Original Research Article

In vitro release behavior of paclitaxel and carboplatin from poly(l-lactide) microspheres dispersed in thermosensitive biodegradable gel for combination therapy

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Abstract

The objective of the current work was to design an injectable, sustained release formulation of a combination of anticancer drugs, carboplatin and paclitaxel, for localized delivery. In this combination formulation, carboplatin was encapsulated into poly(L-lactide) (PLA) microspheres and paclitaxel was dissolved in thermosensitive biodegradable gel of PLGA-PEG-PLGA (poly (DL-lactide-co-glycolide- polyethylene glycol- poly (DL-lactide-co-glycolide)); no external solvent like cremophorEL was used in the formulation, further, these carboplatin microspheres were dispersed in the gel containing paclitaxel to achieve a single delivery system. The combined formulation was assessed for various parameters for sustained release of both the drugs. Release profiles of carboplatin from PLA microspheres; paclitaxel from hydrogel alone and in combination with carboplatin and carboplatin microspheres dispersed in paclitaxel loaded gel were studied. In vitro release of both the drugs from PLGA-PEG-PLGA hydrogel showed that carboplatin was released with 40-50% burst release and paclitaxel was released in biphasic manner for 50-60 days. Initial burst of carboplatin was controlled by incorporating it in PLA microspheres which were then dispersed in paclitaxel loaded hydrogel and the new formulation did not exhibit any burst release of the drug. Release pattern of combination formulations revealed that the two drugs were co-eluting from a single delivery system and the rate of release of each of the individual drugs was significantly affected.

Thus, a novel injectable combination formulation for sustained and simultaneous delivery of carboplatin and paclitaxel was developed which provided sustained release of each of the drugs and could be further explored in tumor models.

Keywords: Thermosensitive hydrogels, Microspheres, controlled release, combination chemotherapy.

Introduction

Combination chemotherapy is today being explored as one of the most attractive approaches to ensure total cell kill and complete remission in various cancerous diseases. Skipper and Schabel first described the importance of combination therapy on the basis of log kill hypothesis [1]. Combination of paclitaxel and platinum analogues (cisplatin and carboplatin) is already employed in clinical practice and this is a FDA

approved combination for the treatment of different types of cancers; viz. advanced ovarian cancer and lung cancer.

Carboplatin is second generation platinum analog and acts as an alkylating agent. It is a cell cycle-non specific agent having antiproliferative action. It is having molecular weight of approximately 371.29 and solubility of 14 mg/ml. It is mainly used in treatment of ovarian cancer, and also in other types of cancers like lung, head, neck, endometrial cancers. More than 30% of the patients taking carboplatin experience side effects like low blood counts, thrombocytopenia, hair loss, peripheral neuropathy, diarrhea, general weakness etc. [2]. Paclitaxel is a water insoluble drug (aqueous solubility 4 µg/ml) and belongs to BCS class IV. It is soluble in organic solvents like alcohols, DMSO, chlorinated solvents. Its molecular weight is 853.9. Paclitaxel is having anti-proliferative, antimetastatic and anti-invasive actions [3]. It is mainly used in the treatment of ovarian, breast and non small cell lung cancer. Most serious side effect of paclitaxel is bone marrow depression especially neutropenia. Considering the individual efficacies of these two drugs in the treatment of cancer, carboplatin with paclitaxel have been combined based upon-

i) Carboplatin and paclitaxel show promising single agent activities in metastatic breast cancer, and efficacy in various other cancers due to their complementary mechanisms of action [4].

ii) Existence of inverse relationship between carboplatin and paclitaxel; as observed in resistant cell models where resistance to one led to sensitivity to the other [5].

iii) When paclitaxel/carboplatin are used in combination; paclitaxel appears to have more platelet sparing action that reduces thrombocytopenia induced by administration of carboplatin alone [6].

Considering the potential of this combination of anticancer drugs, it is of wide clinical utility, whereby conventional procedure for administration of paclitaxel and carboplatin combination involves 3 h infusion of paclitaxel followed by 30 min infusion of carboplatin or vice versa. Time required for reaching C_{max} for carboplatin (C_{max} 37 µg/ml) and paclitaxel (C_{max} 4.265 µg/ml) is 1 h and 3 h after infusion respectively [7, 8]. This cycle is repeated 3-4 times after every 3 weeks. This procedure is not patient complaint and is also associated with certain delivery and toxicity problems of each of the molecules due to systemic administration. Conventionally, paclitaxel is administrated as taxol® which is a marketed formulation of the drug containing cremophorEL to enhance its solubility (aqueous solubility of paclitaxel is 4µg/ml) [9]. The use of cremophorEL is associated with serious side effects, particularly life threatening hypersensitivity reactions [10].

In order to obviate the problems associated with systemic delivery of anticancer agents, localized delivery at the tumor site appears to be an attractive approach because this maximizes the therapeutic effect and minimizes the adverse effects. Polymeric controlled drug delivery systems particularly in situ depot forming systems have recently gained enormous interest to accomplish this task and triblock co-polymer, PLGA–PEG–PLGA is one such thermosensitive polymer explored widely. This system has been proved useful for delivery of single agents particularly for hydrophobic molecules [11-15]. Considering hydrophilic molecules, such as carboplatin are expected to exhibit burst release from this system. In order to overcome this problem, incorporation of hydrophilic drug into particulate carrier followed by dispersion into the gel may circumvent burst release along with prolonging the duration of release. Among particulate carriers, microparticles have certain advantages, such as easy method of preparation, scalability, high entrapment efficiency etc. Microparticles of biodegradable polymers like PLA are widely used because of its extensive safety profile and non toxicity [16].

To the best of our knowledge, no attempt has been made to design a controlled release formulation of paclitaxel and carboplatin which could release both the drugs in simultaneous and sustained manner. In this study, we developed a combination formulation of these two drugs in in situ depot forming system PLGA-PEG-PLGA, where carboplatin microspheres (CP MS) were dispersed in biodegradable copolymer gel of PLGA-PEG-PLGA containing paclitaxel. This system is injectable and could be injected into the tumor directly. This would help in maintaining high locoregional concentrations of both the drugs, also eliminating the systemic adverse effects caused by these two potent molecules. However, designing such a formulation was a challenging task owing to the opposite physicochemical nature of the two drugs; paclitaxel being highly hydrophobic and carboplatin being highly hydrophilic. Further, the focus of the paper was to understand the release behavior of these two opposite nature drugs releasing simultaneously from a single delivery system and to optimize the system so as to obtain sustained release of both the drugs.

