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Dendimer-Mediated Solubilization, Formulation Development and *in Vitro*−*in Vivo* Assessment of Piroxicam

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Abstract: The present investigation was aimed at exploring dendrimer-mediated solubilization and formulation development followed by *in vitro, in vivo* assessment of piroxicam (PXM) nanocomposite. For this, two dendrimer generations (3.0G and 4.0G) were synthesized and characterized by IR, ^{1}H NMR spectroscopic and electron microscopy techniques. The optimized formulations containing 0.2% w/v of PXM loaded PAMAM dendrimer at pH 7.4 referred to as 0.2-$D_3P_{7.4}$ (3.0G) and 0.2-$D_4P_{7.4}$ (4.0G) resulted in significant enhancements of PXM solubility approximately by 107- and 222-fold, respectively. The *in vitro* release behavior of PXM from the formulation in medium-I (PBS 7.4) and medium-II (PBS with 1% albumin) and stability studies were also favorable. Pharmacokinetic study showed higher area under curve ($AUC_{0\rightarrow t}$; μg/mL/h) of 293.78 $\pm$ 2.04 and 321.54 $\pm$ 2.37 with optimized 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ formulations, respectively, as opposed to 279.11 $\pm$ 1.48 with plain PXM. The elimination half-life of the drug encapsulated in the formulation was significantly higher (0.2-$D_3P_{7.4}$, 36.6 and 0.2-$D_4P_{7.4}$, 41.1; h) than that of pure drug (33.7 h; $p < 0.005$), and the overall elimination rate constant of formulations was also less as compared to free drug ($p < 0.005$). Pharmacodynamic assessment by rat-paw model of 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ formulations displayed inhibition levels of 54.21 $\pm$ 1.25% and 59.33 $\pm$ 0.63%, respectively, which are higher than those of plain PXM (41.81 $\pm$ 2.9) formulations, after the sixth hour of administration. The second, fourth and eighth hour organ distribution data showed significantly higher recovery of PXM in rat paw with dendrimer-based formulations in comparison to plain PXM. However, comparison of overall data suggested 4.0G-based formulations to be superior to 3.0G as well as pure PXM.

Keywords: Dendrimers; piroxicam; solubilization; formulation development

Introduction

Solubility is an intrinsic physiochemical property of a drug, which influences drug action, structure activity relationship, drug transport kinetics and site specific drug release.[1] Approximately, 50% of new chemical entities (NCEs) discovered today bear hydrophobic character, and solubility issues chiefly complicate this aspect. Solubilization techniques have been shown to enhance absorption and biological activity and reduce hydrolysis rate. It has been a constant ambition of formulation scientists to formulate and optimize an efficient delivery system for hydrophobic drug candidates. Cyclodextrins (CDs) have been extensively investigated for enhancing solubilization,[2,3] but high costs and nephrotoxicity on parenteral administration limit the use of CDs in this

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perspective. In addition to this, there are numerous reports on micelle and polymeric-micelle mediated solubilization. However, formation and stability of these systems are both concentration and pH dependent, and are thermodynamically unstable at concentrations below their critical micellar concentration (CMC). The disruption of micellar structure on dilution with body fluids below its CMC exaggerates this problem by bursting the release of entrapped drugs with unwanted drug precipitation.[4]

Dendrimers signify a class of carriers that bear promising properties in this context.[5] Their hydrophobic core and hydrophilic periphery exhibit micelle-like performance along with drug loading properties in solution.[4] Newkome first proposed the engagement of dendrimers as unimolecular micelles,[6] and this analogy highlighted the utility of dendrimers as solubilizing agents.[7] Dendrimers are now well proven as a tool in the solubilization of insoluble drugs. In 1993 Hawker and co-workers reported the solubilization of pyrene in polyether dendrimers.[8] In the year 2000, solubilization of ibuprofen was reported using PAMAM dendrimers, and from then almost more than 50 drugs have been reported to be solubilized using dendrimers.[9−13]

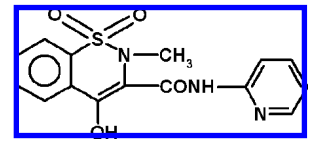

Figure 1. Structure of piroxicam.

Piroxicam (PXM; Figure 1) belongs to the class of acidic, nonsteroidal anti-inflammatory drugs (NSAIDs). It is quite efficient in the short or long-term treatment of rheumatoid arthritis, osteoarthritis and other painful inflammatory disorders.[14−16] But there are a number of aspects that limit its pharmaceutical application, the chief being its water insolubility that immensely creates formulation tribulations.[17] Apart from this, oral administration shows low absorption,[18] considerably high food interaction, GI tract associated adverse effects, which displays poor bioavailability, limiting its oral usage. Moreover, PXM shows extensive plasma protein binding and hence unavailability at desired site in therapeutic dose.[19]

The present study was aimed at exploring dendrimer-mediated solubilization of PXM followed by formulation development and *in vitro* as well as *in vivo* assessment in albino rats. It was envisaged that the PXM shall get incorporated inside dendritic microcavities and this entrapment will not only improve the solubility but will also help in sustaining blood plasma level. It was also anticipated that these dendrimer-based macromolecules may cross through the wall of blood vessels into interstitial spaces, and consequently would generate higher drug concentration inside inflamed body area as compared to that of free drug, and therefore may provide better therapeutic efficacy at lower dose.

Materials and Methods

Materials. Ethylenediamine (EDA) and methyl methacrylate were purchased from CDH, Mumbai, India, while methanol was procured from Qualigens Fine Chemicals,

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Mumbai, India. Piroxicam was received as a generous gift sample from Sun Pharma, Vadodara, India. All remaining chemicals were of analytical reagent (AR) grade.

Synthesis of PAMAM Dendrimers. Amine-terminated PAMAM dendrimers of third and fourth generations (3.0G and 4.0G) were prepared using EDA as initiator core, following reported methods.[20−22] Briefly, methanolic methyl methacrylate solution (5% molar excess) was reacted with EDA in a light-resistant environment to form ester-terminated dendrimer, followed by removal of excess methanol in a rotary vacuum evaporator (Superfit, Mumbai, India). The ester-terminated dendrimer was treated with methanolic EDA (10 molar times) for 55 h in the dark. The excess of EDA was removed under high vacuum (5 mmHg) to yield 0.0G dendrimer. This reaction sequence was repeated for the required number of times to produce PAMAM dendrimers up to 3.0 and 4.0 generations. Completion of every reaction step in the synthesis was confirmed with copper sulfate color reaction.[22,23] The synthesized dendrimers were characterized by FT-IR (Shimadzu 8201 PC, Japan) and ^{1}H NMR (solvent, D_2O; at 300.1301665 MHz using Bruker Advanced DRX, 300 MHz) and transmission electron microscopy (TEM). Briefly in TEM analysis a drop of 0.001% w/v of methanolic solution of 3.0G and 4.0G PAMAM dendrimer was added to a carbon grid and stained with osmium tetroxide (negative stain) and viewed at an acceleration voltage of 50 kV in a transmission electron microscope (Philips CM12 Electron Microscope, Eindhoven, The Netherlands).

