Published in final edited form as:

J Pharm Sci. 2010 August ; 99(8): 3381–3388. doi:10.1002/jps.22098.

***In vivo* absorption of steroidal hormones from smart polymer based delivery systems**

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Abstract

The purpose of this study was to develop smart polymer based controlled delivery systems to deliver steroidal hormones after single subcutaneous injection at predetermined rates over extended period of time. *In vivo* absorption and pharmacokinetics of levonorgestrel and testosterone were investigated from the thermosensitive and phase sensitive polymeric controlled delivery systems. A selective, reliable and rapid method for determination of serum levonorgestrel concentration was developed using High Performance Liquid Chromatography - Tandem Mass Spectrometry with Atmospheric Pressure Chemical Ionization Interface, while testosterone in serum samples was detected and quantified by a competitive immunoassay. The delivery systems controlled the absorption of levonorgestrel in rabbits up to 6 weeks from thermosensitive and ~4 weeks from phase sensitive polymeric delivery systems. *In vivo* study of testosterone delivery systems in castrated rabbits controlled the release of testosterone for at least 2 months from both thermosensitive and phase sensitive polymers. Thermosensitive and phase sensitive polymer formulations significantly ($p < 0.05$) increased relative bioavailability of steroidal hormones compared to control. In conclusion, thermosensitive and phase sensitive polymer based delivery systems controlled the release *in vivo* in rabbits for longer duration after single subcutaneous injection.

Keywords

Controlled release; biodegradable polymers; pharmacokinetics; polymeric drug delivery systems; Poly (DL-lactic) acid; bioavailability; Levonorgestrel; Testosterone; Phase sensitive; Thermosensitive

INTRODUCTION

Control of fertility constitutes a global health issue, as overpopulation and unintended pregnancy have both major personal and societal impact. The contraceptive revolution in the 1960s following the development of hormonal-based oral contraceptives for women has had a major impact on societal dynamics in several cultures and laid the foundations for women's liberation, and are now widely used form of hormonal contraception in U.S.^{1–2}

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The main drawback of oral contraceptives is that they must be taken daily. This drawback has over the past half-century fuelled a quest for alternatives that could be taken less frequently. Controlled-release drug delivery systems have been found useful for long-term medications that require continuous administration of a drug in therapeutically effective doses. Contraceptive implants were originally conceived for long-term continuous release of a steroid hormone in order to avoid over and under dosing and to liberate the users from the daily attention associated with intermittent administration.³ The first implantable contraceptive Norplant[®], which provides subcutaneous controlled delivery of levonorgestrel (LNG), is an example of such an application. It has been approved by FDA in United States in 1990 and successfully marketed since long-term unattended use is indeed one of its most appealing features for many users.^{2, 4} However; it requires surgery for administration and removal of this implant by professionals due to its non-biodegradable nature and hence has been withdrawn from the market in 2002 due to the complications associated with its removal.²

Another steroidal hormone, testosterone (TSN) is the main androgenic hormone which controls many physiological processes like, sexual functions and secondary sex characteristics, muscle protein metabolism, erythropoiesis, plasma lipid and bone metabolism.⁵ Androgen deficiency, also known as hypogonadism, occurs in about 1 in 1000 newborn males.⁶ It is necessary for patients with hypogonadism to be supplied with the required amount of TSN from outside the body over a long period. All have attempted to overcome the pharmacological limitations of TSN as therapeutic drug, namely its very low oral bioavailability and short plasma half-life. These features led to the development of orally active as well as depot TSN formulations. Although subcutaneous implantation of TSN pellets has its favorable pharmacokinetics, this preparation is not widely used and is unlikely to be acceptable for androgen replacement therapy for many hypogonadal men because of the need to undergo a minor surgical procedure to implant the pellets and some associated side effects including bleeding and pellet extrusion as well as the risk of local hematoma formation, inflammation, infection, and fibrosis.⁷ The ideal route of TSN administration should be convenient, have long dosing intervals to foster compliance, and be free of local pain or irritation.