Materials and methods
Materials
dl-Lactide and glycolide dimers were purchased from Purac and were used without further purification. Polyethylene glycol (MW 1000 Da; PEG_{1000}) and stannous 2-ethylhexanoate were procured from Sigma Aldrich (Germany) and were used as received. Paclitaxel was obtained as a gift from Prof. Avi Domb, Hebrew University of Jerusalem, Israel and carboplatin was generously gifted by Getwell Life Sciences (New Delhi, India). CremophorEL was purchased from Sigma Aldrich (Germany). All other chemicals used were of analytical grade.

Synthesis and Characterization of PLA
PLA polymer was synthesized by ring opening polymerization of dl-lactide using stannous-2-ethylhexanoate as catalyst [17]. Synthesized polymer was characterized by GPC using Shimadzu LC-10AD HPLC and Shimadzu RID 6A refractive index detector to know the weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity.

Preparation and characterization of microspheres
Preparation of microspheres
Microspheres were prepared by acetonitrile/light mineral oil emulsion and solvent evaporation method. Carboplatin (10 mg) was dispersed in acetonitrile (1 ml) and sonicated with probe sonicator for 10 min to reduce the particle size. PLA (90 mg) solution in acetonitrile (1 ml) was added to the above dispersion. This internal phase (carboplatin + PLA + acetonitrile) was added to the external phase i.e. 40 ml of light liquid paraffin oil containing 1% w/w of surfactant (Span 80), while stirring at 500 rpm. The solvent (acetonitrile) was allowed to evaporate for 4 h. The particles obtained were washed in hexane and dried under vacuum.

Size and surface morphology
Size analysis of microspheres was performed by optical microscopy (Meiji Technology TC5500), images were captured by infinity camera and size was measured using "i solution" software (Version 7.2, iMTechnology). Surface morphology of microspheres was studied by Scanning Electron Microscopy (S-3400N, Hitachi Japan).

Entrapment efficiency
Weighed amount of microspheres (5 mg) were dissolved in methylene chloride followed by addition of water (5 ml) to this solution. This solution was vortexed using cyclomixer for 3 min to extract out the carboplatin into water. The drug content was then analyzed using HPLC analytical method [18].

In vitro release study of carboplatin loaded microspheres
Microspheres (~10 mg) were taken in dialysis bag (10000 M.W. cut off). This dialysis bag was transferred to a vial containing 5 ml of phosphate

buffer (pH 6.8; 100 mM) and placed in reciprocal shaking water bath maintained at 37ºC. An aliquot (1 ml) of the release medium was withdrawn at preset time points and volume withdrawn was replaced with fresh release medium. Drug content in samples was analyzed by HPLC.

The release data was evaluated by model dependent analysis using the generic equations that mathematically translates the dissolution curve into a function of some parameter related to the pharmaceutical dosage form. There are several models where the amount of the drug (Q) is quantitatively interpreted as a function of test time (t) (Equation 1). Some of the commonly used analytical definitions of the Q (t) function are Zero-order, First-order, Higuchian and Hixson-Crowell's models.

$$Q = f(t) \qquad \text{…Eq. 1}$$

In vitro release data was fitted into these models and regression analysis was carried out. The measure for selecting the most suitable model was based on best goodness-of-fit.

Synthesis and characterization of triblock copolymers

Triblock copolymers (PLGA-PEG-PLGA) were synthesized following the method reported by Zentner et al. (15). In brief, ring opening polymerization was employed to bulk polymerize dl-lactide (LA) and glycolide (GA) using PEG as an initiator and stannous-2-ethyl hexanoate as catalyst. The copolymers were designated as PLGA-PEG-PLGA (x/y) where x/y is the ratio of lactide to glycolide. Molecular weight and molecular weight distribution were determined by GPC. M_n and lactide to glycolide ratios (LA/GA) were determined from ^{1}H NMR. Test tube inverting method was used to determine gelation temperature of aqueous solutions (0.5 g) of polymers (25% w/w).

Development of formulations

Details of the formulations and their respective nomenclature have been enlisted in table 1.

Table 1. Details of formulations and their respective nomenclature.

Formulation	Description	Copolymer	Abbreviations used
Formulation 1	Carboplatin alone in gel	LA/GA 3/1	--
Formulation 2	Paclitaxel alone in gel	LA/GA 3/1	--
Formulation 3	Carboplatin and Paclitaxel combination in gel	LA/GA 3/1	--
Formulation 4	Carboplatin alone in gel	LA/GA 8/1	--
Formulation 5	Paclitaxel alone in gel	LA/GA 8/1	--
Formulation 6	Carboplatin and Paclitaxel combination in gel	LA/GA 8/1	--
Formulation 7	Carboplatin microspheres in gel	LA/GA 3/1	CP MS in gel
Formulation 8	Carboplatin microspheres in gel	LA/GA 8/1	CP MS in gel
Formulation 9	Carboplatin microspheres in paclitaxel loaded gel	LA/GA 3/1	CP MS in paclitaxel containing gel
Formulation 10	Carboplatin microspheres in paclitaxel loaded gel	LA/GA 8/1	CP MS in paclitaxel containing gel

* PLGA-PEG-PLGA of two different LA/GA ratios 3/1 and 8/1 were studied.

Formulation 1-6 (Carboplatin and/or paclitaxel in PLGA-PEG-PLGA): Copolymer was first dissolved in acetone followed by addition of the drug/s. Acetone was then evaporated under vacuum using Buchi rotavapor to form a drug-polymer matrix which was dissolved in purified water to obtain 25 % w/w of copolymer concentration.

Formulation 7-8 (Carboplatin microspheres dispersed in PLGA-PEG-PLGA): Gel was prepared by dissolving 25 % w/w of copolymer in purified water. Microspheres equivalent to 1 mg of carboplatin were then dispersed in the gel at low temperature ($< 5°C$).

Formulation 9-10 (Carboplatin microspheres dispersed in PLGA-PEG-PLGA containing paclitaxel): Copolymer and paclitaxel were dissolved in acetone separately and the two solutions were then mixed. Acetone was evaporated under vacuum to form paclitaxel-polymer matrix which was then dissolved in purified water to obtain 25 % w/w copolymer concentration.

Microspheres equivalent to 1 mg of carboplatin were dispersed uniformly in gel containing paclitaxel.

In vitro release studies
For in vitro release studies, two different release media were employed to maintain sink conditions- phosphate buffer (pH 6.8; 100mM) was used for carboplatin alone formulations while phosphate buffer (pH 6.8; 100mM) with 10% w/v cremophorEL was selected as release media for paclitaxel containing formulations due to its limited solubility in phosphate buffer.

Formulation (~1 g) was taken in a vial and kept at 37°C to equilibrate for 10 min to form gel. After 10 min, 2 ml of release medium was added and sampling was done at regular time intervals wherein whole of the release media was withdrawn and replaced with the fresh media. The amount of drugs in the release samples was determined by a simultaneous HPLC method developed and validated in lab [18], and the release data was evaluated by model dependent analysis as mentioned above in section 2.3.4.

