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Original Research Article

Evaluation of *In vitro* and *In vivo* Performance of Granisetron *In situ* Forming Implants: Effect of Sterilization, Storage Condition and Degradation

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Abstract

Purpose: *To investigate the effect of various solvent systems and gamma irradiation on the in vitro and in vivo performance of granisetron HCl injectable phase-sensitive in situ forming implants (ISFIs).*
Methods: *ISFIs were prepared by mixing and sterilized by gamma irradiation. Effect of solvent system was studied. Injectability, polymer degradation and stability studies (4 and 25 ^{o}C for 4 months), viscosity measurements, as well as in vitro and in vivo (in rabbits) drug release, and also histological examinations for biocompatibility studies (in rabbits and rats) were carried out.*
Results: *ISFIs showed good injectability from 20-gauge needle and their in vitro drug release increased in the following rank order of solvent/solvent combinations: dimethylsulphoxide (DMSO) > DMSO:prophylenecarbonate (PC) > DMSO:triacetin(TA) > DMSO:benzylbenzoate (BB). DMSO:PC incorporating ISFI gave zero order (r^2 = 0.9503) drug release for 21 days; application of gamma irradiation accelerated drug release with a difference factor (f_1) of 53 but zero order release (r^2 = 9690) was maintained. Following test results for DMSO:PC including ISFI as decrease in molecular weight of polymer was descriptive for drug release behavior and sterilization effect, additionally dynamic viscosities decreased in line with polymer degradation and all forms of this ISFI showed plastic flow (fresh, irradiated, aged at 4 and 25 ^{o}C for 4 months). In vivo performance showed steady state plasma drug concentrations between 2 to 21 days with value of 0.55 ± 0.03 µg/ml and biocompatibility was confirmed by histological results obtained at specific stages of tissue reactions, and also by lack of fibrous capsule formation.*
Conclusion: *An ISFI for long-term antiemetic therapy achieved in this preliminary study is promising and, therefore, further investigations are required.*

Keywords: *Implant, Poly(DL-lactide-co-glycolide), Granisetron, Gamma irradiation, Sterilization, Degradation, Viscosity, Stability, Pharmacokinetic, Biocompatibility.*

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INTRODUCTION

The development of injectable ISFIs based on biodegradable phase-sensitive polymer solutions has received considerable attention in recent years due to advantages in manufacturing process and administration and avoidance of surgical intervention, thus improving patient compliance [1,2]. Formulation parameters for ISFIs such as amount and properties of drug, polymer and solvent are all active areas of research, and although delivery of high molecular weight (M_w)

therapeutics widely investigated, little research has been conducted to optimize ISFIs design for delivery of low M_w drugs [1-3].

Problems such as poor *in vitro–in vivo* correlations, potential solvent toxicity and the possibility of initial burst effect leading to poor loading of drug may cause local or systemic side effects. Drawbacks associated with this system are still under investigation [1-3]. In the case of sterilization of ISFIs, the most preferable sterilization method is gamma irradiation which characteristically highly with a low dose rate (kGy/hour) [4]. Biocompatibility of ISFI systems is of utmost importance for clinical application. A desirable response of an implanted system shows a short-lived inflammatory response with a minimum of fibrosis resulting from the normal healing response of wounds [5].

Granisetron HCl is a highly water soluble low M_w drug which is effective and well-tolerated in the management of chemotherapy-induced nausea and vomiting. In this study, the effect of various solvent systems and gamma irradiation on the *in vitro* performance of granisetron HCl ISFIs was investigated with regard to release behavior, stability, viscosity as well as *in vivo* performance.

EXPERIMENTAL

Materials

The materials used include granisetron HCl (Cipla Limited, India); poly(D,L-lactide-co-glycolide (Resomer RG 504H, Boehringer Ingelheim GmbH, Ingelheim, Germany); ketamine hydrochloride (Ketalar®, Pfizer, Turkey); dimethylsulphoxide, disodium hydrogenphosphate, potassium dihydrogenphosphate, sodium chloride, N-(1-naphthyl) ethylenediamine dihydrochloride, acetonitrile, sodium hydroxide, sodium dihydrogen phosphate, toluene, ortho-phosphoric acid, formaldehyde (Merck, Darmstadt, Germany); propylene carbonate, tetrahydrofuran (Sigma-Aldrich, Steinheim, Germany); triacetin, benzyl benzoate (Sigma, Germany), polystyrene analitical standard (Fluka,UK). All other chemicals were analytical grade.