Liquid formulations turning into a semi-solid depot after subcutaneous injection is an attractive delivery system for parenteral application of steroidal hormones. Smart polymers that display a physiochemical response in nonlinear fashion to external stimuli (temperature, pH, solvent, magnetic field, electric field, ultrasound, etc.) are widely explored as potential drug delivery systems.⁸⁻⁹ Biodegradable, biocompatible, phase sensitive and thermosensitive smart polymer based drug delivery systems offer several advantages such as they release the drug in a predefined controlled manner, can be used for hydrophilic and hydrophobic or small and large molecular weight therapeutic agents, low burst release, low batch-to-batch variation in comparison to implants or microspheres, achieve high drug loading (up to 90%) in comparison to microspheres, and ease of preparation. The injectable biodegradable phase sensitive delivery system utilizes a miscible blend of a water insoluble polymer and a water miscible biocompatible solvent such as N-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO), glycofurol, triacetin, ethyl benzoate, and benzyl benzoate.¹⁰⁻¹⁴ Upon injection into an aqueous tissue environment, the water soluble solvent diffuses out of the polymer, which

then precipitates, resulting in a solid implant *in vivo*, from which the drug is released in a controlled fashion.¹⁵ Phase sensitive polymeric formulations containing poly(D,L-lactic acid) in hydrophobic (benzyl benzoate) and hydrophilic (benzyl alcohol) solvent mixture have been investigated for the controlled release of proteins such as lysozyme¹⁶ and insulin.¹⁷ The use of block copolymers in drug delivery was first proposed in early 1980s.¹⁸ Copolymers consisting of hydrophobic and hydrophilic blocks tend to form micelles in water to reduce free energy mainly from hydrophobic interactions; their aqueous solutions show thermoreversible gelation with temperature changes. Triblock copolymers consisting of A-blocks and B-blocks, arranged as BAB or ABA, where A is hydrophobic (eg. PLGA) and B is hydrophilic moiety (eg. PEG) exhibit reversible thermal gelation property. Aqueous solutions of these polymers undergo a reversible gel-sol transition and form a free-flowing sol at room temperature, becoming a transparent gel at body temperature.^{19–20}

Our previous studies demonstrated a zero-order *in vitro* release kinetics of TSN and LNG from phase sensitive and thermosensitive delivery systems.^{21–22} *In vitro* release of TSN and LNG was controlled by optimizing the formulation parameters such as polymer concentration, drug loading and solvent composition for phase sensitive delivery systems; and block length of triblock copolymers for thermosensitive delivery systems.²³ We have also demonstrated that the phase sensitive and thermosensitive polymers were biocompatible *in vitro* and *in vivo*.^{17, 24} In this study, we investigated the controlled absorption of TSN and LNG and their pharmacokinetics *in vivo* in rabbits from phase sensitive and thermosensitive polymeric delivery systems.

MATERIALS AND METHODS

Materials

LNG and TSN were purchased from Spectrum Chemical & Laboratory Products Lab (Gardena, CA). Benzyl benzoate (BB) and Benzyl alcohol (BA) were obtained from Sigma-Aldrich (St. Louis, MO). Poly (DL-lactic) acid with an intrinsic viscosity of 0.20 dl/g (MW 11,000) was purchased from Polysciences, Inc. (Warrington, PA). PLGA-PEG-PLGA triblock copolymer (MW 1400–1000-1400) was synthesized and characterized in our laboratory and used in this study. TSN ELISA kit was obtained from Peninsula Labs (San Carlos, CA). All other reagents used were of HPLC grade.

Preparation of delivery systems

Thermosensitive polymer delivery system: Polymer solutions were prepared by dissolving the thermosensitive copolymer PLGA-PEG-PLGA (30%, w/v) into ice-cold water for 24 h. LNG (12 mg/mL) or TSN (300 mg/mL) was added to the aqueous copolymer solution and homogenized at 8000 rpm for 2 min to make thermosensitive polymer formulation.