Statistics
The data obtained was analyzed by Student's t-test at all points using SPSS statistics (version 17.0) software and a significance level of $p < 0.05$ was denoted significant in all cases.

Results and discussion
Microspheres
**Synthesis and characterization of PLA**
PLA synthesized was white in color appearance, solid in nature, and yield obtained was more than 80%. PLA was synthesized by ROP and characterized by GPC. M_w and M_n were found to be 55.06 kDa and 49.88 kDa respectively and polydispersity index was 1.1.

Preparation and characterization of microspheres
Carboplatin is a hydrophilic drug and is expected to show burst release from the gel matrix. To avoid the burst release, drug was first formulated in microspheres of hydrophobic polymer (i.e. PLA) by emulsion solvent evaporation method. Particle size analysis results suggested that carboplatin microspheres had a mean particle size of 28.22 ± 6.20 µm and SEM images of the microspheres showed that these were spherical and had smooth surfaces (Fig. 1). Microspheres were prepared at two different drug loadings 10% and 20% w/w and the corresponding entrapment efficiencies obtained were 85.6% and 62% respectively. A decrease in entrapment efficiency was observed as theoretical drug loading was increased. This might be due to the decrease in polymer concentration with corresponding increase in drug loading, resulting in formation of droplets with less viscous polymeric phase at a fixed internal phase ratio. This caused increase in escape of drug from the microspheres. Thus, increase in theoretical drug loading shows decrease in entrapment efficiency.
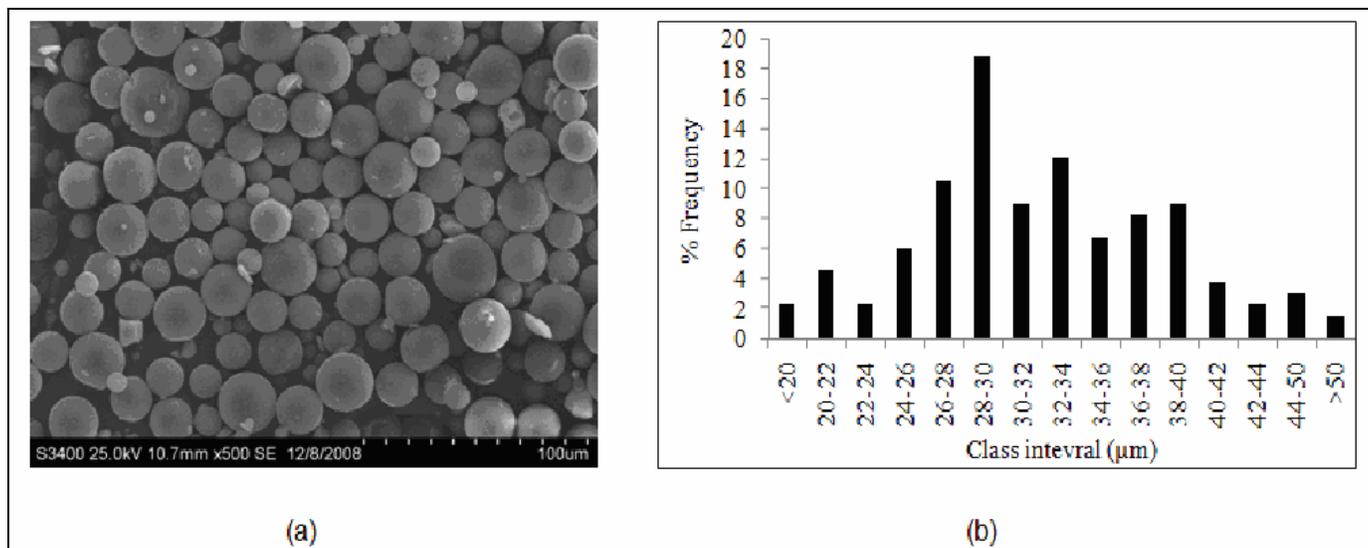
Figure 1. Carboplatin microspheres (CP MS) a) SEM image b) particle size distribution.
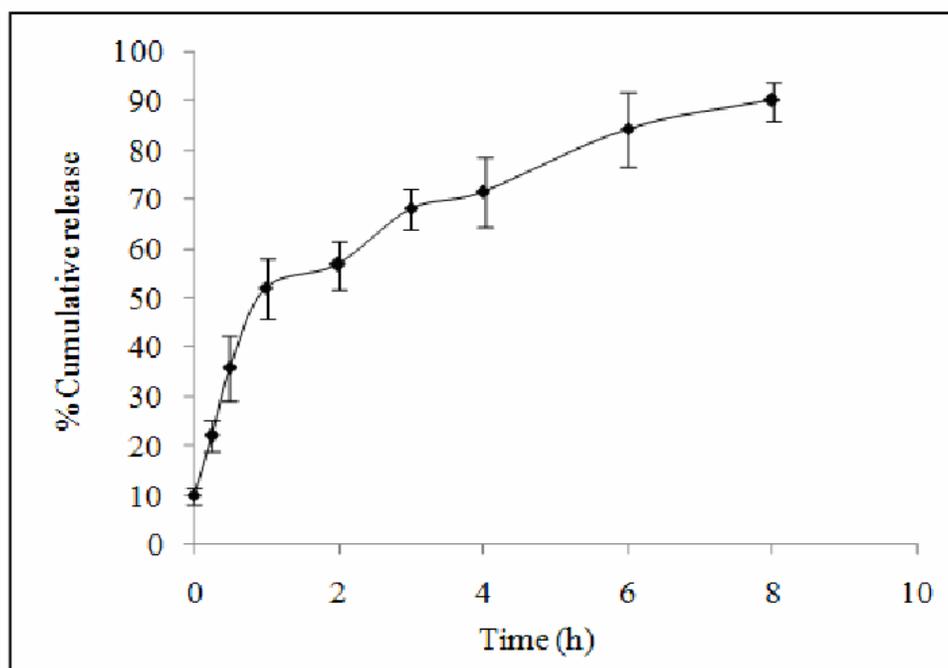
Figure 2. In vitro release profile of carboplatin from CP MS (carboplatin loaded PLA microspheres).

Carboplatin release from PLA microspheres showed a release for 8 h (Fig. 2) and followed Higuchian kinetics (table 3), thus, it is concluded that drug release from microspheres mainly followed diffusion dependent kinetics; this along with hydrophilic nature of the drug (aqueous solubility of 10-14 mg/ml) might be the reason for shorter duration of drug release. However, a more sustained release of carboplatin was desired for the intended application and to meet this end; these carboplatin microspheres were dispersed into PLGA-PEG-PLGA gel, which would provide an additional barrier for the drug to diffuse out of microspheres to reach the external environment.

