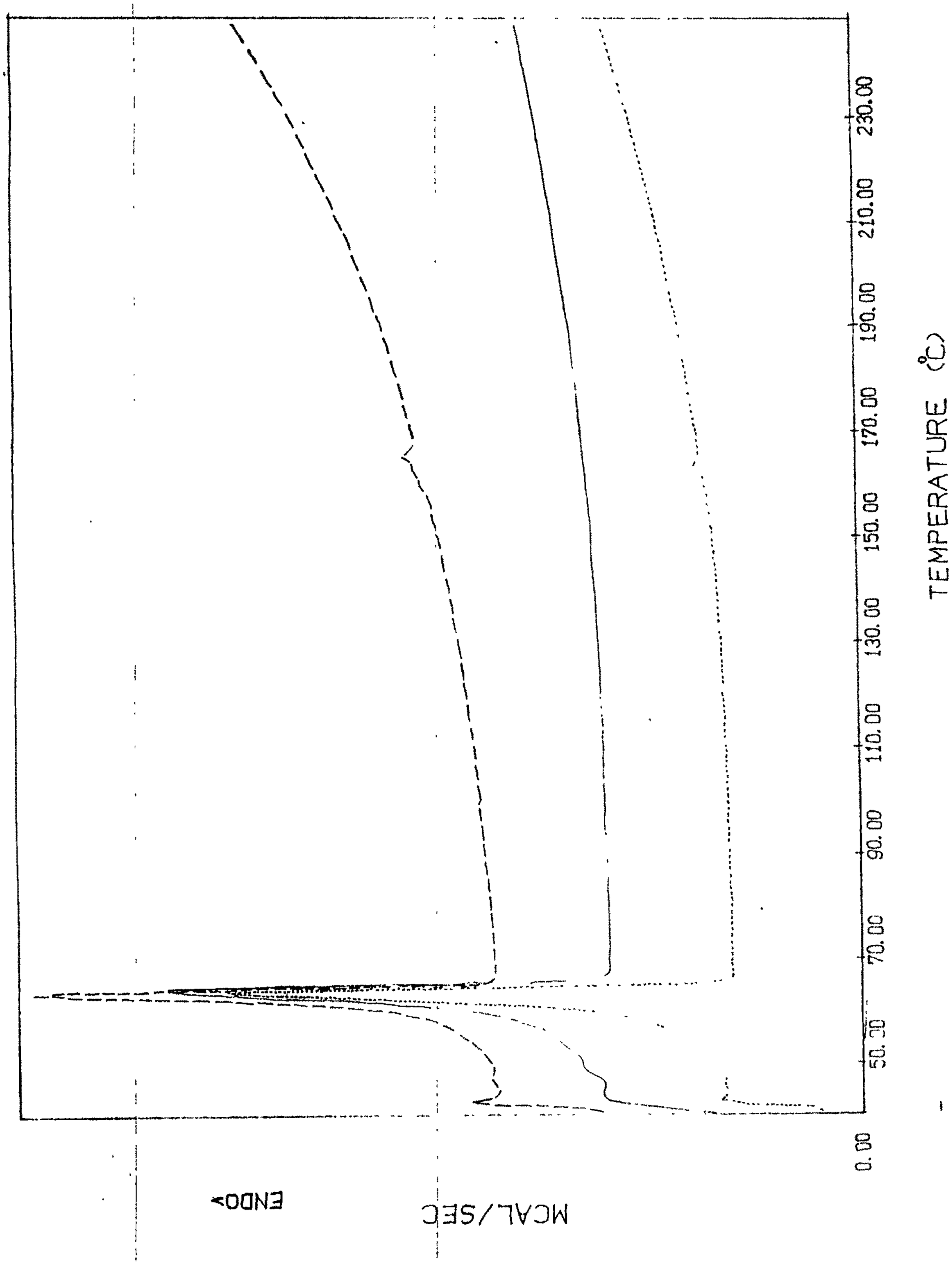


Fig - 11. M_w V/S % Area plots of PLGA before and after subjecting to different doses of gamma radiation

———— 0.0 Mrads; ...... 1.5 Mrads; ----- 2.5 Mrads

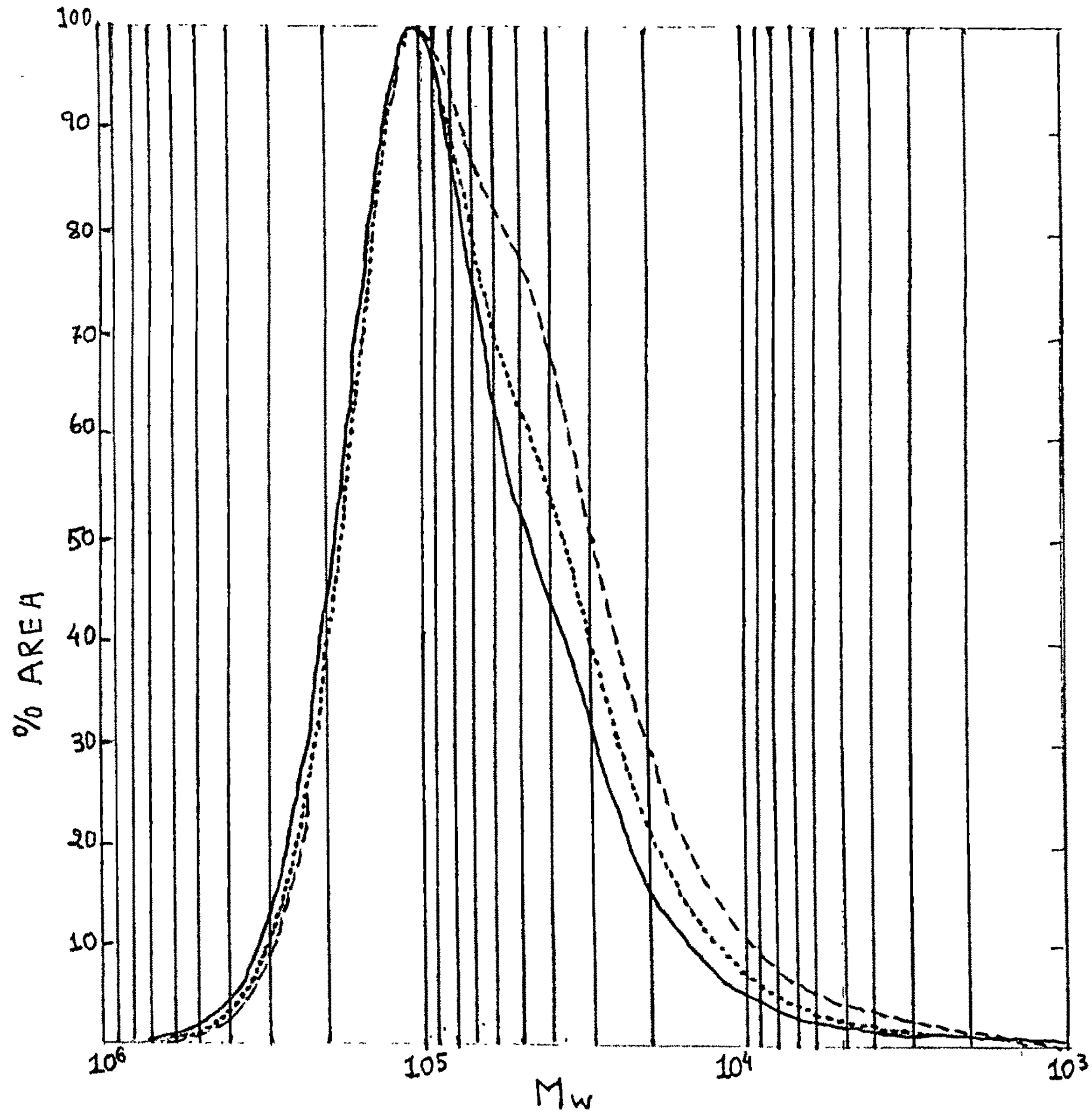
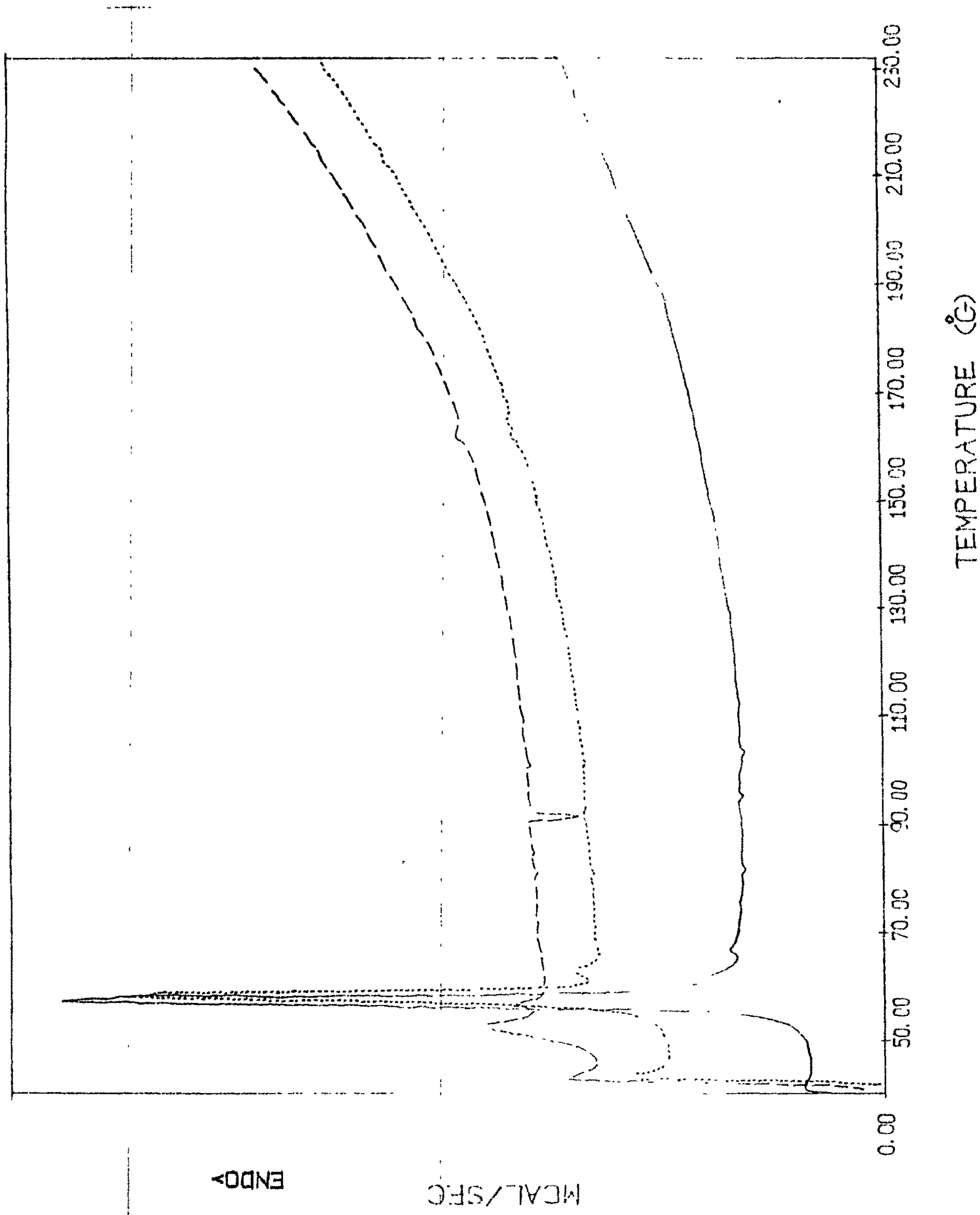


Fig - 12. DSC scans of PLGA before and after subjecting to different doses of gamma radiation

——— 0.0 Mrads; ...... 1.5 Mrads; ----- 2.5 Mrads



2B Development of Injectable and Implantable Dosage Forms

The present investigation describes the development & evaluation of long acting injectable (microspheres) and implantable formulations of LNG using biodegradable polymers. The study was initiated with the optimization of process parameters for the preparation of microspheres. The drug loaded microspheres were then prepared under optimized process conditions. Levonorgestrel containing microspheres thus prepared, were evaluated for their physicochemical characteristics and *in-vitro* release profile. Selected batches of microspheres were then characterized for their polymer properties. Three different polymers were evaluated for their suitability as a suspending agent for dispensing the microspheres. Levonorgestrel containing implants were then prepared from the microspheres by a reported method. These implants were evaluated for their physicochemical properties as well as *in-vitro* release pattern. The selected formulations (microspheres as well as implants) were subjected to accelerated stability testing after radiation sterilization. The biocompatibility and pharmacodynamic activity of the selected formulations were evaluated in suitable animal models.

2B.1 Development of an Injectable Dosage Form

2B.11 Optimization of process parameters for the preparation of microspheres

An emulsion solvent evaporation technique was used for the preparation of microspheres.[231] This method involves three interrelated processes namely (i) droplet formation, (ii) droplet stabilization and (iii) droplet hardening.[232-234]

Process parameters such as stirring rate, volume and viscosity of the dispersed as well as continuous phase have been reported to influence the particle size distribution of microspheres by virtue of their effect on droplet formation and droplet stabilization.[235,236] Additionally, change in particle size distribution is reported to influence the rate of drug release as it alters the total surface area of microspheres.[237-238] Further, microspheres with particle size ranging between 10 to 250 µm have been reported to be suitable for subcutaneous administration.[234] It was, therefore, necessary to identify critical process variables and optimize them for the preparation of levonorgestrel loaded microspheres with particle size ranging between 10 to 250 µm.

Poly-ϵ-caprolactone microspheres without drug were prepared to study the influence of process parameters, such as stirring rate, emulsifier concentration in the aqueous phase, type of emulsifier, polymer concentration in organic phase, and ratio of organic to aqueous phase, on the particle size distribution of the microspheres.

<u>Materials</u> :

Poly-ϵ-caprolactone, methylene chloride (analytical grade), fused calcium chloride (laboratory grade), poly vinyl alcohol (s-d Fine chemicals) and hydroxypropyl methylcellulose (Methocel E4M) & methylcellulose (Methocel A4M) [courtesy Colorcon, U.K] were used during the study.

Experimental Methods :

Poly-ϵ-caprolactone was dissolved in methylene chloride, in a stoppered conical flask. Aqueous solution of poly vinyl alcohol (PVA) or methylcellulose (MC) or hydroxypropyl methylcellulose (HPMC) was prepared separately in distilled water, in a beaker. Poly-ϵ-caprolactone solution was poured into the beaker, containing aqueous phase, under continuous stirring. The stirring was continued, at ambient temperature (27°C ± 2°C) and atmospheric pressure until a stable emulsion was formed. This emulsion was transferred into a round bottom flask attached to a rotary evaporator. Methylene chloride was removed from the emulsion by gradually decreasing the pressure to 600 mm of Hg. Microspheres were filtered on a 0.45 micron filter, washed with water and dried over fused calcium chloride for 48hrs.

Different batches of blank poly-ϵ-caprolactone microspheres prepared for the optimization of process parameters, are shown in Table-6. Particle size of 300 microspheres, from each batch was measured by optical microscopy.

Results & Discussions :

Mean particle diameters and particle size distribution of different batches of blank microspheres are shown in Table-7. Microspheres with smaller mean particle diameter and narrow size distribution were obtained with increasing stirring rate and emulsifier concentration. On the other hand with increase in poly-ϵ-caprolactone concentration in

Table - 6 : Process conditions employed in the preparation of different batches of blank microspheres

B.NO.	Stirring rate (rpm)	Type of Emulsifier	Emulsifier Conc. (% w/w)	PCL Conc. (% w/v)	Organic to aqueous phase ratio
BM-1	4000	PVA	0.5	10	1:40
BM-2	2000	PVA	0.5	10	1:40
BM-3	1000	PVA	0.5	10	1:40
BM-4	4000	PVA	0.2	10	1:40
BM-5	4000	PVA	0.1	10	1:40
BM-6	4000	PVA	0.5	10	1:20
BM-7	4000	PVA	0.5	10	1:10
BM-8	4000	PVA	0.5	5	1:20
BM-9	4000	PVA	0.5	2.5	1:20
BM-10	4000	MC	0.5	5	1:20
BM-11	4000	HPMC	0.5	5	1:20

Table - 7 : Particle size distribution different batches of blank microspheres

B.NO.	Mean particle diameter (μ)	< 10μ (%)	10-25μ (%)	25-50μ (%)	50-150μ (%)	150-200μ (%)
BM-1	23.3	18.30	44.70	28.00	9.00	-
BM-2	28.3	10.00	47.00	27.00	16.00	-
BM-3	37.2	9.67	38.67	28.67	21.67	1.33
BM-4	36.6	8.67	40.33	24.67	24.33	2.00
BM-5	31.6	13.00	38.67	29.33	18.00	1.00
BM-6	25.9	15.33	44.67	30.33	9.67	-
BM-7	31.7	5.00	41.33	36.33	17.33	-
BM-8	17.8	13.67	64.67	21.00	-	-
BM-9	11.7	25.67	74.00	-	-	-
BM-10	25.8	1.00	46.67	57.33	1.00	-
BM-11	15.9	15.33	74.00	10.67	-	-

Table - 8 : One way anova for the particle size distribution of the blank microspheres

Sr. No.	Variable studied	Calculated F Value	F (0.05,2,6)	Conclusion $p < 0.05$
1	Stirring rate	9.06	5.14	S
2	Emulsifier Conc.	9.78	5.14	S
3	Organic to aqueous phase ratio	5.67	5.14	S
4	Polymer Conc.	28.41	5.14	S
5	Emulsifying agent	14.23	5.14	S

organic phase, and organic to aqueous phase ratio, the mean particle diameter increased. Microspheres with smaller mean particle diameter were obtained with PVA and HPMC as compared to those with MC. Furhter, it was observed that the HPMC and MC produced elongated microspheres, which were adhering to each other. Statistical analysis (one way ANOVA) showed (Table-8) significant effect of each variable on the particle size distribution of the microspheres at $P < 0.05$.

Following process parameters were therefore selected for the preparation of LNG loaded microspheres :

stirring rate - 4000 rpm;

emulsifier - PVA at 0.5% w/v concentration;

polymer concentration - 5% w/v and

organic to aqueous phase ratio -1:10.

2B.12 Preparation & evaluation of levonorgestrel loaded microspheres

Materials :

Levonorgestrel B.P./U.S.P., poly-ε-caprolactone, poly (dl-lactide-co-glycolide), poly vinyl alcohol, methylene chloride (analytical grade) and fused calcium chloride (laboratory grade) were used for the preparation of drug loaded microspheres. Absolute alcohol, Karl Fischer reagent and tween 60 (analytical grade) were used for the characterization of drug loaded microspheres.

Experimental Methods :

Levonorgestrel (0.1 - 0.4 gm), PCL (0.36 - 1.9 gm) and/or PLGA (0.36 - 1.44 gm) were dissolved in 40 ml methylene chloride. This drug polymer solution was poured into 400 ml distilled water containing 0.5% w/v polyvinyl alcohol as an emulsifier and stirred at 4000 rpm at 27 ± 2°C and atmospheric pressure. Once a stable emulsion was formed, methylene chloride was removed at 600 mm of Hg. Microspheres formed were filtered on 0.45 micron PVDF filter, washed with 500 ml distilled water and dried over fused calcium chloride for 48hrs. The dried microspheres were stored at -20°C in airtight containers in a desiccator. The compositions of different batches of drug loaded microspheres are shown in Table-9.

All batches of levonorgestrel containing microspheres were evaluated for :

(i) Drug content :

Levonorgestrel content of the microspheres was determined by UV spectrophotometry as described under stability of drug - polymer(s) mixtures in preformulation studies (section 2A.3).

(ii) Moisture content :

Moisture content of the microspheres was determined by Karl Fischer method.

An accurately weighed sample (about 100 mg) of the microspheres was dispersed in previously neutralized

Table - 9 : Composition of different batches of LNG loaded microspheres

B.No.	LNG content (% w/w)	PCL content (% w/w)	PLGA content (% w/w)
DLM-1	05	95	-
DLM-2	10	90	-
DLM-3	20	80	-
DLM-4	10	72	18
DLM-5	10	54	36
DLM-6	10	36	54

methanol and titrated against Karl Fischer reagent on an Elico Karl Fischer Titrator.

(iii) Particle size distribution :

Microspheres were dispersed in a 0.05% v/v aqueous solution of tween 60 and particle size was determined by laser diffraction particle size analyzer. (Malvern -X)

(iv) Surface properties :

The surface morphology of the microsphere was examined by scanning electron microscope (SEM). The dried microspheres were mounted on metal stubs, coated under an argon atmosphere with gold-palladium and then observed under SEM.

Results & Discussions :

Levonorgestrel loaded microspheres, prepared by emulsion solvent evaporation method, were obtained as white, loosely agglomerated masses. However, after passing through ASTM sieve no.40, these masses became free flowing powder, yields of the process were good (about 83%).

The standard plot of LNG in absolute alcohol, was linear between 0-15 μg/ml concentration. The coefficient of correlation, r was found to be 0.9993 and regression equation was

$$ABS = 0.0561 * CONC. - 0.0002$$

The physicochemical properties of the different LNG loaded microspheres are given in Table-10. Levonorgestrel content of different batches of microspheres ranged between 97.32 and 99.28% of theoretical drug loading and moisture content of the microspheres ranged between 0.53 to 0.85%. The mean volume diameter of the microspheres, with 5% w/w and 10% w/w drug loading, was larger than that of the microspheres with 20% drug loading. At 10% w/w drug loading, the mean volume diameters of the microspheres prepared with combination of PCL and PLGA were smaller than that of the microspheres prepared with PCL alone.

The electron photo-micrographs of the microspheres are depicted in Fig.13 and Fig.14, respectively. The microspheres were seen as aggregated small spherical particles. Surface appearance was rough with presence of pores. Better encapsulation of LNG was seen in PCL microspheres prepared with 5% and 10% drug loading than with 20% drug loading. At 10% w/w drug loading, micropheres prepared with PCL alone showed better encapsulation than those prepared with combination of PCL and PLGA.

