AAPS PharmSciTech

AAPS PharmsciTech 2001; 2 (3) article 16
(http://www.pharmscitech.com)

In Vitro and In Vivo Evaluations of Biodegradable Implants for Hormone Replacement Therapy: Effect of System Design and PK-PD Relationship

Submitted: December 1, 2000; Accepted: August 20, 2001; Published: September 21, 2001

Senshang Lin,[1*] Pi-Yun Chao,[2] Yie W. Chien,[2,3] Amyn Sayani,[4] Sandeep Kumar,[4] Michelle Mason,[4] Thomas West,[4] Alice Yang,[4] and Donald Monkhouse[4]

[1]College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, NY 11439
[2]Controlled Drug-Delivery Research Center, Rutgers University, Piscataway, NJ 08854
[3]Current address: Kaohsiung Medical University, College of Pharmacy, Kaohsiung, Taiwan
[4]R & D Division, Therics, Inc, Princeton, NJ

ABSTRACT

This investigation evaluated the feasibility of using subdermally implantable devices fabricated by nonconventional 3-dimensional printing technology for controlled delivery of ethinyl estradiol (EE_2). In vitro release kinetics of EE_2 and in vivo pharmacokinetics/pharmacodynamics in ovariectomized New Zealand White rabbits were carried out to study 3 implant prototypes: implant I (single-channel EE_2 distribution in polycaprolactone polymer core), implant II (homogeneous EE_2 distribution in polycaprolactone polymer matrix), and implant III (concentration-gradient EE_2 distribution in polycaprolactone and poly(dl-lactide-co-glycolide) (50:50 matrix). EE_2 was found to be released from all the implants in a nonlinear pattern with an order of implant III > implant II > implant I. The noncompartmental pharmacokinetic analysis of plasma EE_2 profiles in rabbits indicated a significant difference ($p < .05$) in C_{max}, t_{max}, and mean residence time between implant I and implants II and III, but no difference in the area under the plasma concentration time curves calculated by trapezoidal rule (AUC) among the implants. For pharmacodynamic studies, endogenous follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were observed to be suppressed following implantation of all implants, which demonstrated that a therapeutically effective dose of EE_2 had been delivered. Furthermore, the noncompartmental analysis of plasma FSH and LH profiles in rabbits showed a significant difference ($p < .05$) in AUC and the mean residence time between implant III and implants I and II. A good in vivo/in vitro relationship was observed between daily amounts of EE_2 released and plasma profiles of EE_2 for all implants. This relationship suggests that plasma profiles of EE_2 could be predicted from in vitro measurement of daily amount of EE_2 released. Therefore, performing in vitro drug release studies may aid in the development of an EE_2 implant with the desired in vivo release rate.

KEYWORDS: 3-dimensional printing, ethinyl estradiol, implant, pharmacokinetic, pharmacodynamic, rabbit

INTRODUCTION

Subcutaneous implants have been increasingly recognized as a useful drug delivery system that provides greater assurance of patient compliance and a better therapeutic outcome than conventional drug therapies, particularly for chronic medication [1-3]. A survey of the literature showed that numerous studies have been performed to investigate the use of polymer-controlled drug delivery systems [4-7], efficacy [8-10], and pharmacokinetics and pharmacodynamics [11-13] of subdermal implants for the controlled delivery of drugs for long-term therapy. The science and the engineering approaches in the development of implantable therapeutic systems have been well described in the literature [14].

The most common method for manufacturing matrix-type implants is to blend the drug with the polymers and then use a compression or injection molding technique to fabricate the device [15]. Recently, a novel 3-dimensional fabrication technology [16-19] has been developed for the manufacture of biodegradable implants in a rapid, highly reproducible manner [20].

***Corresponding Author:** Senshang Lin, College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, NY 11439; Telephone: 718-990-5344; Facsimile: 718-990-6316; E-mail: linse@stjohns.edu

The in vitro release kinetics of polymeric drug delivery systems fabricated by this technology have been evaluated, and a complex drug delivery pattern has been demonstrated. The release of multiple drugs or multiphasic release of a single drug from the same implant may be achieved [20]. However, in vivo evaluation of implants fabricated by this technology has not been conducted to date.

In this investigation, 3 prototype subdermal implants using ethinyl estradiol (EE_2) as the model drug were fabricated using this novel 3-dimensional fabrication technology. In addition to in vitro kinetic studies, the in vivo release kinetics of EE_2 and its pharmacokinetic profiles and pharmacodynamic responses from each of the implants were conducted in ovariectomized New Zealand White rabbits to establish the in vitro/in vivo relationship. The pharmacokinetic-pharmacodynamic relationship and the effects of system design were also investigated.

MATERIALS AND METHODS

Materials

All polymers used in the fabrication of the devices were obtained from Birmingham Polymers, Inc (Birmingham, AL). Ethinyl estradiol was obtained from Spectrum Chemicals (New Brunswick, NJ). All chemicals used in the analysis were obtained from Fisher Scientific (Pittsburgh, PA).