Thermosensitive gel PLGA-PEG-PLGA
Synthesis and characterization of triblock copolymers

The copolymers with a predetermined molecular weight and LA to GA (3/1 and 8/1) ratios were synthesized. These copolymers were golden yellow in color and sticky in nature. Table 2 shows the data obtained from ^{1}H NMR and GPC. The feed ratios vs. practical ratios of LA/GA were also calculated by ^{1}HNMR and results have been summarized in table 2. All these values were on expected lines and indicated the quality

of the synthesized polymer. It has been reported that in order to exhibit the thermogelling properties, the overall molecular weight of these triblock copolymers should lie in the range of 2000-4990 Da [13]. Results as obtained by both NMR and GPC indicated that all the copolymers were in the desired molecular weight range. Table 2 also shows the lower gelation temperature (T_{lower}) and upper gelation temperature (T_{upper}) for the copolymer solutions (25% w/v).

Table 2. Characterization of the copolymers.

Triblock copolymer (PLGA-PEG-PLGA)	Yield (%)	GPC			NMR			Sol-gel transition temperature (°C)	
		$M_n{}^a$	$M_w{}^b$	$M_w/M_n{}^c$	$M_n{}^a$	LA/GA^e	% PEG	$T_{lower}{}^f$	$T_{upper}{}^g$
Copolymer 1 (LA/GA 3/1)	82.4	3534	4584	1.29	3126	2.98:1	31.97	16	50
Copolymer 2 (LA/GA 8/1)	81.4	3860	4840	1.25	2946	7.86:1	33.94	14	65

^{a} Number average molecular weight
^{b} Weight average molecular weight
^{c} Polydispersity
^{e} Molar ratio of lactic acid to glycolic acid (LA/GA)
^{f} Lower transition temperature from sol to gel
^{g} Upper transition temperature from gel to precipitate

Table 3. Model-fitting of the in vitro release profiles of carboplatin from different formulations.

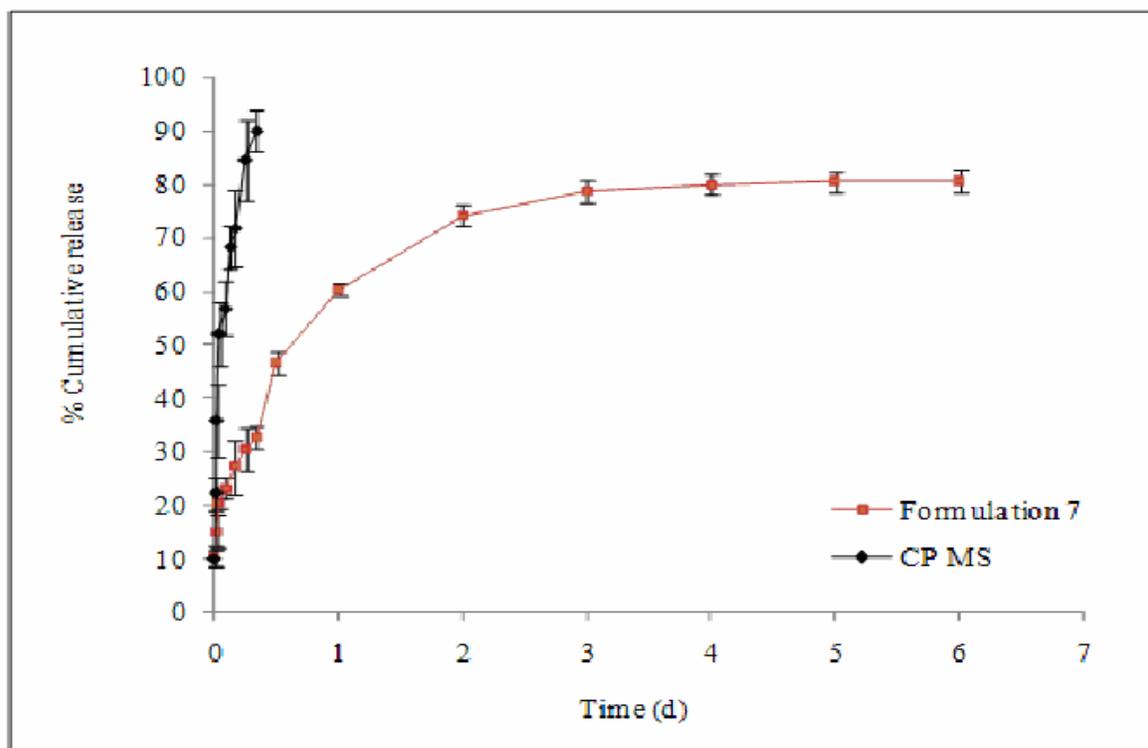|			Carboplatin							
		CP MS	Formulation 1	Formulation 3	Formulation 4	Formulation 6	Formulation 7	Formulation 8	Formulation 9	Formulation 10
Zero order	Intercept	30.2	77.48	61.439	61.099	65.806	28.7	25.4	31	26.48
	Slope	8.895	4.439	9.0629	8.0773	2.9817	11.55	9.466	12.28	9.79
	R^2	0.816	0.4131	0.7765	0.7029	0.7351	0.763	0.675	0.744	0.677
First order	Intercept	1.422	1.2876	1.5814	1.5929	1.5306	1.406	1.128	1.433	1.1.230
	Slope	0.086	-0.1291	-0.1768	-0.1753	-0.047	0.114	0.130	0.114	0.131
	R^2	0.579	0.6229	0.9021	0.9227	0.8185	0.617	0.40	0.612	0.353
Higuchi model	Intercept	14.28	70.718	55.677	51.488	62.853	16.46	12.91	19.04	14.65
	Slope	28.78	13.182	19.948	21.561	8.0133	31.48	28.54	33.06	29.09
	R^2	0.964	0.6544	0.9362	0.8996	0.8947	0.922	0.865	0.916	0.869
Hixon-Crowell model	Intercept	3.034	4.2485	3.9386	3.9203	4.0349	2.983	2.706	3.050	1.938
	Slope	0.228	0.0807	0.1735	0.1567	0.0579	0.302	0.296	0.309	0.378
	R^2	0.667	0.3816	0.741	0.6311	0.7089	0.676	0.522	0.664	0.716
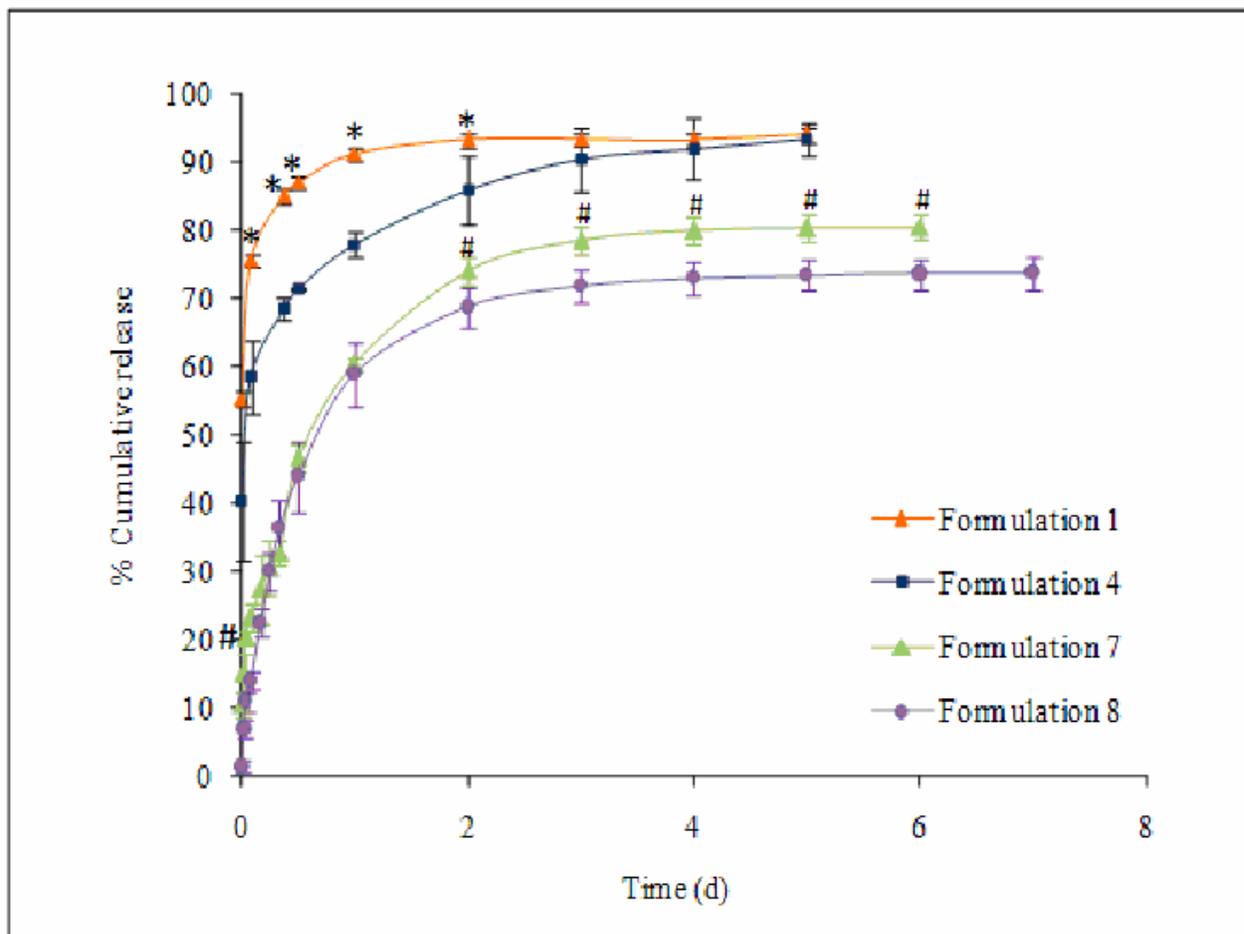
Figure 3. In vitro release of carboplatin from formulation 1, 4, 7 and 8. Each point represents the mean ± SD; n= 3. Significant difference (p< 0.05) was observed at all time points denoted with an asterisk mark (*) (between formulation 1 and 4) and hash mark (#) (between formulation 7 and 8).

