RESEARCH PAPER

Accepted 21 December 2002
Revised 25 November 2002
Received 23 April 2002
Indian J. Pharm. Sci., 2003, 65(2): 139-145

Amphiphilic Copolymeric Micelles for Delivery of Nimesulide: Preparation, Optimization and Characterization

SULEKHA BHADRA, D. BHADRA AND G. P. AGRAWAL*
Department of Pharmaceutical Sciences
Dr. Harisingh Gour Vishwavidyalaya, Sagar-470 003.

In the present study five copolymeric blends of polycaprolactone-polyethylene glycol were prepared by ring opening polymerization technique. These were characterized by IR and NMR studies and by determination of critical micelle concentration. The copolymeric blends were optimized from the entrapment capacity, release rate and stability studies of the nimesulide loaded-micelles prepared using such copolymers. These micellar preparations had shown better stability at refrigerated temperature. The formulation prepared by dialysis method using copolymer, having molar ratio of ε-caprolactone to methoxypolyethylene glycol fifty-to-one, was used for further studies in rats. Mean residence time and bio-distribution studies showed that the micelles have higher circulation time in blood and lesser distribution in other tissues as compared to plain drug solution.

Nimesulide, a selective COX-2 inhibitor[1], is a commonly used new generation NSAID. Treatment of inflammatory diseases using sustained release dosage forms is advantageous as this helps in maintaining the therapeutic concentration of the drugs for a longer duration, particularly throughout the night and thus alleviating morning stiffness[2]. Many novel drug delivery systems were tried to enable sustained drug delivery. Most of these systems like liposomes and nanoparticles have short circulation life in the blood as they are taken up by the reticuloendothelial system of the body due to their size and nonpolar surface. In order to increase their circulation time, a new concept of coating these carriers with polar molecules e.g. polyethylene glycol (PEG) has been proposed[3-6]. Polymer micelles prepared from amphiphilic copolymers are based on this concept of increasing the circulation time as they have polar coat. These polymer micelles can also be categorized as 'stealth' nanoparticles[7]. Amphiphilic copolymers are soluble macromolecules composed of hydrophobic and hydrophilic segments. Thus, in aqueous medium, they form micellar structures. Since the inner core of the polymer micelles is of hydrophobic nature, they may incorporate hydrophobic drugs in their core and hence are suitable for the delivery of water insoluble drugs like nimesulide.

***For correspondence**
E-mail: bhadrasb28@yahoo.com

In the present study polycaprolactone-methoxypolyethylene glycol (PCL-MPEG) copolymers, containing ε-caprolactone (ε-CL) as hydrophobic and methoxypolyethylene glycol (MPEG) as hydrophilic segment, were used as the amphiphilic copolymers. PCL-MPEG copolymers can be synthesized by ring opening polymerization technique (fig. 1) without any catalyst, using MPEG as initiator[8]. The drug nimesulide was chosen for present study with an intention to solve the problem of solubility and liver toxicity associated with its delivery[1].

MATERIALS AND METHODS

Methoxypolyethylene glycol was procured from Sigma Chemical Co., St. Louis, Mo. Nimesulide was obtained as gift sample from Alkem, Mumbai. ε-Caprolactone was purchased from Merck, Germany. Cellulose dialysis membrane tubing of 2.4 nm pore size and 12000 MWCO was purchased from HiMedia labs, Mumbai. All other chemicals used were

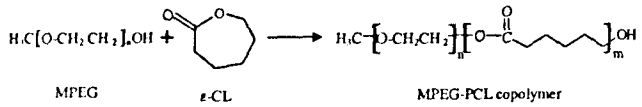

Fig. 1: Reaction of Methoxypolyethylene glycol (MPEG) and ε-Caprolactone (ε-CL).

procured from CDH, India and were of analytical grade.

Synthesis and purification of copolymer:

Different polycaprolactone-methoxypolyethylene glycol (PCL-MPEG) diblock copolymers were synthesized by ring opening polymerization technique[8] using different molar ratios of ε-CL and MPEG. Weighed amounts (Table 1) of ε-CL and MPEG were taken in a round bottom flask. This mixture was first degassed and cooled using rotary vacuum evaporator and then heated in vacuum oven at 180°. The product mixture was dissolved in 5 ml of dichloromethane. This preparation was dialyzed against dichloromethane. The content inside the dialysis tube was concentrated and precipitated using excess amount of diethyl ether. The precipitate was then collected by filtration and then dried in vacuum oven at 40° for 3 d.