Solubilization and Formulation Development. Solubility study was carried out by the phase solubility technique as reported earlier by Higuchi and Connors, with slight modifications.[24] This study was performed under three different pH conditions (4.0, 7.4 and 10.0) to interpret the effect of pH on dendrimer-mediated solubilization of PXM. Briefly, an excess of PXM was added into amber-colored screw-capped glass vials (10 mL) containing varying concentrations (0.02 to 0.2% w/v) of 3.0G and 4.0G PAMAM dendrimers in acetate phthalate buffer (pH 4.0). Separately excess drug was added in a vial containing only acetate phthalate buffer (pH 4.0) and was treated as control. The vials were shaken at room temperature for 24 h in a metabolic shaker (Indian Equipment Corporation, Mumbai, India) and allowed to stand for 12 h to attain equilibrium. The insoluble drug was removed by filtration though a 0.45 μm membrane filter (Sigma, Germany) and washed thrice with double distilled water. The undissolved drug was solubilized in methanol and analyzed indirectly by spectrophotometer (UV−vis 1601 Shimadzu, Japan) at 334 nm for drug content using water−methanol (1:1) as solvent. The amount of drug solubilized was calculated by following eq I:

$$\text{amount of drug solubilized} = \text{amount of drug added} - \text{amount of drug undissolved} \quad \text{(I)}$$

A similar procedure was followed at pH 7.4 and 10.0, and the dendrimer formulation that showed the maximum drug loading/solubilization was selected for further characterization.

Formulation Characterization. Drug loaded dendrimer solution (2% w/v) was analyzed over the UV range between 200 to 600 nm in a UV visible spectrophotometer (UV−vis 1601 Shimadzu, Japan) to analyze the effect of solubilization as well as drug loading at λ_{max}. The drug−dendrimer complex was lyophilized (Heto Lyophilizer, Germany), and FT-IR (Shimadzu 8201 PC, Japan) analysis was performed using the KBr pellet method.

Hemolytic Toxicity. Hemolytic studies were performed as per previously reported studies.[22,25,26] Whole human blood was collected in anticlot blood collection vials (Himedia Laboratories, Mumbai, India) and centrifuged at 600*g* for 5 min; supernatant was removed continually 3 times, and the red blood cells (RBCs) collected at the bottom of tube were washed by normal saline (0.9% w/v) until a clear, colorless supernatant was obtained. The cells were resuspended in normal saline. The RBC suspension so obtained was used further for hemolytic study. To 1 mL of RBC suspension separately, double distilled water (5 mL) was added, and was considered as 100% hemolytic. Similarly, 5 mL of normal saline was added to 1 mL of RBC suspension and assumed to produce no hemolysis and considered as negative control (0% hemolytic). To 1 mL of RBC suspension, a mixture of normal saline (4.5 mL) and drug−dendrimer formulation (0.5 mL) was added. A similar procedure was followed for pure dendrimer and plain drug solution. All the samples were incubated at 37 ± 2 °C for 4 h and centrifuged at 3000 rpm for 15 min. The supernatant was analyzed spectrophotometrically at 550 nm (UV−vis 1601 Shimadzu, Japan). The degree of hemolysis was estimated by eq II,

$$(\%)\ \text{hemolysis} = (\text{Ab}_s - \text{Ab}_s0/\text{Ab}_{100} - \text{Ab}_s0) \times 100 \quad \text{(II)}$$

where Ab_s, Ab_s0 and Ab_s100 are the absorbances of formulation treated sample, a solution of 0% hemolysis and a solution of 100% hemolysis, respectively.

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***In Vitro* Release.** *In vitro* release studies were performed under sink conditions in two media, one comprising PBS (pH 7.4; medium-I) and the other comprising PBS with 1% of albumin (medium-II). Five milliliters of drug−dendrimer solution was filled in a cellulose tubing (MWCO 2 KD, Anaspec, USA), which was pretreated with PBS (pH 7.4). The tubing was suspended in 100 mL of medium-I under constant stirring using a magnetic stirrer (Remi Equipments, Vasai, India) at 37 ± 2 °C. Two milliliter aliquots were withdrawn from the external solution and replenished with the same volume of fresh PBS to maintain perfect sink conditions, and the amount of drug released was determined spectrophotometrically at 334 nm against blank, indirectly. The same procedure was followed for the *in vitro* release study in medium-II.

***In Vivo* Anti-Inflammatory Activity.** All the animal studies were conducted in accordance with the protocol approved by the Institutional Animal Ethical Committee of Dr. H. S. Gour University, Sagar, India (Registration No. 379/01/ab/CPCSEA).

There are a number of established methods for screening anti-inflammatory activities, viz., nonimmunological[21,27−30] and immunological methods.[31,32] In the present study, a pharmacodynamic study was performed using nonimmunological carrageenan induced hind paw edema method, which had been previously reproduced by our group. [21,30] In the screening of anti-inflammatory activity 0.1 mL of 1% carrageenan was taken as phlogistic agent. Acute inflammatory activity was determined by measuring change in the volume of inflamed paw produced by injection of carrageenan. The paw volume was measured using a plethysmometer (UGO, Basile, Italy).

On the basis of efficient solubilization as well as better *in vitro* performance, formulation 0.2-$D_4P_{7.4}$ (0.2% w/v PXM loaded 4.0G PAMAM dendrimer formulation at pH 7.4) was selected for *in vivo* evaluation. Albino male rats (Sprague−Dawley strain) were weighed, numbered and marked on the right hind paw, just behind the tibia−tarsal junction. Each time the paw was dipped in the plethysmometer up to the fixed mark to ensure constant paw volume. A constant temperature was maintained in the animal house, and stresses of any kind were avoided. Every time, the study was carried out at daytime to avoid any variation due to circadian rhythms.

Animals were divided into three groups (including one control group), each group comprising four animals. Test formulation (0.2-$D_4P_{7.4}$) and plain dendrimer solution were solublized in PBS (pH 7.4; 0.2% w/v). The plain drug (PXM) was solubilized in PBS pH 7.4 employing a minimum quantity of DMSO as cosolvent. The dose of 2 mg/kg (equivalent to PXM) body weight was administered through an intravenous route in albino rats of the respective group, precluding the control.

A dose of 0.1 mL solution of carrageenan (1% w/v in normal saline) was injected in the right hind paw of the test animals, 10 min postadministration of the test formulation. The paw volume was measured every hour until the eighth hour, the last two observations were recorded at the 12th and 24th h, and a graph was plotted between percentage inhibitions of edema vs time (h). Percentage inhibition of edema was calculated for each group by eq III:

$$\%\ \text{inhibition of edema} = \left(\frac{V_{\text{control}} - V_{\text{treated}}}{V_{\text{control}}}\right) \times 100 \quad \text{(III)}$$

where V_{control} and V_{treated} are the mean edema volume of rats in control group and test group, respectively.