Fabrication of Implants

All implants used in these studies were manufactured using Therics' (Princeton, NJ) proprietary 3-dimensional fabrication technology (TheriForm) [19]. Briefly, the TheriForm technology is a solid, free-form fabrication process in which objects are built in a laminated fashion (Figure 1). Polymer (or excipient) powder is first spread on a powder bed in a thin layer. The printhead assembly then scans over the powder bed, depositing the binder droplets through a nozzle onto selected regions. Different materials may be dispensed through single or multiple nozzles as particulate matter in a liquid vehicle or as dissolved matter in a liquid carrier. In regions where the binder is printed, the powder particles are held together through a variety of material-specific interactions, creating regions consolidated by solid bridges within the 2-dimensional slice. A new layer of powder is spread after the floor of the powder bed is lowered. Information for each layer is relayed from the computer

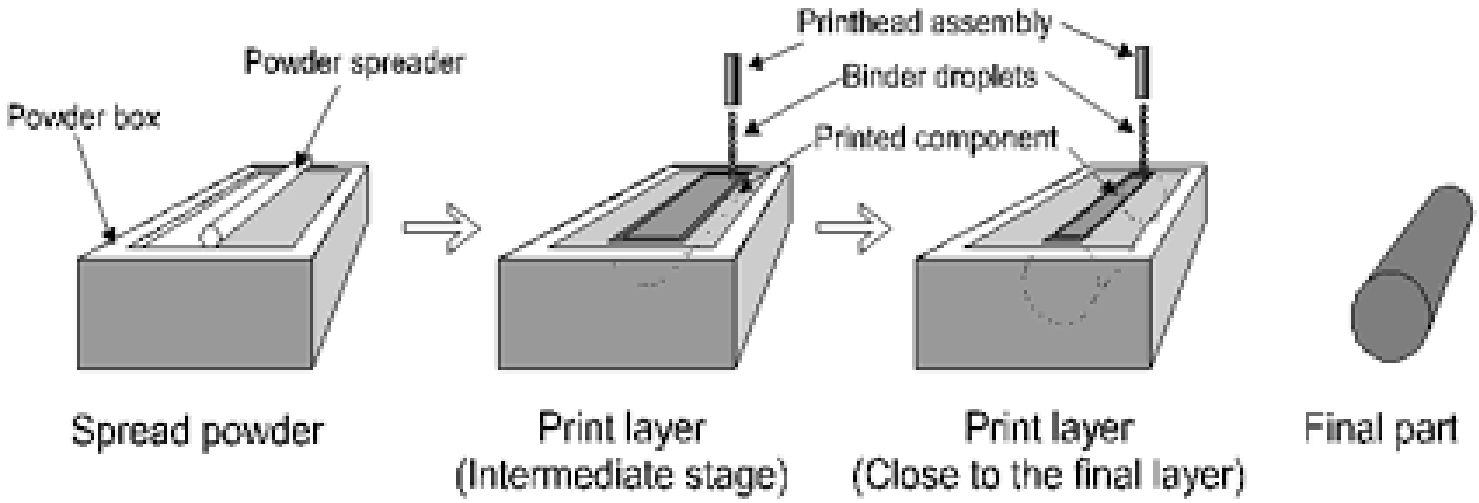

Figure 1. Schematic illustration of the 3-dimensional printing fabrication process.