Characterization of copolymers:

The structural confirmation of the copolymer thus formed was done by FT-IR analysis of the copolymer. The spectra of the polymers were compared with the FT-IR of MPEG. The actual ratio of MPEG and ε-CL in each copolymer was determined using the NMR spectrum of the copolymer samples. The ratio of MPEG:ε-CL of each copolymer was calculated from the relative intensities of methylene peaks due to MPEG and ε-CL at 3.75 and 4.79 ppm, respectively.

In order to determine the critical micellar concentration (CMC) of the prepared copolymer, the surface tension of copolymer solutions was determined at its various concentrations. Solutions of the copolymer were prepared in the concentration range of 1-8 μg/ml. The surface tension of each solution was determined by drop number method[9], using stalagmometer. A graph between the concentration and surface tension was plotted, and the concentration at which there is a sharp change in surface tension was taken to be the CMC of the copolymer.

Drug loading:

Drug loading was accomplished *in situ*, i.e. during micelle formation by dialysis method and emulsification method. In first method 100 mg copolymer and 10 mg nimesulide were dissolved in 10 ml dimethylformamide (DMF) and stirred for 24 h. This solution was dialyzed against 1000 ml distilled water for 24 h using cellulose dialysis membrane tubing. The resultant product from the dialyzing tube was then sonicated for 1 min and centrifuged for 5 min. The supernatant containing the micelles were separated by decantation. Micelles, made from copolymers MC10, 35, 50, 70 and 100, were named as D_1-D_5, respectively.

In emulsification method, an o/w emulsion was prepared by slowly introducing the organic phase (containing 60 mg copolymer MC50 and 6 mg nimesulide in 2 ml dichloromethane) in 100 ml distilled water under high-speed stirring. The stirring was continued till the organic phase gets

TABLE 1: COMPOSITION OF MPEG/ εCL BLOCK COPOLYMER.

Copolymer name assigned	Feed molar ratio ε-CL/MPEG	Quantity taken		
		MPEG[1] (g)	ε-CL[2]	
			Weight (g)	Equivalent volume[3] (ml)
MC10	10	5	1.14	1.1
MC35	35	5	3.99	3.7
MC50	50	5	5.71	5.3
MC70	70	5	7.99	7.5
MC100	100	5	11.41	10.7

[1]calculated from molecular weight of MPEG (i.e. 5000) [2]calculated from molecular weight of ε-CL (i.e. 114.15) [3]calculated from density of ε-CL (i.e. 1.07 g/ml).

evaporated completely. This was then sonicated for 1 min and centrifuged for 5 min. This supernatant was then separated by decantation and named as E_3.

***In vitro* characterization of the copolymeric micelles:**

The shape of the micelles could not be seen through ordinary light microscope. Thus transmission electronic microscopy (TEM) of the polymeric micelles was done to characterize these systems after drying on beryllium grid and staining negatively at 100,000X.

Drug loading capacity of the copolymeric micellar system was determined by lysis of 1 ml preparation with dimethylformamide. The amount of drug present in this 1 ml preparation was determined spectrophotometrically at 397 nm, after appropriate dilution with DMF:0.1 N sodium hydroxide (1:9) mixture.

For the drug-release profile study, appropriate volume of the preparation was taken in the dialysis tube and dialyzed against 20 ml phosphate buffer (pH 7.4) with continuous stirring using magnetic stirrer. At appropriate time intervals, dialysate was analyzed spectrophotometrically at 393.4 nm, after appropriate dilution. Each time the dialysing medium was completely replaced with fresh buffer. This study was continued up to 96 h.

Stability studies:

The stability study of the micellar preparations (D_1, D_3, D_5) was performed at various temperatures (refrigerated temperature, rt, room temperature RT and at 50°) for a period of 5 w. Physical parameters like precipitation or crystallization, change in colour, appearance of any turbidity and the drug leakage from the micelles were checked every week. The drug leakage was determined as the percentage increase in release rate profile of the preparations.