Table - 10 : Physicochemical characteristics of LNG loaded microspheres

Sr. No.	Characteristics	Microsphere Batch No.					
		DLM-1	*DLM-2*	*DLM-3*	*DLM-4*	*DLM-5*	*DLM-6*
1	LNG:PCL:PLGA ratio	05:95:00	10:90:00	20:80:00	10:72:18	10:54:36	10:36:54
2	Colour, odour & visual appearance	---------- odourless, white, free flowing powders --------					
3	Moisture content (% w/w)	0.77	0.76	0.81	0.79	0.58	0.59
4	Drug content (% w/w) (mean ± sd) (n=3)	97.32 (2.10)	98.57 (1.39)	99.28 (2.34)	98.15 (1.70)	98.39 (2.69)	98.69 (3.01)
5	Mean volume diameter (µ)	54.80	56.80	24.30	31.90	24.20	25.10
6	Particle size distribution (%)						
	a) < 10 µ	10.1	11.8	18.8	14.1	13.9	15.8
	b) 10 - 50 µ	39.2	37.0	39.7	41.0	46.5	43.4
	c) 50 - 150 µ	35.1	31.9	26.0	28.2	24.6	27.8
	d) 150 - 235 µ	14.4	17.1	14.4	15.2	13.6	12.2
	e) > 235 µ	1.2	2.2	1.1	1.5	1.4	0.8
	f) Sp. surface area (sq.m/cc)	0.244	0.263	0.363	0.310	0.325	0.337

Fig - 13. SEM photomicrographs of microspheres prepared with only PCL at different drug loadings

Batch No. DLM - 1. *Batch No. DLM - 2.* *Batch No. DLM - 3.*

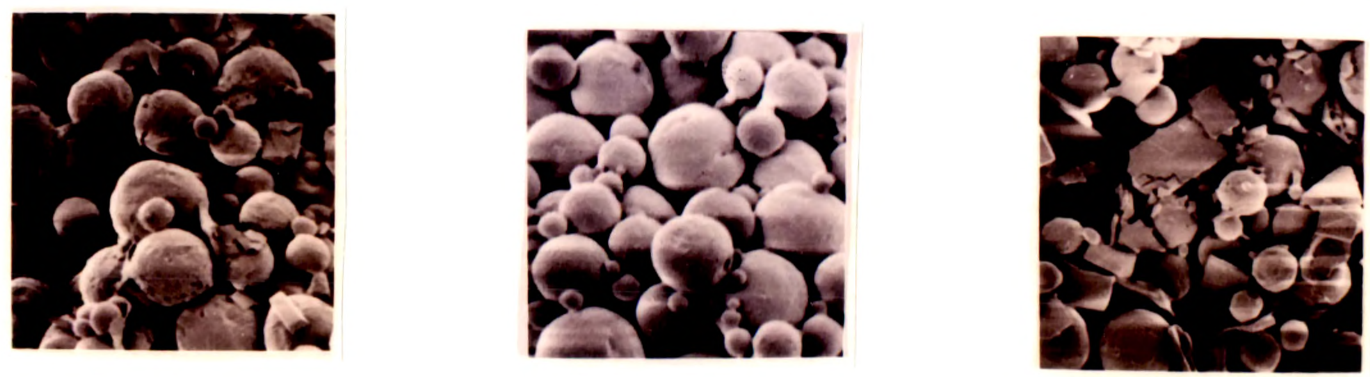
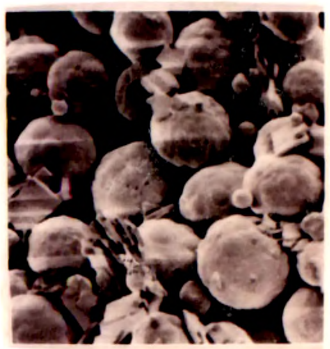
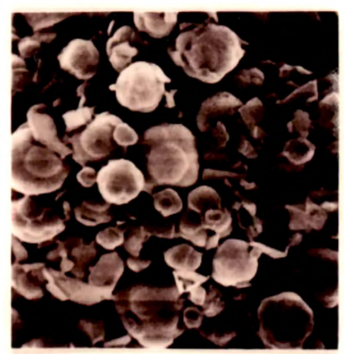


Fig - 14. SEM photmicrographs of microspheres prepared with PCL & PLGA at 10% w/w drug loading

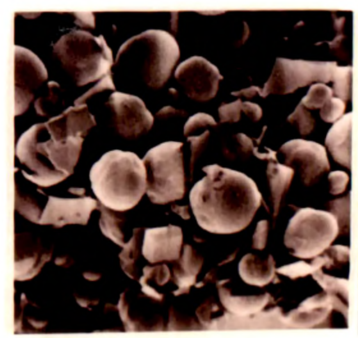
Batch No. DLM - 4.



Batch No. DLM - 5.



Batch No. DLM - 6.



2B.13 *In-vitro* release studies of drug loaded microspheres

There are no set guidelines as regards to the volume of dissolution media, type of dissolution media, dissolution apparatus and other conditions to study *in-vitro* release rate of drugs from the injectable microsphere dosage forms. However, some methods are reported for the assessment of *in-vitro* release pattern of prolonged action parenteral system. These include flow through cells, rotating bottle method, static method and paddle method.[206,207,239] In continuous flow type of apparatus, the delivery system is placed in a compartment and continuously exposed to a stream of dissolution medium at 37°C. The constant rotation apparatus consists of a plexiglass holder (in which the device is placed), which is rotated at a constant speed in the elution medium. In the static method, the drug delivery system is incubated in dissolution medium, in flasks or tubes, at 37°C. The whole amount of medium is replaced periodically and analysed for drug content to follow drug release pattern. However, static method shows boundary layer effect, and, therefore, it is not preferred.[240] In the method used in the present study, screw capped bottles containing dissolution medium and microspheres, were agitated at 100 strokes per minute to eliminate boundary layer effect on the dissolution of drug. This method has been used by several researchers, to study *in-vitro* release of drug from microspheres.[241-243]

Ethanolic phosphate buffered saline pH 7.4 (EPBS) was used as a dissolution medium in the present investigation. The dissolution media with different alcohol content i.e. 10% v/v and 50% v/v were selected for *in-vitro* release studies. The method of estimating drug concentration in the selected dissolution media was standardised.

The *in-vitro* release patterns of levonorgestrel from different microspheres were then determined in 10% v/v EPBS and 50% v/v EPBS.

<u>Materials</u> :

Levonorgestrel B.P./U.S.P., di-sodium hydrogen orthophosphate, potassium dihydrogen orthophosphate and sodium chioride (analytical grade) and absolute alcohol I.P. were required for the dissolution study.

<u>Experimental Methods</u> :

(a) <u>Determination of wavelength of maximum absorption of levonorgestrel in different dissolution media</u>

Solution of levonorgestrel, having concentration of 10 µg/ml, were prepared in 10% and 50% v/v EPBS. The UV spectra of these solutions were recorded using a double beam UV-visible spectrophotometer.

(b) <u>Preparation of standard plots of levonorgestrel in 10% v/v EPBS</u>

Standardized LNG 5.0 mg, accurately weighed, was dissolved in 10 ml absolute alcohol in a 100 ml volumetric flask. This solution was diluted to 100 ml, with phosphate buffered saline pH 7.4. The solution obtained was used to prepare a series of standard solutions of the drug in 10% v/v EPBS. Concentrations of the standard solutions ranged from 1.0 to 15 µg/ml. The absorbances of standard solutions were determined at 247 nm, on a double beam UV-vis spectrophotometer, against 10% v/v EPBS as a blank.

(b) <u>Preparation of standard plots of levonorgestrel in 50% v/v EPBS</u>

Standardized LNG, 5 mg, was accurately weighed and dissolved in 50 ml absolute alcohol, in a 100 ml volumetric flask. This solution was diluted to 100 ml, with phosphate buffered saline pH 7.4. The solution obtained was used to prepare a series of standard solutions of the drug in 50% v/v EPBS. Concentrations of the standard solutions ranged from 1.0 to 15 µg/ml. The absorbances of standard solutions were determined at 244 nm, on a double beam UV-vis spectrophotometer against 50% v/v EPBS as a blank.

The graphs of absorbance against concentration (µg/ml) were plotted. They were used as the standard curve for determination of drug content in samples withdrawn in the *in-vitro* drug release studies.

(d) **<u>Release studies in 10% v/v EPBS</u>** :

Microspheres, equivalent to 5 mg of LNG, were tied in small pieces of nylon cloth (400 mesh). These were immersed in 100 ml of 10% v/v EPBS, (prewarmed to 37

± 1°C), in screw capped bottles. These bottles were maintained at 37 ± 1°C and agitated (100 strokes per minute) on horizontal water bath shaker, during the entire period of the study, i.e. 60 days. Daily, 25 ml of the dissolution medium, from each bottle was collected in tubes and replaced by an equal volume of the fresh dissolution medium (prewarmed to 37 ± 1°C). The drug concentrations in the periodically sampled solutions were determined by measuring their absorbances at 247 nm against suitably prepared blanks.

(e) Release studies in 50% v/v EPBS :

Microspheres, equivalent to 5 mg of LNG, were tied in small pieces of nylon cloth (400 mesh). These were immersed in 50 ml of 50% v/v EPBS (prewarmed to 37 ± 1°C), in screw capped bottles. These bottles were maintained at 37 ± 1°C and agitated (100 strokes per minute) on horizontal water bath shaker, during the entire period of the study, i.e. 7 days. At periodic intervals of time (two hourly for the first 12 hours and every 12 hours for the remaining period), 1 ml of the dissolution medium, from each bottle was collected in tubes and replaced by an equal volume of the fresh dissolution medium. The drug concentrations in the periodically sampled solutions were determined, after suitable dilution, by measuring their absorbances at 244 nm against suitably prepared blanks.

Results & Discussions :

Selection of dissolution media is an important parameter of *in-vitro* release studies. The use of co-solvents such as propylene glycol and hydro-alcoholic mixtures have been reported for dissolution studies of steroid drugs (e.g. progesterone, estrone, norethisterone).[241,243-245] Ethanolic phosphate buffered saline pH 7.4 was used in the present study to provide sink solubility condition and to inhibit bacterial growth. The aqueous component was isotonic to simulate any osmotic effects *in-vivo*. Dissolution media containing different percentages of ethanol were used to evaluate the influence of formulation variables, effect of storage conditions and radiation sterilization on the *in-vitro* release pattern. Additionally, the *in-vitro* release studies in different dissolution media helped in predicting possible duration of action of these dosage forms.

The wavelength of maximum absorption for levonorgestrel was found to be 247 nm and 244 nm in 10% v/v & 50% v/v EPBS respectively. The drug solutions were found to obey Beer-Lambert's law in the studied range of concentrations.

The release profiles of uncoated drug and the different batches of microspheres, in 10% v/v EPBS, are as shown in Figs. 15, 16 and 17. The uncoated drug dissolved slowly and complete dissolution was observed after 15 days. This may be attributed to the low solubility of the drug in the dissolution medium (0.1 - 0.11 mg/ml). All the microspheres released about 80% of drug over a period of 60 days, and

Fig – 15.

Dissolution profile of levonorgestrel in 10% v/v EPBS

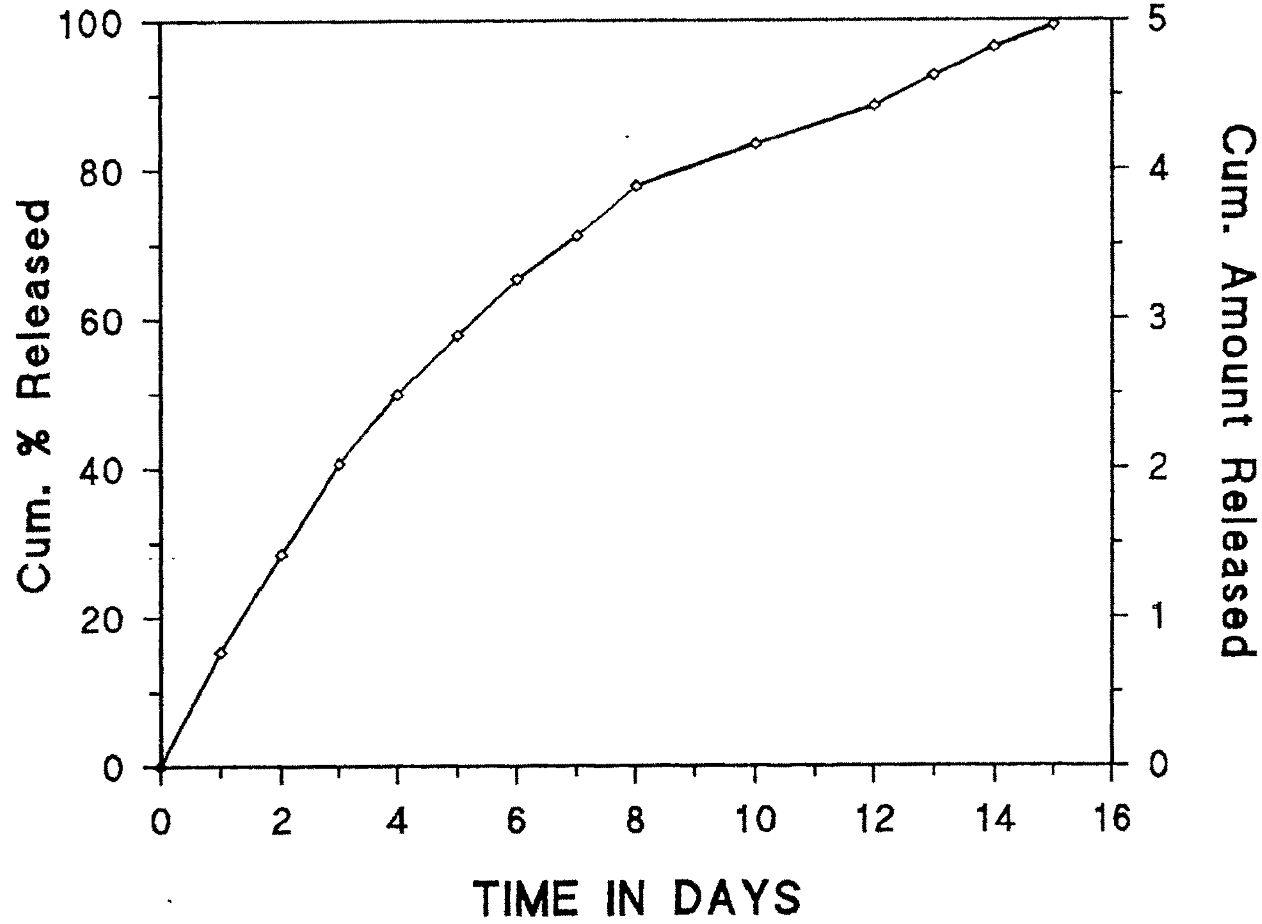
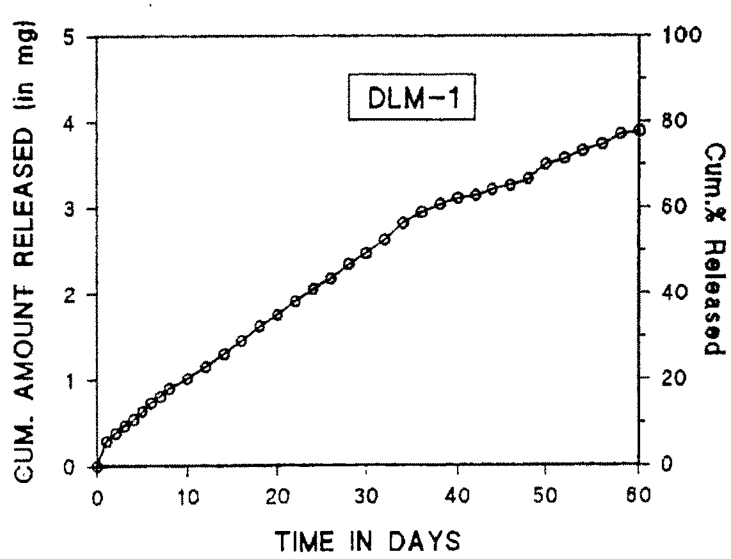
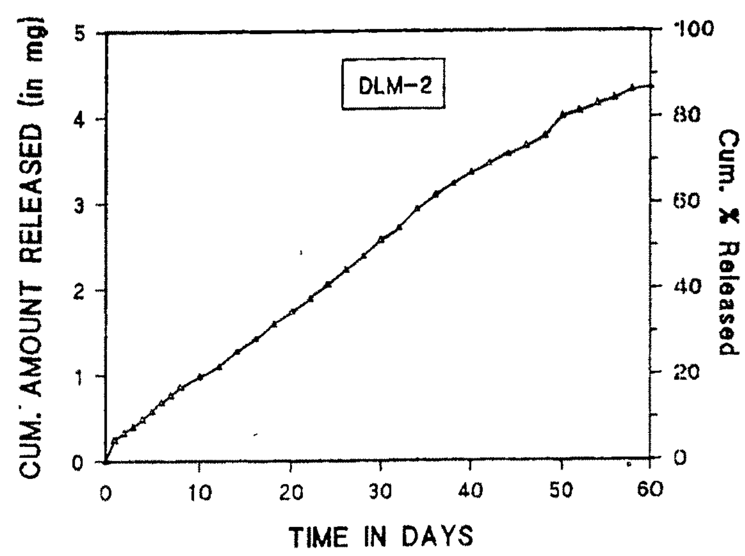
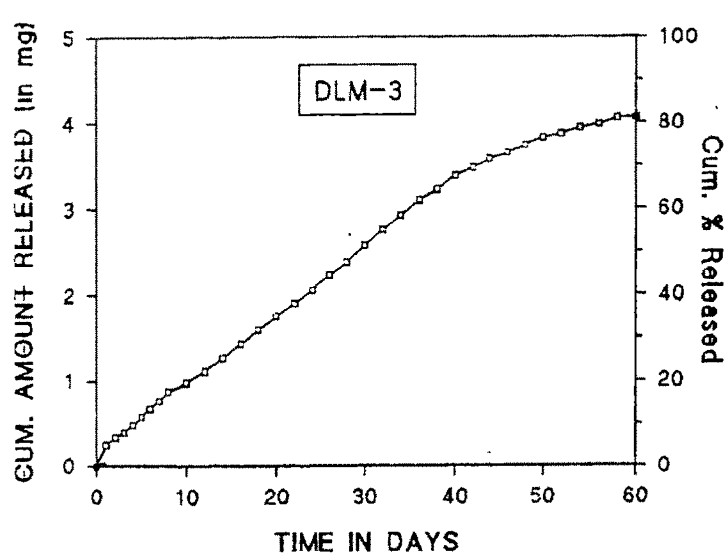


Fig – 16. Release profiles of LNG from different batches of PCL microspheres in 10% v/v EPBS

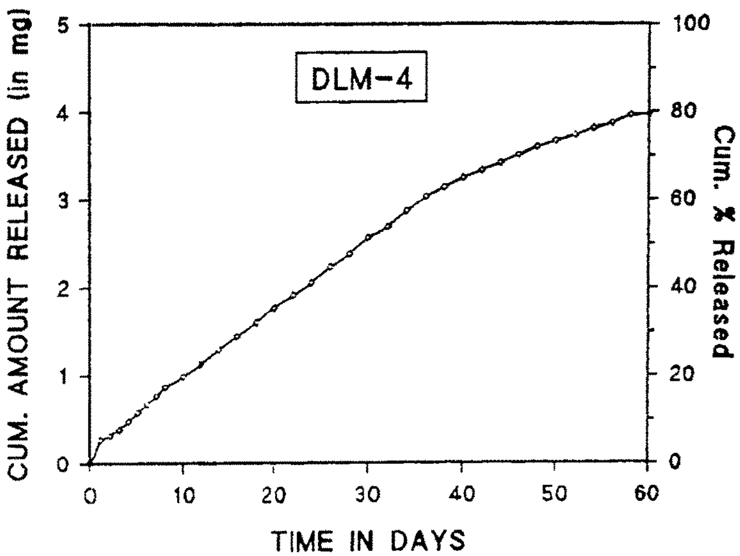


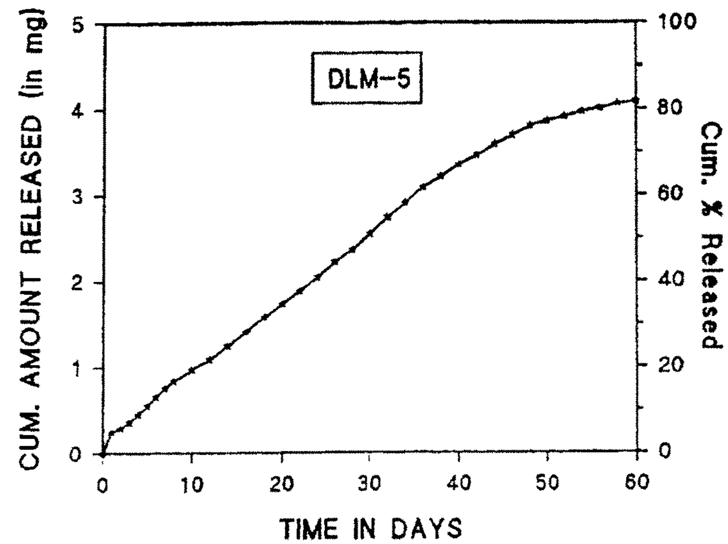


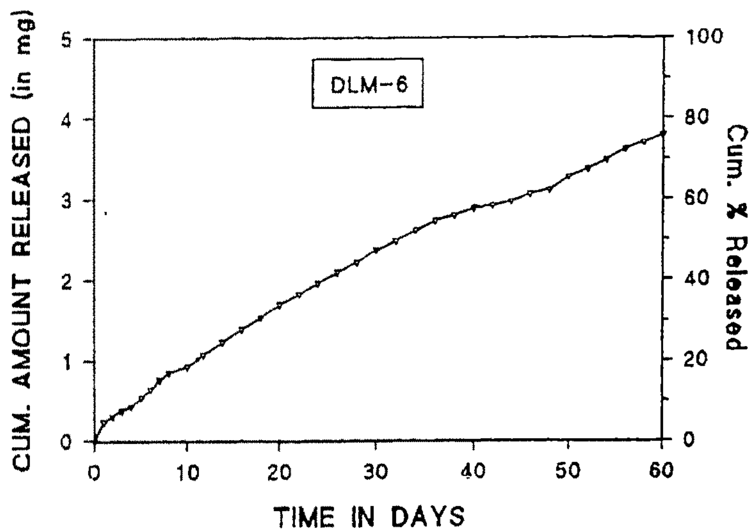


B. No.	Drug Loading	Wt of Microspheres
DLM-1	5% w/w	100 mg
DLM-2	10% w/w	50 mg
DLM-3	20% w/w	25 mg

Fig -- 17. Release profiles of LNG from different batches of PCL-PLGA microspheres in 10% v/v EPBS
(Wt. of Microspheres 50 mg)





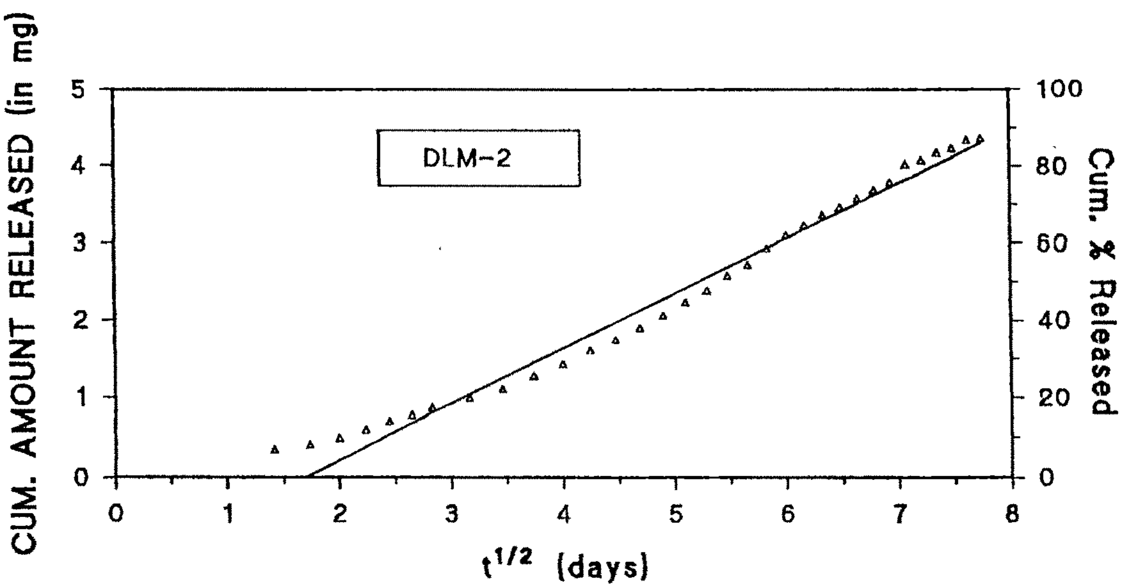


B. No.	LNG : PCL : PLGA Ratio
DLM-4	10 : 72 : 18
DLM-5	10 : 54 : 36
DLM-6	10 : 36 : 54

their release profiles were similar. Higuchi plots [percent released v/s $(\text{time})^{1/2}$] are reported to be used to assess mechanism of release of drugs from microspheres.[245-248] Higuchi plots of the microspheres with LNG:PCL:PLGA ratios of 10:90:0 and 10:36:54 are shown in Fig.18. It was observed that the plots, for both the batches of microspheres, were linear, indicating that drug release occurred by diffusion only.