Phase sensitive polymer delivery system: The phase sensitive polymer delivery system was prepared by mixing polymer (PLA, 5% w/v) and solvents (BB and BA, 85/15 v/v) in shaking water bath at 37°C for 24 h. LNG (12 mg/mL) or TSN (300 mg/mL) was dissolved in the above polymer solution by homogenization for 2 min at 8000 rpm.

***In vivo* absorption of steroidal hormones in rabbits**

New Zealand White (NZW) rabbits, 10 weeks old and about 2.0–3.0 kg body weight, were used in the study. All animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) at NDSU. NZW rabbits were housed in a 12-hour light/12-hour dark cycle at constant environment of 22°C. Food and water were supplied *ad libitum* during the period of whole study.

***In vivo* absorption of LNG delivery systems:** The day of the study, rabbits were fasted 12 hours before dosing and were anesthetized with 30 mg/kg pentobarbital sodium (Nembutal®) given intraperitoneally. A 1-mL thermosensitive polymer formulation or phase sensitive polymer formulation containing 12 mg/mL LNG was injected subcutaneously at the shaved back neck of the rabbits. Rabbits in the control group received 1 mL LNG solution (12 mg/mL in DMSO) without polymer. Blood samples were collected from central ear artery up to 3 months for LNG polymer formulations and 72 h for LNG solution, respectively. LNG in rabbit serum was determined by High-Performance Liquid Chromatography-Tandem Mass Spectrometry with Atmospheric Pressure Chemical Ionization (HPLC-MS-MS with APCI). The HPLC instrumentation is a HP 1100 series system and the MS system is a PE Sciex API 3000 system. Table 1 shows the experimental conditions for the HPLC-MS-MS.

One hundred microliter of internal standard (IS, 100 ng/mL Oxandralone in Methanol) was added to 1 mL serum sample, and LNG was extracted with 4 mL of Methyl t-butyl ether. The sample was vortexed for 1 min and centrifuged for 5 min at 2400 rpm and 4°C. The ether phase was then decanted into a dry, silanised tube and evaporated to dryness at 50°C under nitrogen. The residue was reconstituted in 125 µL of reconstitution solution, and an aliquot of 75 µL was injected into the HPLC-MS-MS system. Quality control (QC) samples of three concentration levels (0.5, 10, 100 ng/mL) were prepared and spiked with internal standard. QC samples were extracted and analyzed in the same way as for the analysis of the above samples. The precision and accuracy of the method was assessed by analysis of the relative standard deviation (RSD) of measured QC samples.

***In vivo* absorption of TSN delivery systems:** *In vivo* absorption of TSN delivery systems was performed in castrated male rabbits. The rabbits were fasted for 12 h before surgery. The operative site was prepared by shaving the skin. The skin surrounding the incision site was decontaminated by the application of povidone iodine solution followed by 70% alcohol. The rabbits were castrated under pentobarbital anesthesia (30 mg/kg, IV). A bilateral scrotal incision was made over the scrotum, and the testicles with adjacent epididymis were removed. The incision was sutured and povidone iodine solution was applied topically at the site of incision. Postoperatively, the rabbits were given a single injection of gentamicin at a dose of 10 mg/lb body weight, and 1 mg/lb dexamethasone. The rabbits were allowed to recover on a flat pad and were used for *in vivo* experiments 4 weeks after castration.^{25–26}

The day of the study, rabbits were fasted 12 hours before dosing and were anesthetized by 30 mg/kg pentobarbital sodium (Nembutal®) given intraperitoneally. A 1-mL

thermosensitive polymer formulation or phase sensitive polymer formulation containing 300mg/mL TSN was injected subcutaneously at the shaved back neck of the rabbits. A 1-mL TSN solution without polymer (300 mg/mL TSN in DMSO) was administered subcutaneously as a control. The blood samples were collected from central ear artery at specific time points. Serum TSN concentration was determined with TSN ELISA kit (Peninsula laboratories Inc., San Carlos, CA). A competitive immunoassay for TSN was used to detect and quantify the concentration of TSN in rabbit serum²⁷, which is based on the competitive binding of free TSN and a fixed concentration of alkaline phosphatase-labeled TSN to a mouse monoclonal antibody. After washing steps, the substrate tetramethylbenzidine was added and the concentration of bound alkaline phosphatase-labeled TSN was measured. The concentration of free TSN in serum was inversely proportional to the optical density (OD) at 450 nm, which was determined using a Dynex MRX & MRX Revelation TC model 96 well microplate reader (Vienna, VA).