Pharmacokinetics and Biodistribution Studies. Pharmacokinetic and tissue distribution studies of free drug and dendrimer complexed drug were performed in albino rats with carrageenan-induced inflammation in the right hind paw. Albino rats were divided in two groups, each group comprising 12 rats, numbered and marked. Test formulation of dendrimer drug complex 0.2-$D_4P_{7.4}$ and plain drug PXM in the doses of 2 mg/kg body weight were administered though an intravenous route, followed by the collection of blood samples from the retro-orbital plexus at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 h. In each case the blood sample was allowed to stay for 15 min and then centrifuged at 3000 rpm for 15 min to separate RBCs and serum. The supernatant (serum) was collected with the help of micropipet (Microlit, 5−50 μL, Germany) and analyzed for drug content employing HPLC (Shimadzu LC-10 ATVP, HPLC, Japan) for the determination of various pharmacokinetic parameters.

For organ distribution studies, animals were divided into two groups, and each group was administered with the same intravenous dose (2 mg/kg) of 0.2-$D_4P_{7.4}$ (equivalent to PXM) and plain PXM. Four rats from each group were sacrificed at 2, 4, and 8 h, and the organs, viz., paw, kidney, liver and spleen, were removed immediately and weighed. The organs were wrapped in aluminum foil and were refrigerated at −20 °C. The organs were homogenized, centrifuged at 4000 rpm for 15 min followed by collection of supernatant and assayed for PXM by the HPLC method as reported earlier, with slight modifications[33,34] employing a C_{18} 150 mm × 4.5 mm

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internal diameter, 5 μm particle size (Thermo Hypersil, ODS) analytical column protected by a compatible guard column. The HPLC system (Shimadzu LC-10 ATVP, Japan) consisted of a variable UV−visible detector, and the elution was carried out by using water:methanol:acetic acid (44:50:6) as the mobile phase. The flow rate was kept at 1.0 mL/min throughout the process.

Stability Testing. The stability testing of formulation was based on the principle of chemical kinetics. Stability studies were carried out under accelerated temperature and light conditions. The samples (10 mL) were kept in amber-colored (dark) and in colorless glass vials (light) at 0 °C, room temperature (25 ± 2 °C) and accelerated temperature (60 ± 2 °C) in a controlled oven for a period of 6 weeks. The samples were withdrawn and analyzed initially and periodically (every week) up to six weeks for any precipitation, turbidity, crystallization, color change, consistency and drug leakage.

Percent drug leakage was determined by estimating increase in drug release from the formulation during storage, under different conditions. Briefly, formulation samples (4 mL) were kept in cellulose tubing (MWCO 2KD; Sigma, USA) and dialyzed against external medium (50 mL), monitored for drug content spectrophotometrically. The procedure was repeated at weekly interval for up to six weeks. The data was analyzed to infer the conditions suitable for the storage of the formulation.

Results

Synthesis and Characterization of Dendrimer. PAMAM dendrimers of 3.0G and 4.0G were synthesized employing ethylenediamine (EDA) as initiator core and methyl acrylate in the appropriate molar ratio. Completion of each reaction step in the synthesis was confirmed with copper sulfate color reaction,[21,22] wherein full generation dendrimers gave a purple color, while half-generations gave a deep blue color due to copper chelation at the $-NH_2$ terminal groups of the dendrimers, as reported earlier.[23] The dendrimer synthesis was also confirmed by FT-IR, ^{1}H NMR and microscopic studies. In case of 3.0G PAMAM dendrimer, the presence of peaks at 3438.5 cm^{-1} (N−H stretching of primary amine); 1731.1 cm^{-1} (C═O stretching of ester); 1650.6, 1583.2 cm^{-1} (−NH−CO stretching of amide); 1439.9, 1387.8 cm^{-1} (N−H bending of N substituted); 1208.5, 1030.2 cm^{-1} (C−O stretching); and 2670 cm^{-1} (C−H bending peaks) confirmed the synthesis. Similarly in the FT-IR spectrum of 4.0G PAMAM dendrimer, peaks at 3350.2 cm^{-1} (N−H stretching of primary amine); 3190.0 cm^{-1} (N−H stretch antisymmetric of substituted primary amine); 2890.0 cm^{-1} (C−H stretch); 1641.3 cm^{-1} (C═O stretch of carbonyl group); 1566.0 cm^{-1}, 1327.9 cm^{-1} (N−H bending of N substituted amide); and 1198.5 cm^{-1} (C−C bending) confirmed the synthesis. Further, the synthesis was also confirmed from the ^{1}H NMR spectroscopy of 3.0G and 4.0G dendrimer (Table 1). Results of electron microscopy displayed the nanometric size of the synthesized dendrimers, wherein circular nanometric aggregates were observed (Figure 2). This data was in agreement with our published reports.[21−23]

Table 1. ^{1}H NMR Shift and Interpretation of the Spectrum of 3.0G and 4.0G Amine-Terminated PAMAM Dendrimer

dendrimer	δ values (ppm)	interpretation
3.0G	3.3	$-NH_2$ (terminal group)
	2.3−2.9	$-CH_2-NH_2$ (alkyl amine)
	3.05	>N-CH_2-CH_2 CO
	1.69	$-CH-CH_3$ (alkane)
4.0G	2.417, 2.435	carbonyl ($-CH_2$ C═O)
	2.5−2.8	amide-NH
	3.0−3.5	$-CH_2$ NH_2 terminal group
	4.7−5.2	−OH (methanolic)

Phase Solubility Studies, Formulation Development and Characterization. Formulation development as well as solubility studies were carried out at three different pHs (4.0,