The release profiles of uncoated drug and the different microspheres, in 50% v/v EPBS, are as shown in Figs.19 and 20. The uncoated drug dissolved completely within 6 hrs. All microspheres showed initial burst release, releasing about 40-60% of the drug in the first 12 hours. Nuessle et al have also reported burst release of oestrone, in 50% v/v methanolic phosphate buffer solution pH 7.4, from poly (1-lactic acid) microspheres with average diameters of 125-160 µm. This was attributed to the presence of free drug and/or drug on the surface of microspheres.[243] A burst release of norethisterone enanthate, was observed by Beck et al also, from the drug containing poly (dl-lactide) microcapsules. The dissolution medium used in their study was 27.5% w/w aqueous ethanol. However, they have reported that the extent of burst release is inversely proportional to average particle diameter of the microspheres. The microspheres with an average diameter of 45-63 µm showed a burst release of about 75% in the first 10 hrs whereas, those having an average diameter of 150-250 µm showed only 20% of norethisterone enanthate release during the same

Fig – 18. Higuchi plots for DLM-2 and DLM-6 microspheres.



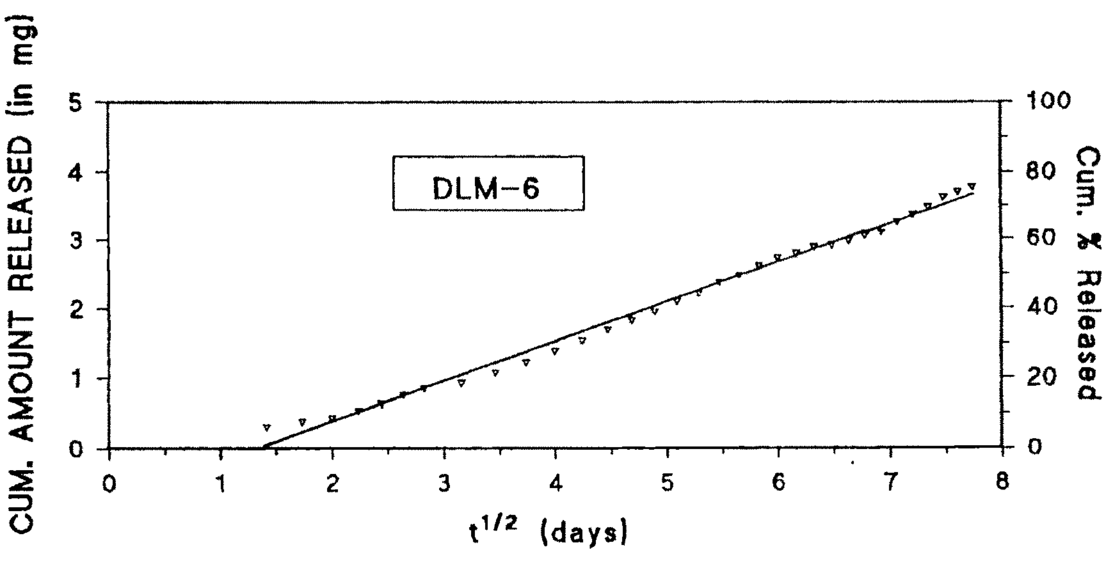


FIG - 19. Release profiles of LNG from different batches of PCL microspheres in 50% v/v EPBS

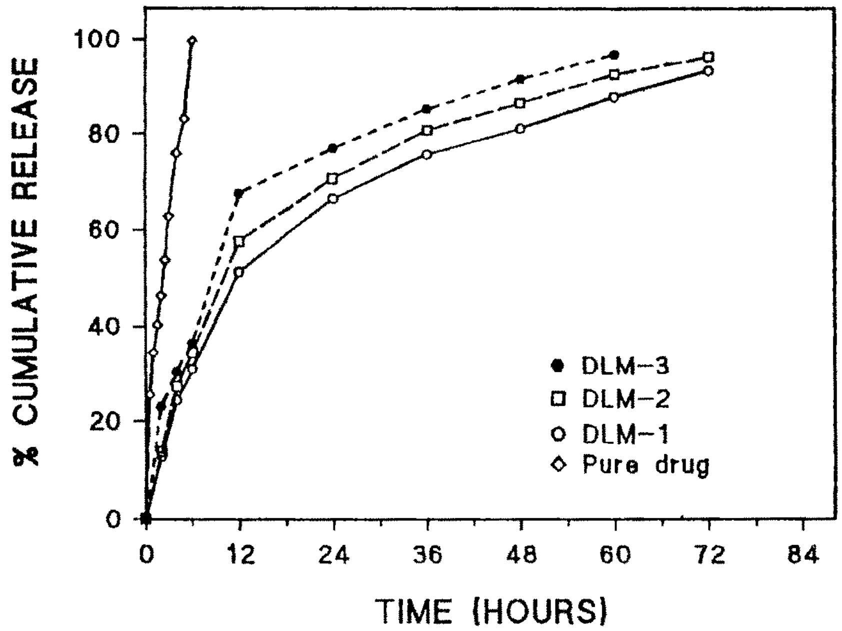
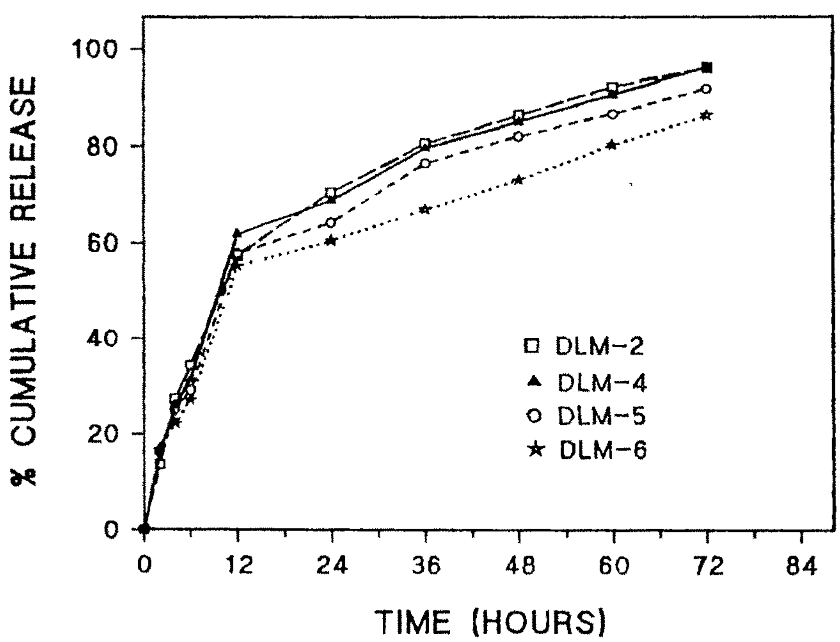


FIG - 20. Release profiles of LNG from different batches of PCL-PLGA microspheres in 50% v/v EPBS



time period. Further, they report that the microcapsules with smaller particle diameter (10-20 μm) helped in achieving therapeutic plasma concentrations rapidly, but were effective for shorter duration when compared to microcapsules with particle size ranging from 10 - 240μm.[249]

Therefore, the initial rapid release of LNG in 50% v/v EPBS may be attributed to the small quantity of free drug that might be present, smaller mean particle diameter of the microspheres and high solubility of drug in the dissolution media (0.9-1.0 mg/ml). This burst release is desirable to rapidly achieve therapeutic concentration of drug in plasma.

An increase in the release rate was observed with increased drug loading. Release of levonorgestrel decreased with increasing PLGA ratio. This could be explained by the low permeability of PLGA to steroids.

Considering drug release pattern and physicochemical properties, microspheres with LNG:PCL:PLGA ratio of 10:90:0 (B.No.DLM-2) and 10:36:54 (B.No.DLM-6) were selected for further investigation.

2B.14 Characterization of polymer properties of selected batches of microspheres

The *in-vitro* release of drug from biodegradable microspheres is governed by the physicochemical properties of the microspheres e.g. particle size distribution, surface area etc and the intrinsic properties of the polymer. The polymer properties, such as molecular weight distribution and glass transition temperature, have been reported to change during the preparation depending upon the processing conditions employed.[250,251] These properties were therefore studied for selected batches of microspheres.

Experimental Methods :

The procedures used for determining molecular weight distribution and T_g or T_m were same as those described under characterization of polymers in preformulation studies. However, DSC system (Perkin Elmer DSC7) was calibrated at a heating rate of 2°C per minute and samples were also analysed at the same heating rate.

Results & Discussions :

The average molecular weights and T_g or T_m of the polymer(s), in the selected batches of microspheres are listed in Table - 11. It was observed that weight average molecular weight (M_w) of PCL present in microsphere batch no. DLM-2 decreased from 155500 daltons to 105971 daltons but, the number average molecular weight (M_n) increased from 39000 daltons to 65000 daltons as a result, polydispersity (M_w/M_n) decreased from 3.95 to 1.61. Thus, molecular weight distribution of the polymer became narrow during microsphere preparation. This may be attributed to (i) partial hydrolysis of high molecular weight fraction of the polymer leading to relatively low molecular weight fraction and/or (ii) solubilization of very low molecular weight fractions of the polymer in the aqueous phase during the preparation of microspheres. Similar changes were observed in molecular weight distribution of polymers present in microsphere batch no. DLM-6. This decrease in molecular weights (M_w) was associated with corresponding decrease in T_m or T_g of the polymers. T_m of PCL decreased from 63°C to 61°C in DLM-2 whereas, T_g of PLGA decreased from 57°C to 49°C in DLM-6. However, there was no change in T_m of PCL in DLM-6. Difference in hydrophilicity is a probable reason for this, PLGA being more hydrophilic is affected more as compared to PCL. DeLuca et al have reported similar changes in T_g and T_m of poly (l-lactide) during the preparation of progesterone loaded microspheres.[170]

Table - 11 : Polymer properties of the selected batches of drug loaded microspheres

Sr. No.	Characteristic	DLM - 2	DLM - 6
1	GPC		
a)	M_w (Daltons)	105971	95680
b)	M_n (Daltons)	65753	54039
c)	PD	1.61	1.70
2	DSC T_m or T_g (°C)	61.51	49.04 & 63.47

2B.15 Selection of vehicle for dispensing microspheres :

Since the microspheres prepared using PCL and PLGA are to be provided as dry powders, to be dispersed in suitable dispensing medium at the time of administration, the selection of vehicle also formed part of development of an injectable dosage form.

Different suspending agents, solubilized in normal saline were evaluated as vehicle for dispensing microspheres. 10% w/v suspensions of microspheres (B.No. DLM-2 & DLM-6) were prepared in different vehicles. The performance of these vehicles was evaluated on the basis of suspendibility, sedimentation volume, ease of redispersion and syringeability.

Materials :

Microsphere B.No. DLM-2 & DLM-6, HPMC, MC, Sod. CMC and sodium chloride AR were used in this study.

Experimental Methods :

The vehicles employed in the study were:

- Normal saline containing 0.5% w/v sod.CMC.
- Normal Saline containing 0.5% w/v HPMC
- Normal saline containing 0.5% w/v MC

All these vehicles were clear solutions and were sterilized by filtration, through 0.22 micron PVDF filter, in a sterile area.

Suspensions were prepared by transferring 10 ml of the vehicle (i.e. normal saline containing 0.5% w/v of sod. cmc or HPMC or MC) into a sealed vial containing 100 mg of the microspheres (DLM-2 or DLM-6). The pH values and viscosities of suspensions were recorded using pH meter (Elico, Model LI 120) and Brookefield Viscometer (Model DV II^{+}). Suspendibility was judged to be good if suspension was achieved without any problems of wetting, agglomeration or lump formation, otherwise, it was judged to be bad. These suspensions were then evaluated for :

a) Sedimentation volumes :

Ten millilitre of the suspensions of microspheres in each of the three vehicles were carefully poured into 10 ml graduated cylinders. The suspensions were shaken to achieve a uniform dispersion, and then left undisturbed on a flat surface. At periodic intervals of time the volume of sediment (Hv) in each case was noted. A plot of Hv/Ho against time was plotted. (Hv-height of sediment at time 't', Ho-height of sediment at zero time).[252]

b) Ease of redispersion :

At the end of 24 hours of preparation of suspensions, mentioned above, the volume of sediment in case of each sample was noted. After this, the number of swirls required to redisperse the sediment particles, and obtain a uniform dispersion, was noted.[252]

c) Syringeability :

About 1 ml of each suspension was withdrawn into a tuberculin syringes using various gauge needles (26, 24, 22 & 21 gauge). The contents in the syringe were then expelled by applying pressure on the plunger. The suspensions were withdrawn after agitation of the suspension and the ease with which contents were expelled from the syringe was noted.[252]

Results & Discussions :

Physicochemical characteristics of the various suspensions are listed in Table-12. The sedimentation behaviours of the suspensions are depicted in Fig.21.

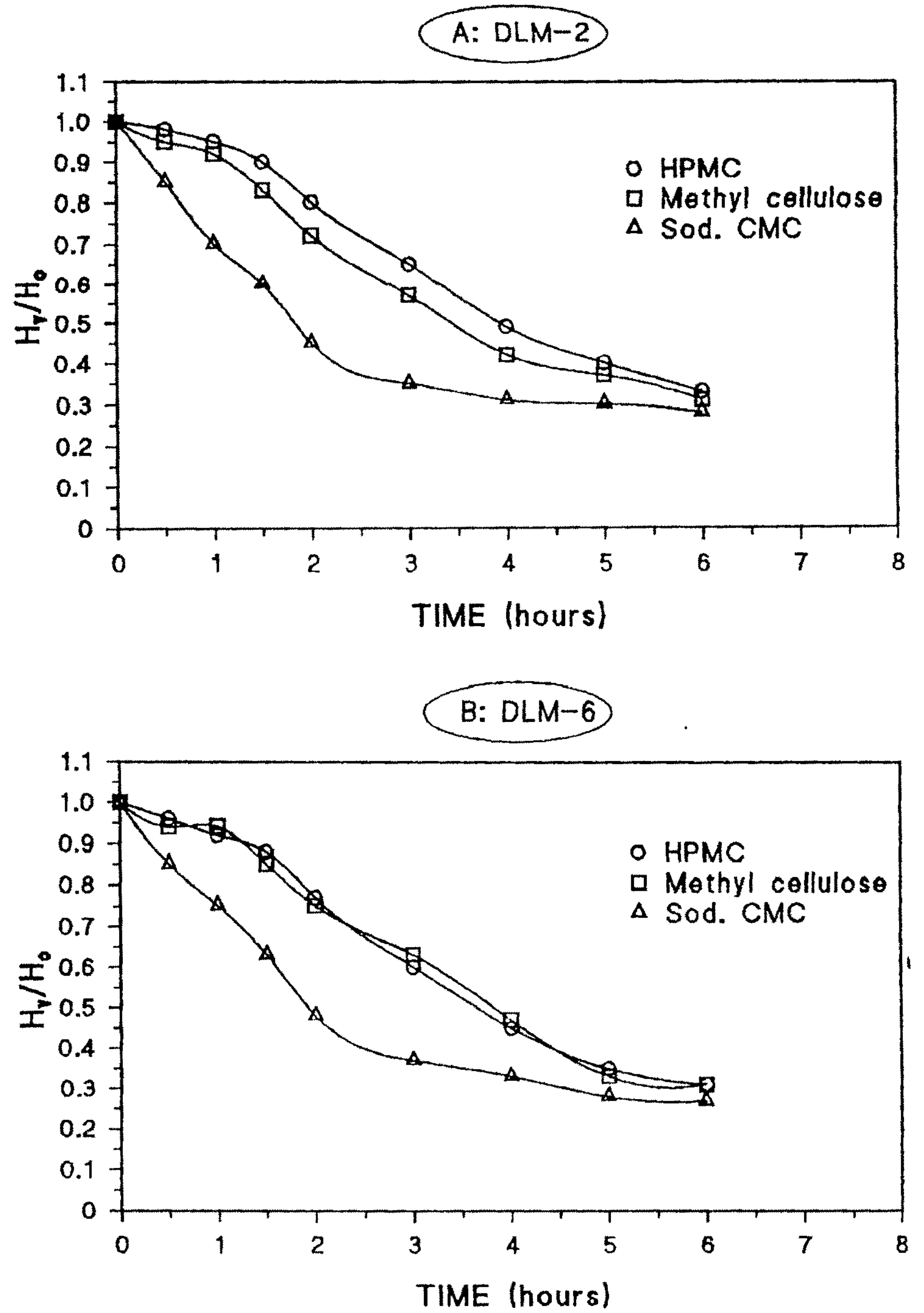
Aqueous vehicles were selected due to their ease of preparation and sterilization.[252] Furthermore, all the vehicles were found to have pH of 6.5, which being close to the physiological pH would not cause discomfort to the subject.

Suspendibility was found to be good in vehicles containing HPMC or MC. However, in Sod.CMC, particles agglomerated and showed a problem of wetting. Additionally, sedimentation volume was noticed to be better for vehicles containing HPMC or MC than for vehicle containing sod.cmc. The high viscosity of HPMC and MC are probably the reason for better sedimentation volume. However, the rate of sedimentation of microspheres was more retarded in vehicle containing HPMC than that containing MC for first 3 to 4 hrs.(Fig. 21).

Table - 12 : Characteristics of selected batches of microspheres in normal saline containing different suspending agents

Sr. No.	Characteristic	0.5% w/v HPMC		0.5% w/v MC		0.5% w/v Sod.CMC	
		DLM-2	*DLM-6*	*DLM-2*	*DLM-6*	*DLM-2*	*DLM-6*
1	Suspendibility	good	good	good	good	fair	fair
2	Sedimentation volume (ml)	3.3	3.1	3.1	3.1	2.9	2.7
3	Ease of redispersion (No. of swirls)	good (6)	good (7)	good (8)	good (8)	fair (12)	fair (11)
4	Syringeability (21 gauge needle)	----easy----		----easy----		----easy----	
5	Viscosity (25°C) cps	100		100		60	
6	pH	6.5		6.5		6.5	

Fig – 21. Sedimentation studies of microspheres in normal saline with different suspending agents



Redispersibility was observed to be good with HPMC and MC. The suspensions were easily syringeable only through needles of gauge 21. In case of 22 gauge needle blockage occurred at times, whereas syringeability through gauge 26 and 24 needles was poor.

Based on the results of this study, normal saline containing 0.5% w/v HPMC was selected as vehicle for further studies.

