Department of Pharmaceutical Sciences, Dr. Harisingh Gour University, Sagar, India

Dendrimer grafts for delivery of 5-flurouracil

P. K. TRIPATHI, A. J. KHOPADE, S. NAGAICH, S. SHRIVASTAVA, S. JAIN and N. K. JAIN

Polyamidoamine (PAMAM) dendrimers were prepared by linking methyl methacrylate and ethylenediamine successively on an amine core. Surface modification of PAMAM dendrimer was done by fatty acid grafting converting them to a unimolecular micellar system (Dendrimer grafts). IR, ^{1}H NMR, ^{13}C NMR studies confirmed the structure. The drug 5-fluorouracil (5-FU) was entrapped in dendrimer grafts. The effects of various solvents (ethanol, dichloromethane, tetrahydrofuran), pH and ionic strength on solubilization of 5-FU were determined. Phospholipid was further coated on the dendrimer grafts. The product was lyophilized and obtained as yellowish-white powder. Average particle size was ca. 375 nm as determined by Malvern's Mastersizer 4. Drug loading was ca. 53% by weight. Stability studies were conducted for 1 month at room temperature and 40 °C, where the systems were relatively stable. Release rate was sustained across cellulose tubing in PBS. *In vivo* studies were performed in albino rats and pharmacokinetic parameters and bioavailability were determined from the plasma profile of 5-FU. The phospholipid coated dendrimer graft formulation was found to be more effective orally than free drug. The lymphatic uptake was also increased indicating absorption of the developed formulation through the lymphatic route.

1. Introduction

Dendrimer, a unique hyperbranched polymer, has attracted attention from pharmaceutical scientists for targeting and controlled drug delivery due to its core-shell property [1, 2]. PAMAM dendrimers are one of the simplest types of dendritic systems synthesized by repeated of exhaustive Michael addition and exhaustive amidation. Dendrimers have a wide range of applications, which include their use as vaccines, magnetic resonance imaging aids, delivery system for drug and genes, immunoassays etc [3, 4]. Their highly uniform size, globular structure, functional specificity and large number of functional groups distinguish them from conventional delivery systems. Their surface functionality allows modifications to produce unimolecular micellar or reverse micellar dendrimer cores, where the bio-actives can be incorporated. In 1996, host guest properties of inverted unimolecular dendritic micelles synthesized by modification of end groups of hydrophilic poly (propyleneimine) dendrimers with hydrophobic alkyl chains were investigated by Stevelman et al. [5]. Tomalia et al. [6] reported the use of hydrophobically modified PAMAM dendrimers as container molecules for copper sulphate in an organic solvent.

In the present study an effort was made to covalently link fatty acids on to the surface of PAMAM dendrimers to form a hydrophilic-hydrophobic core-shell structure [7] wherein a water soluble drug can be entrapped and released slowly. The phospholipids were then coated in order to make them biomimics of lipoproteins similar to an approach reported for supramolecular biovectors [8]. A water soluble drug, 5-fluoronracil (5-FU), was selected which is one of the most commonly used drugs for various tumors. Its a application, however as a number of limitations such as short biological half-life, host toxicity, non-specific distribution, incomplete and non-uniform oral absorption.

Supramolecular bio-vectors [8] and reverse micelles [9] are used to enhance absorption of water-soluble drugs, which suffer from low gastrointestinal. absorption due to enhanced lymphatic uptake. Therefore, it was envisaged that the dendrimer grafts of fatty acid may be regarded as covalently fixed models of reverse micelles [6] with the advantage that this structure may not be affected by the gastric environment like a fluid reverse micellar structure. They also resemble supramolecular biovectors in having a hydrophilic core and a hydrophilic fatty acid grafted shell. The present paper explores the potential of these phospholipid coated dendrimer grafts for the oral delivery of encapsulated 5-FU.