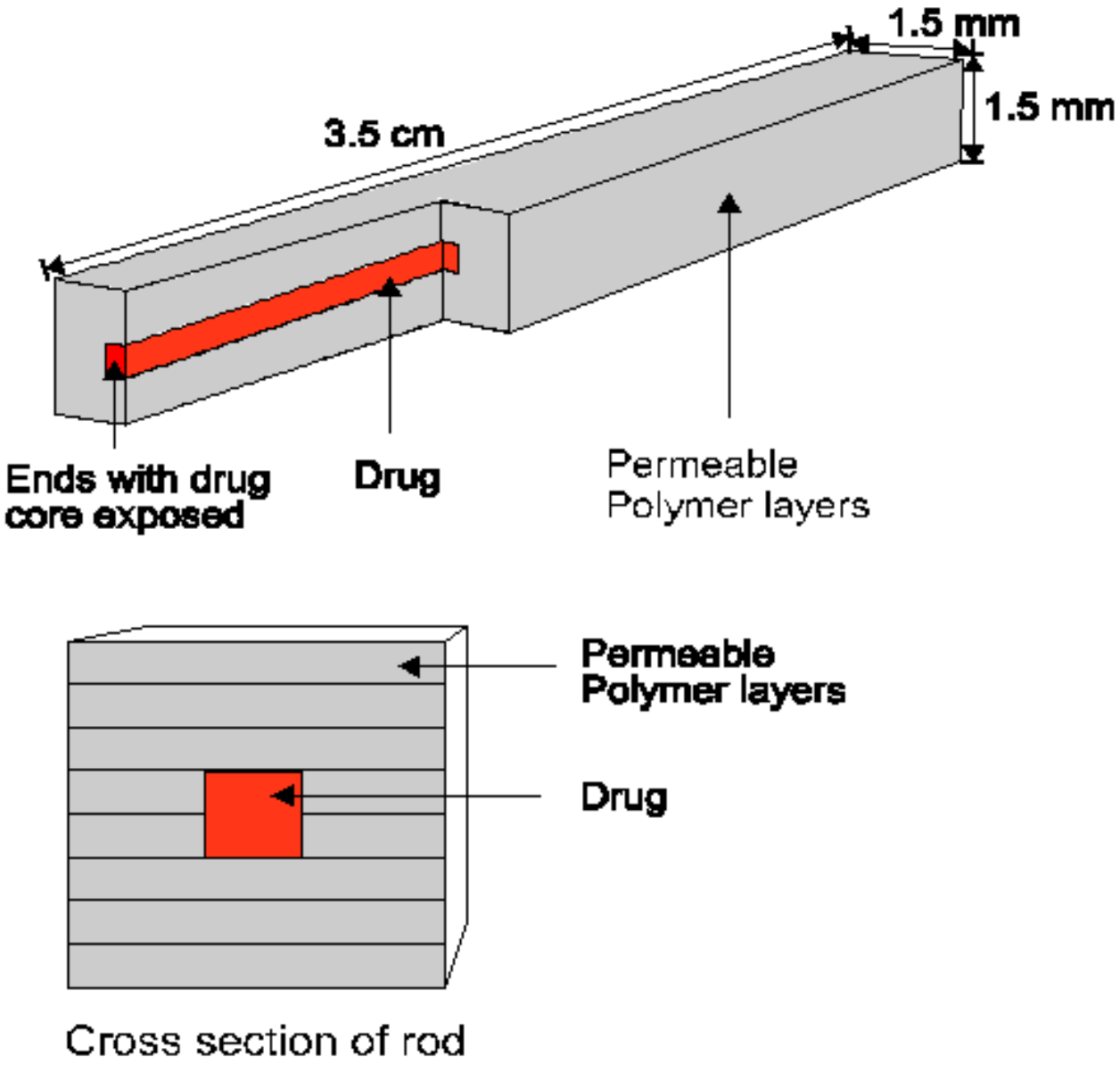

Figure 2. Schematic illustration of the single-channel design prototype EE_2 implant, in which the drug is incorporated into a single-channel drug loading surrounded by layers of poly-e-caprolactone.

(which uses a computer-aided design rendition for its instructions) and then printed. The process of powder spreading and printing is repeated until construction of the object is completed. A more thorough explanation of the TheriForm technology and its applications in drug delivery systems has been published previously [19,20].

Implant Designs

With the accurate spatial distribution possible with TheriForm technology, drug content and release rates can be well-defined and achieved with low variation. Using these features, 3 implant prototypes were designed and fabricated. One such design is shown in Figure 2. This implant (implant I) comprised a single channel of EE_2 embedded in a matrix of poly-e-caprolactone (PCL; MW 100,000 Da; IV 1.0-1.3 dL/g; Tg < -60). Additionally, 2 other designs were fabricated: implant II, in which EE_2 was homogeneously distributed in a PCL matrix, and implant III, in which EE_2 was placed in a concentration gradient in a polymer matrix comprising a blend of PCL and poly(lactide-co-glycolide) (PLGA 50:50; MW 50 000 Da; IV 0.55-0.75; Tg 45°C-50°C) (Table 1). Each of the implants has a 1.5 ' 1.5 ' 35 mm^3 dimension and 1.8 mg of EE_2 in loading.

In Vitro Evaluations

In Vitro Release Studies

Release studies for each of the implant designs (n = 6 for each) were conducted in isotonic phosphate buffer (5 mM; pH 7.4). Implants were placed in 8-mL glass vials filled with the buffer and incubated in a shaking water bath at 37°C. At predetermined time intervals, the solution in the vial was removed and replenished. The sample was filtered through a 0.22-µm filter and analyzed using the fluorospectrophotometric method.

Analytical Instrument and Methods

For the in vitro release samples, EE_2 was analyzed using a fluorospectrophotometer at an excitation wavelength of 288 nm and emission wavelength of 311 nm. A standard curve was established before each quantitative analysis. Residual drug content was analyzed using a high-performance liquid chromatography (HPLC) method. A rapid, sensitive HPLC method for determining EE_2 content in the implants was developed. A Hewlett-Packard 1100 HPLC device was used. The active component was separated from excipients on reverse-phase C8 column (Alltech, Lichrosorb RP-8, 5 m, 4.6 × 250 mm) by elution with water-acetonitrile (40:60) at a flow rate of 1 mL/min. An ultraviolet detector set at 280 nm was used. The samples were extracted with dioxane and diluted with mobile phase. The detection limit was 1 µg/mL.

Table 1. Comparison of Ethinyl Estradiol (EE_2) Subdermal Implants*

	EE2 Implant		
	I	**II**	**III**
Polymer type	PCL†	PCL	PCL/PLGA‡
Device design	Capsule type	Matrix type	Matrix type
EE2 distribution	Single-channel	Homogeneous	Concentration-gradient

*Each implant fabricated by the 3-dimensional fabrication technology [16-19] has 1.8 mg EE2 in loading.
†Polycaprolactone (MW 100 000 Da, IV 1.0-1.3 dL/g, Tg < –60).
‡Polyglycolic acid-polylactic acid copolymer (50:50) (MW 50 000 Da, IV .55-.75, Tg 45°C-50°C).

In Vivo Evaluations

Ovariectomized Rabbit Model

Twelve female New Zealand White rabbits, surgically ovariectomized, were purchased from Covance Research Products Inc (Denver, PA). All animals were recovered after the surgery for at least 3 weeks before the initiation of studies. The animals were housed individually in cages under environmentally controlled conditions (temperature 20°C ± 1°C, relative humidity 50%, and a 12-hour lighting cycle). These animals were fed once daily with a standard rabbit diet that is commercially available and had access to water ad libitum. The ovariectomized rabbit model was used to simulate actual postmenopausal conditions, in which natural estradiol production is suppressed.