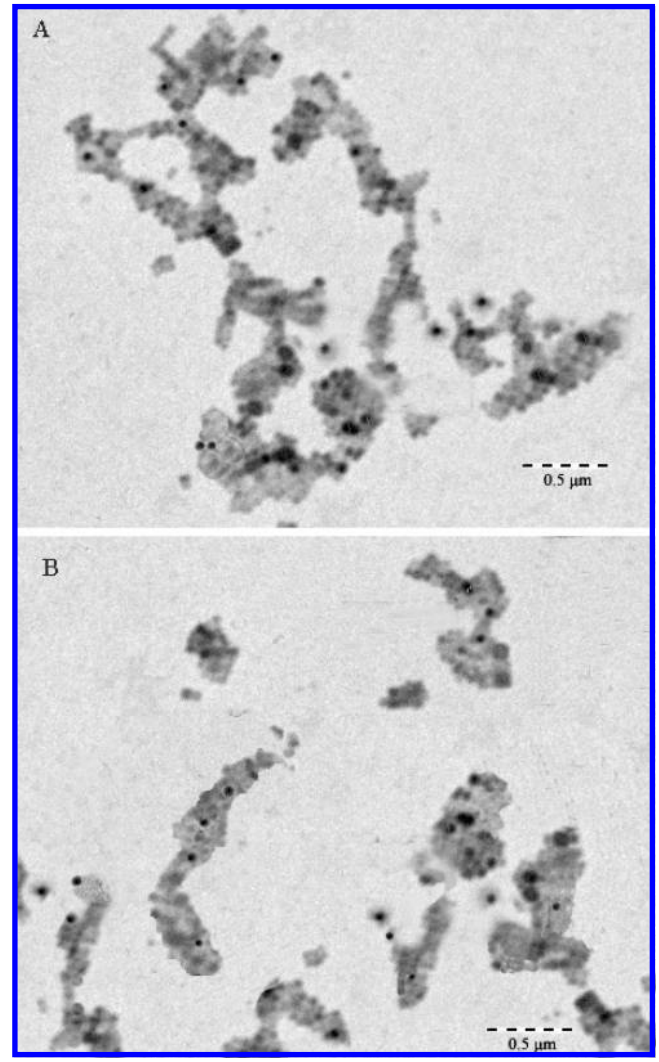

Figure 2. TEM images: (A) 3.0G (B) 4.0G PAMAM dendrimer.

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Table 2. Effect of pH on Solubility of PXM at Various Concentrations of 3.0 and 4.0 Generations of PAMAM Dendrimer[a]

		dendrimer concentration (w/v %)						
pH	type of dendrimer	0	0.025	0.05	0.1	0.15	0.2	0.25
4	3.0G	0.5 ± 0.01	0.75 ± 0.09	1.26 ± 0.66	1.171 ± 0.5	1.89 ± 0.75	2.05 ± 0.26	2.35 ± 0.36
	4.0G	0.5 ± 0.05	0.97 ± 0.25	1.79 ± 0.24	2.95 ± 0.52	3.15 ± 0.97	3.69 ± 0.85	3.98 ± 0.78
7.4	3.0G	4.1 ± 0.75	33.78 ± 0.65	72.98 ± 4.55	315.2 ± 10.87	395.4 ± 17.12	439.1 ± 12.88	452.3 ± 21.25
	4.0G	4.1 ± 0.08	62 ± 1.85	155.87 ± 7.15	642.8 ± 18.97	901.46 ± 27.89	1004.12 ± 39.14	1015 ± 27.64
10	3.0G	41.02 ± 3.87	43.97 ± 2.54	99.75 ± 5.87	375.1 ± 6.78	425.3 ± 10.89	498.75 ± 8.9	579.9 ± 2.87
	4.0G	41.02 ± 1.87	99.01 ± 3.88	201.34 ± 4.63	724.55 ± 20.78	801.64 ± 27.89	910.47 ± 38.45	940 ± 23.75

[a] Results are represented as mean ± SD ($n = 3$).

7.4 and 10.0), to discern the effect of pH, if any, on dendrimer-mediated solubilization of PXM. Results suggested that solubility of PXM increased with increase in dendrimer concentration as well as generation. The increase in solubility was found linear and was highest at dendrimer concentration of 0.2% w/v. Under optimized condition of dendrimer concentration (0.2% w/v) at pH 7.4 and 10.0, solubility of PXM was remarkably greater than at pH 4.0. In the case of 4.0G, at pH 10.0 the solubility was found to be enhanced approximately 24-fold. The solubility of PXM increased in the order of pH 7.4 > 10.0 > 4.0 in both 3.0G- and 4.0G-based dendrimer formulations. The optimized 3.0G and 4.0G dendrimer-based formulations were selected for further studies and were assigned as 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$, respectively, in further discussion. The data showed that 0.2-$D_4P_{7.4}$ formulation enhanced aqueous solubility of PXM by 222-fold compared with 0.2-$D_3P_{7.4}$ formulation where only 107-fold increment in solubility was observed (Table 2).

The optimized formulations were analyzed by FT-IR spectroscopy to authenticate the drug−dendrimer complexation. It was found that the FT-IR spectra of 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ formulations displayed close resemblance with that of FT-IR spectra of respective dendrimer subtypes, with a prominent broad peak at around 3000−3600 cm^{-1} (Figure 3). The formulations were also scanned over the entire UV−visible range, wherein maxima corresponding to 258.3, 330.3 nm (0.2-$D_3P_{7.4}$), and 255.1, 330.5 nm (0.2-$D_4P_{7.4}$) were recorded in contrast to 253.3, 288.2, and 340.3 nm with plain PXM scan, inferring drug complexation (Figure 4).

In Vitro **Release Studies.** The *in vitro* release of PXM from 0.2-$D_4P_{7.4}$ and 0.2-$D_3P_{7.4}$ was explored in two media: medium-I (PBS pH 7.4) and medium-II (PBS pH 7.4, containing 1% of albumin) (Figure 5). In medium-I, after 2 h, the release was nearly 42.74 ± 1.56 and 52.35 ± 2.2%, as opposed to 63.49 ± 1.9 and 69.58 ± 1.5%, after 6 h, from 0.2-$D_4P_{7.4}$ and 0.2-$D_3P_{7.4}$ formulations, respectively. In the same medium, after 24 h, the release from 0.2-$D_4P_{7.4}$ and 0.2-$D_3P_{7.4}$ formulations was nearly 85.36 ± 2.33 and 93.87 ± 2.15%, respectively. These observations in particular indicated an initial rapid release, followed by the delayed release of the drug from the formulations.

The release study was also conducted in the presence of a model protein (albumin) to qualitatively assess the fate of formulations upon injection in body fluids, which are rich in this protein content. As compared to medium-I, faster release rate of PXM was observed in medium-II, by more than 50% (50.67 ± 4.54 and 54.97 ± 3.78%) and 90% (92.04 ± 2.25 and 94.28 ± 2.14%) from 0.2-$D_4P_{7.4}$ and 0.2-$D_3P_{7.4}$ formulation after 2 and 9 h, respectively. It is also quite evident that the release profile was more sustained and controlled with 0.2-$D_4P_{7.4}$ formulation.

Hemolytic Toxicity. The hemolytic toxicity studies of the formulations revealed PXM to be relatively least cytotoxic bioactive, with 1.7, 2.31, 3% hemolysis at 0.1, 0.2 and 0.4% w/v formulation concentrations, respectively (Figure 6). It was also observed that drug−dendrimer formulations displayed relatively lower toxicity (0.2-$D_3P_{7.4}$, 6.3, 8.8, 10.5%, and 0.2-$D_4P_{7.4}$, 7, 8.9, 11.5% hemolysis at 0.1, 0.2 and 0.4% w/v concentrations, respectively) as compared to plain dendrimer (3.0G PAMAM, 6.2, 9.2, 11.1%, and 4.0G PAMAM, 7.8, 11, 13.5% hemolysis at 0.1, 0.2 and 0.4% w/v concentration, respectively. However the difference in the hemolytic toxicity of 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ was insignificant. Based on this report along with better solubilization and drug loading potential, which are prerequisite for development of parenteral formulation, the 4.0G dendrimer-based formulation (0.2-$D_4P_{7.4}$) was selected for further studies.