2. Investigations, results and discussion

The synthesis of starburst PAMAM dendrimers was started using ammonia as core by the reported two-step repeated process involving exhaustive Michael addition and exhaustive amidation of the resulting esters [10]. A novel inverted unimolecular micellar dendritic molecule was prepared by the modification of end groups of hydrophilic PAMAM dendrimers with hydrophobic fatty acid moieties [11]. The modifications consist of the conversion of 4° G generation (48 primary amines) of the PAMAM dendrimers into their amide analogue with palmitoyl chloride in tetrahydrofuran in the presence of triethylamine as a base. Both water-soluble and water-insoluble products were obtained. These product characteristics made separation and characterization very easy. The yield was 78% for the water insoluble compound. The water-insoluble product was used for further characterization. Structural validation was done using IR, ^{1}H NMR, ^{13}C NMR spectroscopy. The carbonyl IR peaks at 1701 cm^{-1}, amide-NH stretching peak at 3377 cm^{-1}, peaks of C−N at 2920 cm^{-1} and N−H bending at 1490 cm^{-1} confirm the formation of fatty acylated dendrimer. Peaks for protons were also obtained in ^{1}H NMR at various δ values viz. 0.901 ppm for CH_3 (triplet), 1.635 ppm for $-CH_2$ of fatty acids (multiplet), 3.6 ppm for $-CH_2-NHCO$ and 4.40 ppm for $-NHCO$ alone, confirming the acylation. ^{13}C NMR showing characteristic peaks of carbon at δ equal to 24.7 ppm, 29.1–29.7 ppm, 76.6–77.4 ppm for fatty acids CH_2 groups and at 25 ppm, 34.1–34.4 ppm, 60.2 ppm and 180.5 ppm for $NHCO-CH_2-CH_2$ group also provided the evidence of fatty acylation of the dendrimers. The analysis shows that more than 95% palmitoyl chloride was attached to the dendrimer.

The drug was solubilized in dendrimer-grafts (DG), which also served as an evidence for their capability to act as host-guest system. The dendritic core is relatively hollow

and contains free channels extended from core to the periphery, where the drug gets encapsulated. Different solvent systems were chosen for entrapment of drug in the inner hydrophilic core in DG. In ethanol and tetrahydrofuran solution both drug and DG were soluble therefore entrapment takes place by equilibration. In methylene chloride, the aqueous solution of DG exists analogous to reverse micelles in which drug is partitioned. The entrapment was relatively less than the ethanolic solution, perhaps due to a slow partitioning process through layer of fatty acid chains (Table 1). The λ_{max} of encapsulated 5-FU shifted from 266 nm to 278 nm. This suggests some interaction between the DG core and the drug. The encapsulation was determined by extracting the drug from the inverse micellar system using toluene, as it was not possible to extract drug by simple washing with water. The solubilization reached saturation values and was independent of the drug concentration used for encapsulation. This is in contrast with a conventional micellar system where increasing amounts can be solubilized with increasing concentrations of drug to a limit that destroy micellar structures. There was a linear relationship between the amount of 5-FU dissolved and the concentration of DG (Table 1). Solubilization ability of DG was observed at a concentration as low as 1×10^{-5} mol $\cdot$ l^{-1}. This marks the contrast with traditional micellar behavior where essentially no solubility enhancement occurs below the critical micelle concentration e.g. 8.1×10^{-3} mol $\cdot$ l^{-1} for sodium lauryl sulphate [12].

Effect of pH was studied as interior of DG system is shielded from the solvent by apolar palmitoyl barrier and to study if there is unique guest selectivity as function of pH. The solubility of 5-FU was 0.147 mg $\cdot$ ml^{-1}, 0.135 mg $\cdot$ ml^{-1} and 0.117 mg $\cdot$ ml^{-1} at pH 1.2, 7.0 and 9.0, respectively. The increase in the internal volume of DG at lower pH, due to spreading of branches as a result of ionization of secondary and tertiary amines, could be the reason for the slightly higher solubility [13]. The possibility of ionic interaction is remote, as both drug and dendrimer core would be positively charged at low pH. There was a slight effect of ionic concentration at 0.1 M NaCl, but a significant decrease in the solubility of 5-FU was noted at 0.5 to 1.0 M NaCl. The solubility was 0.126 mg $\cdot$ ml^{-1}, 0.093 mg $\cdot$ ml^{-1} and 0.034 mg $\cdot$ ml^{-1} for 0.1, 0.5 and 1.0 M NaCl concentrations respectively. The solubility was 0.104 mg $\cdot$ ml^{-1}, 0.075 mg $\cdot$ ml^{-1} and 0.013 mg $\cdot$ ml^{-1} for 0.1, 0.5 and 1.0 M $CaCl_2$ concentrations respectively showing the decreasing effect being more prominent for divalent cationic salts. This may be due to enhanced folding of dendrimer branches [14].