Animal Study

On the day of implantation, the procedure was carried out as described elsewhere [21]. In brief, the hair over the lower lumbar dorsal site of each rabbit was clipped and the skin was cleaned by alcohol swab. Before implant insertion, the animals were anesthetized by subcutaneous injection of lidocaineand all apparatus and tools were sterilized. After placing each EE_2 implant inside a 10-gauge hypodermic needle, the

needle was introduced, in parallel with the vertebral column, into the subdermal tissue. The EE_2 implant was then gently pushed through the needle into the subdermal tissue while the needle was withdrawn and the implant was left inside the subdermal tissue. A piece of Band-Aid was then applied over the insertion site to prevent infection.

Following the implantation, serial blood samples (~4 mL each) were drawn from the rabbits' marginal veins at 1, 2, 4, 7, 10, 14, 18, 22, 26, and 30 days and once a week thereafter for a total of 13 weeks. The blood samples were centrifuged, and plasma was immediately transferred into a vacuum tube containing NaF and then stored in a freezer at -20°C until assay of EE_2, estradiol (E_2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) was completed. A capillary GC/MS-method, using negative ion chemical ionization with selective ion monitoring, was used to determine the plasma concentrations of EE_2, while specific radioimmunoassays (RIAs) were used to determine the plasma levels of E_2, FSH, and LH [22,23].

Three prototypes of EE_2 implants (Table 1) were administered randomly into 3 groups of rabbits (4 rabbits in each group). A total of 12 experiments were performed. The mean body weight of rabbits was ~2.5 kg at time of implantation and ~3.5 kg at completion of the 13-week implantation studies.

Pharmacokinetic Analysis

For comparison studies, pharmacokinetic parameters (peak concentration observed during the dosing period [C_{max}], time to reach the peak concentration [t_{max}], the area under the plasma concentration time curves calculated by trapezoidal rule [$AUC]_{last}$, and mean residence time [$MRT]_{last}$) were calculated by using the PCNonlin program [24]. To quantitatively assess the pharmacodynamic effect of EE_2 on gonadotropic response, the values of AUC and MRT were further determined from the plasma profiles of E_2, FSH, and LH by PCNonlin.

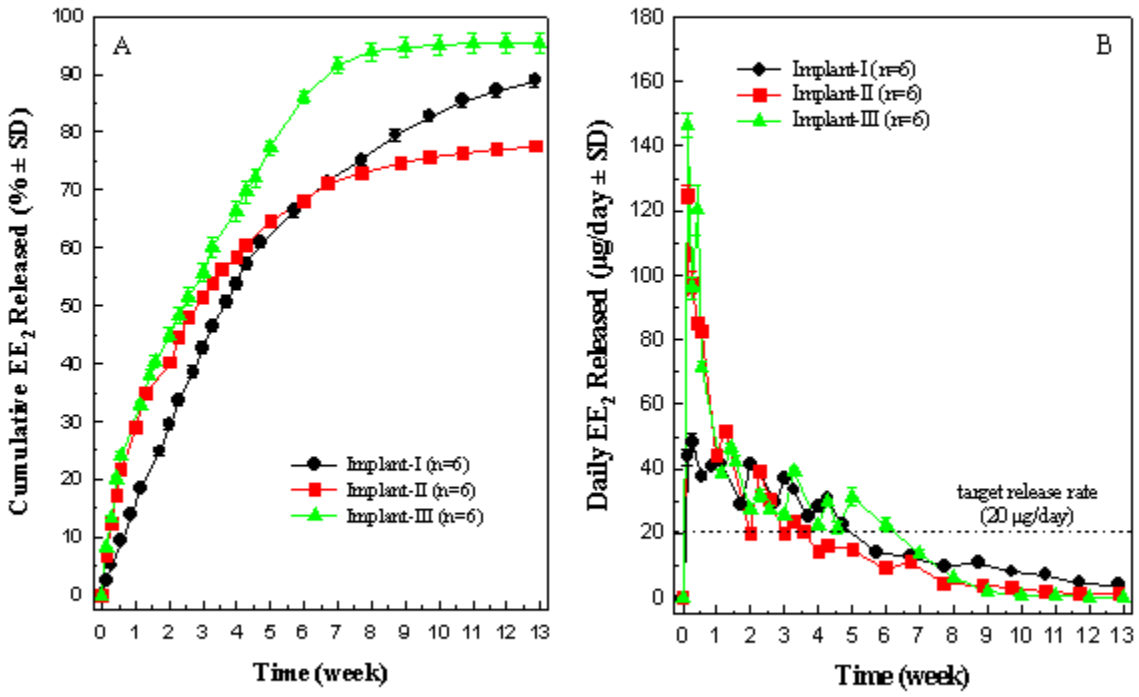

Figure 3. Comparison of the (A) cumulative release profiles and (B) daily dose of ethinyl estradiol (EE_2) released from the various designs of subdermal EE_2 implants (1.8 mg/implant) fabricated by the 3-dimensional printing fabrication technology.

Statistical Analysis

The analysis of variance (ANOVA) was used in this investigation to determine the statistical significance of the differences among the EE_2 implants for each parameter obtained from the in vitro and in vivo studies. If the result of ANOVA indicated a significant difference ($p < .05$), these were then, on a pairing basis, analyzed using the Student-Newman-Keuls test.