Pharmacokinetic Studies. From the pharmacokinetic profile, it can be inferred that the blood levels of PXM in edema induced paw tissues were much higher with 0.2-$D_4P_{7.4}$ formulation as compared to that of plain drug PXM, under all the monitored time intervals ($P < 0.05$; Figure 7, Table 3).

The plasma pharmacokinetic parameters of 0.2-$D_4P_{7.4}$ and free drug were also calculated and are shown in Table 4. The area under the plasma concentration time profile ($AUC_{0\rightarrow t}$; μg/mL/h) with 0.2-$D_4P_{7.4}$ was 321.54 ± 2.37 μg/mL/h, which was 1.15- and 1.094-fold compared to free PXM and 0.2-$D_3P_{7.4}$, respectively. Also, the elimination rate constant of PXM observed with 0.2-$D_4P_{7.4}$ formulation was 0.0169 as against 0.0206 and 0.0185 in the case of PXM and 0.2-$D_3P_{7.4}$, respectively (Figure 8, Table 4). The half-life of PXM experienced with 0.2-$D_4P_{7.4}$ formulation was significantly higher compared to free drug (1.21-fold, $p < 0.05$) and 0.2-$D_3P_{7.4}$ (1.13-fold, $p < 0.05$). However the increment in half-life does not seem to be significant practically. Along with this the increased $AUC_{0\rightarrow t}$ *vis à vis* a diminished elimination rate as observed with 0.2-$D_4P_{7.4}$ suggest extended plasma level of PXM.

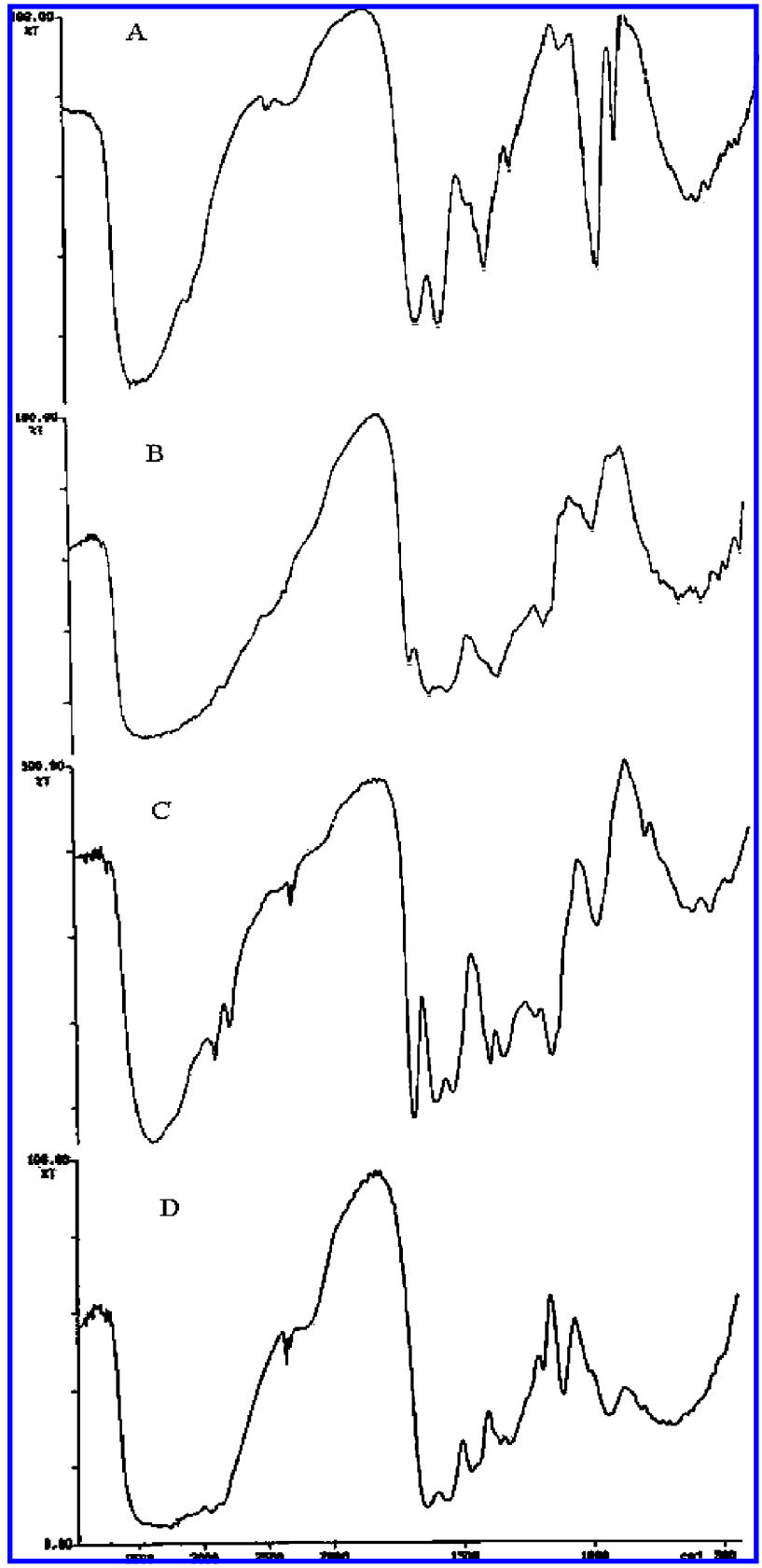

Figure 3. FT-IR spectrum of (A) 3.0G PAMAM, (B) 3.0G PAMAM–PXM complex, (C) 4.0G PAMAM, (D) 4.0G PAMAM–PXM complex.

With free PXM formulation, the percentage of drug recovered in paw increased from 1.02 ± 0.25 (2 h) to 2.1 ± 0.29 (4 h), which was found to diminish significantly after 8 h ($p < 0.001$) with just 1.09 ± 0.96% recovery.