Phospholipid was coated on dendrimer grafts (DGP) using a mixture of phospholipid and a hydrophilic surfactant octylglucoside to avoid the formation of liposomes and to obtain an uniform dispersion. Without hydrophilic surfactant it was not possible to disperse the DGP uniformly in water. This led us assume that the DG is coated with a bilayer forming as a result of coating step and dialysis (Fig. 1): first layer with head groups inserted in fatty-acyl chains towards the hydrophilic dendrimer core is formed in methylene chloride (polar interactions are favored in non-polar solvent) and a second layer is formed during dialysis against water when excess surfactant (octylglucoside) diffuses out leaving some surfactant and phospholipid molecules in a conformation with tails embedded in fatty chains of DG dendrimer and heads outside. The assumption is, however, speculative and need to be proved further. Formulation was obtained in both lyophilized powder and suspension form.

Table 1: Effect of solvent and concentration on drug-dendrimer solubilization

Solvent	Dendrimer solution ($\times 10^{-5}$ M)	5-FU dissolved (mg $\cdot$ ml^{-1})
Ethanol	1	0.156
	2	0.257
	3	0.396
Methylene chloride	1	0.136
	2	0.257
	3	0.396
Tetrahydrofuran	1	0.125
	2	0.252
	3	0.384

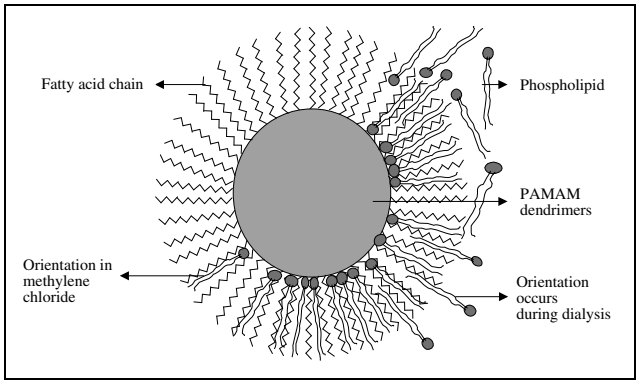

Fig. 1: Proposed phospholipid coating of dendrimer solubilizate system (Dendrosomes)

The drug loading of DGP formulation was found to be $52.9 \pm 1.3\%$ w/w. The shape and size of the DGP was examined by Transmission Electron Microscopy (TEM). The particles were spherical and their size was in the nanometric range. Some aggregates were also seen perhaps due to drying effects. The size was determined by a particle size analyzer, which confirmed that particles are in the 100–700 nm range. The average size was found to be 375 nm. The size is sufficiently large as for an assumed ideal phospholipid coated dendrimer graft, which clearly indicates the presence of aggregates due to hydrophobic interaction in water. There is a sharp peak in the distribution curve indicating low polydispersity. Release studies

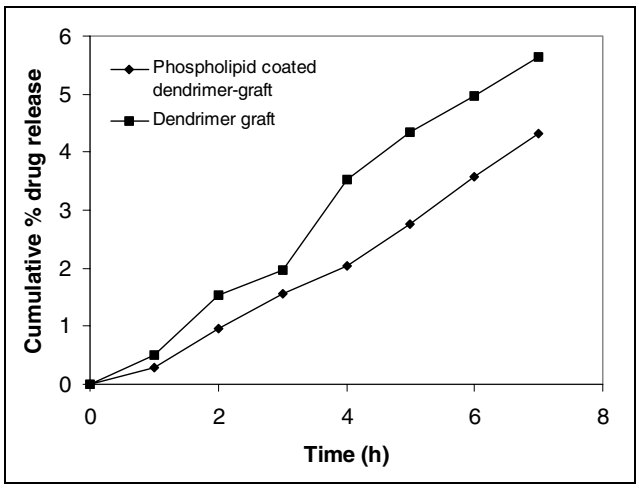

Fig. 2: Release profile of 5-FU from dendrimer graft (DG) and phospholipid coated dendrimer graft (DG-P) formulation