RESULTS

In Vitro Evaluations

To gain a better insight into the mechanisms underlying the controlled release of EE_2 from the subcutaneous tissue implants and their role in the systemic delivery of EE_2, the release kinetics of EE_2 were investigated. The in vitro release profiles compared in Figure 3A indicate that all the implants released EE_2 continuously, but at nonlinear kinetics, throughout the 13-week study for implant I and for about 7 weeks for implants II and III. The release profiles were observed to tap off afterward. The maximal levels, 76% for implant II and 95% for implant III, of the incorporated EE_2 were released during the 7-week period.

The daily amounts of EE_2 released from the implants were also calculated. The results compared in Figure 3B indicate that implants II and III have released a higher daily dose of EE_2 than did implant I within the first week. Then the release rates of EE_2 from implants II and III declined to the same dose range as that of implant I from week 2 to week 8, and further reduced thereafter to below the dose range for implant I until the end of the study. On the other hand, the daily dose of EE_2 released from implant I is observed to stay at a steady level for the first 4 to 5 weeks before declining gradually thereafter throughout the 13-week study.

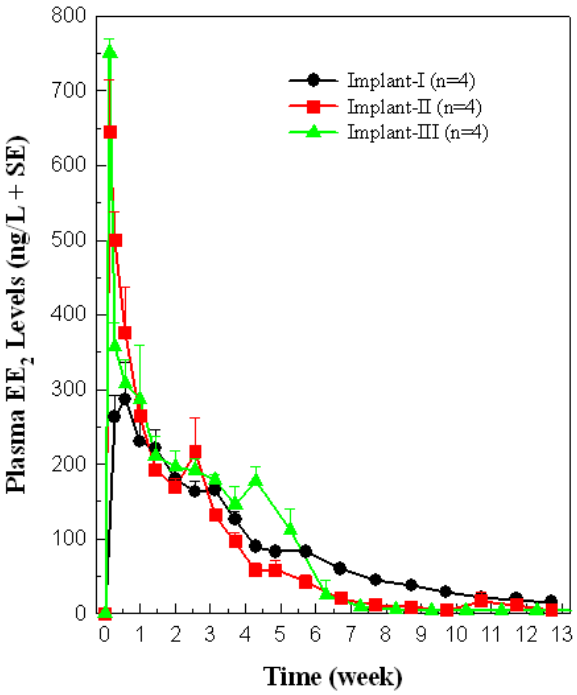

Figure 4. Comparative plasma profiles of ethinyl estradiol (EE_2) achieved by the various EE_2 implants (1.8 mg/implant) following their subcutaneous implantation in the ovariectomized rabbits.

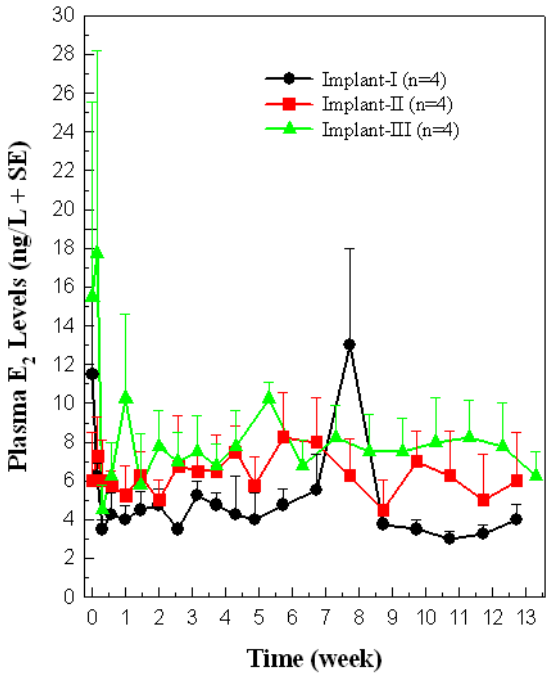

Figure 5. Basal plasma profiles of estradiol (E_2) after the subcutaneous implantation of the various EE_2 implants (1.8 mg/implant) in the ovariectomized rabbits.

In Vivo Evaluations

The plasma profiles of EE_2 following the subcutaneous implantation of EE_2 implants in 3 groups of ovariectomized rabbits are compared in Figure 4. The results indicate that plasma EE_2 concentrations reached a peak level within 4 days following the implantation, and then declined gradually toward the baseline in the following 6 weeks for implants II and III. On the other hand, implant I was observed to achieve a lower peak level of EE_2 than were implants II and III, but more steady and higher concentrations were maintained throughout the 13-week study, especially the final 6 weeks.

Pharmacokinetic parameters obtained from the noncompartmental analysis of plasma EE_2 profiles (displayed in Figure 4) are compared in Table 2. The results indicate that the differences between implant I and implants II and III are statistically significant ($p < .05$) in the mean values of C_{max} (300 vs 645 and 725 ng/L), t_{max} (3.5 vs 1.0 and 1.0 day), and MRT_{last} (25.4 vs 14.8 and 16.9 days). However, no difference was observed in the AUC_{last} values (8067, 7356, and 8462 ng/L × day for implants I, II, and III, respectively) among the 3 groups of implants. The results suggest that all 3 implants were able to deliver similar amounts of EE_2 to the systemic circulation. However, implant I has achieved a better controlled delivery of EE_2 than implants II and III, as judged from the attainment of a lower C_{max} and a longer MRT value.