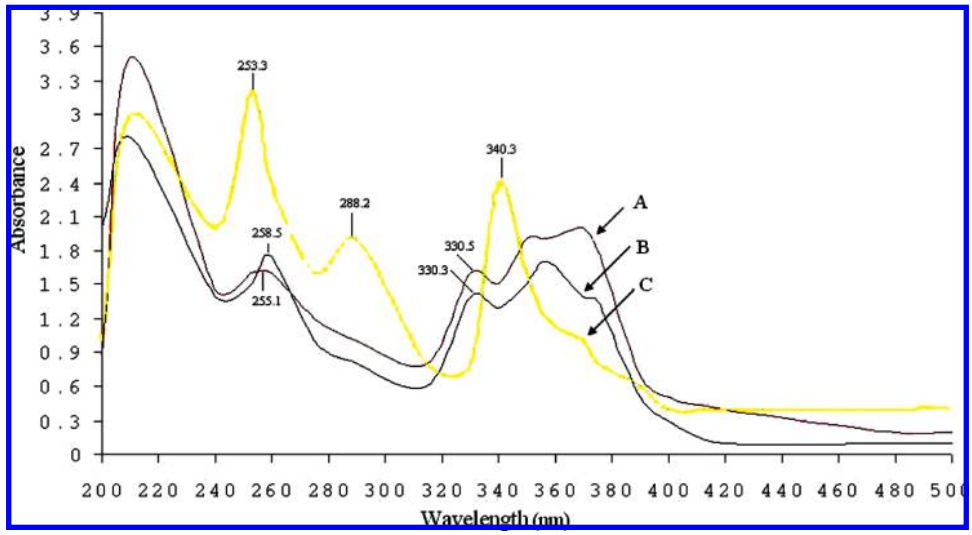

Figure 4. UV−visible scan of drug−dendrimer complex: (A) 3.0G-PXM, (B) 4.0G-PXM, and (C) plain PXM.

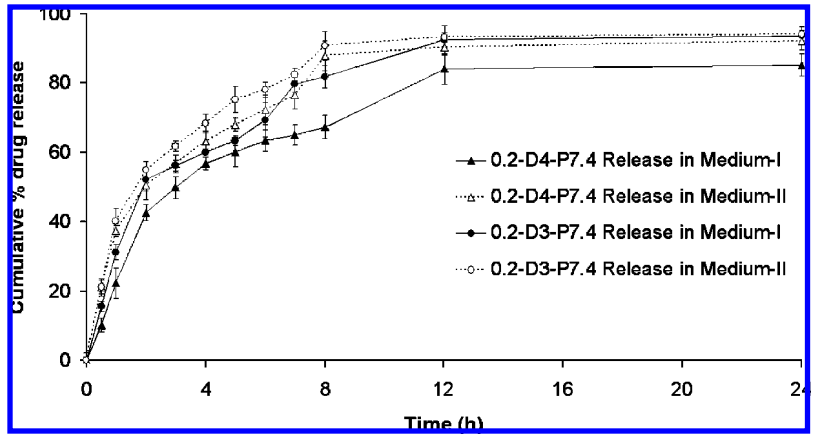

Figure 5. Cumulative drug release from 0.2-$D_3P_{7.4}$, 0.2-$D_4P_{7.4}$ formulations in medium-I (PBS pH 7.4) and medium-II (PBS pH 7.4, with 1% of albumin; *n = 3)*.

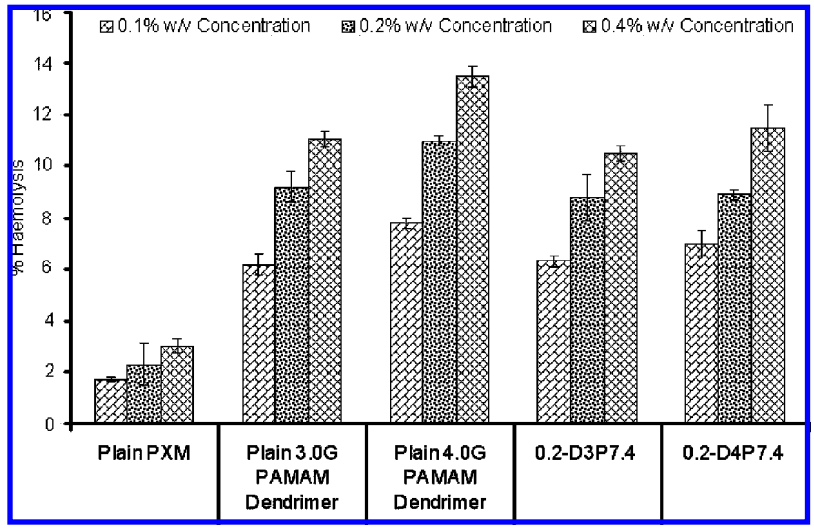

Figure 6. Hemolytic toxicity profile of different formulations (*n* = 3).

On the other hand, with 0.2-$D_4P_{7.4}$ formulation a continuous rise in percentage of drug recovered from paw was observed, with 2.75 ± 0.89, 3.66 ± 0.99 and 3.92 ± 1.25% after 2, 4, and 8 h, respectively. The same pattern of drug

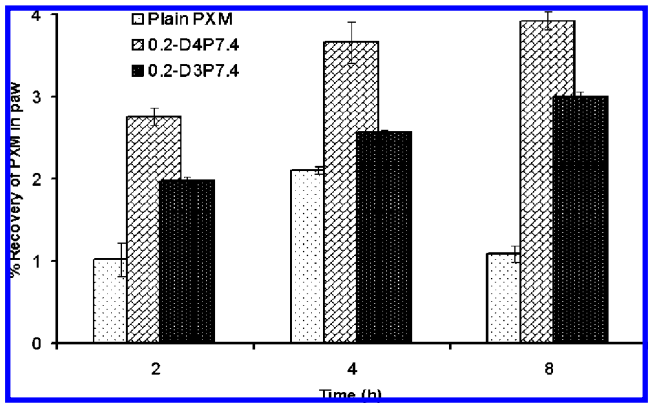

Figure 7. Percent drug recovery in paw after intravenous administration of different PXM formulations ($n = 6$).

Table 3. Percentage Drug Recovered from Various Organs after Intravenous Administration of the Plain PXM, 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ Formulations[a]

		percentage of drug recovered at different time intervals (h)		
organ	system	2	4	8
paw	PXM	1.02 ± 0.25*	2.10 ± 0.29**	1.09 ± 0.96**
	0.2-$D_3P_{7.4}$	1.98 ± 0.74*	2.57 ± 1.0*	3.42 ± 1.9**
	0.2-$D_4P_{7.4}$	2.75 ± 0.89**	3.66 ± 0.99*	3.92 ± 1.25**
kidney	PXM	1.90 ± 1.02*	1.01 ± 0.42*	0.60 ± 0.28^{ns}
	0.2-$D_3P_{7.4}$	2.99 ± 1.84**	1.5 ± 0.95*	0.42 ± 0.2^{ns}
	0.2-$D_4P_{7.4}$	3.52 ± 3.00**	2.20 ± 1.07*	0.65 ± 0.50^{ns}
liver	PXM	3.29 ± 1.36**	3.01 ± 0.90**	1.72 ± 0.03*
	0.2-$D_3P_{7.4}$	4.37 ± 2.01**	2.98 ± 0.75**	1.29 ± 0.3*
	0.2-$D_4P_{7.4}$	5.52 ± 4.00**	3.12 ± 1.05*	1.39 ± 0.69*
spleen	PXM	0.55 ± 0.18^{ns}	0.41 ± 0.12^{ns}	0.27 ± 0.50^{ns}
	0.2-$D_3P_{7.4}$	0.84 ± 0.31*	0.61 ± 0.29^{ns}	0.33 ± 0.06^{ns}
	0.2-$D_4P_{7.4}$	1.01 ± 0.01*	0.82 ± 0.08^{ns}	0.43 ± 0.62^{ns}

[a] Results are represented as mean ± SD ($n = 6$).**, $p < 0.001$ (very significant); *, $p < 0.05$ (significant); ns, $p > 0.05$ (nonsignificant).