***In vivo* studies:**

All the studies were conducted using Sprague Dawley rats of approximately equal body weights. The animal experiment protocols have been approved by the Institutional Animals Ethics Committee (CPCSEA Reg. No. 379/01/ab/CPCSEA). The optimized D_3 copolymeric micellar formulation (having molar ratio of ε-caprolactone to methoxypolyethylene glycol 50:1) was taken for *in vivo* studies.

Standard curves of nimesulide in blood serum and other tissue extracts were prepared. The blood of the rat was collected in a vial containing potassium oxalate solution and centrifuged at 3000 rpm in order to get the plasma (supernatant). Then it was deproteinized with equal volume of acetonitrile and again centrifuged at 3000 rpm for 15 min. Two millilitres of this supernatant (blood serum) was diluted to 10 ml with PBS (pH 7.4). Drug solutions of known concentrations (1-20 μg/ml) were prepared in PBS (each containing 1 ml of this diluted serum) and absorbance was measured against suitable blank at 393.6 nm. Standard curve in liver, lung and spleen extract was prepared to determine biodistribution of nimesulide. For this one rat was anaesthetized with chloroform and dissected and its liver, spleen and lungs were taken out. The whole spleen (0.6 g) and 1 g each of liver and lungs were homogenized separately with 5 ml PBS (pH 7.4). Then these organ-homogenates were deproteinized with equal volume of acetonitrile and centrifuged at 5000 rpm. Drug solutions of known concentrations were prepared in PBS (each containing 0.2 ml of supernatants). Absorbance was measured against appropriate blank at 393.6 nm.

Nine Sprague Dawley rats were divided into three groups having three rats each. One group was kept as control. Remaining 2 groups were given separately plain drug solution (in PBS, pH 7.4) and micellar preparation (D_3) containing 100 μg equivalent of nimesulide, intravenously through the tail vein. After every 1 h time interval, 0.5 ml blood sample was collected from the retro-orbital plexus into a vial containing potassium oxalate solution and then centrifuged and deproteinized. These were then analyzed spectrophotometrically (using internal standards) at 393.6 nm against similarly treated blood samples of control rats. The serum level data was used to determine the bioavailability and the mean residence time (MRT) of different formulations. The bioavailability (area under concentration-time curve, AUC) was calculated by trapezoidal rule[10]. The MRT was calculated using the formula: MRT=AUMC/AUC, where AUMC is the area under first moment curve[11].

For biodistribution studies, micellar solution (equivalent to 500 μg nimesulide) was administered to nine rats of nearly equal weights (90-100 g) by intravenous route. Rats were sacrificed after 1, 3 and 8 h of administration of dosage form (n=3). Their blood, liver, spleen and lungs were taken out. The tissues were extracted with PBS. The tissue extracts were treated with 1 ml of dimethylformamide (in order to rupture whole of the micellar preparation accumulated in the organs) and analyzed spectrophotometrically at 393.6 nm, after proper dilutions.

Tail flick method using analgesiometer was used to

compare the analgesic activity of the free drug with the drug entrapped in the micellar system. Nine rats of average weight 100 g were selected for the study. They were divided into three groups including one control group. Each rat was placed in the rat holder and tail was protruded out through the slot in the lid and placed on a hot wire, which was heated with 3 A current. The normal reaction time i.e. the time taken to flick the tail was noted. Again the reaction time at different time intervals after the administration of plain drug solution (made by dissolving 10 mg nimesulide in 100 ml of PEG:water::1:1 solution) and copolymeric micellar preparation (D_3), equivalent to 100 μg of nimesulide were also noted.

RESULTS AND DISCUSSION

The MPEG/εCL copolymers were synthesized by ring opening polymerization of ε-CL without any catalyst using MPEG as initiator. A series of copolymers were prepared by taking different ratio of ε-CL and MPEG. Completion of synthesis was confirmed by precipitation of copolymer with diethyl ether. This fact was further used to purify the reaction products. The products were first dissolved in minimum volume of dichloromethane and purified from unreacted MPEG by dialysis against plain dichloromethane. Then ice-cold diethyl ether was added into these solutions to precipitate the copolymers.

The success of synthesis procedure was confirmed by analyzing infrared spectrum (FT-IR) of both MPEG homopolymer and MPEG/ε-CL copolymer. The appearance of a new strong carbonyl band at 1733.1 cm^{-1} in the IR spectrum of the product obtained after synthesis and purification process, confirmed the synthesis of MPEG/ε-CL copolymer. This peak is attributed to the formation of ester linkage between the free hydroxy group of MPEG and carboxyl group of caprolactone, giving rise to formation of the block copolymer. Other peaks remained unaffected.