Note: Formulations 1 and 4 contain carboplatin alone in copolymer 3/1 (LA/GA 3/1) and copolymer 8/1 (LA/GA 8/1) respectively. Formulations 7 and 8 contain carboplatin loaded microspheres in copolymer 3/1 (LA/GA 3/1) and copolymer 8/1 (LA/GA 8/1) respectively.
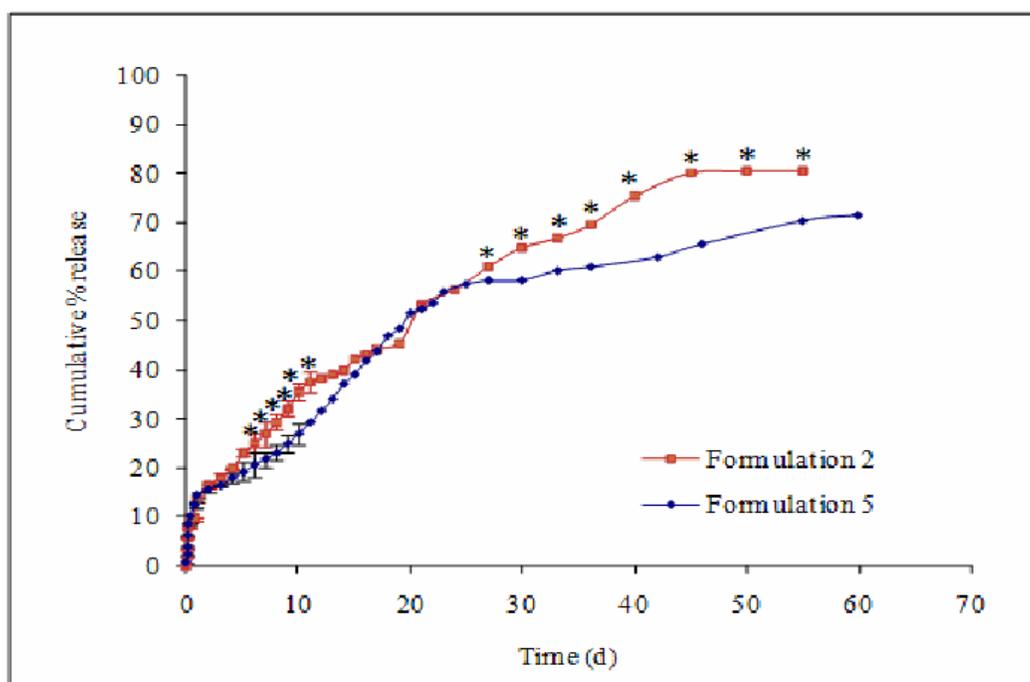
In vitro release

It was a difficult task to design a matrix which would provide a sustained release for a combination of two opposite natured drugs like paclitaxel and carboplatin where one is hydrophobic and the other is hydrophilic in nature, respectively. To design such a delivery system, PLA microspheres were dispersed in thermosensitive in situ gelling system. In order to completely understand the release behavior of the two opposite nature drugs in combined formulation, selected formulations (as given in table 1) were designed and evaluated extensively for their drug release behavior.