Table 4. Pharmacokinetic Parameters of PXM and Dendrimer-Based Formulations in Serum of Male Albino Rats[a]

system	C_{max} (μg/mL)	T_{max} (h)	K_{el}	$t_{1/2}$ (h)	AUC (μg/mL/h)
PXM	6.869	0.5	0.0206	33.7	279.11 ± 1.48
0.2-$D_3P_{7.4}$	6.871	0.48	0.0185	36.6	293.78 ± 2.04
0.2-$D_4P_{7.4}$	6.218	0.5	0.0169	41.1	321.54 ± 2.37

[a] C_{max}, peak plasma concentration; T_{max}, time of peak constant; K_{el}, elimination rate constant; $t_{1/2}$, half-life; AUC, area under the curve ($n = 6$).

recovery in paw was observed with 0.2-$D_3P_{7.4}$ formulation, but the percentage drug recovered in paw with 0.2-$D_4P_{7.4}$ was statistically higher ($p < 0.05$) as compared to that of 0.2-$D_3P_{7.4}$.

It should be noted that, after 2 h of administration of naked PXM, higher concentration was observed in kidney and liver than in paw, and at this time point similar observations were made with 0.2-$D_3P_{7.4}$ and 0.2-$D_4P_{7.4}$ formulations. After 4 h, both naked and 0.2-$D_3P_{7.4}$ formulation continued to show higher recovery in liver (3.01 ± 0.90; 2.98 ± 0.75) than in paw (2.10 ± 0.29; 2.57 ± 1.0), respectively. However, with 0.2-$D_4P_{7.4}$ formulation after 4 h higher concentration of PXM

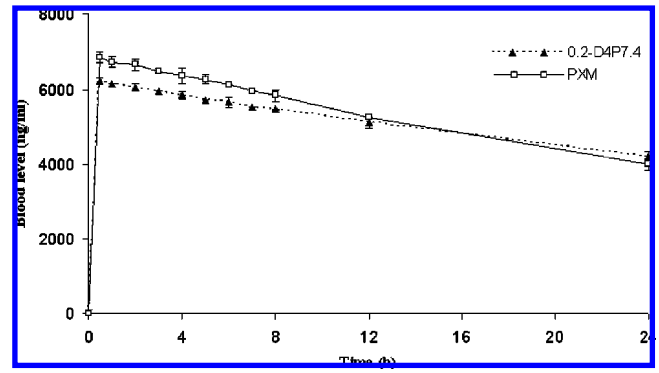

Figure 8. The blood plasma concentration of plain PXM and dendrimer-based formulation ($n = 6$).

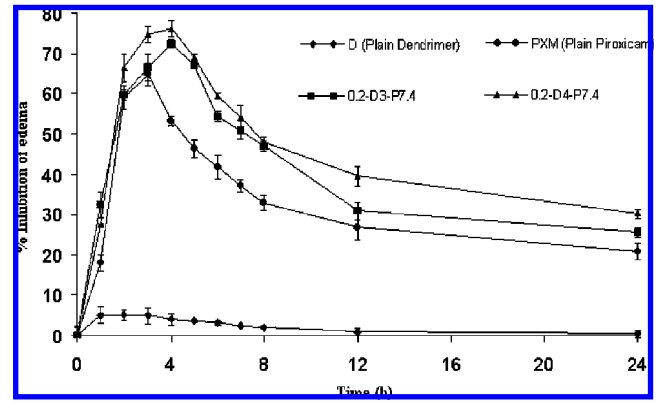

Figure 9. Anti-inflammatory activity of various formulations ($n = 6$).

was recovered from paw (3.66 ± 0.99) as against 2.20 ± 1.07 and 3.12 ± 1.05 in kidney and liver, respectively.

Pharmacodynamic Studies. Pharmacodynamic studies were performed employing carrageenan-induced paw-edema model.[21,30] In the case of plain drug, the inhibition level was found to be 26.9 ± 2.0% and 20.9 ± 0.76%, after 12 and 24 h, respectively. The formulation under investigation (0.2-$D_4P_{7.4}$) displayed maximum inhibition after 4 h (76.19 ± 2.2%), and the inhibition level was maintained at 39.42 ± 1.1% and 30.17 ± 2.3%, even after 12 and 24 h, respectively (Figure 9).

Stability Studies. The stability assessment of drug−dendrimer complex (0.2-$D_4P_{7.4}$) was performed under different conditions of temperature (0 °C; room temperature, 25 ± 2 °C; and 60 ± 2 °C). The designed dendrimer-based system was found to be sufficiently stable even at elevated temperatures up to 60 ± 2 °C, dark (amber-colored vials). However, a slight change in color as well as a sign of precipitation was noted after six weeks when kept at 60 ± 2 °C, in the presence of light (transparent vials). Considerable drug loss was observed under higher temperature stipulations in the presence of light (Figure 10, Table 5).

Discussion

In the present study, the solubilization potential of $-NH_2$ terminated PAMAM dendrimers was explored. PAMAM

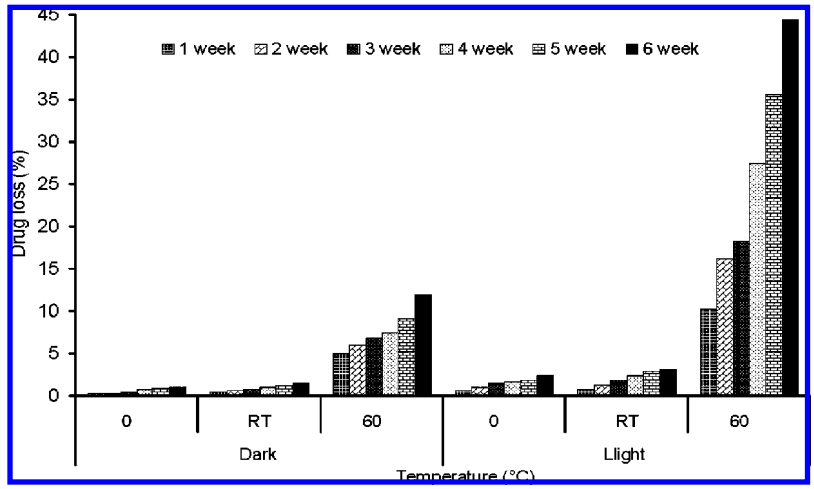

Figure 10. Drug loss from drug−dendrimer formulation (0.2-$D_4P_{7.4}$) under different storage conditions.