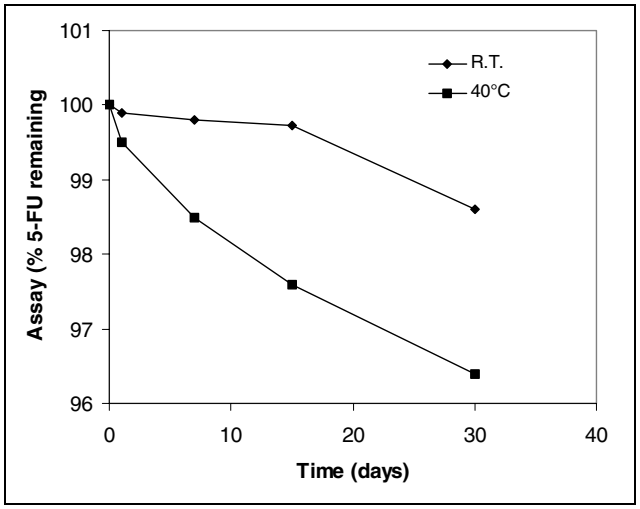

Fig. 3: Stability studies of phospholipid coated dendrimer graft (DG-P) formulations

were carried out in PBS at pH 7.4 (Fig. 2). There was no release of 5-FU up to 48 h. Only 4.3% were released in 7 days which shows that the release of the hydrophilic drug is extremely retarded. The very slow release was also reported by Zhou et al. [15] with a 5-FU dendrimer conjugate where the release was due to hydrolysis of the conjugate. Our results indicate an even slower rate perhaps due to a slow partition across the fatty layer compared to the hydrolysis rate of the dendrimer-5-FU conjugate reported by Zhou et al. Stability studies show less than 2% degradation for lyophilized powder while about 4% for suspension in 30 days indicating relatively good shelf-stability of the formulations (Fig. 3).

The *in vivo* performance is shown in Fig. 4 as plasma/lymph profile of 5-FU. The pharmacokinetic parameters were calculated from these curves (Table 2). The AUC was found to be 6.64 μg ml^{-1} h^{-1} for i.v. administration of 500 μg of 5-FU, which increased to 12.42, and 21.52 μg ml^{-1} h^{-1} for oral administration of 2500 μg of free 5-FU and an equivalent amount of DGP formulation, respectively. The elimination of drug was lowered and subsequently the half-life increased. The results indicated a bioavailability of about 37.4% for the free drug through the oral route, which resulted in a 64.8% or 1.7 fold increase. The high 5-FU concentration in lymph (solid circles in Fig. 4) indicated that absorption of drug through lymphatic pathway was significant. The uptake and translocation of hydophobically modified poly(L-lysine) dendrimer through G.I. tract was investigated by Florence et al. [16]. They reported that dendrimers were directly absorbed through G.I. tract but uptake is lower than that of 50 nm latex particles.

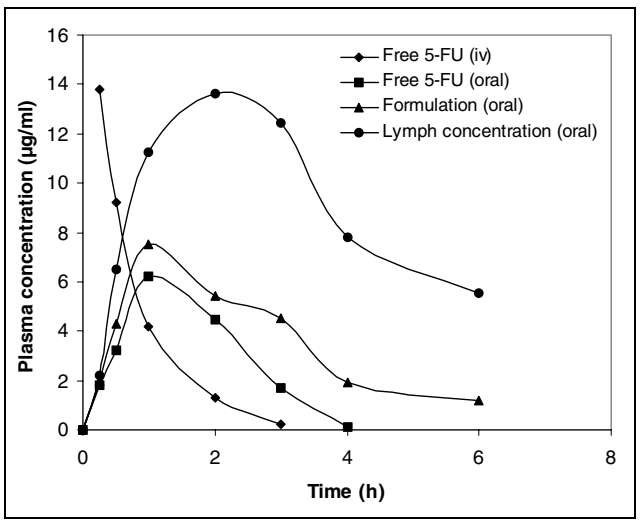

Fig. 4: Plasma/lymph profile of 5-FU from various formulations (indicated in figure).