2B.2 Development of an Implantable Dosage form

According to Blackshear,[253] the scientific concept of implantable drug delivery systems originated in 1937 when R. Deansby and A.S. Parkes presented a paper, describing the effects of subcutaneous implant of various hormone preparations on the growth of livestock, at the Royal Society of Medicine in London. A year later, pure estrogen compressed into a pellet was implanted subcutaneously in a young woman, at Guy's Hospital in London, for the treatment of premature menopause. The drug release from such implants was primarily dependant on the surface area of the implant, particle size & solubility of the drug in the body fluids.[254] Consequently, attempts were made to prepare implants with polymers which controlled the rate of release of the drug. In the past two and half decades, great progress has been made in the development of implantable dosage forms and few implants have reached the stage of clinical trials (e.g. Capronor[R], Implanon[R] and Uniplant[R]).[21,255] One such implant, Norplant[R], is presently available commercially.[25]

Currently, subdermal implants of various type namely, pellets, rods and fibers, are being developed using biodegradable polymers for prolonged delivery of bioactive materials.[256] The present study describes the preparation and evaluation of levonorgestrel containing biodegradable pellets. The method used for the fabrication of pellets has been reported by Gangrade et al.[257]

2B.21 Preparation and characterization of implants :

Experimental Methods :

Pellets (circular discs) containing 5, 10 or 20 mg of LNG were prepared from the microspheres prepared earlier. The microspheres equivalent to 5, 10 or 20 mg of LNG were transferred in a 13 mm die and compressed under 12 tonnes pressure using IR press. Time required to obtain an intact pellet was optimized previously and was found to be 15 minutes. The compositions of different batches of pellets are listed in Table-13.

TABLE - 13 : Composition of different batches of levonorgestrel pellets

B.No.	Weight of Implant (mgs)	LNG content (% w/w)	PCL content (% w/w)	PLGA content (% w/w)
IMP-1	25	20	80	-
IMP-2	50	10	90	-
IMP-3	100	5	95	-
IMP-4	100	10	90	-
IMP-5	100	20	80	-
IMP-6	50	10	72	18
IMP-7	50	10	54	36
IMP-8	50	10	36	54

All the batches of pellets were evaluated for :

a) Drug content :

Levonorgestrel content of the pellets was determined by the method described under stability of drug - polymer mixtures in the preformulation studies (Section 2A.3).

b) Friability :

Six pellets, were placed in the Roche friabilator. Pellets were allowed to fall from a distance of 6" for 10 times. Weight of the intact implants was noted. Percent friability was calculated by the formula

W2/W1 x 100; where

W1 = the weight of six pellets before test

W2 = the weight of intact pellets after test.

c) Thickness :

Thickness of the pellets was measured with a Vernier calliper having a least count of 0.001 cm.

Results & Discussions :

The drug content, friability and thickness of the different pellets prepared are shown in Table-14. The diameter of all pellets was found to be 13.0 ± 0.1 mm. The drug content of all the bathces of pellets ranged between 97% w/w to 100% w/w of the theoretical drug load. Thickness of batch no. IMP-1 could not be determined due to its high friability. Thickness of the pellets, having total weight of 50 mg, was found to be 1.1 mm whereas those having total weight 100 mg were of 2.0 mm. Friability of the pellets decreased with increase in pellet weight but, it increased with increase in PLGA quantity.

Table - 14 : Physicochemical characteristics of different batches of Implants

B.No.	Weight of implant (mg)	LNG:PCL:PLGA	Drug content (% w/w)	Friability (%)	Thickness (mm)
IMP-1	25	20:80:00	99.83	100	ND
IMP-2	50	10:90:00	99.36	15.50	1.1
IMP-3	100	05:95:00	98.18	14.50	2.0
IMP-4	100	10:90:00	97.62	13.90	2.0
IMP-5	100	20:80:00	98.30	13.40	2.0
IMP-6	50	10:72:18	97.31	18.31	1.1
IMP-7	50	10:54:36	99.30	20.41	1.1
IMP-8	50	10:36:54	98.61	24.36	1.1

ND - NOT DETERMINED

2B.22 *In-vitro* release studies of drug loaded implants

Experimental Methods :

The pellets were placed in screw capped bottles containing 50 ml of 50% v/v EPBS (prewarmed to 37 ± 1°C). These bottles were maintained at 37 ± 1°C and agitated (100 strokes per minute) on horizontal water bath shaker, during the entire period of the study, i.e. 7 days. At periodic intervals of time (two hourly for the first 12 hours and every 12 hours for the remaining period), 1 ml sample was withdrawn from each bottle and collected in stoppered glass test tubes. An amount equal to each withdrawal was replaced by fresh dissolution medium. The drug concentration in the periodically sampled solutions was determined, after suitable dilution, by measuring their absorbance at 244 nm against suitably prepared blanks.

Results & Discussions :

In-vitro release profiles of the different batches of pellets are shown in Figs.22, 23 and 24. It was observed that at 5 mg dose, the rate of drug release decreased with increasing implant thickness. The 1.1 mm thick implants (IMP-2) showed desired rate of drug release (70 µg/hr) and 2.0 mm thick implants (IMP-3) showed much lower rate of drug release (30 µg/hr) (Fig.22). When the drug load of 2.0 mm thick implant was increased from 5 mg to 10 mg, the rate of drug release also increased. It was found to be higher (90 µg/hr) than the desired rate of drug release (60-80 µg/hr) (Fig.23). Therefore, 1.1 mm thick implants, containing 5 mg of levonorgestrel, were prepared with different PCL:PLGA ratios. The rate of LNG release decreased with increase in PLGA quantity.(Fig.24).

The implant with LNG:PCL:PLGA ratio 10:90:0 and 1.1 mm thickness was selected for subsequent investigation based on the results of this study.

Two batches of the microsphere (DLM-2 and DLM-6) and one batch of the implant (IMP-2) were found to have desired physicochemical properties and release profile. Hence, these formulations were selected and subjected to additional investigation such as sterilization studies, accelerated stability testing and *in-vivo* studies.

Fig - 22. Release profiles of LNG from PCL implants prepared with different thickness at 5 mg dose

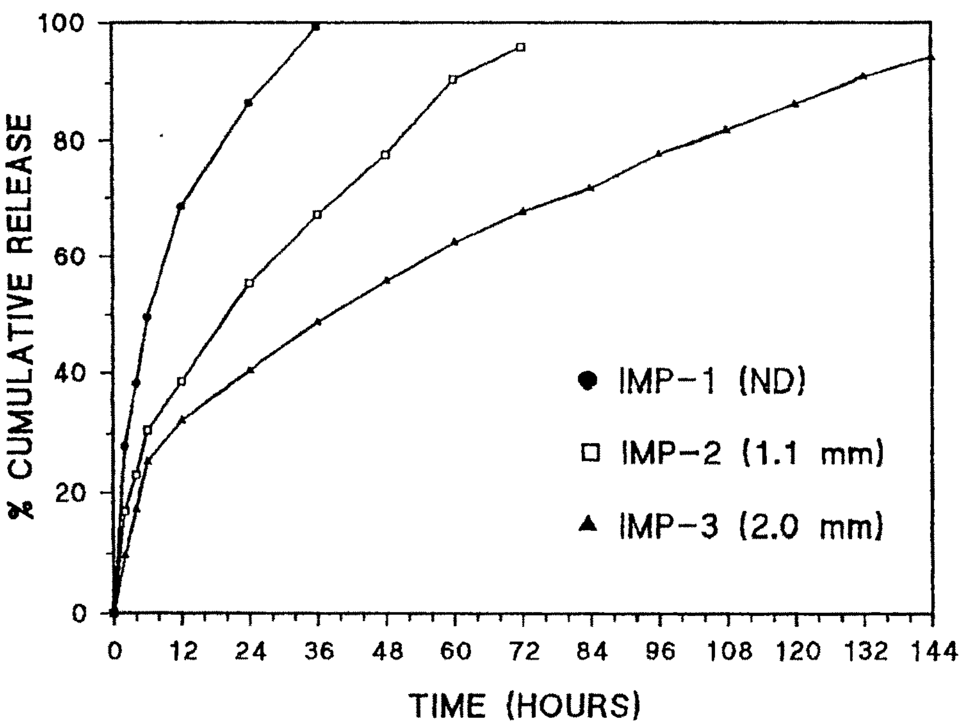


Fig - 23. Release profiles of LNG from PCL implants prepared with different drug load and 2.0 mm thickness

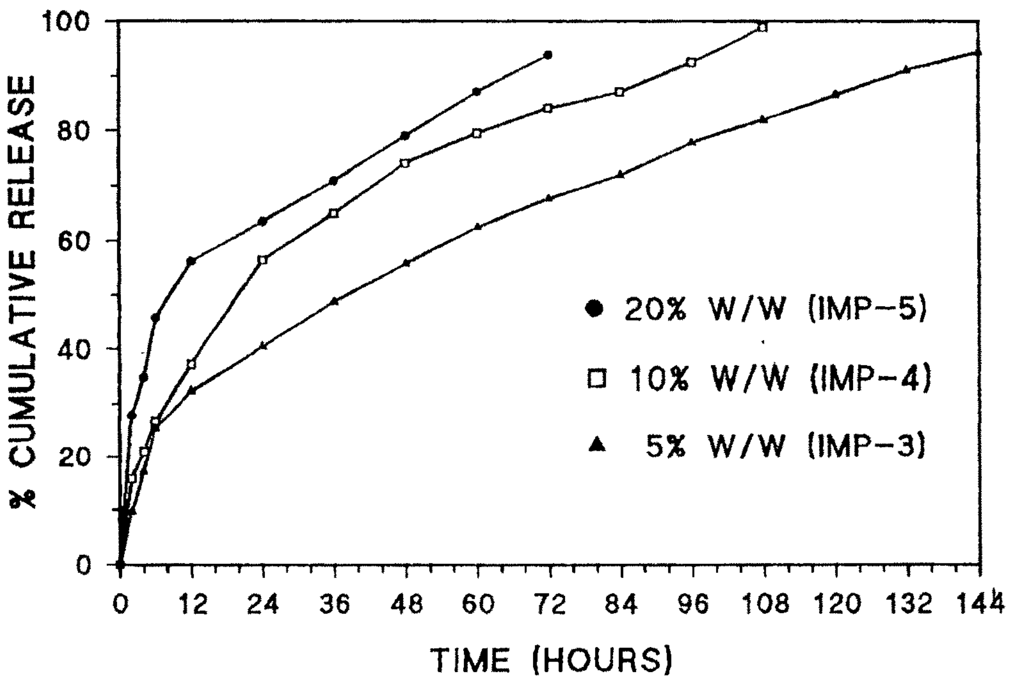
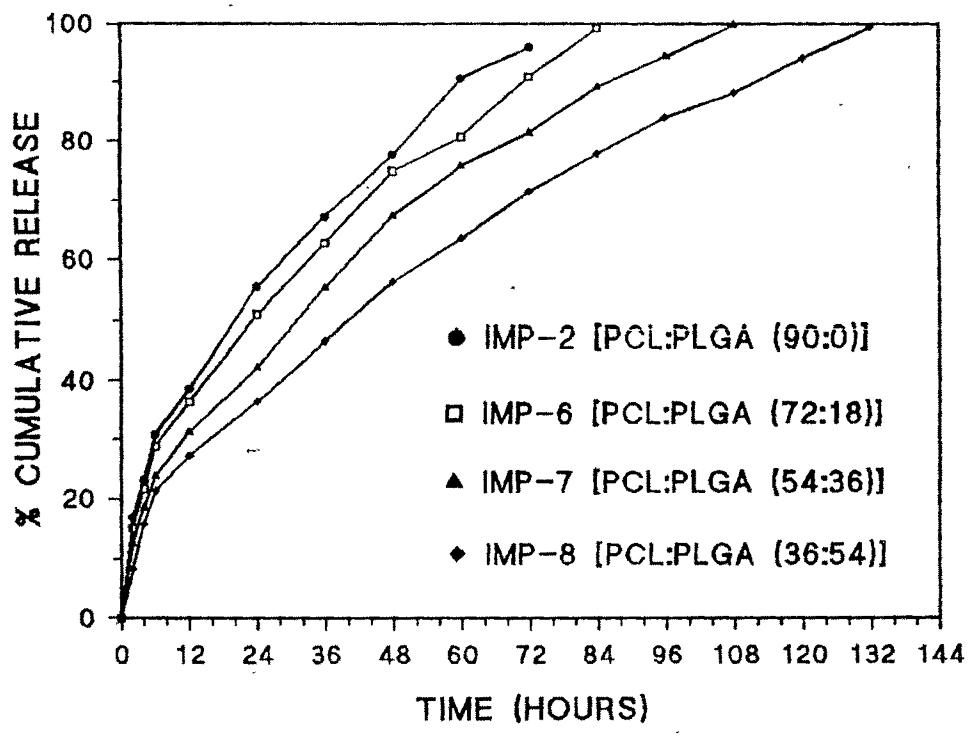


Fig - 24. Release profiles of LNG from implant prepared with different polymer composition (thickness 1.1 mm, dose 5 mg)



2B.3 Radiation Sterilization of the Selected Batches of Microspheres & Implants

Since the terminal sterilization processes have higher sterility assurance level as compared to aseptic processes it was decided to sterilize the selected formulations by gamma radiation. Further, gamma radiation dose of 2.5 Mrads was selected for sterilization of the selected formulations because; i) it is a generally recommended dose for sterilization of pharmaceutical products by the US FDA; ii) during preformulation studies no significant changes were obserevd in the properties of PCL when exposed to this dose and iii) though significant changes were observed in the avearge molecular weights (M_w & M_n) and T_g of the PLGA, these changes were considered to be beneficial since the initial release rate of LNG from one of the selected formulation (i.e. DLM-6) was just meeting the minimum required release rate i.e. 60 µg/hr in 50% v/v EPBS or 60 µg/day in 10% v/v EPBS. The present study was undertaken to characterize the effects of radiation sterilization on some of the important properties of the selected formulation.

<u>Experimental Methods</u> :

Microspheres or pellets, sealed in type I vials under nitrogen, were exposed to gamma radiation (Dose 2.5 Mrads). Radiation sterilized microspheres were evaluated for LNG content, molecular weight of the polymers, *in-vitro* release pattern in 50% v/v EPBS and suspendibility by the procedures described under development of injectable dosage form. Pellets subjected to radiation sterilization were characterized for LNG content, friability and *in-vitro* release profile by the methods described in section 2B.2.

Sterility of the microspheres and implants, exposed to gamma radiation, was determined by direct inoculation technique described under radiation sterilization of LNG in preformulation studies (section 2A.41).

<u>Results & Discussions</u> :

The drug content of all the selected formulations, before and after gamma irradiation, was found to be similar and irradiated formulations passed the test for sterility. The suspensions of gamma irradiated microspheres were formed without any problems of particle agglomeration. Molecular weight distributions of the polymers, before and after sterilization of microspheres (DLM-2 and DLM-6) are shown in Figs.25 and 26. *In-vitro* release profiles of LNG from these microspheres are shown in Figs.27 and 28.

It was seen that molecular weight distributions and *in-vitro* release profile of DLM-2 microspheres remains unchanged after radiation sterilization. On the other hand, *in-vitro* release rate of LNG from DLM-6 microspheres increased after radiation sterilization. A similar observation has been made by Aso et al in release of progesterone from poly (dl-lactide) microspheres after radiation sterilization.[258] Kissel at al have also reported an increase in release of captopril from poly (dl-lactide-co-glycolide) microspheres following radiation

Fig - 25. M_w V/S % Area plots of DLM-2 microspheres.

——— before radiation sterilization
------ after radiation sterilization

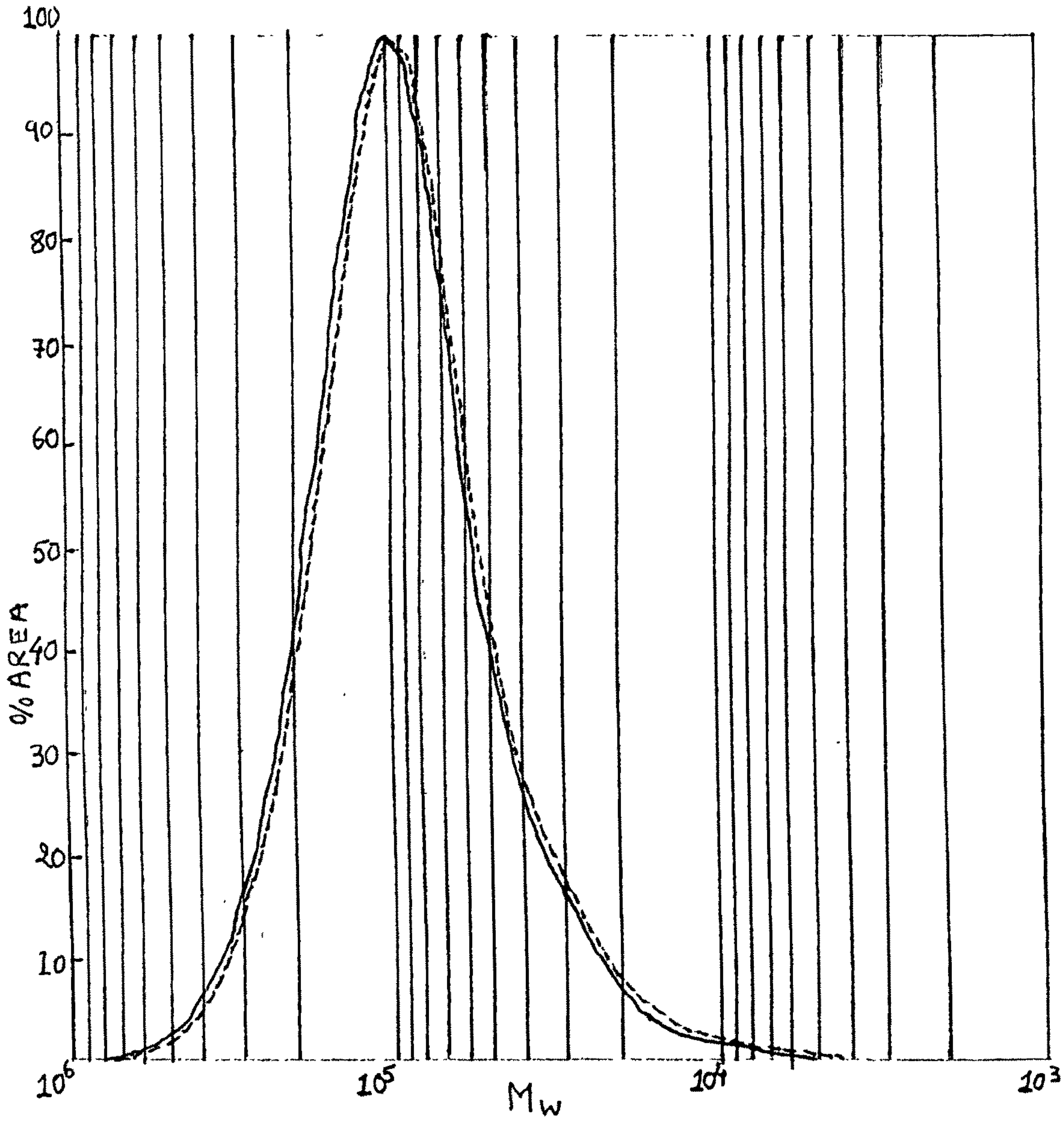


Fig - 26. M_w V/S % Area plots of DLM-6 microspheres.