Preparation of ISFIs

ISFIs (470 mg) were prepared by mixing PLGA (32 %w/w) and solvent or mixture (1:1) of two solvents (64 %w/w) - DMSO or DMSO:BB, DMSO:TA, DMSO:PC) in glass vials until formation of a clear solution; granisetron HCl 4 %w/w was added and homogenized (Bandelin Sanoplus HD 2070, Germany). Liquid implant formulations coded with incorporating solvent and FD containing DMSO, FDB containing DMSO:BB, FDT containg DMSO:TA and FDP containig DMSO:PC were then sealed and heated to 65 ^{o}C in a water bath to remove trapped air bubbles [6].

Sterilization process

ISFI (FDP) in the sealed vials were irradiated with a ^{60}Co source (Tenex Issledovatel, TAEK, Ankara, Turkey) and coded as RDP. A 25 kGy dose was applied following the European Pharmacopoeia recommendations for an effective sterilization [7].

Injectability studies

Injectability of ISFIs, coded as FD, FDB, FDT, FDP and RDP, from 20-gauge needle attached to a 2 ml injector and with an application of 20 psi force was determined.

In vitro drug release studies

ISFIs (470 mg) were injected into 10 ml phosphate buffer saline pH 7.4 containing vials and *in vitro* dissolution test carried out in a shaker bath (GFL 1086, Germany) at horizontal strokes of 30/min and 37 ^{o}C (n = 3). Replenished, filtered (0.22 µm cellulose acetate membrane, Sartorius, Germany) and collected dissolution media at predetermined time points (1st, 4th, 24th hour and once on each of days 2 to 21) were analyzed using a UV spectrophotometer (Shimadzu 1240, Japan) at 301 nm. The mechanism of drug release was analyzed kinetically by zero order, first order, Higuchi and Korsmeyer-Peppas models. Comparison of drug release profiles were evaluated by dissolution f_1 and f_2 parameters [8].

Polymer molecular weight studies

Polymer degradation in ISFIs was investigated by gel permeation chromatography (GPC) (Agilent 1100 HPLC System GPC/RID, Tübitak Atal, Ankara, Turkey) to determine the weight average polymer (M_w). In this determination, ISFIs (fresh, irradiated, taken out during dissolution test and irradiated form stored at 4 and 25 ^{o}C for 4 months) were dissolved in tetrahydrofuran (0.5 %w/v) and analysed with polystyrene standards (580 - 370000 Da). Polydispersity index (PDI) of either plain PLGA or PLGA in various ISFI forms wasere calculated.

Rheological measurements

Dynamic flow properties of liquid ISFIs (fresh, irradiated and irradiated form stored at 4 and 25^{o}C for 4 months) were measured (n = 3) by using rotation type programmable viscometer (Brookfield Engineering Laboratories Inc., Model

DV-2, USA) at 24 ± 0.1 °C with spindle TF no: 96. The shear rate ranged from 10 to 200 s^{-1} corresponding to 5 to 100 rpm with 10 s between each two successive speeds and then determined also in a descending order. Equilibration of the sample was for 5 min following loading of the viscometer. Ramp time for each viscosity step was 20 s. Data were computed from constructed rheograms.

Ethical approval

Ethical approval was obtained for the animal studies from Ankara University Animal Welfare and Ethics Committee, Turkey (approval no. 2006/29). The animals (New Zealand rabbits, Wistar rats) were treated according to the principles of the care and use of laboratory animals [9].