The plasma profiles of E_2 in the 3 groups of ovariectomized rabbits, in response to the subcutaneous implantation of EE_2-implants, were also measured by RIA. Results compared in Figure 5 appear to suggest that relatively steady plasma E_2 levels have been achieved and maintained throughout the 13-week study period. The pharmacodynamic parameters obtained from a noncompartmental analysis of the plasma E_2 profiles (displayed in Figure 5) are shown in Table 3, which indicate that there are no differences in the mean values of AUC_{last} (435, 563, and 729 ng/L × day for implants I, II, and III, respectively) and MRT_{last} (42.9, 42.2, and 46.5 days for implants I, II, and III, respectively) among the 3 groups of EE_2 implants. The observations imply that the effect of difference in the delivery rates of EE_2 on the pharmacodynamic responses-in terms of the secretion of endogenous E_2-in the ovariectomized rabbit model is very minimal, if any.

Table 2. Comparison in Pharmacokinetic Parameters of Plasma Ethinyl Estradiol (EE_2) Delivered from Various EE_2 Implants in Ovariectomized Rabbits

	EE2 Implant*		
Parameters	**I**	**II**	**III**
Body weight (kg)			
at beginning of implantation	2.5 (.1)	2.7 (.1)	2.6 (.1)
at 13th week of implantation	3.5 (.1)	3.7 (.1)	3.3 (.1)
Cmax (ng/L)†	300 (48)	645 (69)	725 (31)
tmax (day)†	3.5 (.5)	1.0 (.0)	1.0 (.0)
AUClast (ng/L ×day)	8067 (420)	7356 (603)	8462(505)
MRTlast (day)†	25.4 (1.5)	14.8 (.6)	16.9 (.9)

*Data were presented as mean (± SE) of 4 rabbits.

†The results of ANOVA tests indicated a significant difference ($p < .05$) between implant I and implants II and III, but not between implants II and III. The differences were substantiated by pairwise comparisons using the Student-Newman-Keuls test.

Cmax indicates the peak concentration observed during the dosing period; tmax, time to reach the peak concentration (Cmax); AUC, the area under the plasma concentration time curves calculated by trapezoidal rule; MRT, the mean residence time; ANOVA, analysis of variance.

Table 3. Comparison in Pharmacodynamic Responses of Plasma Ethinyl Estradiol (EE_2) Delivered from Various EE_2 Implants in Ovariectomized Rabbits

	EE2 Implant*		
Parameters	**I**	**II**	**III**
Estradiol (E2)			
AUClast [(ng/L)×day]b	435 (40)	563 (127)	729 (145)
MRTlast (day)c	42.9 (2.1)	42.2 (2.6)	46.5 (1.4)
Follicle Stimulating Hormone (FSH)			
AUClast [(µg/L)×day]b, d	2114 (158)	2407 (93)	4523 (483)
MRTlast (day)c, d	40.1 (1.5)	46.2 (2.0)	60.5 (1.1)
Luteinizing Hormone (LH)			
AUClast [(µg/L)×day]b, d	2.4 (2.2)	5.6 (1.4)	55.4 (1.9)
MRTlast (day)c, d	23.5 (13.9)	43.8 (15.8)	73.5 (2.7)

The plasma profiles of FSH and LH in the 3 groups of ovariectomized rabbits, in response to the subcutaneous controlled delivery of EE_2 from the various EE_2 implants, were also measured by RIA. Results compared in Figures 6A and B indicate that the EE_2 delivered has shown similar effect on the secretion of endogenous FSH and LH, as demonstrated by the similarity in the pattern of plasma profiles throughout the entire course of 13-week implantation. The plasma concentrations of FSH and LH decrease rapidly from the basal levels immediately following the implantation to the minimum in less than a week. The suppression is maintained for 6 to 12 weeks, depending on the type of implant, and then increased again. The duration of

suppression and the extent of rebound in FSH and LH appear to depend on the rate of EE_2 disappearance from the systemic circulation. The basal levels of FSH and LH are recovered within the 13-week study period for implant III, but may require a longer period for implants I and II.

The pharmacodynamic parameters obtained from a noncompartmental analysis of the plasma FSH and LH profiles are reported in Table 3. The results indicate that the differences between implant III and implants I and II are statistically significant ($p < .05$) in the mean values of AUC_{last} (4523 vs 2114 and 2407 µg/L × day) and MRT_{last} (60.5 vs 40.1 and 46.2 days) for FSH. The mean values of AUC_{last} (55.4 vs 2.4 and 56 µg/L × day) and MRT_{last} (73.5 vs 23.5 and 43.8 days) for LH are also statistically different among the various implant designs. The observations imply that the EE_2 implants have different extents of effect on the secretion of FSH and LH as a result of the variation in implant design, which yields a difference in the rate of EE_2 release.

DISCUSSION

In Vitro Evaluations

Because of the different designs of these implants, as shown in Table 1, the release pattern of EE_2 from the implants was found to vary (Figure 3A). Based on the mechanisms of bioerosion-modulated drug release reported in the literature [14], the release of EE_2 from the various subdermal implants is essentially controlled by a combination of passive diffusion and polymer erosion from the biodegradable polymer matrix. It has been reported that implants fabricated from PCL are capable of delivering an incorporated drug for several months when a lower molecular weight polymer (eg, 30 000 Da) is used, to more than 1 year when a higher molecular weight polymer (eg, 56 000 Da) is used [25]. On the other hand, the implants fabricated from PLGA were found to be degraded almost completely before the end of the 8-week studies [3,5,26]. The results appear to suggest that PGLA has a higher rate of hydrolytic degradation than does PCL.