It is well known that drug release from the hydrogels occurs by two principal mechanisms: (i) drug diffusion from the hydrogel during the initial release phase and (ii) release of drug by the erosion of the hydrogel matrix during the later phase. In this case, two drug molecules showed characteristically different release profiles from the copolymer gels. Carboplatin showed an initial burst release and a shorter duration of release (formulations 1 and 4) (Fig. 3) whereas paclitaxel exhibited a completely different release profile with no burst effect and a very prolonged duration of release (formulation 2 and 5) (Fig. 5).

Figure 4. In vitro release study of carboplatin from formulation 7 and CP MS. Each point represents the mean ± SD; n= 3. Significant difference was observed at all the time points between CP MS and formulation 7.
Note: Formulations 7 contains carboplatin microspheres in copolymer 3/1 (LA/GA 3/1) and CP MS contain carboplatin microspheres only.

Figure 5. In vitro release study of paclitaxel from formulations 2 and 5. Each point represents the mean ± SD; n= 3. Significant difference (p< 0.05) was observed at all time points denoted with an asterisk mark (*).
Note: Formulations 2 and 5 contain paclitaxel in copolymer 3/1 (LA/GA 3/1) and copolymer 8/1 (LA/GA 8/1) respectively.

Release profiles of formulations containing carboplatin alone

Formulation 1 vs. 4: In vitro release profile of carboplatin alone from copolymer gels showed three phases; an initial burst release phase, a sequential high release phase and a plateau region where very small amount of drug release was seen (Fig. 3). In this case, burst effect was more likely due to syneresis in the polymer. At 37°C, the gel contracted due to packing and aggregation of the micelles. This resulted in decrease in the system's volume and expulsion of water. During this process, a substantial fraction of the dissolved drug came out with water leading to burst phenomenon. This was in agreement with reported results wherein, this push out effect was observed with drugs present in the hydrophilic domain of the gel (drug soluble in water) (19). PLGA-PEG-PLGA consists of hydrophobic PLGA domains and hydrophilic PEG domains; where carboplatin tends to get partitioned into the hydrophilic PEG domain because of its hydrophilic nature. Continuous release of carboplatin in second phase resulted from diffusion through hydrophilic channels of the hydrogel matrix. In this diffusion-predominant release phase, strength of hydrogel also created an impact upon the release behavior. A significant difference was observed in the release profiles from copolymer gels with LA to GA ratio 8/1 as compared to 3/1 at most of the time points. As strength of the gel increased (increase in LA/GA ratio from 3/1 to 8/1), a sustained release with the decrease in initial burst effect (from 55% to 40%) and absence of plateau phase was observed.

Formulation 7 vs 8 : When carboplatin was dispersed directly in the gel (formulation 1 & 4), a substantial fraction of the drug was expelled out of the system as burst release. In order to prevent this burst release, carboplatin microspheres were dispersed in gel (formulation 7 and 8). In vitro release profile of these formulations showed no burst release while a prolonged release of 6 and 7 days was observed with 78.62 % and 71.83 % being released in first 3 days from formulation 7

and 8, respectively. It was then followed by plateau phase where lesser amount of drug was released continuously (Fig. 3). No significant difference was observed in release profiles with copolymers having different LA/GA ratios. This is understood, as in this case carboplatin is entrapped in the PLA microspheres and is not present in PEG domains of the gel, thus the release is not affected by the contraction and hence the strength of the gel did not show any influence on the initial release.

Formulation 7 vs plain carboplatin microspheres: Release study of carboplatin from microspheres dispersed in gel (formulation 7) showed drug release up to 6 days which is significantly longer than 8 h release of carboplatin as shown by PLA microspheres (Fig. 4). This might be due to the fact that gel structure provided an additional barrier for diffusion of the drug from microspheres into the release media. Carboplatin, after diffusing out from microspheres, gets located in PEG domains of copolymer thus giving a significantly longer release.

Summarizing release behavior of carboplatin from all these formulations, it is concluded that due to its hydrophilic nature, it gets located in to PEG domains of the copolymer and show faster and shorter duration of release. Copolymers with higher LA/GA ratio showed reduced burst effect. In order to prevent this burst release, CP MS were dispersed in gel, where absence of burst release was seen since drug is not present in soluble form but as solid reservoir in PLA microspheres. CP MS in gel significantly sustained the release of carboplatin as compared to the plain carboplatin microspheres due to additional barrier provided by the gel, after diffusing out from the microspheres. Thus, microspheres dispersed in gel provided a better delivery system for obtaining sustained release of such a hydrophilic molecule without any burst release.

Release profiles of formulations containing paclitaxel alone

Formulation 2 vs 5: No burst release of paclitaxel was observed from these formulations; however, a biphasic release profile was seen (Fig. 5). Paclitaxel is a hydrophobic drug and thus it is expected that it will remain in hydrophobic PLGA domains of the gel. Due to this reason, it is not affected by the contraction of the gel and corresponding syneresis; resulting in the absence of burst release from the formulations. Initial phase of paclitaxel release is diffusion-controlled while degradation predominates in the later phase, hence, no significant difference was observed during the initial phase of release from the copolymer gels having different LA/GA ratios at individual time points, though, % release was higher in formulation 2 having lower LA/GA ratio, but later phase exhibited a significant difference where both diffusion and rate of hydrogel erosion were decreased with increasing LA/GA ratio (12, 15).