***In vivo* drug release studies**

Following sedation with intramuscular 15 mg/kg ketamine hydrochloride, adult male New Zealand rabbits (weighing 3.0 - 3.5 kg, n = 4) were given a single subcutaneous injection of ISFI (470 mg) at their signed dorsal region using a 20-gauge needle and polypropylene rod shaped plungers injector. One rabbit was used as control. Blood (1 ml) obtained from the dorsal ear vein of the rabbits was collected into Lithium-heparin tubes and separated by centrifugation (Nüvefuge CN180) at 3000 g for 3 min to obtain plasma on the 1st and 4th hours, and also on 1 st, 2 nd, 3 rd, 4 th, 7 th, 10 th, 14 th, 18 th, 21 st days). The plasma samples were frozen at - 45 °C until high pressure liquid chromatography (HPLC, Shimadzu LC-10AD, Japan) analysis was carried out to determining drug concentration using the method of Pinguet *et al* [14]. The column used was SGE SS Wakosil II C18 RS (250 x 4.6 mm) with a mobile phase consisting of acetonitrile:buffer (adjusted to pH 4.5 with 0.1M NaH_2PO_4 and o-phosphoric acid) in a ratio of 15:85 at a flow rate of 1 ml/min. Detection was carried out at λex of 305 nm and λem of 365 nm. The developed method was evaluated for various system suitability parameters and validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) per ICH guidelines.

Biocompatibility studies

Biocompatibility studies were accomplished in two parts. In the first part, rabbits that received ISFI fin the *in vivo* drug release studies were euthanized after final blood sampling on the 21st day. In the second part, ISFI was injected to dorsal signed region of six adult male Wistar rats (weighing 250 - 350 g) to evaluate biocompatibility in terms of specific stages of tissue reactions. Two rats were used as control. For observation of tissue reactions on the 1st, 3rd and 21st days (following injection), a pair of rats was euthanized (intraperitoneal overdose ketamine hydrochloride) on each of these days. Tissue sampling and microscope analysis were the same for the rabbits and rats, and it was carried out by removing tissue samples from the injection sites using a scalpel, fixed by immersion in 10 % buffered formalin solution and then embedded in paraffin. Transverse sections (5 - 6 μm) were cut using a microtome. Slides of the tissue sections were prepared and stained with hematoxylin and eosin. The slides were observed under the light microscope (Olympus BX, DP25, Australia) and evaluated.

Statistical analysis

The results were expressed as mean ± standard deviation. Statistical comparison was made using one-way ANOVA and $p < 0.05$ was considered statistically significant. Correlation analysis was performed by least square linear regression method and correlation coefficient examined for significance by Student's t-test using GraphPad Instat 3.0 software. Statistical analyses were conducted using SSPS software version 9.0 for Windows, SPSS Inc, Chicago, IL, USA).

RESULTS

All ISFIs showed good injectability into phosphate buffer saline. Drug release profiles are presented in Figure 1(a). DMSO caused burst release from FD. Solvent combinations prevented initial burst and provided slow and similar drug release until 300 h later from FDP, FDT and FDB. Comparison of drug release profiles of FDP and its irradiated form, RDP, are presented in Figure 1(b). Among kinetic models investigated for drug release mechanism from FDP and RDP, the highest r^2 values (0.9503 and 0.9690, respectively) were obtained for zero order kinetic model. Difference between FDP and RDP profiles (Figure 1(b)) was indicated by the difference factor, f_1 = 53 and similarity factor, f_2= 33 which are outside the respective ranges of f_1 = 0 - 15 and f_2 = 50 - 100 [8] meaning that irradiation altered drug release.

Determined M_w and PDI values of either plain PLGA or PLGA in various ISFI forms (fresh, irradiated and irradiated form aged at 4 and 25 °C for 4 months) showed that M_w of PLGA was decreased by preparation, irradiation and ageing processes (Table 1). Decrease in polymer M_w in RDP was kinetically analyzed and degradation kinetics of polymer best fitted to first order kinetic