The actual w/w percentage composition of the copolymers was determined by ^{1}H-NMR analysis of all the copolymers formed. The unit ratio of MPEG and ε-CL was obtained from the peak intensities of $-CH_2-$ proton of PEG chain and the ε- CH_2- proton in ε-CL units, respectively at 3.76 and 4.79 ppm. The calculated ratios for various peak intensities are summarized in Table 2. The peak due to proton attached to C=O group (at 1.2-2.8 ppm) also confirmed the synthesis of copolymer by the formation of ester linkage.

For further characterization of the copolymers, the CMC was also determined by the surface tension method. The CMC of MC 50 was found to be 5 μg/ml. Transmission electron microscopic analysis showed that the micellar preparations were spherical in shape (fig. 2). This also had shown a relatively regular outer surface and hollow interiors, where the drug can be entrapped. The drug loading efficiency of the copolymers was determined by rupturing the micelles with DMF:0.1 N sodium hydroxide (1:9) mixture and then analyzing the drug spectrophotometrically at 397 nm. The results showed that the drug loading efficiency of the copolymers increased as the ratio of hydrophobic (caprolactone) chain increased (Table 3). Also the percentage w/w drug loading in the micelles prepared by emulsification technique was found to be greater than in those prepared by dialysis method (using the same copolymer). But the w/v content in the micelles prepared by emulsification technique was found to be less than those prepared by dialysis technique. Thus, if the micellar preparations are lyo-

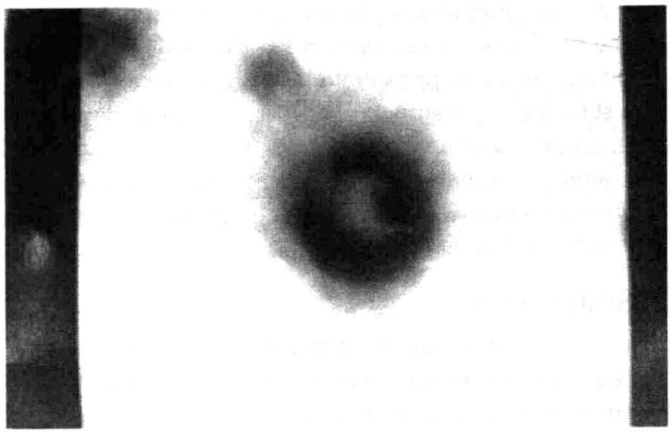

Fig. 2: Transmission Electron Microscopy of copolymeric micelles at 100, 000 X.

TABLE 2: NMR SPECTRAL DATA REPRESENTING COMPOSITION OF MPEG/εCL COPOLYMERS.

Sample	Composition (%w/w)	
	Theoretical[a]	Practical[b]
MC 10	81.4:18.6	80.9:19.4
MC 35	55.6:44.4	54.4:45.5
MC 50	46.7:53.3	45.2:54.1
MC 70	38.5:61.5	36.8:62.4
MC 100	30.5:69.5	29.8:70.2

[a] calculated from the weight of monomers taken using molecular weight of MPEG (5000) and ε-CL (114.15). [b] calculated using intensity of peaks in NMR spectra.

TABLE 3: DRUG LOADING CAPACITY OF MICELLES PREPARED USING DIFFERENT COPOLYMERS.

Sample	Volume of preparation (ml)	Concentration of drug	
		(μg/ml)	% w/w*
D1	10.0	115	1.1
D2	9.5	166	1.5
D3	9.0	404	3.6
D4	9.0	476	4.3
D5	10.5	560	5.9
E3	50.0	23	13.0

* % w/w of copolymer taken; D1-Prepared by dialysis method using MC10, D2-Prepared by dialysis method using MC35, D3-Prepared by dialysis method using MC50, D4-Prepared by dialysis method using MC70, D5-Prepared by dialysis method using MC100, E3-Prepared by emulsification method using MC50

philized, it can be concluded that the emulsification method can yield copolymeric micelles of greater drug loading efficiency than the dialysis method.