Table 2: Pharmacokinetic parameters of 5-FU administered as free drug and dendrimer formulation through oral and intravenous route

S.No.	Parameter	IV administration Free drug 500 μg 5-FU	Oral administration	
			Free drug 2500 μg 5-FU	Formulation 2500 μg 5-FU
1.	$T_{1/2}$ (h)	0.32	0.53	1.7
2.	K_{el} (h^{-1})	2.152	1.297	0.388
3.	V_d (ml)	35.02	155.15	299.04
4.	Cl ($ml \cdot h^{-1}$)	75.30	201.25	117.9
5.	AUC ($\mu g \cdot h^{-1} \cdot ml$)	6.64	12.42	21.52

In conclusion, we have achieved good success in sustaining drug release of a hydrophilic drug *in vitro* and fairly good success in improving absorption of poorly absorbed water-soluble drug through oral route by encapsulating it in phospholipid coated dendrimer grafts. High accumulation in the lymph suggests its potential to control tumour metastasis. Further work in order to better understand absorption mechanisms and to optimize the formulation is required.

3. Experimental

3.1. Materials

Materials used were ammonium thiocyanate, disodium hydrogen orthophosphate, sodium diethyl dithio carbamate (BDH, India), diethyl ether, potassium dihydrogen orthophosphate (CDH, India), chloroform, glacial acetic acid, heptane, sodium chloride, thionyl chloride (Ranbaxy, India), triethanolamine, methacrylate, potassium hydroxide (Loba Chemie, India), strong ammonia solution, ethylenediamine (Qualigens fine chemicals, India), methanol, ethanol, hydrochloric acid (E.Merck, India), palmitic acid (Sisco, India) and dialysis tube (Sigma, USA). The materials were of AR grade and distilled water was used for all experiments.

3.2. Synthesis of dendrimer

Strong ammonia solution (5 ml) was taken in methanol (30 ml) in a light resistant round bottom flask and methyl methacrylate (24.5 ml) was added. This solution was kept overnight. 2 ml of this solution were added to ethylenediamine (1.31 ml) in methanol and kept in the dark for 55 h at 25 °C. Excess of ethylenediamine was removed under vacuum (5 mm Hg), to yield an amine terminated dendrimer which was referred to as zero generation (0.0 G). For next generation, 5.0 ml of amine-terminated dendrimer (0.0 G) was added to methylmethacrylate (8.83 ml) in methanol and kept for 24 h in dark to obtain ester-terminated dendrimer which was referred to as half generation (0.5 G). 2.0 ml of ester-terminated dendrimer (0.5 G) was then added to 0.22 ml of ethylenediamine in methanol and kept in dark for 72 h at 25 °C to obtain amine-terminated dendrimer which was referred to as one generation (1.0 G). Both the reactions were alternately repeated several times for synthesizing successive generations of dendrimers i.e. 1.5 G, 2.0 G, 2.5 G, 3.0 G, 3.5 G and 4.0 G under similar reaction conditions [10].

3.3. Fatty acid grafting on 4th generation dendrimer

To the solution of 2.5 g dendrimer $(NH_2)_{48}$ in 50 ml tetrahydrofuran, 5.0 g triethyl amine and 8.66 g palmitoyl chloride were added, the mixture was stirred for 20 h at room temperature and the solvent was evaporated. The mixture was heated under reflux in 50 ml of diethyl ether for 30 min and filtered to remove an excess of palmitoyl chloride. To the residue, a solution of 2 g Na_2CO_3 in 50 ml H_2O was added. The mixture was shaken for 6 h in order to remove residual ammonium salts and the residue was dried in vacuum. The product was obtained as a white/yellow solid after lyophilization [11].

3.4. Structure validation of dendrimers

3.4.1. Nuclear magnetic resonance spectroscopy

NMR spectroscopy of fatty acid grafted dendrimer was performed on samples taken in $CDCl_3$.

3.4.2. Infrared spectroscopy

IR spectroscopy of all the samples was performed using KBr disc technique. Discs were prepared from a mixture of potassium bromide with sample after grinding, and applying 8 ton/inch^2 pressure for 2–3 min.

3.5. Quantitative estimation of fatty acids

Palmitic acid was assayed by taking in 25 ml chloroform/heptane (1 : 1 v/v) using methods of Lauweyer [17] and Peyrot et al. [9].