**Release profiles of formulations containing combination of drugs**

Release profile of carboplatin from combination formulations (formulation 3 and 6) reveals a significantly more controlled release during initial phase as compared to carboplatin alone formulations (formulation 1 and 4) (Fig. 6). Initial phase of carboplatin release is primarily due contraction of the gel in the aqueous media and syneresis, resulting in expulsion of the drug out of the system. Simultaneous presence of paclitaxel in the system along with carboplatin makes the gels more hydrophobic (due to its hydrophobic nature) and hence stronger. The increase in the hydrophobicity of the system decreases the contraction of the gel structure along with decrease in the affinity of the release media towards the gel thereby controlling the release of the hydrophilic molecule in the initial phase. Change in the hydrophobicity of the system has a strong influence upon the nature of release as has also been observed in the release patterns obtained from copolymer gels containing different LA/GA ratios (formulation 1 vs. 4); as the lactide content increased (LA/GA ratio increased from 3:1 to 8:1, i.e. increasing the hydrophobicity) a significant decrease in the carboplatin release was observed (Fig. 6).
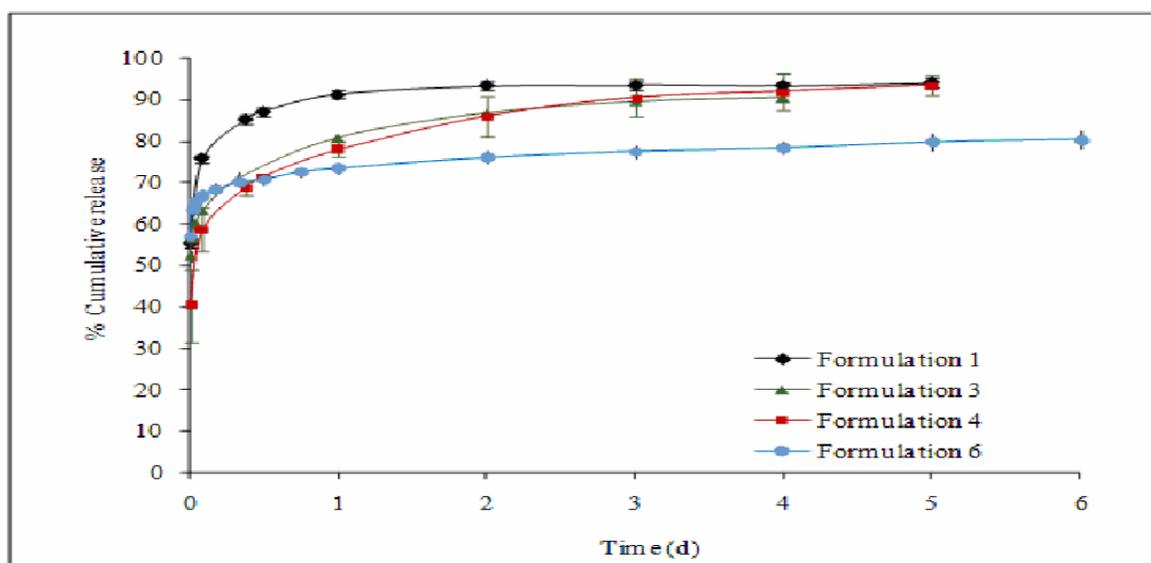

Figure 6. In vitro release study of carboplatin from formulations 1, 3, 4 and 6. Each point represents the mean $\pm$ SD; n= 3. Significant difference ($p< 0.05$) was observed at all time points between formulation 1 and 3 and 4 and 6.
Note: Formulations 1 and 4 contains carboplatin alone in copolymer respectively, formulations 3 and 6 contain carboplatin and paclitaxel combination in copolymer gels 3/1 and 8/1 respectively.
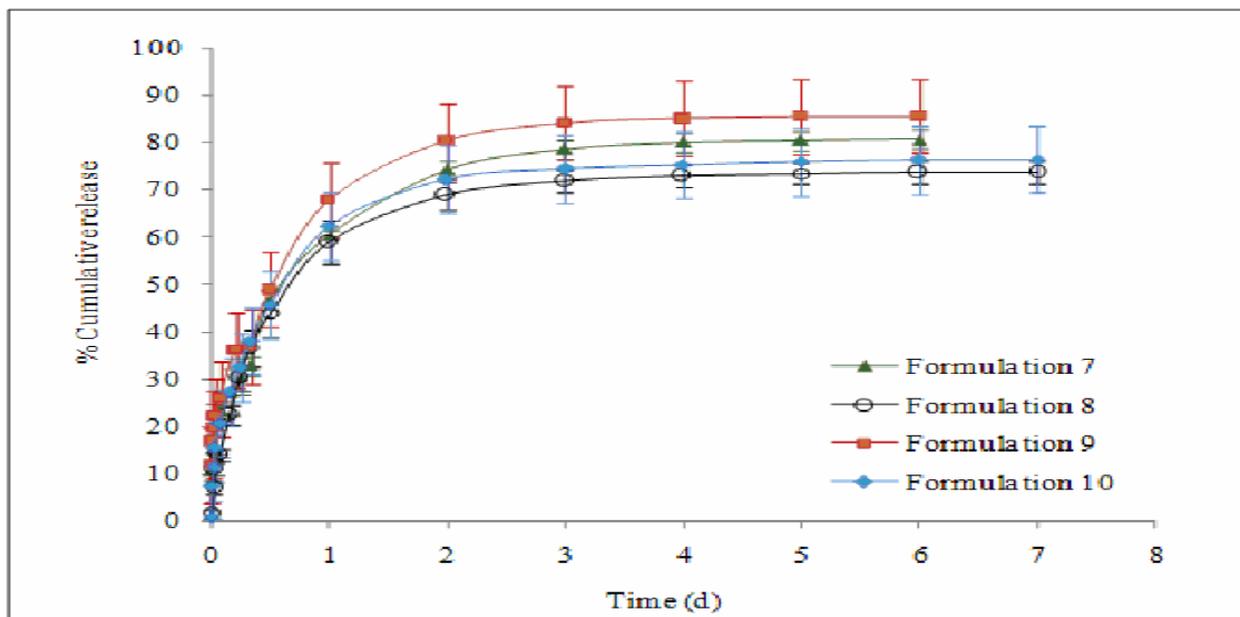
Figure 7. In vitro release study of carboplatin from formulations 7, 8, 9, and 10. Each point represents the mean ± SD; n= 3.
Note: Formulations 7 and 8 contain carboplatin microspheres (CP MS) dispersed in copolymer 3/1 and 8/1 respectively. Formulations 9 and 10 contain CP MS dispersed in paclitaxel loaded copolymer 3/1 and 8/1 respectively.
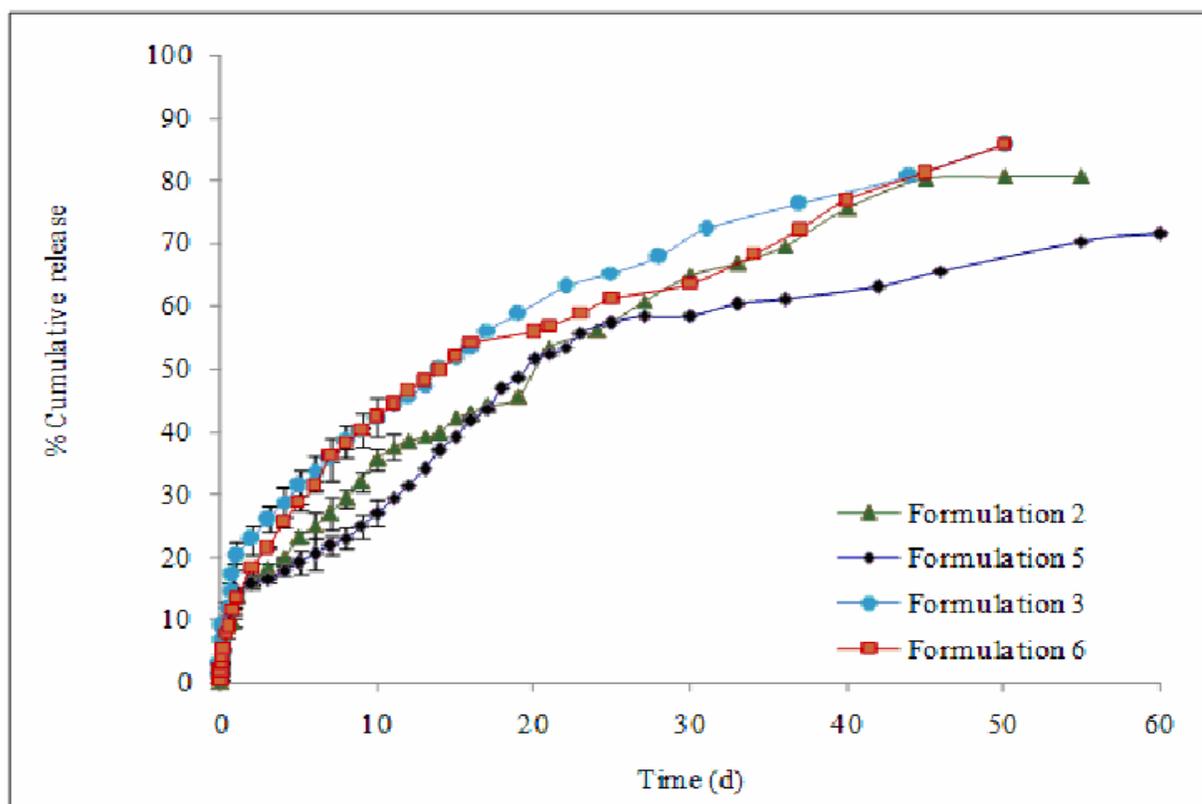
Figure 8. In vitro release study of paclitaxel from formulations 2 and 5 vs. 3 and 6. Each point represents the mean ± SD; n= 3.
Note: Formulations 2 and 5 contain paclitaxel alone in copolymer 3/1 (LA/GA 3/1) and copolymer 8/1 (LA/GA 8/1) respectively. Formulations 3 and 6 contain paclitaxel in combination formulations along with carboplatin in copolymer 3/1 and 8/1 respectively.