——— before radiation sterilization
------ after radiation sterilization

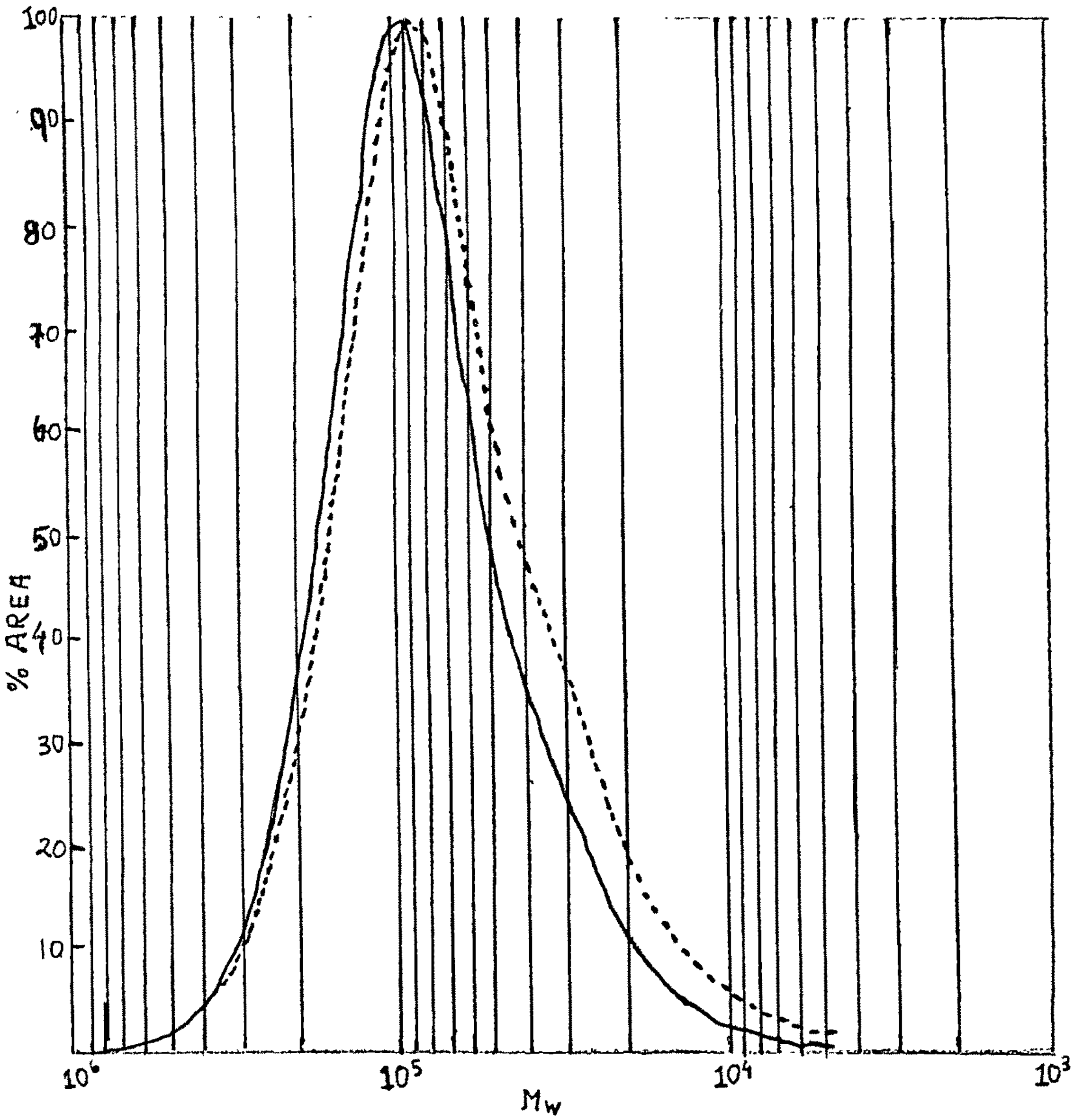


Fig – 27 Release profiles of LNG from DLM-2 & DLM-6 microspheres in 10% v/v EPBS

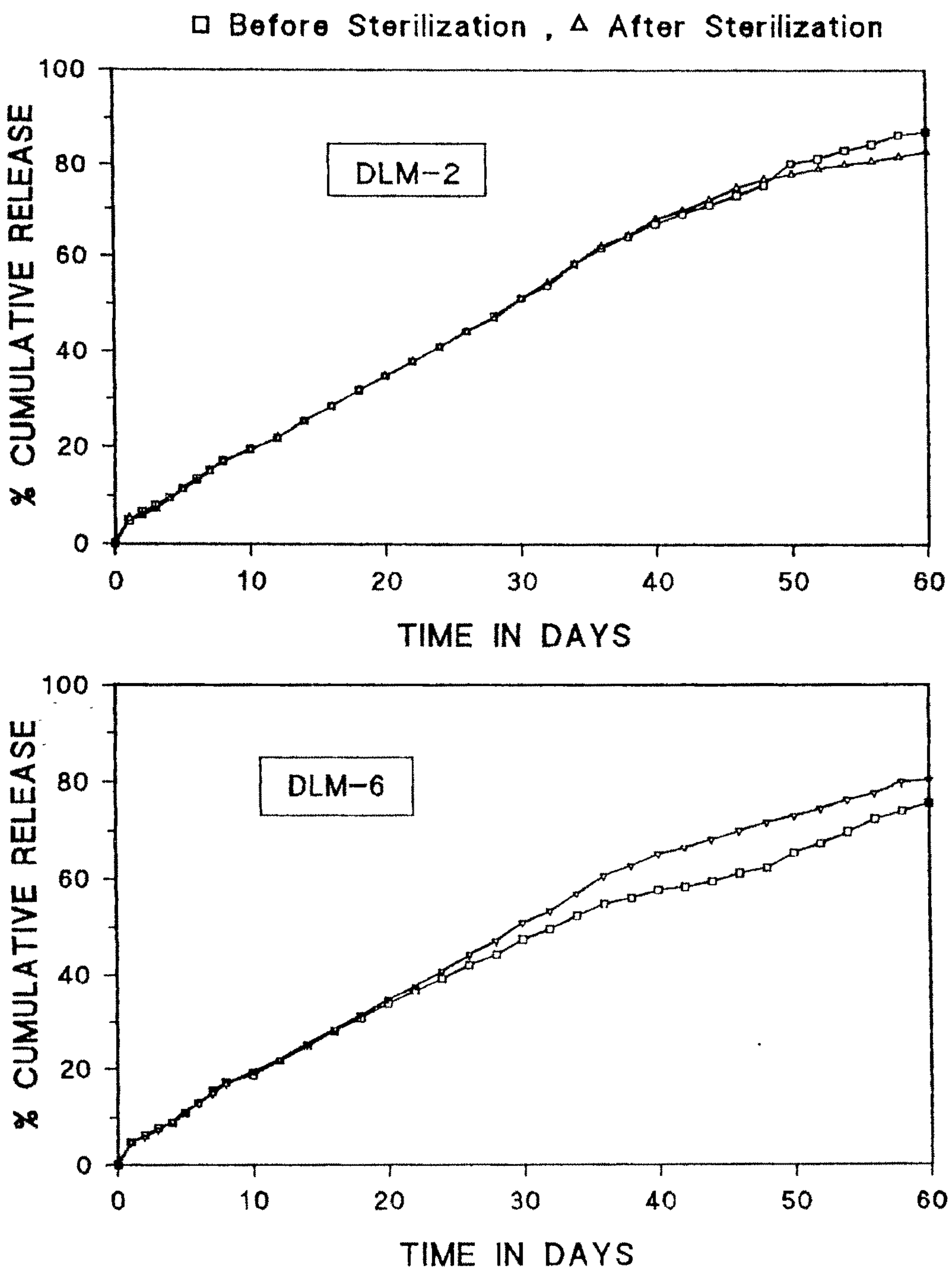
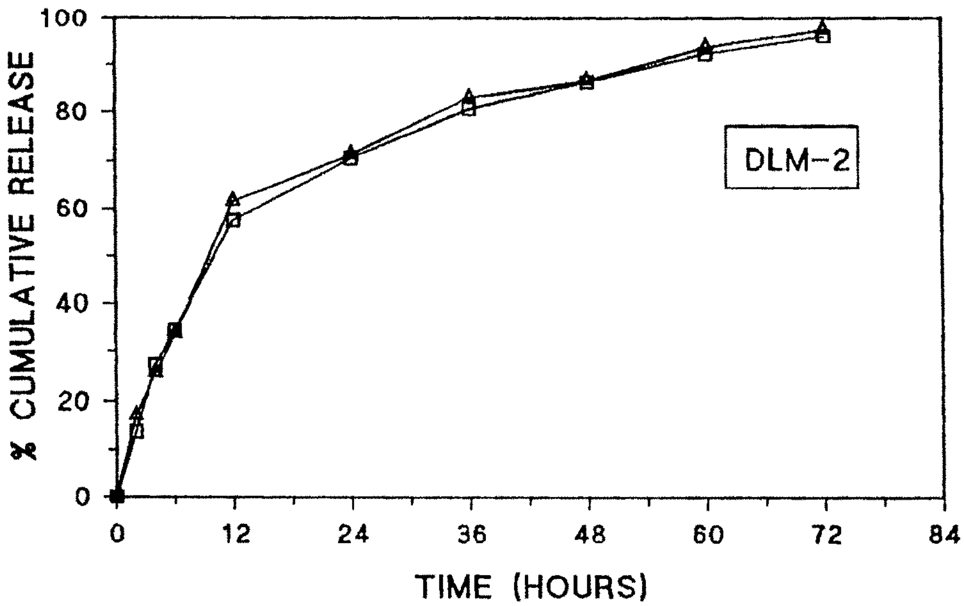
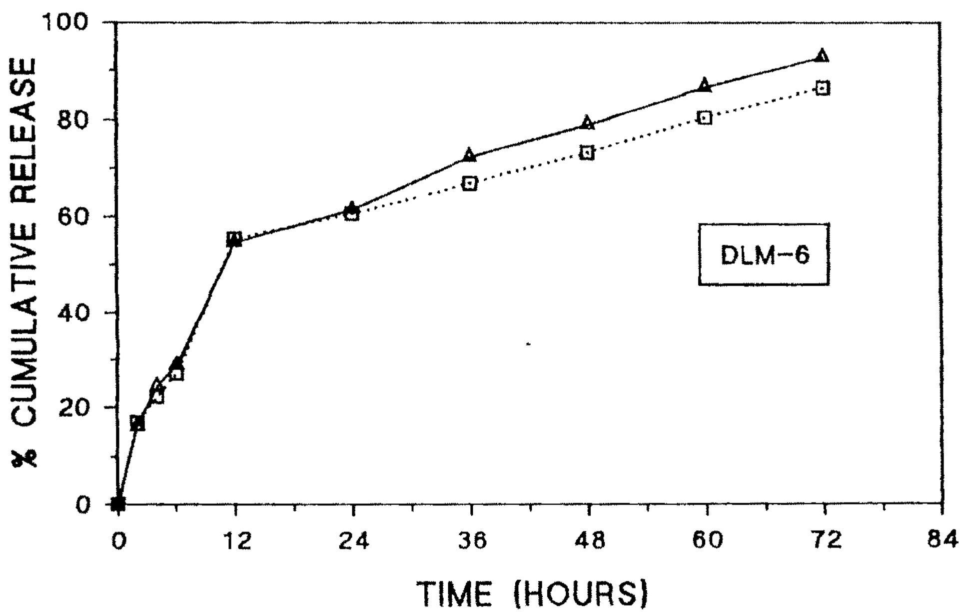


Fig – 28. Release profiles of LNG from DLM-2 & DLM-6 microspheres in 50% v/v EPBS

□ Before Sterilization, △ After sterilization



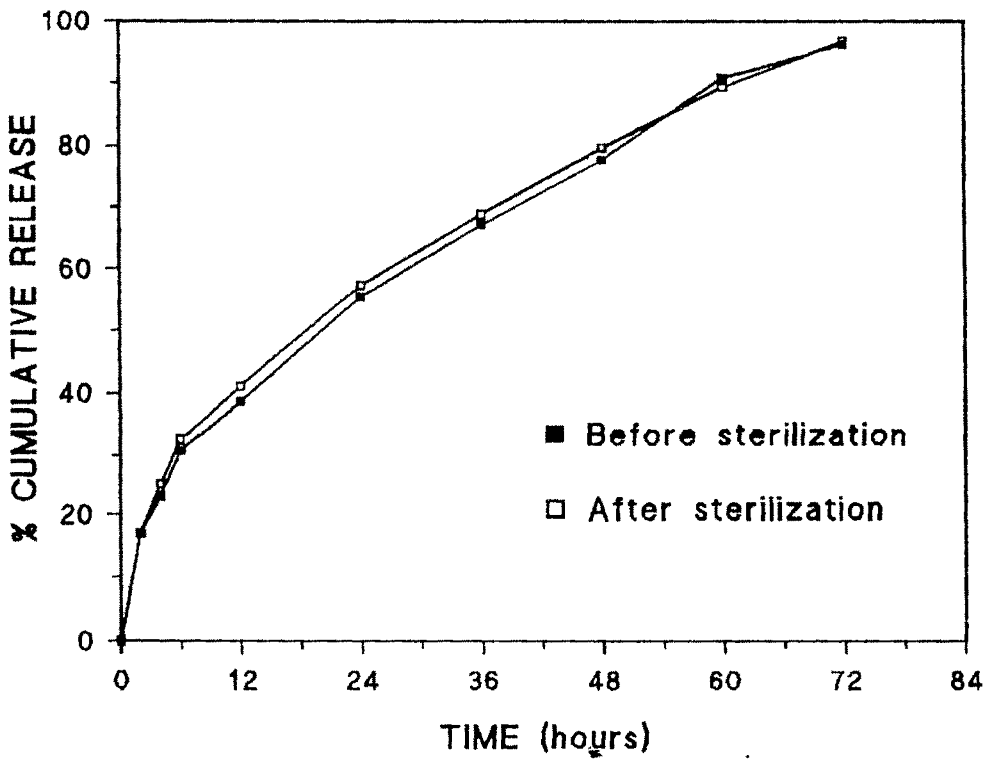


sterilization.[259] The increase in *in-vitro* release rate of LNG from DLM-6 microspheres could be well explained by the corresponding decrease in the average molecular weights of the polymer as indicated in Fig. 26. Further, it has been reported that such changes in molecular weights also result in decrease in T_g of the polymer which in turn increases the free volume within the polymer and hence permeability and/or diffusion of a drug molecule.[170]

The *in-vitro* release pattern of the selected implant remained unchanged after gamma irradiation (Fig.29).

Some of the changes in sterilized materials are time dependant, and become apparent only on storage and ageing. Hence, stability studies of sterilized materials were undertaken.

Fig – 29. Influence of radiation streilization on the release profile of LNG from IMP–2



2B.4 Accelerated Stability Testing of Radiation Sterilized Microspheres & Implants

Experimental Methods

Radiation sterilized samples of the microspheres and implants sealed in type-I vials under nitrogen, were stored at 0-4°C, 25 ± 0.5°C, 37 ± 1°C, 45 ± 1°C and 75% RH at 37 ± 0.5°C for three months. The samples of microspheres, thus stored were evaluated every month, to assess any change in LNG content, moisture content, suspendibility, *in-vitro* release pattern, and polymer properties, whereas, the samples of implants were evaluated to assess any change in LNG content, *in-vitro* release profile and friability. Sterility of the microspheres and implants stored for 3 months at different temperature conditions was also evaluated.

Results & Discussions

Accelerated stability studies of the sterilized samples of DLM-2 carried out at different temperature conditions revealed that LNG content, moisture content did not change on storage(Table 15). However, notable changes in polymer properties were observed for the microspheres stored above 25°C as indicated by the GPC results (Table 16) and DSC thermograms (Fig.30-34). The melting temperature of the PCL was found to increase, as compared to initial value, when stored at higher temperatures. It ranged between 61.41°C to 63.82°C for the samples stored at different conditions. The enthalpy of fusion of PCL was found to increase from 62.82 J/gm (Initial) to about 72.57 J/gm (after 3 months storage

Table - 15 : LNG content & moisture content of DLM-2 microspheres stored at different conditions

Sr.No.	Storage condition	Time (days)	LNG content (mean)(n=3) (% w/w)	Moisture content (%w/w)
1	Initial	0	97.32	0.77
2	0-4°C	30	97.23	0.75
		60	98.91	0.76
		90	99.11	0.75
3	25±0.5°C	30	98.39	0.75
		60	94.31	-
		90	96.91	0.76
4	37±0.5°C	30	98.43	0.74
		60	98.31	-
		90	97.02	0.74
5	45±0.5°C	30	97.34	0.72
		60	97.59	-
		90	97.31	0.71
6	37°C, 75% RH	30	96.99	0.75
		60	98.69	-
		90	97.45	0.76

Table - 16 : Molecular weight distribution of DLM-2 microspheres stored at different conditions

Storage conditions	M_w	M_n	PD
Initial	105726	61576	1.72
0°C, 90 days	99924	62237	1.61
25°C, 90 days	92751	62192	1.49
37°C, 90 days	91706	56830	1.64
45°C, 90 days	89713	56646	1.58
37°C & 75% RH, 90 days	88912	54969	1.61

Fig - 30. DSC scans of DLM-2 microspheres stored at 0-4°C for 90 days

——— INITIAL, ------- AFTER STORAGE

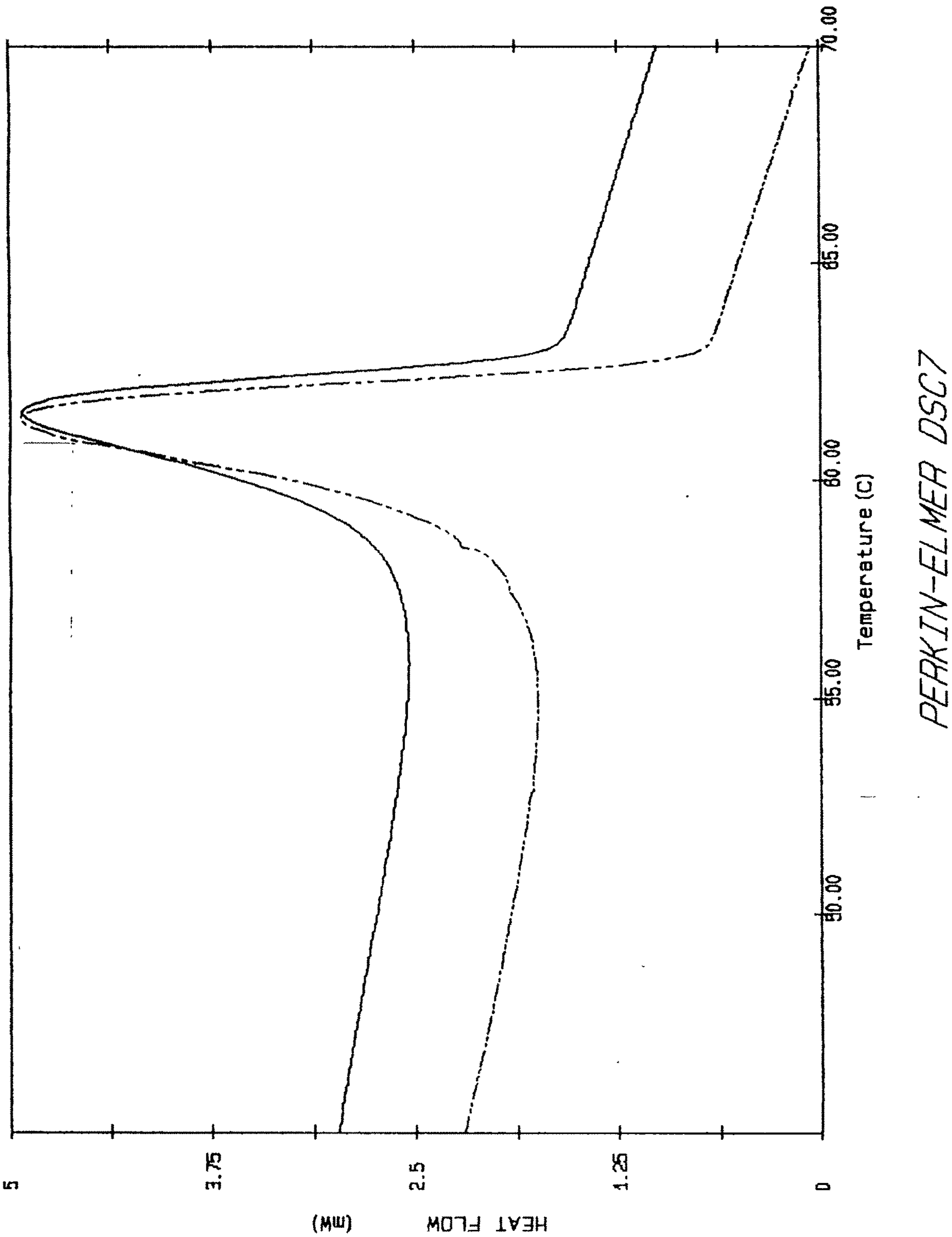


Fig - 31. DSC scans of DLM-2 microspheres stored at 25°C for 90 days

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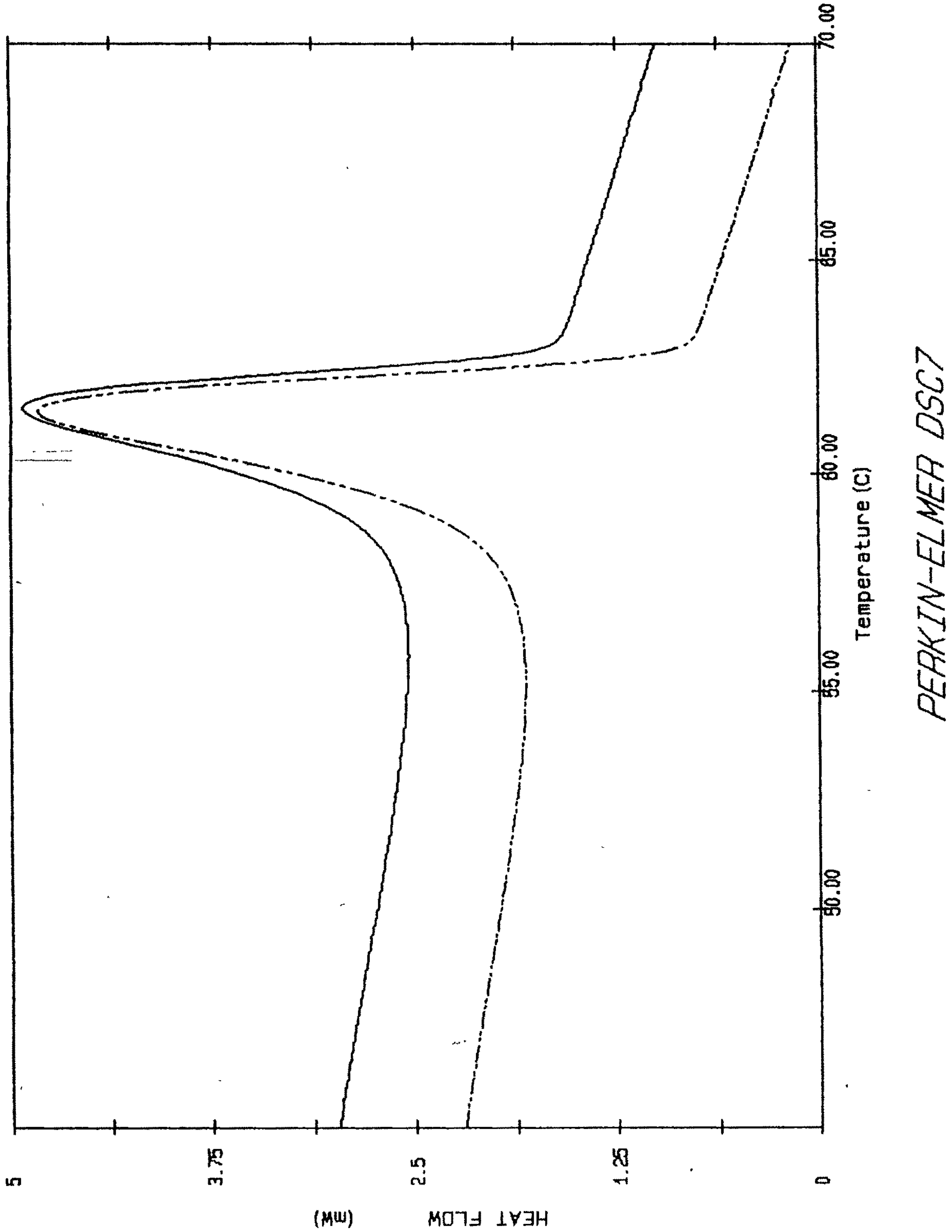