Figure 6A

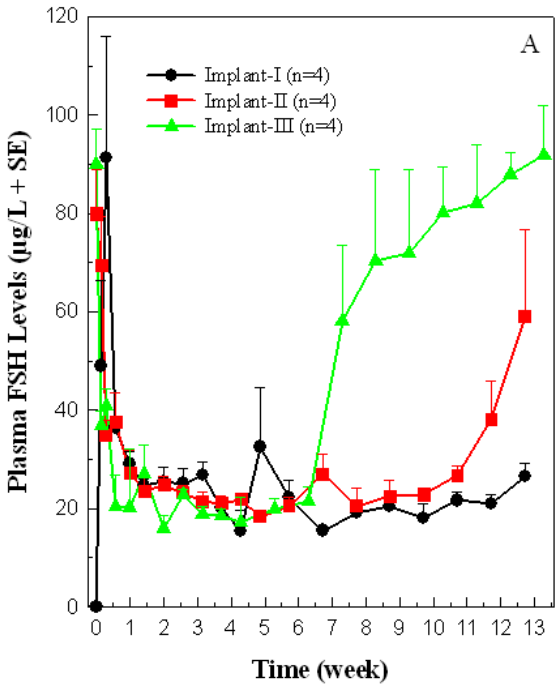

Figure 6B

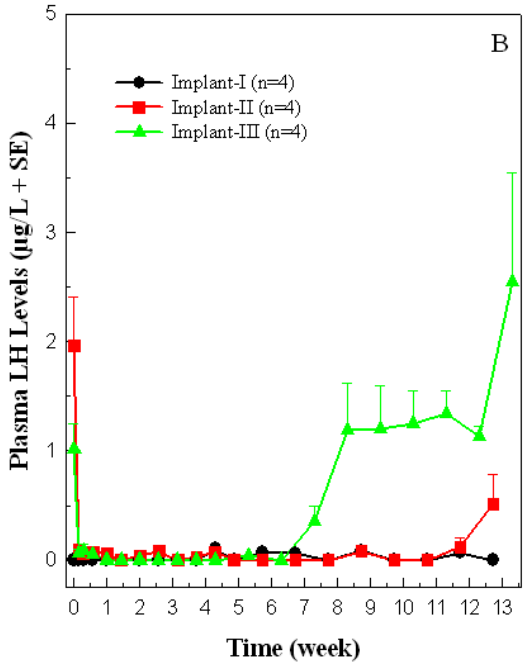

Figure 6. Comparative pharmacodynamic responses to ethinyl estradiol (EE_2) delivered from the various EE_2-Implants as shown by suppression of the endogenous follicle stimulating hormone (FSH) (A) and luteinizing hormone (LH) (B) levels in the ovariectomized rabbits

Because of slow erosion of PCL-based polymeric matrix, the release of EE_2 from implants I and II was apparently controlled primarily by the passive diffusion under a concentration gradient. Therefore, a higher release rate and a shorter delivery duration of EE_2 from implant II (with homogeneous distribution of EE_2) than from implant I (with a single-channel distribution of EE_2) was attained, as expected. Because PLGA-based polymeric matrix has a faster rate of erosion than PCL-based polymeric matrix, the release of EE_2 from implant III appeared to be controlled by a combination of the passive diffusion of EE_2 under the concentration gradient of EE_2 and the erosion of the biodegradable polymer by the hydrolysis of polymer chains. Therefore, implant III releases EE_2 at the highest rate of release and, thus, the shortest duration of delivery among the implants studied (Figure 3A).

In Vivo Evaluations

Following implantation of the implants, the sites of implantation were frequently checked visually. No inflammatory responses, infection, or irritation, which could be due to the administration procedure or the implant itself, were detected throughout the entire course of the 13-week studies. Moreover, the wound caused by the hypodermic needle was noted to heal within the first week after implantation.

Although neither inflammation nor irritation was observed in all the rabbits treated, it was interesting to note that implants I and II appear to be more easily detected than implant III. These observations suggest that implant III might be degraded in the subcutaneous tissue at a rate faster than that of implants I and II. This finding is in good agreement with the fact that PLGA degrades by a bulk hydrolysis of its ester bonds, and is thus broken down into its constituent monomers (lactic and glycolic acids) and then excreted from the body [5]. This finding was further confirmed by the observation that implant III could not be detected or removed at the end of the study, in contrast to implants I and II. A typical picture of implant I, retrieved from the rabbits after 5-month of implantation, is displayed in Figure 7, indicates that the structural integrity of implant I was maintained during the study period.

Moreover, it is interesting to note that the time (~6 weeks after implantation) plasma FSH and LH levels took to return to baseline is similar to the time required for plasma EE_2 levels to reach the end of delivery from

Figure 7. The typical structural integrity of implant I obtained from the rabbits following 5 months of implantation.

implant III. This agreement suggests that, in addition to the attainment of a satisfactory in vivo/in vitro relationship, a good pharmacokinetics-pharmacodynamic relationship has also been established for EE_2 delivered subcutaneously from implant III. A similar correlation and relationship were observed for implant II but not for implant I (possibly due to early termination of the animal study).