Drug release from the micellar preparations was determined by dialysing the micellar preparation against PBS (pH 7.4). The release pattern showed sustained release of drug from micellar preparations as compared to plain drug solution. The results (fig. 3) also showed that as the hydrophobic chain length was increased, the release rate decreased. This may be due to the hydrophobic nature of the drug molecule that favoured its higher retention into the micelles of higher hydrophobic content as compared to those with lower hydrophobic chain length.

In the stability study of the micellar preparations at various temperature, the formulation D_5 was found to be most stable (fig. 4) at refrigerated temperature. The drug leakage was found to be minimum at refrigerated conditions and maximum at 50°. This may be due to the facts that at higher

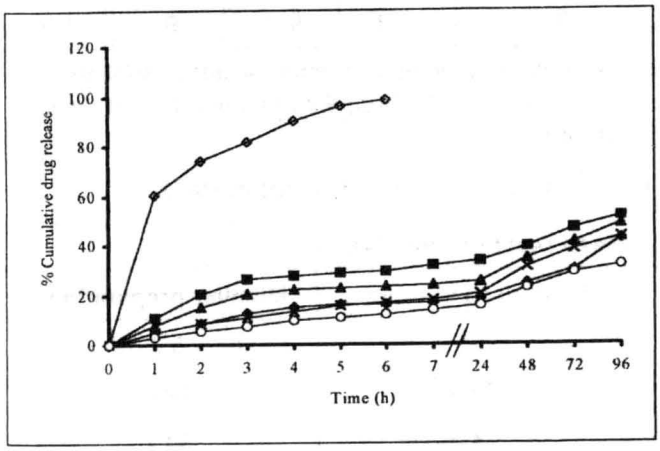

Fig. 3: Release of drug from various micellar formulations

Release of nimesulide from the micellar systems was studied using dialysis against 20 ml pH 7.4, phosphate buffer. Samples were collected and analyzed at various time intervals from Plain Drug Solution (-◇-), Formulation D1 (-■-), D2 (-▲-), D3 (-X-) , D4 (-●-), D5(-○-)

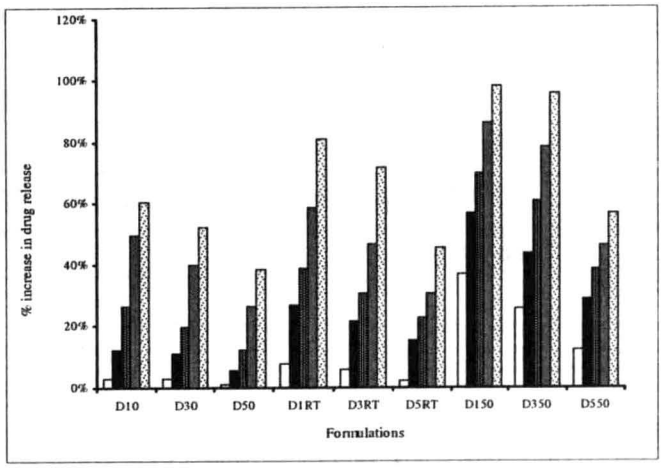

Fig. 4: Stability Studies of various formulations D1, D3 and D5.

Stability studies of formulations D1, D3 and D5 stored at different temperatures such as at refrigerated temperature, $D1_0$, $D3_0$ and $D5_0$ at room temperature $D1_{RT}$, $D3_{RT}$ and $D5_{RT}$ and at 50° $D1_{50}$, $D3_{50}$ and $D5_{50}$, for various time intervals, 1 w (□) 2 w (■) 3 w (■) 4 w (■) 5 w (▨).

temperature, the ring is wide open; the PEG chains of the coating spread in the medium and the mobility of drug molecules entrapped in micelles is high. This causes release of the drug to a greater extent than that at room temperature. So it is advised that the micellar formulations must be labeled as "to be kept at a cool place." Thus, it can be concluded that there will always be some drug leakage if the formulation is stored in solution form. So it is suggested that the micellar preparations should be marketed as lyophilized products.

Again in order to study the pharmacokinetic parameter, the serum level of the drug in rats was determined at different time intervals for free drug solution as well as drug entrapped in copolymeric micellar system. The serum level of the drug after the administration of micellar preparation was maintained nearly constant over a longer period of time as compared to that in case of plain drug solution, where it decreased sharply with time (Table 4). The mean residence time of the copolymeric micellar formulation was found to be more than that for plain drug solution.