Table 5. Stability Data of Drug Dendrimer Formulation 0.2-$D_4P_{7.4}$[a]

	temperature (°C)					
	dark			light		
parameter	0	37 ± 0.5	60 ± 0.5	0	37 ± 0.5	60 ± 0.5
turbidity	−	−	+	−	+	++
precipitation	−	−	−	−	+	+
color change	−	−	++	−	+	++
change in consistency	−	−	++	−	+	+++

[a] Keys: (−) no change; (+) little change; (++) considerable change; (+++) significant change.

dendrimers of generations 3.0G and 4.0G were synthesized and characterized by FT-IR, ^{1}H NMR spectroscopic and microscopic means. The reports of analysis were in accordance with earlier reports[23] (Table 1).

The existence of a broad peak around 3350 cm^{-1}, in FT-IR spectra of drug−dendrimer complex, confirmed the encapsulation of PXM inside hydrophobic cavities of dendrimer possibly due to electrostatic interaction as well as hydrogen bonding. This is because the broad peak at around 3350 cm^{-1} represents the presence of $-NH_3^+$ functionality that can only come into existence as a result of setting up of electrostatic interaction of drug with dendrimer. In addition to this, the downshift in peak from ∼1650 cm^{-1}, in both formulations under study, suggests complexation of drug with dendrimer (Figure 3). Furthermore, the formulations were also scanned over the entire UV range, wherein only the peaks corresponding to nascent dendrimer were observed, confirming complexation as well as core loading of PXM (Figure 4) and which was not a covalent conjugation but rather a simple loading.

The solubilization behavior was found to be a function of pH of milieu, which was found to be maximal at pH 7.4 and minimal at pH 4 (Table 2). The possible reason may be the protonated and deprotonated states of drug candidate and dendrimer at different pH.[35−37] At pH 7.4, both PXM and $-NH_2$ terminated dendrimers exist significantly in ionized state, which offers superior opportunities for interaction and association of PXM with dendrimers. Reports demonstrated that the solubility of drug increased linearly with increasing dendrimer concentrations via electrostatic interaction between amino groups of dendrimer and carboxyl group of drug.[35] The same mechanism of interaction may hold true in the case of PXM, for 3.0G and 4.0G dendrimer-mediated solubility enhancement.

PXM is a weak acid (pK_a 4.6), and its solubility increases with an increase in pH. At lower pH (pH 4.0), the solubility enhancement is only due to electrostatic interaction, whereas at higher pH values (pH 7.4 and 10.0), the observed enhancement is a result of both dendrimeric interaction and its inherent pH dependent solubilization. At pH 7.4, both primary amines of dendrimer and hydroxyl group of piroxicam may be 99% ionized. Therefore, pH 7.4 is an ideal condition at which maximum potential for electrostatic bonding between the drug and dendrimer is expected.[21,30]

At pH 4.0, dendrimer exists in completely protonated state, while at pH 10.0, it exists in depronated state, whereas at pH 7.4, it displays a characteristic 2/3 protonation profile. At pH 4.0, no significant increase in solubility was observed because of predominant protonated states of dendrimers, which precludes any kind of interaction. This protonation of tertiary amines in amine-terminated dendrimers creates an environment with considerable polarity inside the den-

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drimer microcavity, and hence no significant increase in solubility of PXM was observed.

The higher release rate was experienced with medium-II (92 ± 4.4%) as compared to that with medium-I (85.36 ± 2.33%), and the setting up of interaction between drug−dendrimer complex and protein[30] may be one of the reasons for this (Figure 5). The other reasons bring the changed conformational states with modified physiological conditions. It should be realized that altered release profile owing to protein binding of albumin is one of the additional beneficial factors that may contribute passive targeted delivery of PXM to the inflamed region, in agreement with the recent findings on protein binding.[38,39] From the pharmacokinetic profile it was inferred that the blood levels of drug observed with dendritic formulation in edema induced paw tissues was much higher as compared to that of free drug, under all the monitored time intervals (Figure 8; Table 3).

The lower hemolytic toxicity observed in the case of drug-loaded dendrimers may be ascribed to the shielding of free $-NH_2$ groups present on the surface of 4.0G PAMAM dendrimer by lower hemolytic bioactive; PXM, which was found to be 2.31 ± 1.1% hemolytic.[23] However, it is also envisaged herewith that this toxicity can be further brought down by modifying the surface of PAMAM dendrimers by PEGylation.

This could be due to constant drug level in the blood, which was maintained for longer duration in case of 0.2-$D_4P_{7.4}$ formulation as compared to free PXM (Figure 8). The enhanced permeation experienced with 0.2-$D_4P_{7.4}$ formulation may be due to the leaky vasculature in the inflamed paw, redistribution of dendrimer from inflamed area may occur via lymphatic system, unlike tumors where the lymphatic drainage is poor, and is clearly evinced from the high anti-inflamatory potential observed with 0.2-$D_4P_{7.4}$ formulation (Figure 9). One possibility toward localization as well as retention of 0.2-$D_4P_{7.4}$ complex at the inflammatory site is the affinity of dendrimer torwards the glycosaminoglycan, abundantly present in arthitic joints or inflamed areas.[40,41]

Macromolecules do not leak out of the vascular endothelial compartment into the interstitial spaces in the normal tissue; however, the vasculature of inflamed tissue is highly permeable/leaky. Thus it is assumed that these macromolecules based on dendritic systems may extravasate/leak from the blood vessels into the interstitial spaces and consequently higher concentration of drug was found in the inflamed paw in case of dendrimer formulation (Figure 8). The pharmacodynamic assessment revealed that, in the case of plain drug, relatively lower level inhibition was observed (26.9 ± 2.0%, 12 h and 20.9 ± 0.76%, 24 h), as compared to the dendrimeric formulation under investigation (0.2-$D_4P_{7.4}$) that displayed maximum inhibition after 4 h (76.19 ± 2.2%), and a significant inhibition level was observed even after 12 and 24 h that was found to be 39.42 ± 1.1% and 30.17 ± 2.3%, respectively (Figure 9).

Stability studies indicated that the system was stable even at elevated temperatures up to 60 ± 2 °C in the dark. But considerable drug loss was observed under higher temperature stipulations in the presence of light. This may be because of dendritic structure, which is supposed to be more open at higher temperature,[7] and this change in surface characteristics might cause the conformational changes in the structure and release of drug (Figure 10). Also higher temperature reaction kinetics was expected to play a critical role in the presence of light at elevated temperature (60 ± 2 °C). Albeit, no change in turbidity, color and consistency was noticed in formulations when stored at low and normal room temperature conditions, concluding that the formulation should be stored at cool temperatures and in a dark place (Table 5).

In conclusion, dendrimer-based formulation may not only improve the solubility of PXM but also help in localization of drug at the inflammation site and hence provide better therapeutic efficacy at a lower dose. A sustained release and slow elimination of the drug from the formulation, from the body with parallel maintenance of high plasma concentration may be therapeutically promising. The study supports the employment of this nanocarrier for development of safe, effective and biocompatible drug formulation intending therapeutic efficacy with minimal dose.

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