Carboplatin release from MS in gel formulations (formulations 9 vs. 10, 7 vs. 9 and 8 vs. 10, Fig. 7) did not show a significant difference in the release profiles. This could be attributed to the fact that the initial phase of release in this case unlike previous formulations; is not dependent upon the contraction of the gels. Moreover, here, the drug is not surface located rather it is encapsulated into the PLA microspheres that controlled the release during the initial phase. Thus, the change in hydrophobicity of the polymer (either by changing the LA/GA ratio or by the simultaneous presence of hydrophobic molecule like paclitaxel) bears no effect on the release pattern of carboplatin.

In case of release behavior shown by paclitaxel, the combination formulations (formulations 3 and 6) were found to exhibit a significantly faster release as compared to paclitaxel alone formulations (formulations 2 and 5) (Fig. 8). In this case, co-eluting drug carboplatin is hydrophilic in nature and releasing at a faster rate, thus, creating channels filled with release media. These channels would have provided additional passage for the paclitaxel molecules to diffuse out thereby causing faster release. Not much difference in release was observed at day 1, as paclitaxel is located in the hydrophobic PLGA domains and is not affected by the contraction of the gels. However, after one day, significantly faster release was observed which is due to the creation of additional channels by carboplatin during its release.

Formulation (3 and 6) vs (9 and 10): Both the drugs when formulated in PLGA-PEG-PLGA gel, (formulations 3 and 6), showed high burst release of carboplatin (~55 %) (Fig. 6). To overcome the burst release, CP MS in gel formulation was designed, no burst effect was observed and increased duration of release was obtained (Fig. 7). This is due to the fact that carboplatin is present as solid reservoir in PLA microspheres (formulations 9 and 10) and drug has to cross the additional barrier of gel after diffusing out of the microspheres. In case of paclitaxel, no significant difference in release was observed as in both the cases paclitaxel was formulated in the copolymer gel only.

Table 4. Model-fitting of the in vitro release profiles of paclitaxel from different formulations.

		Paclitaxel					
		Formulation 2	Formulation 3	Formulation 5	Formulation 6	Formulation 9	Formulation 10
Zero order	Intercept	13.581	17.571	13.972	15.808	18.30	17.83
	Slope	1.5275	1.693	1.2749	1.6528	1.508	1.352
	R^2	0.9288	0.8726	0.8636	0.876	0.912	0.917
First order	Intercept	1.9643	1.9386	1.9452	1.9473	1.147	1.173
	Slope	-0.0135	-0.0154	-0.0096	-0.0148	0.020	0.018
	R^2	0.9902	0.9849	0.9365	0.9744	0.590	0.606
Higuchi model	Intercept	-0.4008	3.5329	0.8682	1.5707	3.172	3.679
	Slope	11.367	12.011	9.8574	12.01	11.66	9.557
	R^2	0.9915	0.9943	0.9639	0.9931	0.995	0.985
Hixon-Crowell models	Intercept	2.2789	2.4198	2.2848	2.3046	2.454	2.440
	Slope	0.0521	0.0572	0.0466	0.0557	0.048	0.043
	R^2	0.662	0.5803	0.6337	0.6115	0.663	0.550

Model dependent analysis of release data showed release of the drugs from above mentioned formulations, mainly followed Higuchian model (Tables 3 and 4).

Conclusions

An injectable sustained release formulation for localized delivery containing combination of paclitaxel and carboplatin was developed by dispersing carboplatin loaded PLA microspheres in paclitaxel loaded thermosensitive gel. Shorter duration of carboplatin release (up to 6-7 days) along with a continuous and prolonged release of paclitaxel (up to 55- 60 days) was obtained which is in simulation with conventional therapy of this combination, in which carboplatin blood levels get diminished much earlier (25 h) than paclitaxel blood levels (50-55 h). Co-eluting drug molecules have significant effect on the release behavior of each of the individual drugs i.e. presence of hydrophobic molecules in the system, decrease and slow down the release rate of hydrophilic drug, while, presence of hydrophilic drug in the system, causes an increase in the release of hydrophobic drug. Thus, this system holds potential for simultaneous delivery of these anticancer agents which might yield better therapeutic efficacy and decreased side effects and resistance in tumor cells.

Acknowledgements

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