Fig - 32. DSC scans of DLM-2 microspheres stored at 37°C for 90 days

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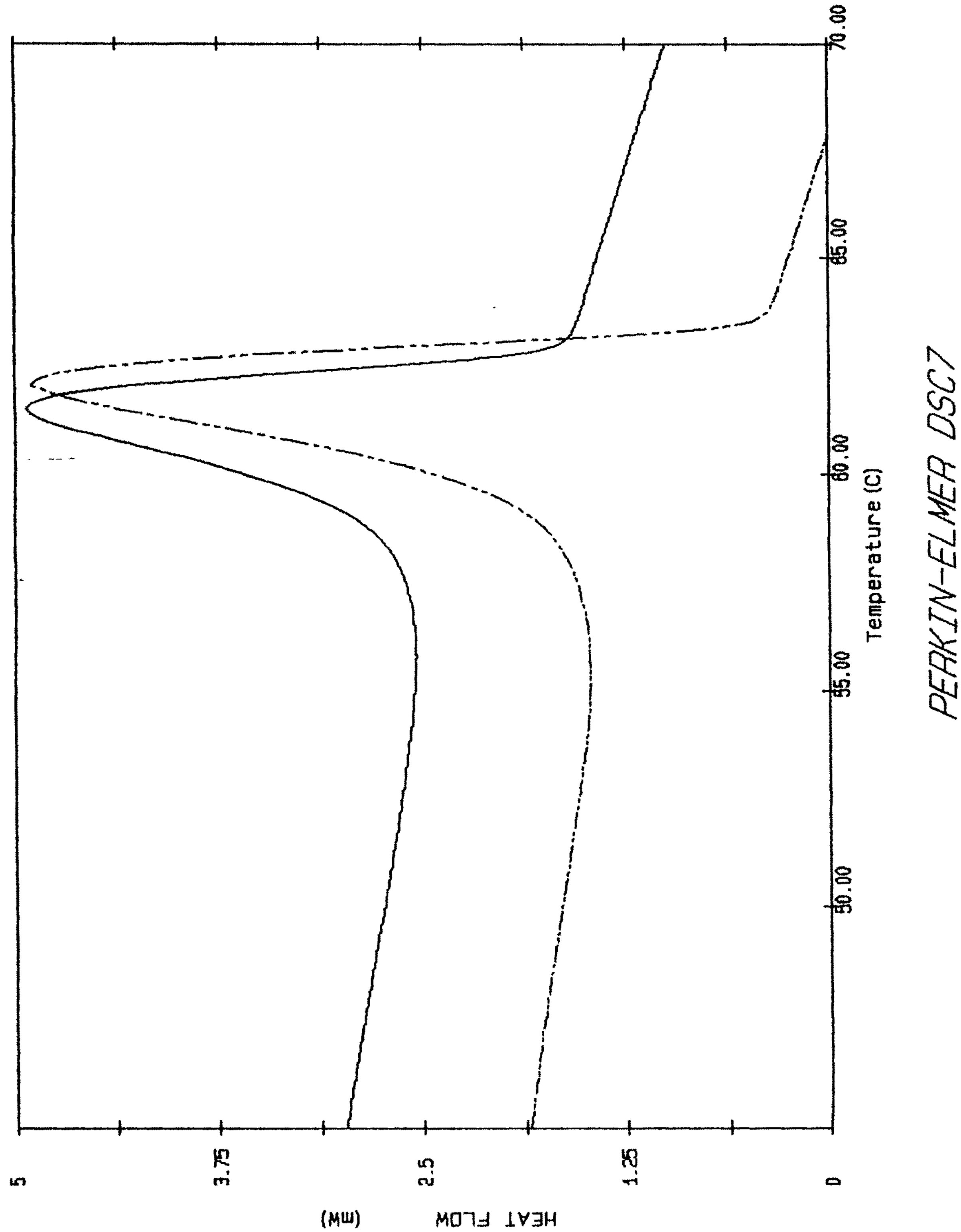

Fig - 33. DSC scans of DLM-2 microspheres stored at 37°C & 75% RH for 90 days

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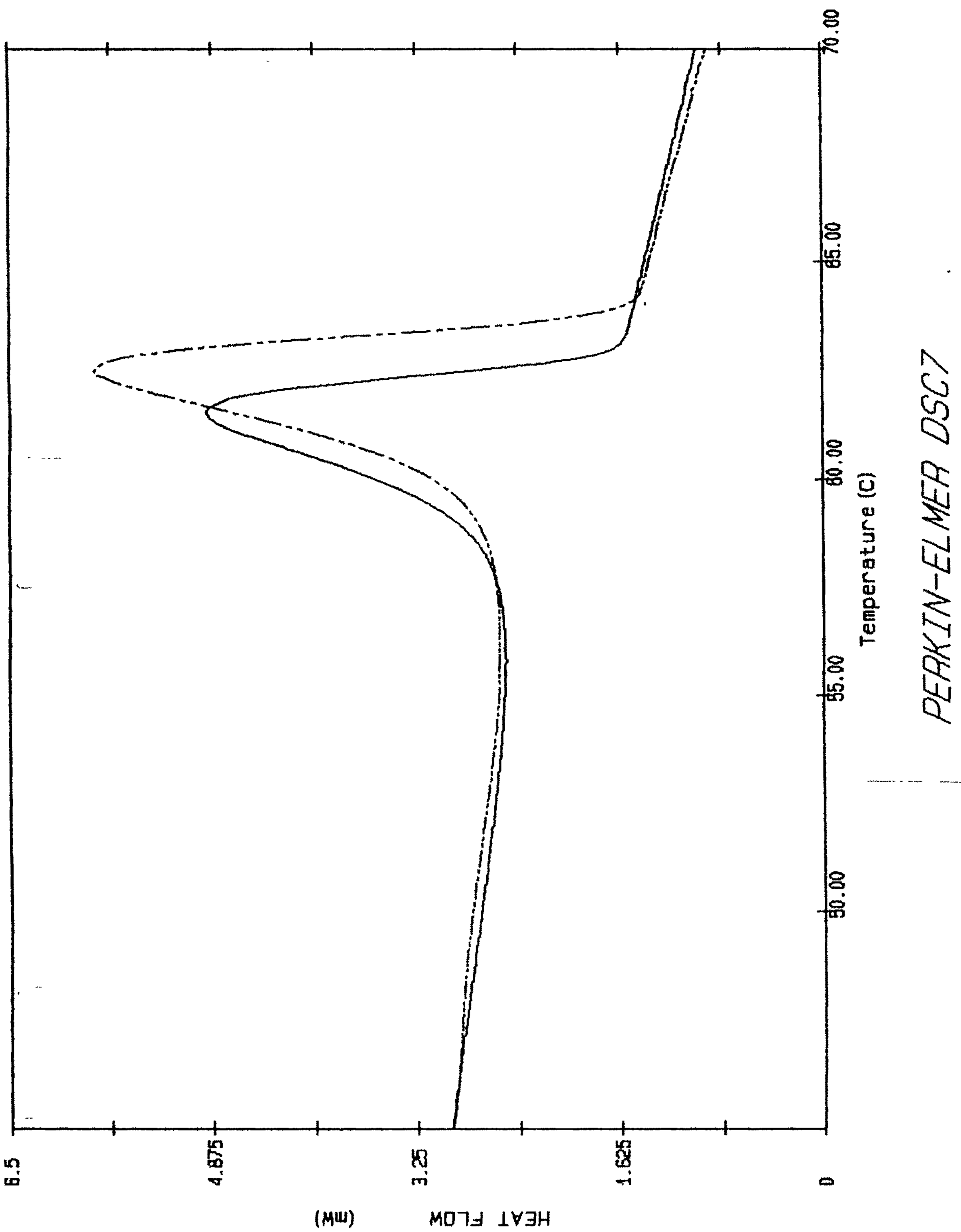

Fig - 34. DSC scans of DLM-2 microspheres stored at 45°C for 90 days

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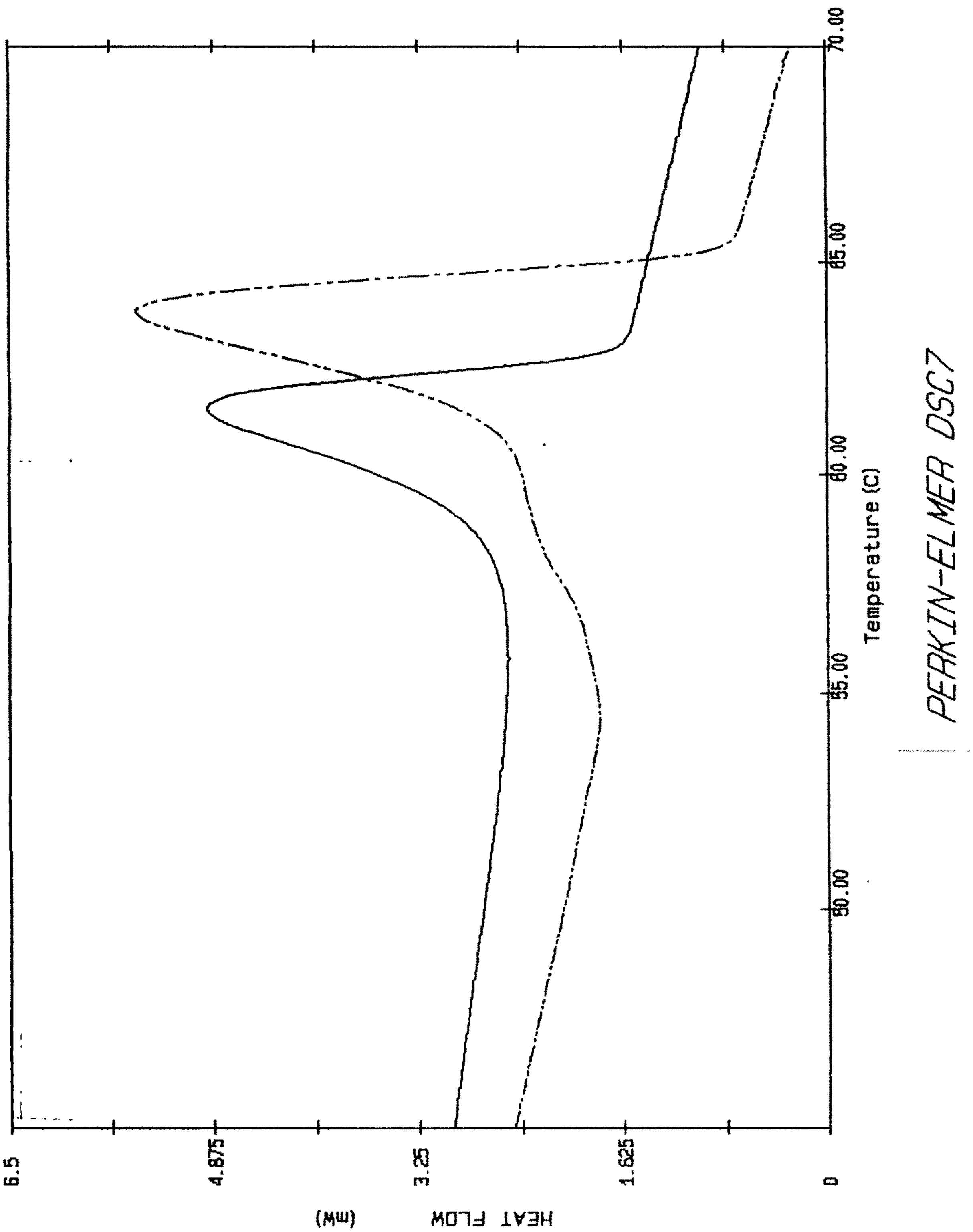
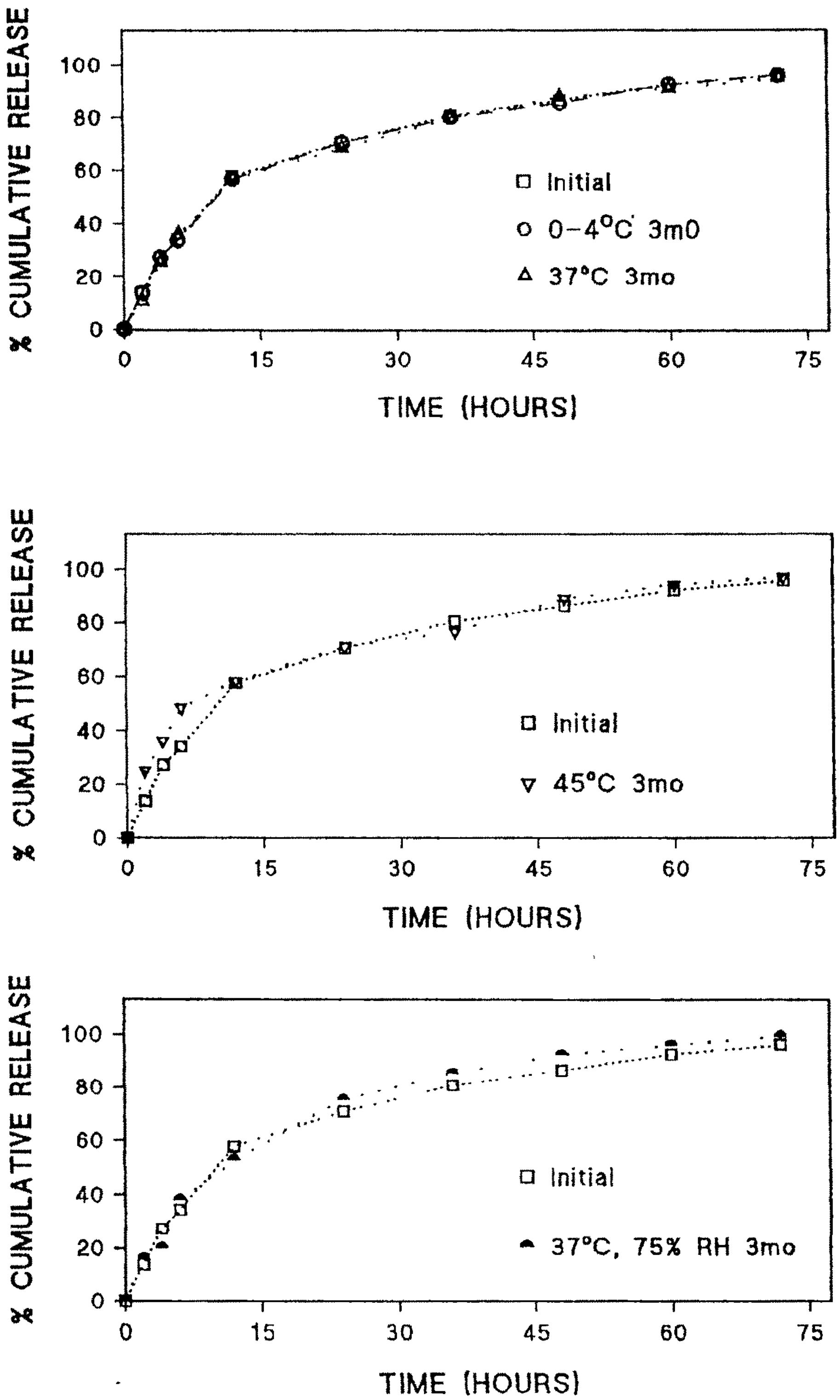


Fig – 35. In-vitro release profiles of DLM-2 microspheres stored at different temperature and humidity conditions



at 37°C or 45°C or 75% RH), indicating about 7% increase in crystallinity (45% to 52%) of PCL. On the other hand the weight average molecular weight of the polymer decreased by about 8 to 14% in the samples stored at temperature above 0-4°C. Pitt et al have also observed an increase in crystallinity of PCL with decrease in molecular weight. Further, it was observed that the *in-vitro* release pattern of LNG from the DLM-2 microspheres stored at different conditions remained unchanged. Similar results have been reported by Pitt et al. They have reporetd that *in-vitro* or *in-vivo* release of steroid drugs, from PCL implants, did not change when the crystallinity of the polymer changed from 45% to 52%. However they observed a significant decrease in release rates when the polymer was 55% crystalline.[260]

LNG content and moisture content of the sterilized samples of DLM-6 microspheres, stored at different conditions, are shown in Table 17. Molecular weight distribution is presented in Table 18, DSC thermograms in Figs.36-40, and *in-vitro* release profile in Fig.41.

The weight average molecular weight of the polymer decreased by about 10 to 13% when the DLM-6 microspheres were stored for 3 months at 25°C, 37°C and at 37°C,75% relative humidity while, a decrease of about 25% was observed in the same at 45°C after the same time period. The molecular weight distribution of DLM-6 microspheres remained unchanged when stored at 0-4°C. The DSC

Table - 17 : LNG content and moisture content of DLM-6 microspheres stored at different conditions

Sr. No.	Storage condition	Time (days)	LNG content (mean) (n=3) (% w/w)	Moisture content (% w/w)
1	Initial	0	98.69	0.59
2	0-4°C	30	99.11	0.60
		60	97.03	0.61
		90	98.24	0.60
3	25°C	30	98.03	0.58
		60	99.32	-
		90	97.12	0.59
4	37°C	30	98.01	0.58
		60	97.05	-
		90	97.12	0.58
5	45°C	30	98.65	0.58
		60	97.04	-
		90	97.31	0.57
6	37°C, 75% RH	30	96.01	0.59
		60	97.09	-
		90	97.06	0.61

Table - 18 : Molecular weight distributions DLM-6 microspheres stored at different conditions

Storage condition	M_w	M_n	PD
Initial	82481	42869	1.92
0-4°C, 90 days	79221	45012	1.76
25°C, 90 days	74888	45753	1.63
37°C, 90 days	73669	44341	1.66
45°C, 90 days	60768	35246	1.72
37°C, 75% RH, 90 days	72292	40731	1.77

Fig - 36. DSC scans of DLM-6 microspheres stored at 0-4°C for 90 days

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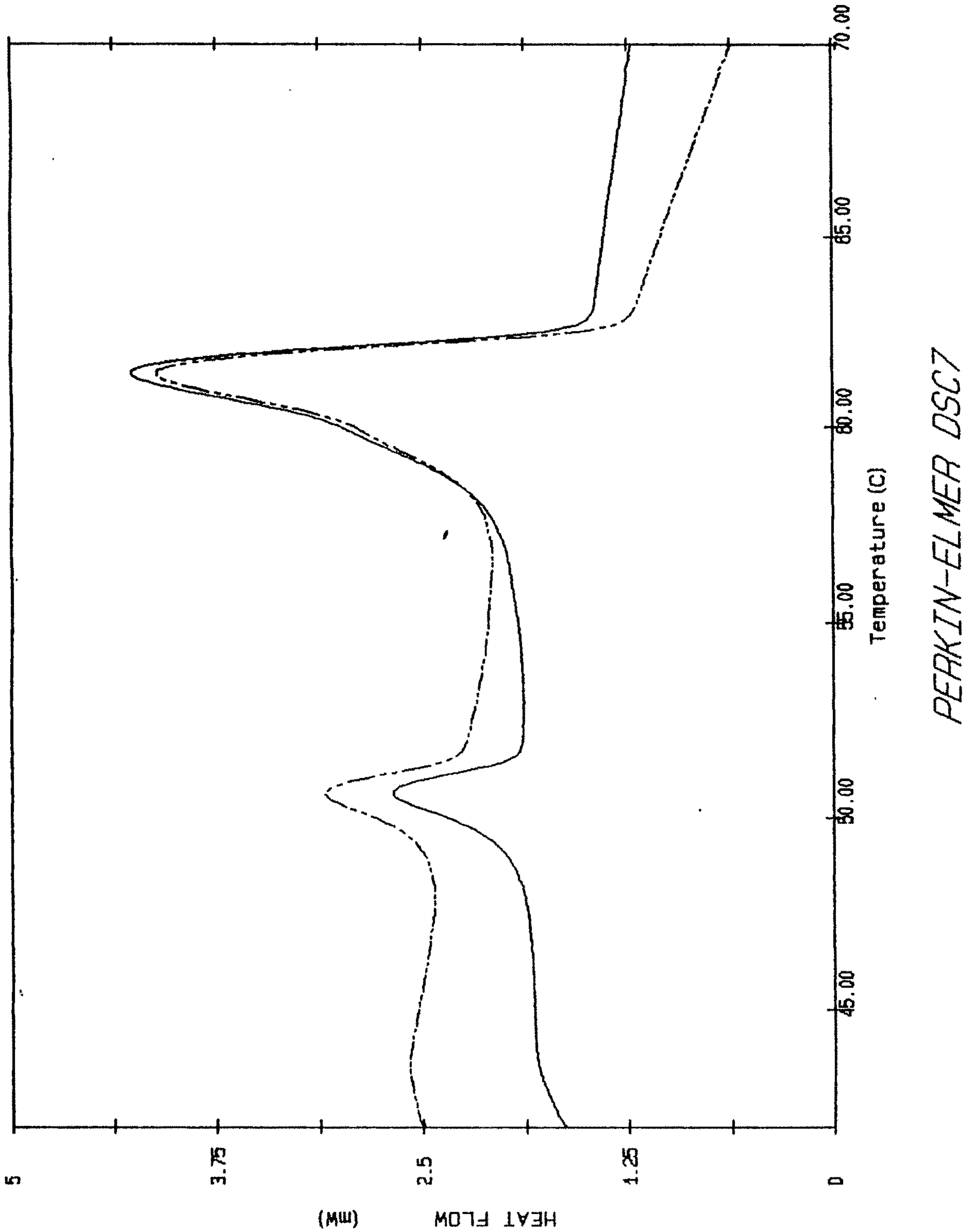