3.6. Solubilization

3.6.1. Effect of solvent

Different concentrations of dendrimer (1, 2 and 3×10^{-5} mol $\cdot$ l^{-1}) were prepared in ethanol or tetrahydrofuran. Excess of drug was added to 100 ml of the above solution and kept for 24 h with intermittent shaking. The solution was precipitated with acetonitrile, filtered, and washed with acetonitrile thrice. The residue was dried in a vacuum desiccator at room temperature. The amount of drug solubilized was determined spectrophotometrically [18]. To the different concentrations of dendrimer solutions in methylene chloride, 1% drug solution was then added in 10 : 1 volume ratio. The flask was shaken and the methylene chloride layer was separated and evaporated in a vacuum evaporator at 40 °C. The residue was dialyzed against acetone-water (95 : 5) to remove un-entrapped drug. The amount of drug encapsulated was determined spectrophotometrically.

3.6.2. Effect of pH

To the dendrimer solution (1×10^{-5} mol $\cdot$ l^{-1}) an excess of drug was added and the pH value was adjusted to 1.2, 7 and 9. The solubility studies were performed as for ethanol.

3.6.3. Effect of ionic concentration

Three different 0.1, 0.5 and 1.0 M solutions of NaCl and $CaCl_2$ were prepared and the drug was dissolved in each of them. Solubility of 5-FU was determined as explained earlier.

3.7. Formulation studies

3.7.1. Coating by phospholipid and encapsulation of drug

Egg lecithin (5 mg) and octylglucoside (2 : 1 w/w) were dissolved in 100 ml $(C_2H_5)_2O : CH_2Cl_2$ (1 : 1). Dendrimer graft containing drug (100 mg) was dispersed in the above solution and dried in rotary vacuum evaporator. Resulting powder was dialyzed against 400 ml of water with intermittent exchanges. A milky white suspension obtained was transferred in vial and kept in refrigerator. This suspension was lyophilized to obtain white powder. An amount of drug present in suspension was measured taking 10 mg of formulation and dissolving it in 10 ml of n-hexane and 5 ml of toluene. This mixture was shaken for 2 h with 30 ml of water in a separating funnel and layers were allowed to separate. Aqueous layer was removed and analyzed through UV spectroscopy after suitable dilution against blank prepared similarly.

3.7.2. Size distribution

Size distribution was measured using a particle size analyzer (Malvern Mastersizer 4, UK) in reverse Fourier mode. The suspension of phospholipid coated dendrimer-drug solubilizate was filled in a 15 ml sample cell after initialization of instrument and stirred with magnetic stirrer.

3.7.3. Transmission electron microscopy (TEM)

TEM measurements were performed at 5 KV. Samples for TEM were prepared by deposition of 3 µl of formulation upon a carbon coated copper grid.

3.7.4. Release rate

A treated dialysis tube was used for *in vitro* release studies. Formulation equivalent to 10 mg drug was introduced into a prewashed dialysis tube and placed in a beaker containing 200 ml freshly prepared PBS (pH 7.4). The sink condition was maintained by constantly stirring the buffer with a magnetic stirrer. Sample aliquots (5 ml) were withdrawn periodically and replaced with a equal volume of fresh PBS. Each sample was analyzed at 266 nm.

3.7.5. Stability studies

Vials containing powder alone (20 mg) and suspension (20 mg powder reconstituted with buffer) were kept for stability studies. Stability studies were conducted over a month at room temperature and at 40 °C. All the vials were analyzed for drug content at definite intervals.

3.8. Animal Studies

Albino rats (Sprague Dawley strain) of either sex weighing 200–250 g were maintained in cages at room temperature for 15 days prior to study and were allowed to have free access to food and drinking water. Animals were fed with standard feed pellets and observed for their behavior, diseases and fatality. Healthy rats were given an oral dose of soyabean oil (40 mg/kg) 4 days prior to study and were starved for 10 h prior to dosing.