The primary objective of applying subcutaneous implantation for drug delivery is to control the delivery of drug to subcutaneous tissue and to maintain the therapeutic effect throughout the treatment duration of interest [1,2]. Implant I appeared to best achieve these goals. As shown in Figures 4 and 6, implant I not only demonstrated a controlled release of EE_2 throughout the period of subcutaneous implantation, but also the amount of EE_2 delivered is capable of maintaining its pharmacological effect by effectively suppressing the secretion of endogenous FSH and LH. These in vivo findings have substantiated the in vitro results on the usefulness of the controlled-release subdermal implants fabricated by the 3-dimensional printing fabrication technology.

In Vivo/In Vitro Relationships

To establish the in vivo/in vitro relationship between the daily amount released and the plasma profiles of EE_2, the results displayed in Figures 3B and 4 were compared and replotted in Figures 8A, 8B, and 8C for implants I, II, and III, respectively. A good in vivo/in vitro relationship was attained for all the implants studied. The agreement may suggest that the plasma profiles of EE_2 could be predicted by measuring the daily amount of EE_2 released from the implants by the in vitro studies. Therefore, an implant with a desired in vivo rate of EE_2 release could be developed by simply performing the in vitro drug release studies in combination with system optimization. A significant reduction in the number of animals used in the in vivo test as well as substantial savings in time and cost could be realized.

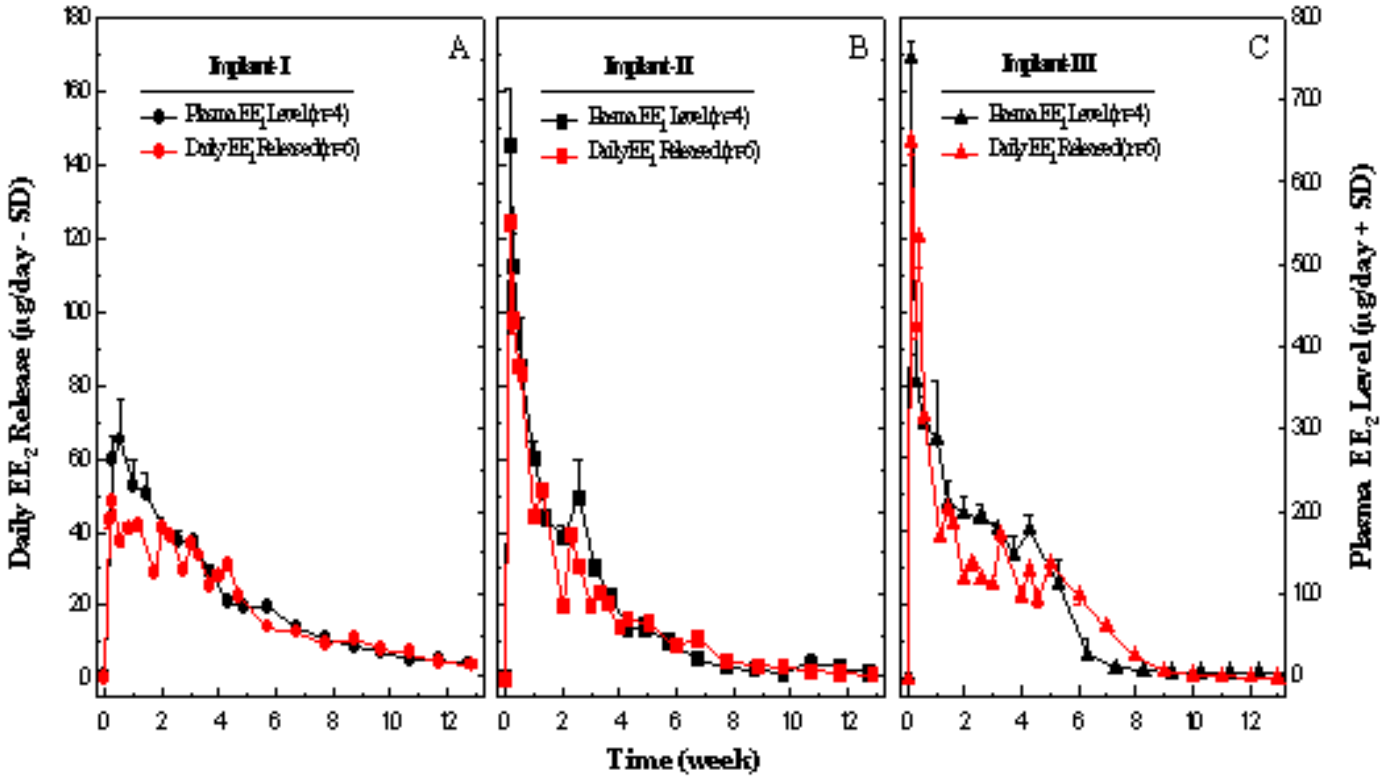

Figure 8. Comparison of the *in vivo-in vitro* relationship between the daily dose released and the plasma profile of ethinyl estradiol (EE_2) delivered from Implant-I (A), II (B) and III (C).

ACKNOWLEDGEMENTS

The authors wish to acknowledge Therics Inc for its financial support of the pharmacokinetics and pharmacodynamics studies; PPD Pharmaco Inc, Richmond, VA, for the analyses of EE_2 by GC-MS method; Dr David Hess and his colleagues at the Oregon Regional Primate Research Center of the Oregon Health Sciences University for the assays of E_2, FSH, and LH by RIA method; and Dr Albert Parlow and his colleagues at the National Hormone and Pituitary Program of the National Institutes of Health for providing reagents for the RIAs.

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