Fig - 37. DSC scans of DLM-6 microspheres stored at 25°C for 90 days

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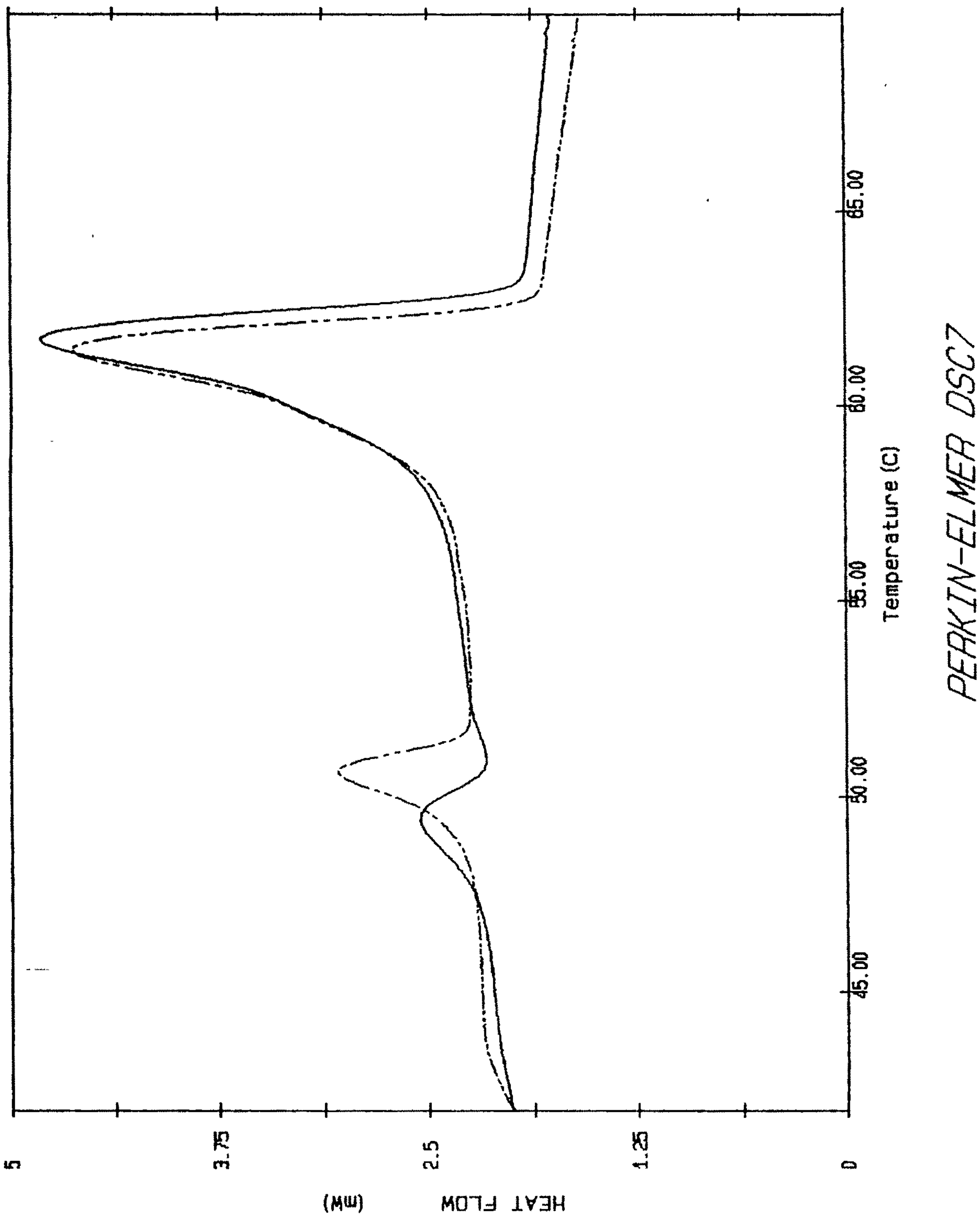

Fig - 38. DSC scans of DLM-6 microspheres stored at 37°C for 90 days

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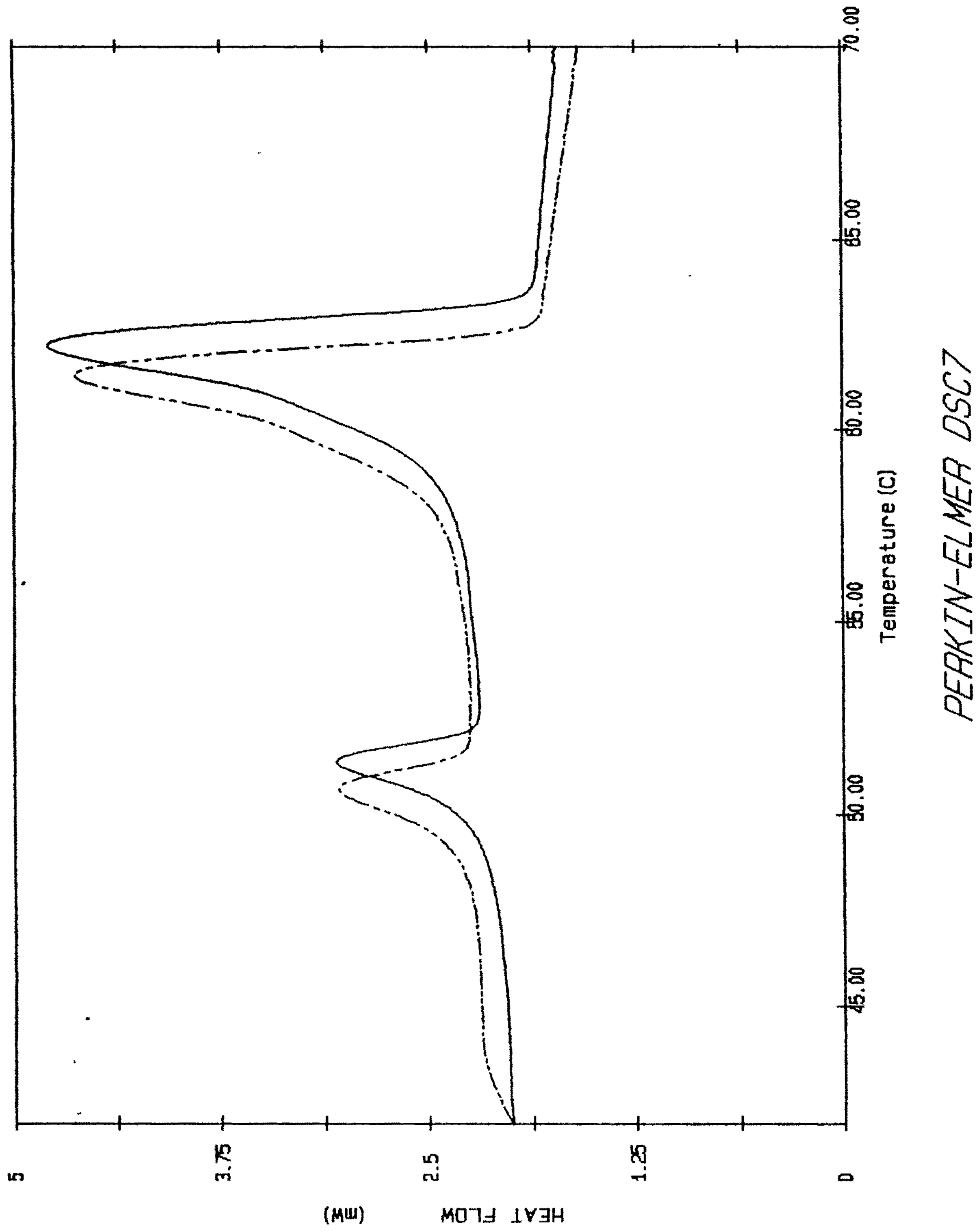

Fig - 39. DSC scans of DLM-6 microspheres stored at 37°C & 75% RH for 90 days

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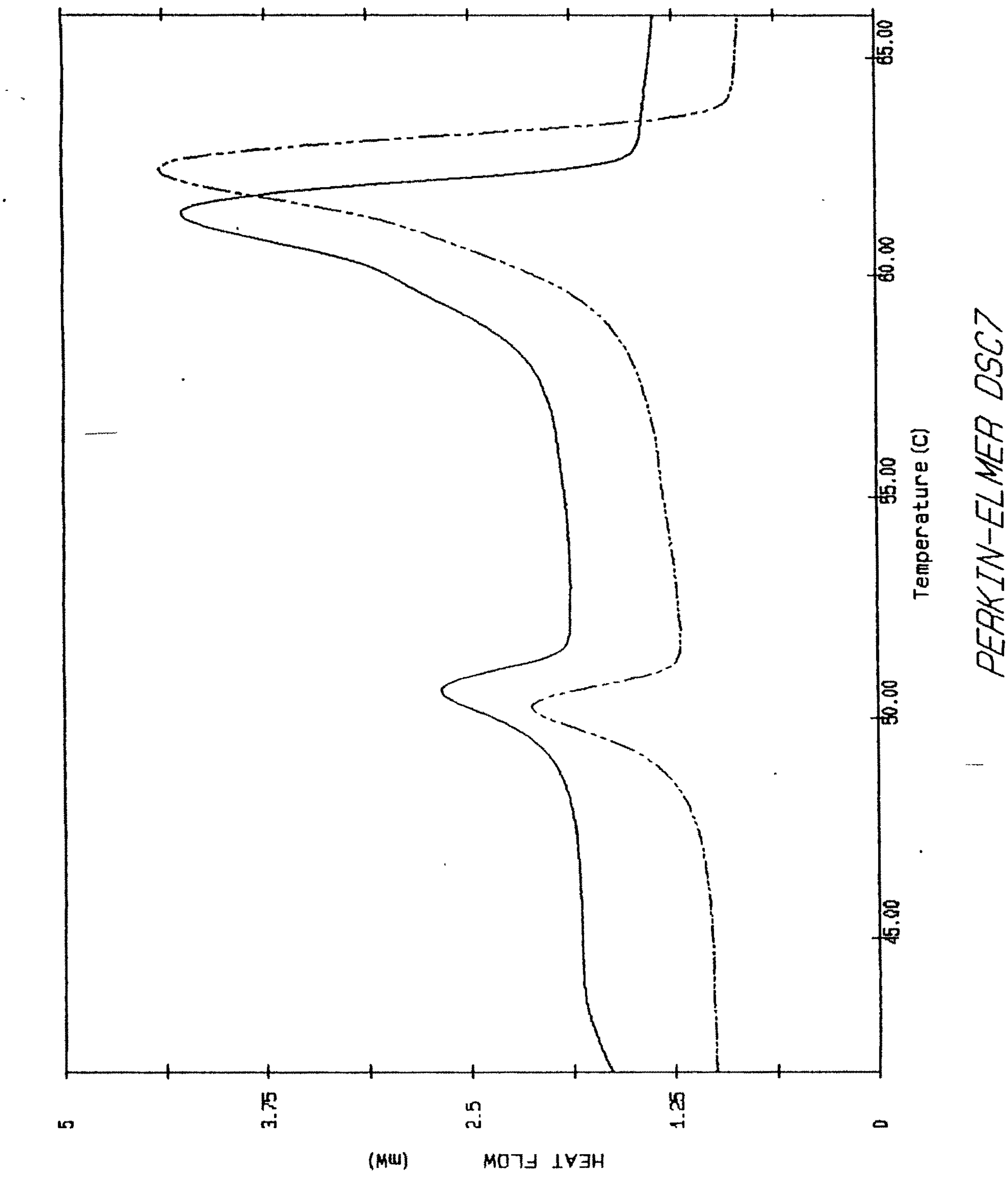

Fig - 40. DSC scans of DLM-6 microspheres stored at 45°C for 90 days

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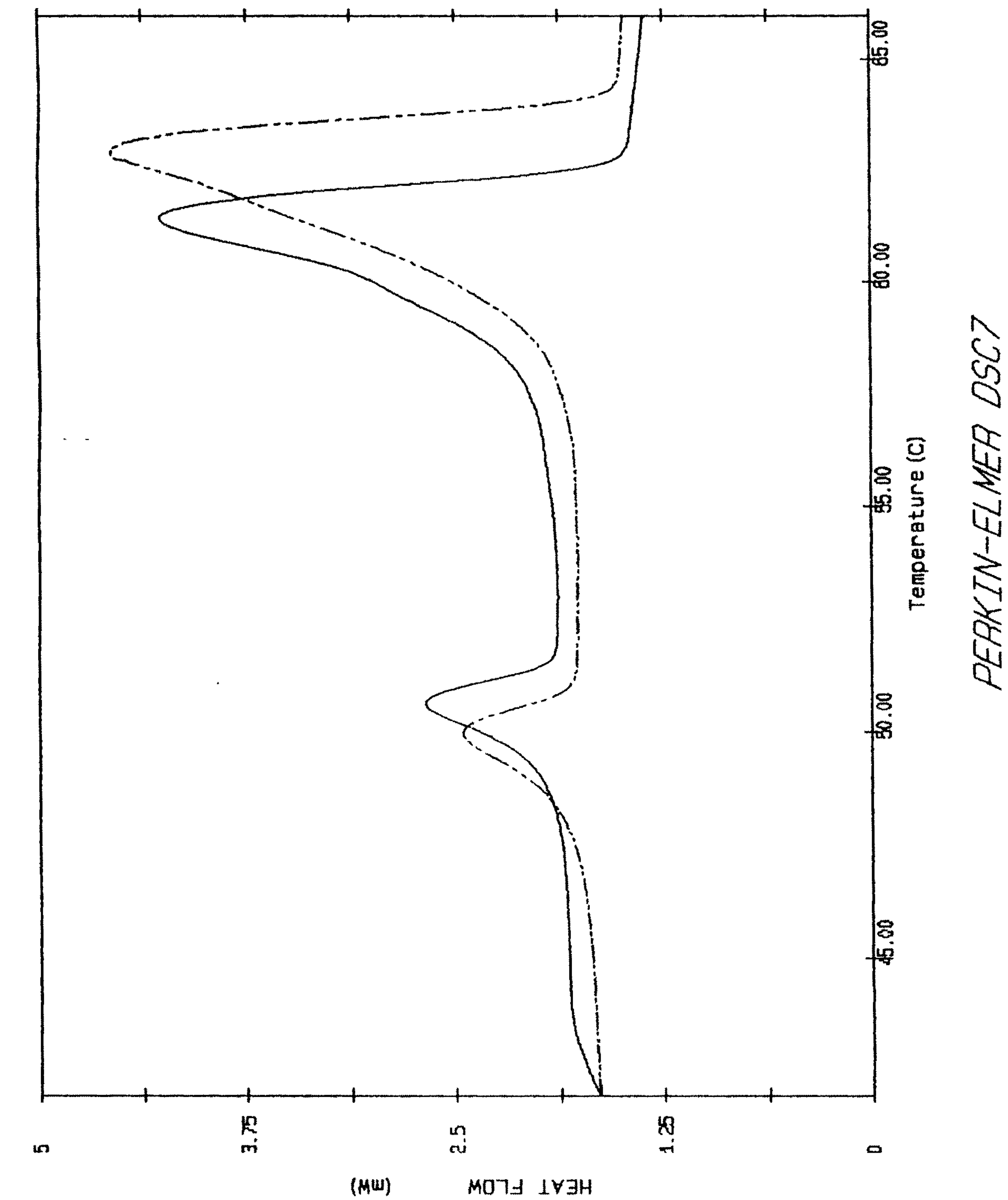
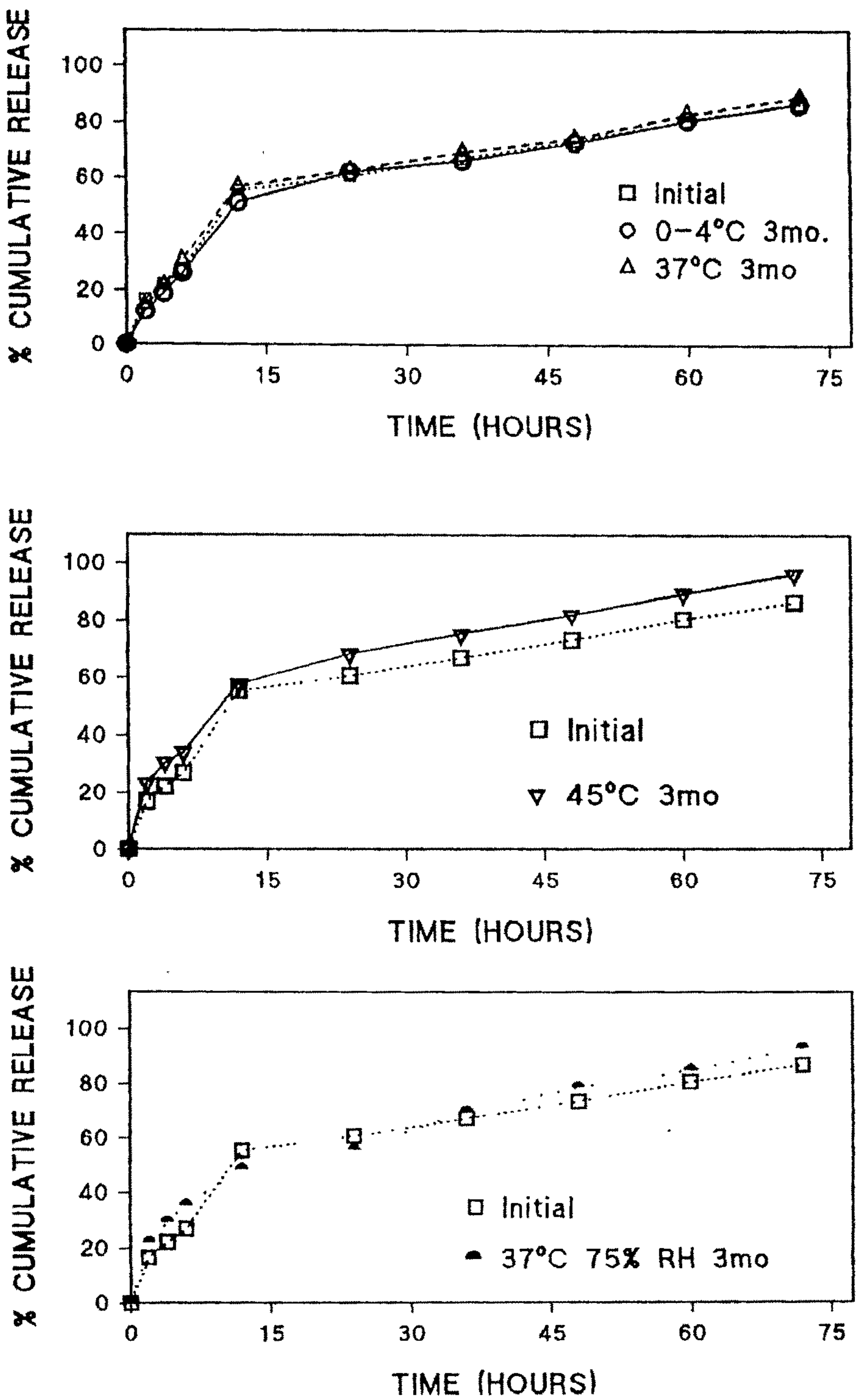


Fig – 41. In-vitro release profiles of DLM-6 microspheres stored at different temperature and humidity conditions.



thermograms showed two endothermic peaks corresponding to T_g of PLGA and T_m of PCL. The T_g of PLGA was decreased by about 2°C in samples stored at 45°C, in all other samples it was found to be similar to the initial value i.e. 49.86°C. The melting temperature of PCL for the samples stored at 37°C or 45°C or 75% RH was around 62.87°C, slightly higher than the initial value i.e. 61.43°C. This increase in T_m of PCL was also associated with increase in crystallinity of the polymer by about 2 to 3%. The T_g & T_m of the samples stored at 0-4°C were similar to those before storage. The *in-vitro* release rate of LNG increased only for the samples stored at 45°C. A greater decrease in weight average molecular weight of the polymer at 45°C explains such a release profile. LNG content and moisture contents of the samples stored at different conditons were unchanged.

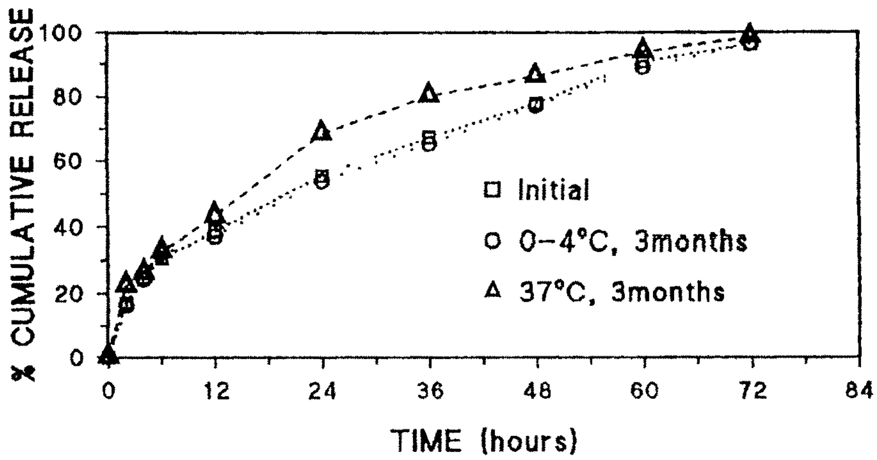
DLM-2 and DLM-6 microspheres stored at different temperature conditions were easily dispersed in the dispensing vehicle. These microspheres were sterile after 90 days.

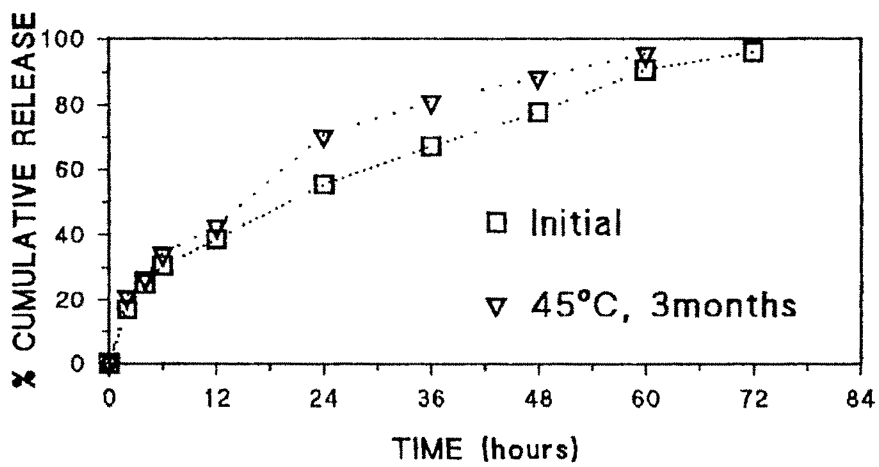
Levonorgestrel content, moisture content and friability of the implant stored at different temperature conditions are shown in Table-19. *In-vitro* release profiles are shown in Fig.42. Accelerated stability studies revealed that drug content, moisture content and friability of the implants were same at all the storage conditions. However, implants stored at 37°C or above showed deformity in shape. *In-vitro* release pattern of LNG remained same for the implants

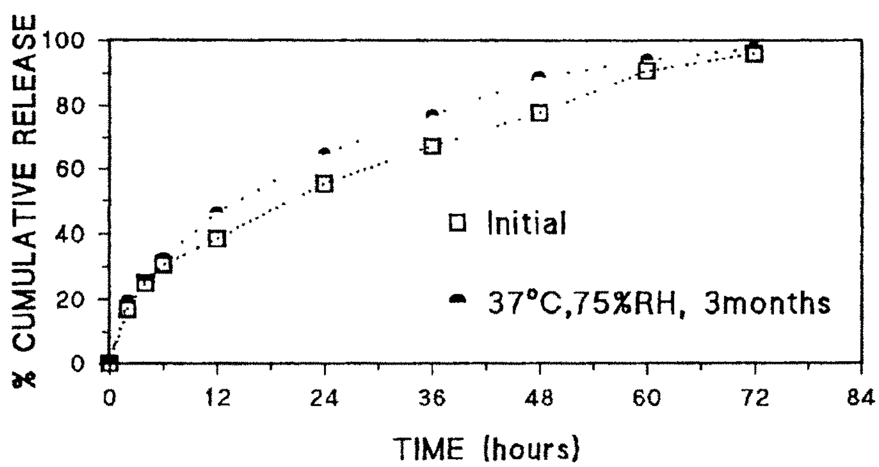
Table - 19 : Characterization of Implant (IMP-2)stored at different conditions

Sr. No.	Storage Condition	Time (Days)	LNG content (mean) (n=3) (% w/w)	Moisture content (% w/w)	Friability (%)
1	Initial	0	99.36	0.53	15.5
2	0-4°C	30	99.31	0.36	-
		60	98.52	-	-
		90	99.38	0.51	15.4
3	25±0.5°C	30	98.31	0.49	-
		60	97.62	-	-
		90	99.51	0.61	15.7
4	37±0.5°C	30	96.31	0.63	-
		60	99.91	-	-
		90	98.45	0.59	15.3
5	45±0.5°C	30	99.36	0.45	-
		60	98.02	-	-
		90	97.96	0.39	15.4
6	37°C, 75% RH	30	98.02	0.61	-
		60	97.69	-	-
		90	96.49	0.75	15.6

Fig – 42. In-vitro release profiles of LNG from IMP-2 at different temperature and humidity conditions







stored below 37°C. An increase in release rate of LNG from the implants stored at or above 37°C might be due to observed deformity in shape.