TABLE 4: PLASMA LEVEL OF NIMESULIDE AFTER I.V. ADMINISTRATION OF DIFFERENT FORMULATIONS.

Time interval (h)	Drug concentration (μg/ml)	
	Plain drug solution	Micellar preparation
1	5.3	1.7
2	2.7	1.9
3	1.8	2.0
4	1.3	1.6
5	0.9*	1.6
6	0.7*	1.6
7		1.4
8		1.4

* values obtained by extrapolation.

The accumulation property of the micellar system was determined by biodistribution study in rats. The results showed that the micellar system was less accumulated in different organs of the reticuloendothelial system (RES) like the liver, spleen and lungs. Even after 8 h of intravenous administration of the micellar system, only 20% of the administered dose was found to be accumulated in these organs (Table 5).

The pharmacological property like the analgesic activity of the nimesulide was evaluated for both, the free drug

TABLE 5: BIODISTRIBUTION OF NIMESULIDE.

Time interval (h)	% drug accumulation*			
	Blood	Liver	Spleen	Lungs
1	96.2	1.9	1.1	0.4
3	82.7	7	6.2	1
8	70.3	9.2	9	1.5

Biodistribution of nimesulide after i.v. administration of micellar preparation; *% Drug with respect to total dose administered.

TABLE 6: EVALUATION OF ANALGESIC ACTIVITY OF DIFFERENT FORMULATIONS IN RATS.

Time interval (h)	Mean reaction time (sec.)		
	Control	Plain drug solution	Micellar preparation
0*	5.3	5.3	5.7
0.5	5.0	38.0	32.3
1	5.3	34.3	34.7
2	5.3	25.7	35.0
3	5.7	15.0	34.3
4	5.0	9.7	29.7
5	5.3	6.7	24.0

*Normal reaction time

solution and the drug given in copolymeric micellar carrier system. This was done to compare the extent and duration of drug-activity when administered in different formulations. This study showed that the extent and duration of drug activity were increased when the drug was administered in micellar preparation. It is evident that even after 5 h the activity of micellar preparation was approximately 4 times that of simple drug solution (Table 6).

Thus, it can be concluded from the *in vitro* and *in vivo* studies that the copolymeric micelles can more effectively be used as a novel drug delivery system with long circulation life in the blood. Also, this type of systems may help in reducing the dose of drug because the major part of the dose administered remain in the blood, as their accumulation in other organs was very less.

ACKNOWLEDGEMENTS

The authors are pleased to express their gratitude to the Head, Department of Pharmaceutical Sciences and UGC, New Delhi for facilitating the present research work.

REFERENCES

1. Singla, A.K., Chawla, M. and Singh, A., **J. Pharm. Pharmacol.,** 2000, 52, 467.
2. Robinson, J.R. and Lee, V.H., Eds., In; Controlled Drug Delivery: Fundamentals and Applications, 2nd Edn., Marcel Dekker Inc., New York, 1987, 33.
3. Veronese, F.M., Calicetti, P., Pastorino, A., Schiavon, O. and Sartore, L., **J. Control. Release,** 1989, 10, 145.
4. Papahadjopoulus, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K, Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J., **Proc. Natl. Acad. Sci.,** 1991, 88, 11460.
5. Phillips, W.T., Klipper, R.W., Awasthi, V.D., Rudolph, A.S., Cliff, R., Kwasiborski,V. and Goins, B.A., **J. Pharmacol. Exp. Ther.,** 1999, 288, 665.
6. Bhadra, D., Bhadra, S., Jain, P. and Jain, N.K., **Pharmazie,** 2002, 57, 5.
7. Yokoyama, M., Miyauchi, M., Yamada, N., Okano, T., Sakurai, Y., Kataoka, K. and Inoue, S., **J. Control. Release,** 1990, 11, 269.
8. Shin, IL.G., Kim, S.Y., Lee, Y.M., Cho, C.S. and Sung, Y.K., **J. Control. Release,** 1998, 51, 1.
9. Yadav, J.B., Eds., In; Advanced Practical Physical Chemistry, 12th Edn., Goel Publishing House, 1992,75.
10. Brahmankar, D.M and Jaiswal,S.B., Eds., In; Biopharmaceutics and Pharmacokinetics (A-Treatise), 1st Edn., Vallabh Prakashan, Mumbai, 1995, 224.