3.8.1. Pharmacokinetic profile

Animals were divided in three groups each containing six rats. The first group received 500 µg of free 5-FU solution i.v. Group II and III received with 2500 µg of free 5-FU solution and equivalent dendrimer formulation orally. Blood was collected from the retro-orbital plexus at 0, 0.25, 0.5, 1, 2, 3, 4 and 6 h. The samples were centrifuged, plasma was collected, extracted with an ethyl acetate : propanol (7 : 3 v/v) mixture and analyzed spectrophotometrically [18]. The bioavailability was calculated according to following formula:

$$\text{Bioavailability} = \frac{\text{AUC}_{\text{oral}}/\text{dose}_{\text{oral}}}{\text{AUC}_{\text{iv}}/\text{dose}_{\text{iv}}} \times 100$$

3.8.2. Lymphatic uptake study

The procedure of administration of formulations was the same as described under. Lymph was collected from the thoracic lymph duct at similar intervals as described by Bollman et al. [19] to estimate drug content.

Acknowledgements: Authors would like to acknowledge Central Drug Research Institute, Lucknow and SPARC Baroda for providing some of the necessary facilities. A. J. Khopade and S. Jain acknowledge CSIR, New Delhi for research associateship. Surekha Nagaich, Shalini Shrivastava and Pushpendra Tripathi acknowledge UGC, New Delhi for the grant of Junior Research Fellowship.

References

1 Liu, M.; Frechet, J. M. J.: Pharm. Sci. Tech. Today, **2**, 393 (1999)
2 Bosman, A. W.; Jansen, H. M.; Meijer, E. W.: Chem. Rev. **99**, 1665 (1999)
3 Gandhi, R.; Khopade, A. J.; Jain, N. K.: Indian Drugs, **34**, 593 (1997)
4 Zeng, F.; Zimmerman, S. C.: Chem. Rev. **97**, 1681 (1997)
5 Stevelman, S.; van Hest, J. C. M.; Jansen, J. F. G. A.; van Boxtel, D. A. F. J.; de Brabander-van der Berg, E. M. M.; Meijer, E. W.: J. Am. Chem. Soc. **118**, 7398 (1996)
6 Sayedsweet, Y.; Hedstrand, D. M.; Spinder, R.; Tomalia, D. A.: J. Mater. Chem. **7**, 1119 (1997)
7 Ramzi, A.; Bauer, B. J.; Scherrenberg, R.; Froehlig, P, Joosten, J.; Amis, E. J.: Macromolecules, **32**, 4983 (1999)
8 Peyrot, M.; Sautereau, A. M.; Rabanel, J. M.; Mguyen, F.; Tocanne, J. F.; Samain, D. Int. J. Pharm., 102, 25 (1994)
9 Dangi, J. S.; Vyas, S. P.; Dixit, V. K.: Drug. Dev. Ind. Pharm. **24**, 631 (1998)
10 Tomalia, D. A.; Baker, H.; Dewald, J. R.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P.: Polym. J. (Tokyo) **17**, 117 (1985)
11 Baars, M. W. P. L.; Froehlig, P. E.; Meijer, E. W.: Chem. Commun. 1959 (1997)
12 Tomalia, D. A.; Naylor, A. M.; Goddard III, W. A.: Angew. Chem. Int. Ed. Engl., **29**, 138 (1990)
13 Chen, W.; Tomalia, D. A.; Thomas, J. L.: Macromolecules, **33**, 9169 (2000)
14 Boris, D.; Rubinstein, M.: Macromolecules, **29**, 1278 (1996)
15 Zhuo, R. X.; Du, B.; Lu, Z. R.: J. Controlled Rel. **75**, 249 (1999)
16 Florence A. T.; Sakthivel, T.; Toth, I.: J. Controlled Rel. **65**, 253 (2000)
17 Lauwreys, R. R.; Anal. Biochem., **32**, 331(1969)
18 Sawant, K. K.; Murthy, R. S. R., Ind. J. Pharm. Sci. **55**, 221 (1993)
19 Bollman, J. L.; Cain, J. C.; Grindlay, J. H.: J. Lab. Clin. Med. **33**, 1349 (1948)

Received April 26, 2001
Accepted November 7, 2001

Prof. N. K. Jain
Department of Pharmaceutical Sciences
Dr. H. S. Gour University
Sagar (M.P.) 470003
India

Dr. A. J. Khopade
Max-Planck Institute
of Colloids and Interfaces
D-14424 Potsdam
ajay.khopade@mpikg-mpg.de