2B.5 In-vivo studies

The safety and efficacy of the selected batches of microspheres and implant were evaluated in adult female wistar rats.

2B.51 Safety studies

The safety of microspheres and implants was evaluated in terms of biocompatibility of these dosage forms with the surrounding tissue.[261] Biocompatibility of polymeric devices are described in terms of acute and chronic inflammatory responses and the subsequent formation of a fibrous capsule around it. Events which occur at the interface of the tissue and the polymeric delivery system are; an initial adhesion of macrophages, followed by phagocytosis of wound debris and erosion, invasion and phagocytosis of the polymer by macrophages and giant cells. Thereafter fibroblasts and connective tissue proliferate and encapsulate the device. Several factors like shape, surface, particle size of dispersed materials, electric phenomena at interface of tissue and device, and leaching of additives from polymers affect the type and degree of tissue injury.[262-265]

The degree of tissue injury caused by the developed dosage forms was studied by following methods

Experimental Methods

Biocompatibility studies of the microspheres :

Group of eighteen adult female wistar rats were injected with samples of LNG (5 mg) or microspheres (equivalent to 5 mg of drug) suspended in 0.5 ml aqueous vehicle [normal saline containing 0.5% w/v HPMC]. A group of eighteen rats were injected with the blank aqueous vehicle, subcutaneously in the hind leg.

At intervals of 3, 7, 30, 60 and 90 days, three rats from each group were sacrificed. The skin around the injection site was excised, cleaned and fixed in buffered formalin. The tissues were then embedded in paraffin wax blocks. Thin sections (5μ) of the tissues were cut by means of a microtome. The sections well stained with haematoxylin-eosin, and observed microscopically to assess any histopathological changes.

Biocompatibility studies of the implants :

One implant containing 5 mg of drug was placed subcutaneously, in each animal, in a group of four rats. In another group of four rats, one implant (without drug) was placed subcutaneously.

Animals were observed for gross inflammatory response for the first three days. Implantation site was observed for inflammatory response and degradation of implant on 30th, 60th and 90th day after implantation.

Results & Discussions

Results of the subcutaneous tissue injury studies for microspheres are summarized in Table-20 and Figs.43, 44, 45 & 46. It was observed that the vehicle did not produce any inflammation or granuloma, however, the uncoated drug and DLM-2 microspheres produced a mild to moderate inflammatory response which persisted till 7th day. The DLM-6 microspheres produced a moderate inflammatory response which persisted till 90th day. Such inflammatory responses have also been reported in case of subcutaneous administration of d,l - lactide /glycolide co-polymer microspheres and are considered to be safe.[261]

Subcutaneous administration of the implant did not show any gross inflammatory response. The implant was partially degraded at the end of 90 days.(Fig.47)

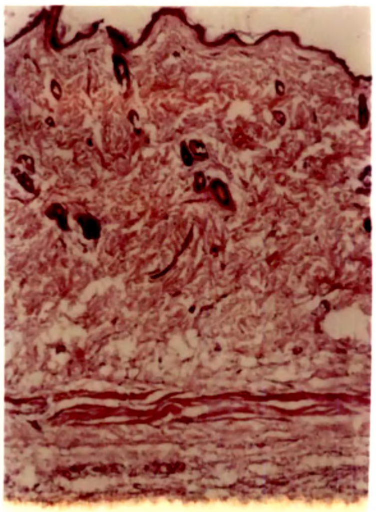
Microspheres (DLM-2) and implant (IMP-2) with LNG:PCL:PLGA ratio of 10:90:0 were selected for the efficacy studies based on the results of biocompatibility study.

2B.52 Efficacy studies

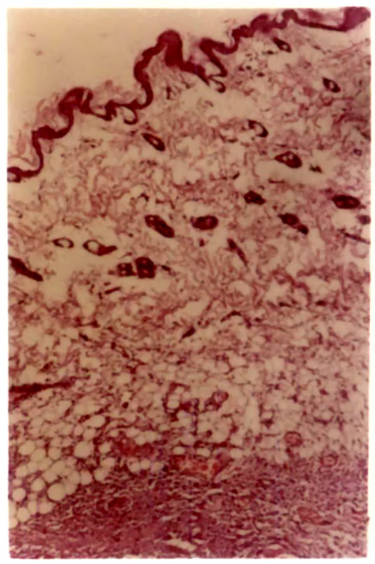
The major biological effect of levonorgestrel is to suppress the secretion of gonadotropins, follicle stimulating hormone and luteinizing hormone from the anterior pituitary gland, thereby inhibiting follicular development, ovarian steroid secretion and ovulation. Unmated laboratory female rats exhibit cyclic changes in

Fig - 43 Photographs of the tissue injury studies *(Day 3)*

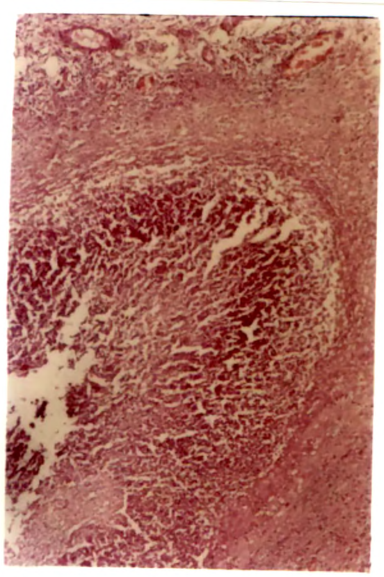
Aqueous Vehicle



Uncoated Drug



DLM-2



DLM-6

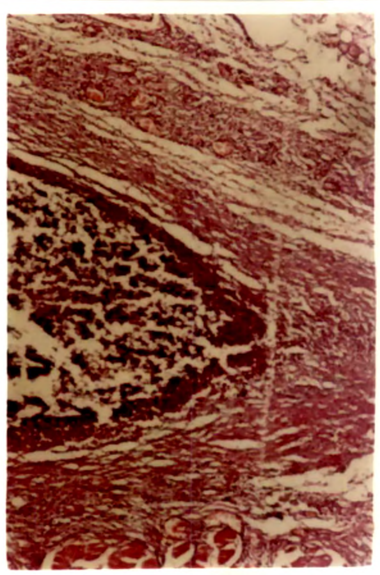
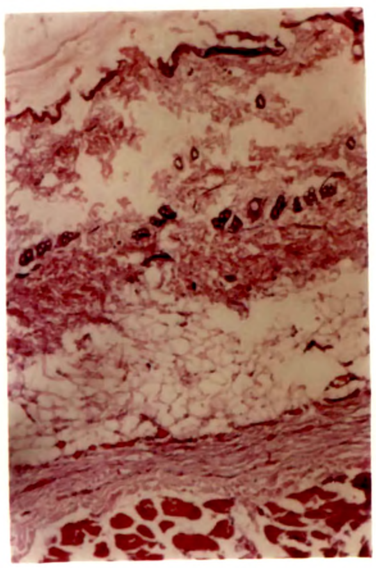
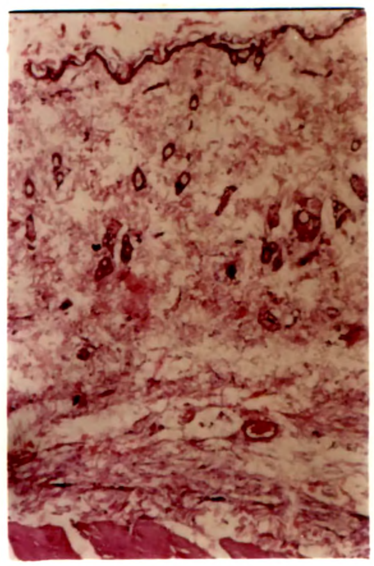


Fig - 44 Photographs of the tissue injury studies *(Day 7)*

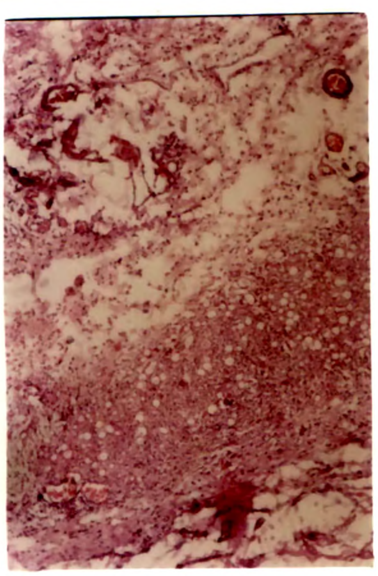
Aqueous Vehicle



Uncoated Drug



DLM-2



DLM-6

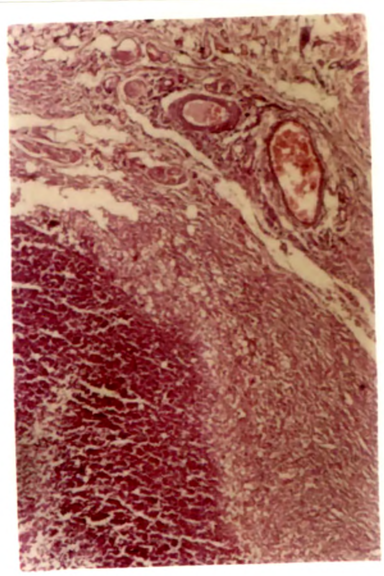
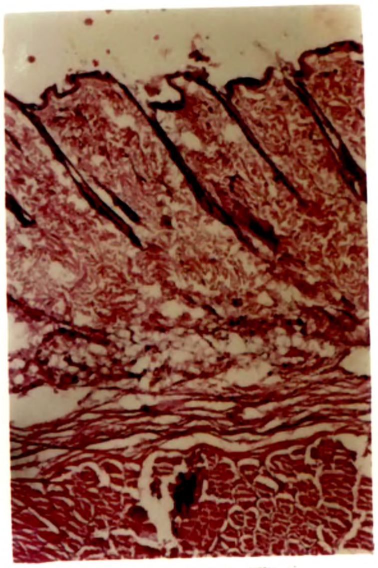
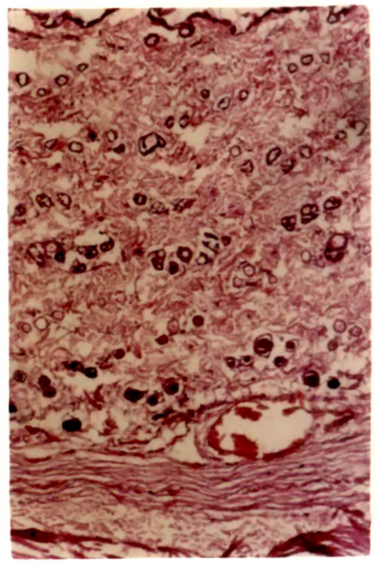


Fig - 45 Photographs of the tissue injury studies *(Day 30)*

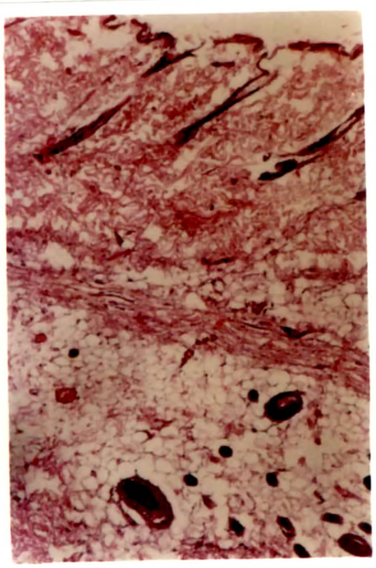
Aqueous Vehicle



Uncoated Drug



DLM-2



DLM-6

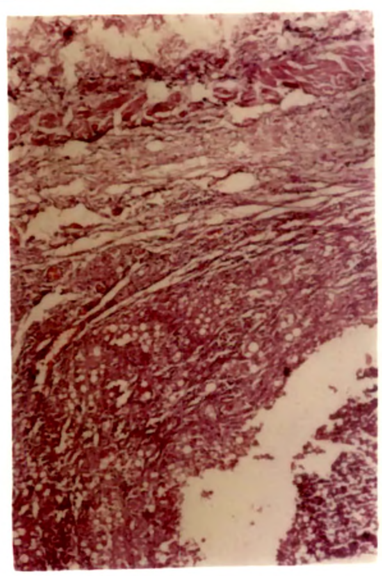
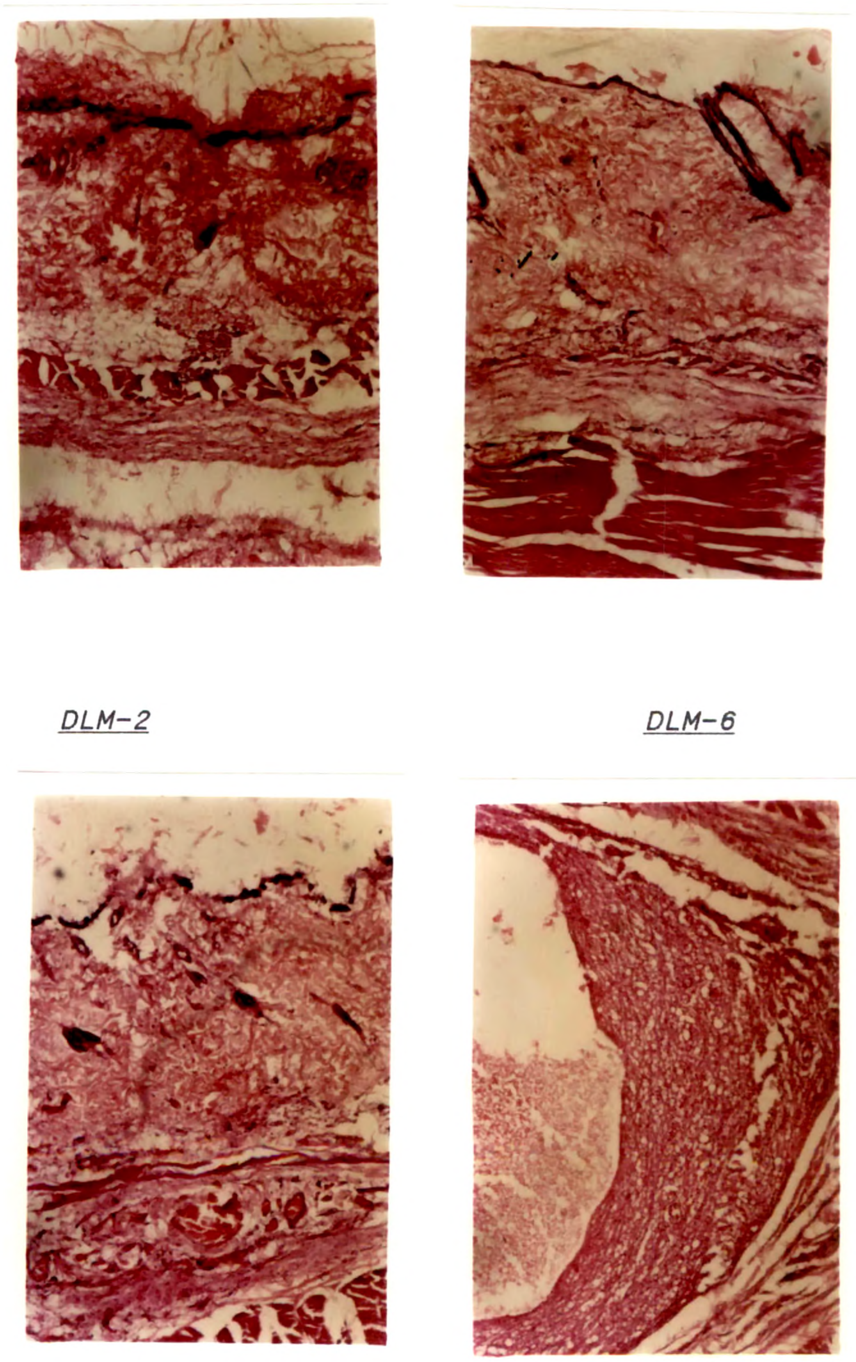


Fig - 46 Photographs of the tissue injury studies *(Day 90)*



Aqueous Vehicle — *Uncoated Drug*

DLM-2 — *DLM-6*

Fig - 47 Photographs of the tissue injury studies *(IMP-2)*

After 30 days

After 60 days

After 90 days

Table - 20 : Results of tissue injury studies

Observation Day	*Formulation*			
	Aq. Vehicle	Uncoated drug	DLM-2	DLM-6
3rd	None - minimal inflammation	Mild inflammatory response L,PMNs & M present	Mild-moderate inflammatory response L, M, D & PMNs present	Moderate inflammatory response L, M, F.C., & PMNs present
7th	None - minimal inflammation	Mild inflammatory response L,PMNs & M present	Mild inflammatory response L,PMNs & M present	Moderate inflammatory response L, M, F.C., & PMNs present
30th	No inflammatory response	No inflammatory response	None - minimal inflammation	Moderate inflammatory response L, M, F.C., G.C., C.D., & PMNs present
60th	No inflammatory response	No inflammatory response	No inflammatory response	Mild-Moderate inflammatory response F.C., G.C., C.D. & PMNs present
90th	No inflammatory response	No inflammatory response	No inflammatory response	Mild-Moderate inflammatory response F.C., G.C. & C.D. present

L - leucocytes; M - macrophages; PMNs - polymorphneucleocytes; D - cell debris;
F.C. - fibrous capsule; G.C. - giant cells; & C.D. - collagen deposition

vaginal cytology over the course of the estrous cycle and these changes are biological indicators of fluctuations in release of ovarian hormones. In most non-pregnant laboratory rats a complete cycle of such changes occurs every 4 or 5 days. The estrous cycle of non-pregnant female rats show four different stages. The first stage is **estrus** which lasts for about 12-18 hrs and is characterized by the predominance of large cornified cells (CC). The second stage is **metestrus** in which the cornified cells become less numerous and small round cells (leucocytes) are present. The metestrus phase lasts for about 10-12 hrs. The next stage is **diestrus** which is characterized by the predominance of leucocytes (LC) and lasts for about 48 hrs. The fourth, **proestrus** stage, is characterized by the presence of many nucleated epithelial cells (NC) and duration of this phase is about 12 hours.[266,267] Subcutaneous administration of progesterone has been reported to delay estrous cycle of rat.[268,269]

Subcutaneous administration of levonorgestrel, blocks the estrus cycle of female rats and retains them in diestrus stage. The developed formulations were, therefore, evaluated for the ability to block the estrus cycle in the diestrus stage.

<u>Experimental Methods</u>

The efficacy of LNG containing formulations were evaluated in gonadally intact, cycling female wistar rats, by subcutaneous administration of the following formulations

- aqueous vehicle
- levonorgestrel (Dose 5mg.)
- microspheres (equivalent to 5mg. LNG)
- implant LNG (equivalent to 5mg. LNG)

Four animals showing normal (4.5 day) estrous cycles in the preinjection period were used in each treatment group. Daily vaginal lavage was taken using standard procedures and then transferred to a slide for microscopic examination of vaginal cell morphological changes in the estrous cycle. Daily observation of the vaginal lavage was continued until the experiment group resumed at least one normal cycle. The smears were scored as to predominance of LC (indicative of diestrus), NC (indicative of proestrus) and CC (indicative of estrus). To quantify the ability of the formulation treatments to block estrus cyclicity, the mean number of consecutive days in the diestrus stage for each animals was scored, for the first seventy five days after treatment with formulations.

Results & Discussions

Results are outlined in Fig.48. The control animals which were treated with aqueous vehicle, maintained normal cyclic changes in vaginal cytology and remained in diestrus stage for normal period of 2 consecutive days. The animals, which were treated with uncoated drug (Dose 5 mg), showed suppression of estrous cycle and remained in the diestrus stage for 12 consecutive days. The group of animals, treated with microspheres equivalent to 5 mg of LNG, showed prolonged suppression of estrous cycle and remained in the diestrus stage for 63 consecutive days. On the other hand, estrous cycle of the group of animals treated with implant was normal for the first 4 days, later on it remained the diestrus condition for 67 consecutive days. This might be due to insuffiecient release of LNG from the implant during initial period. Similar observations has been reported by Gao et al after intramuscular administration of levonorgestrel containing gel formulation.[270]

No deaths occurred during the entire period of study, indicating absence of any lethal effect for all the treatment groups.

Fig – 48 Mean number of consecutive days in diestrous phase