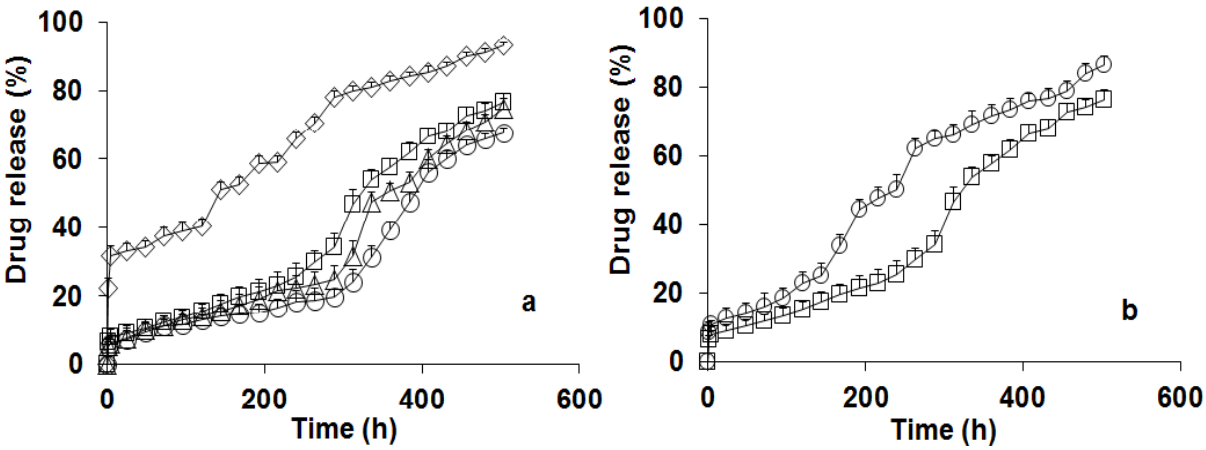

Figure 1: Dissolution profiles of (a) FD(◊), FDP(□), FDT(Δ), FDB(○) and (b) FDP(□), RDP(○) ISFIs.

model (r^2 = 0.9721 vs. Sy.x = 0.1016) with a degradation rate constantof (k) of 0,0030. The relationship between decrease in polymer M_w in RDP and increasing amount of released drug from RDP was investigated by correlation analysis and found significant (r^2 = 0.9935).

The dynamic viscosity of various forms of ISFI (fresh, irradiated and irradiated form aged at 4 and 25 °C for 4 months) decreased compared to fresh form and all forms showed plastic flow behavior (Figure 2).

Table 1: Molecular weight (M_w) and polydispersity index (PDI) of polymer alone, and in FDP, RDP and RDP during dissolution studies over 21 days and following storage at 4 and 25 °C for 4 months

Material	Time (h)	M_w (g/mol)	PDI
Resomer RG504H	0	48452	2.483
FDP	0	46880	2.297
RDP	0	39987	2.429
	4	38398	2.384
	24	37474	2.252
	96	33398	1.997
	168	30436	1.707
	240	22736	1.440
	336	14052	1.823
	432	12310	1.747
	504	8729	1.834
RDP 4°C	4 months	36172	2.370
RDP 25°C	4 months	32998	2.465

HPLC method was validated (r^2 = 0.9998 for linearity and range, LOD=0.0354 µg and LOQ=0.107 µg) and used to determine drug concentration in plasma. The mean plasma profile of RDP in the rabbits (Figure 3) showed that following subcutaneous injection, mean drug plasma concentration was 1.2 µg/ml; it decreased to 0.54 µg/ml at the 48th hour and was steady at 0.55 ± 0.03 µg/ml between 48 - 504 h; these were all greater than the value of LOQ.

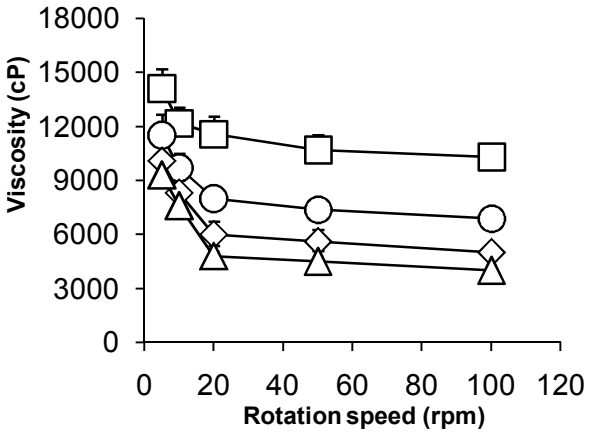

Figure 2: Rheograms of FDP (□), RDP(○) and RDP aged at 4°C (◊) and 25°C (Δ) for 4 months.

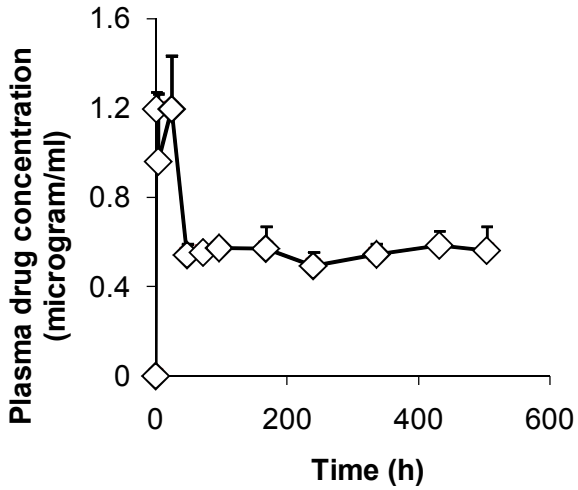

Figure 3: Mean plasma drug profile of rabbits that received RDP formulation (n = 4).

Tissue samples of rats and rabbits that received RDP were similar in terms of microscopical transformations on the 21st day as seen in Figure 4(d) and (e) respectively. Histological sections obtained on the 1st, 3rd and 21st days [Figure 4(b),

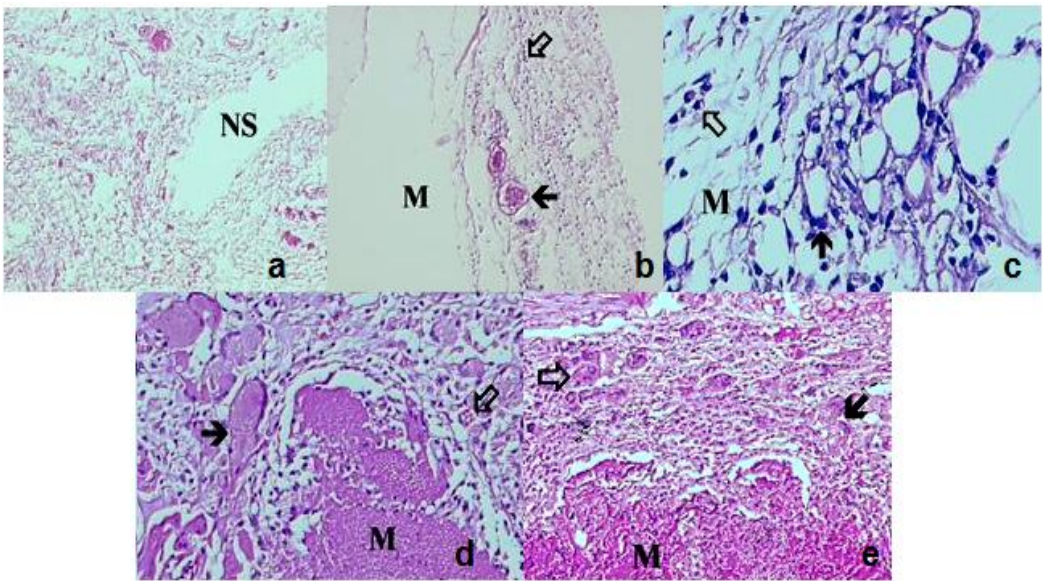

Figure 4: Light photomicrographs of rat tissue sections (a) control group rat received normal saline (NS) on 1st day, (HE, x40), (b) 1st day; distinctive edema with congested vessels (black filled arrow) and neutrophile leucocytes (black hollow arrow) observed around the pale basophilic material (M) in subcutaneous tissue (HE, x100), (c) 3rd day; there is not any congested vessel observed around the basophilic material (M), however lymphocytes and histiocytes (black filled arrow) as well as neutrophile leucocytes (black hollow arrow) observed in subcutaneous tissue, (HE, x400), (d) 21st day; histiocytes (black filled arrow) and multinuclear giant cells (black hollow arrow) stated around the degenerating material (M) in subcutaneous tissue, (HE, x200) and (e) rabbit tissue section obtained on 21st day; histiocytes (black filled arrow) and foreign type giant cells (black hollow arrow) stated around the degenerating material (M), (HE, x100)

(c), (d) from the rats, and on evaluation in terms of specific stages of tissue reactions, acute inflammation on the 1st day and chronic inflammation on the 3rd and 21st days were observed around the ISFI material.

DISCUSSION

Low melting and high boiling points of the solvents [10] in ISFIs allowed them to be liquid at the temperatures of injection (24 ^{o}C) and dissolution (37 ^{o}C), and thus facilitated injectability and *in situ* precipitation. Additionally, solvent levelss in ISFIs were below toxicity limits for human which can be predicted by their values of LD_{50} [10]. The hydrophilicity of solvent systems would be expected to affect the *in vitro* drug release and increase release in the rank order DMSO:PC > DMSO:TA > DMSO:BB, FDP > FDT > FDB. Thus, FDP which incorporates DMSO:PC solvent system with higher hydrophilicity, showed better and regular release profile than the others. Irradiation of FDP to form RDP resulted in a significant increase in drug release.

In the context of achieving sustained release of low M_w granisetron HCl from ISFIs for 21 days, the results show that solvent combination provided a balanced hydrophilicity and thus yielded the desired release profile. The irradiation effect on drug release can be attributed to decrease in M_w and T_g of polymer [11], and aided by the hydrophilic putative solvent system.

During ISFI preparation process, application of sonication and heat probably caused a decrease in the M_w of PLGA; breaking of units from the chain ends of PLGA [12] peobably resulted in increase in its PDI value which is inferred from the results of studies that used magnetic stirring at room temperature [13] and extrusion at 75 ^{o}C [12] for the preparation of PLGA formulations. Application of gamma irradiation to ISFI caused a decrease in M_w of PLGA but not caused a significant change in its PDI value which supported by the study of Friess and Schlapp [14]. During dissolution, PLGA with acid end groups was assumed to have increased the hydrophilicity and ability of water uptake which results in an increase in PLGA degradation time [15].

The degradation mechanism of polymer, based on PDI data could be defined as degradation from the chain ends until the 4th day, random chain scissions between the 7th and 14th day and dominantly random chain scissions between the 14th and 21st day. It could be inferred that due to random chain scissions, polymer was separated into short chains, increased its solubility which catalyzes hydrolysis and thus accelerated the release of hydrophilic drug [15]; this could be attributed to the fast release between 7th and 14th day. Chain scissions are formed in the interior of

an implant due to autocatalysis while chain scission coexists with breaking away of units from chain ends on the surface of implants [15] thus occurred in dominantly chain scissions between 14th and 21st days.

Decrease in polymer M_w in RDP was kinetically best fitted to first order model and this is in agreement with the findings of Kenley *et al* [16] which investigated the decomposition kinetics of PLGA (50:50 lactide:glycolide copoymer). Polymer degradation data correlated with drug release behavior from RDP. Storage of RDP at 4 and 25 ^{o}C for 4 months resulted in less decrease in M_w of PLGA probably due to slower molecular movement and partial phase separation of ISFI that originated from frozen DMSO (melting point +18.5 ^{o}C). The stability results do not agree with those of another study showed that phase sensitive ISFI systems were stable at 4 ^{o}C and acceptably stable at 25 ^{o}C for 300 days [17]. The decreased dynamic viscosities of different forms of ISFI (fresh, irradiated and irradiated form aged at 4^{o}C and 25^{o}C for 4 months) can be attributed to the decrease in the M_w of PLGA (Figure 2).

In vivo performance of DMSO:PC incorporating ISFI in rabbits showed steady state plasma drug concentration between 2 and 21 days. Both *in vitro* and *in vivo* release showed agreement with regard to a burst effect and this is also supported by the findings of Kempe *et al* [18] about good. Biocompatibility results obtained from rabbits and rats were acceptable for RDP and there was no fibrous capsule formation around the degenerating material which could have decreased movement of the released drug from the implant to tissue. The resulting patterns around the implant material were defined as foreign body reaction [5,19] and the implant was considered biocompatible, a finding supported by an earlier work [20].

CONCLUSION

The combination of hydrophilic and hydrophobic solvents may be useful to control the release of high water soluble low M_w drug from phase sensitive ISFI systems, and these systems are easy to prepare and apply up to a period of 3 weeks. *In vivo* performance and biocompatibility data for this system are encouraging but further investigations are required to confirm the present findings.

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