Data analysis

The results of the *in vivo* absorption studies were expressed as a Mean $\pm$ SD (n=4). Statistical comparisons were made using student's *t*-test and one way analysis of variance (ANOVA). The probability value of less than 0.05 was considered as significant. Plasma concentration versus time profiles was evaluated by standard noncompartmental methods. The highest observed plasma concentration and the corresponding time were defined as the C_{max} and T_{max} values, respectively. The area under the plasma concentration curve to the last quantifiable concentration (C_t) at time t (AUC_{0-t}) was determined by linear trapezoidal integration. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/K$; where the overall elimination rate constant (k_{el}) was calculated from the slope of the terminal elimination phase of a semilogarithmic plot of concentration v/s time after subjecting it to linear regression analysis. The area under the first moment of the plasma concentration versus time curve ($AUMC_{0-\infty}$) was obtained by applying the linear trapezoidal method to the product of concentration multiply time versus time up to time t and adding the extrapolated area of $C_t \cdot t / K_{el} + C_t / (K_{el})^2$. Mean residence time (MRT), reflecting the average time a molecule remains in the body, was calculated from $AUMC_{0-\infty} / AUC_{0-\infty}$. The apparent total body clearance after subcutaneous administration (CL) was estimated as dose/ $AUC_{0-\infty}$. All the noncompartmental parameters were calculated manually. The relative bioavailability of LNG and TSN from gel formulations in comparison to reference (subcutaneous solution) was calculated by dividing its AUC with that of subcutaneous solution.

RESULTS AND DISCUSSION

In vivo absorption of LNG delivery systems:

A selective, reliable and rapid method for determination of LNG concentration in rabbit serum was used as described by Wu et al²⁸ with modifications. The precision and accuracy of the method was assessed by analysis of the relative standard deviation (RSD) of measured QC samples. The RSD for both precision and accuracy of the method are <15%. The method has a limit of quantitation (LOQ) of 0.2 ng/ml for levonorgestrel in rabbit serum. The calibration curve of peak area ratios (LNG/IS) to LNG concentration was linear in the range

of 0.25–200 ng/ml ($Y=0.0453X + 0.0756$, $R^2=0.9981$). HPLC - Tandem Mass Spectrometry (HPLC-MS-MS) with APCI interface was used to determine the LNG concentration in rabbit serum. The oxandralone was used as the internal standard (IS). Selected transitions of m/z 313/245 for LNG and m/z 307/289 for oxandralone were monitored using multiple reaction monitoring (MRM) mode for quantitation. Representative MRM chromatograms of LNG and internal standard oxandralone are shown in Figure 1. No endogenous sources of interference were observed at the retention times of the analytes.

Mean serum LNG levels after subcutaneous administration of solution and polymeric formulations containing 12 mg of LNG are shown in Figure 2. Figure 2A shows an immediate increase in LNG concentration after subcutaneous injection of LNG solution with an average maximum serum concentration of 68.18 ± 13.84 ng/mL occurring within 30 min. A high initial concentration of LNG followed by a decrease in concentration over 30 days was observed after application of phase sensitive gel formulation of LNG in rabbits (Figure 2B). In case of thermosensitive polymer formulation, a constant LNG concentration around 5 ng/mL could be observed for about 4 weeks and then gradually decreased over the 6-week period (Figure 2C). Noncompartmental parameters were calculated manually and analyzed. Pharmacokinetic parameters such as C_{max} , T_{max} , area under the curve (AUC), AUMC, MRT and CL are shown in Table 2. We found both phase sensitive and thermosensitive gel formulations significantly ($p<0.05$) increased the AUC value compared to non-polymeric solution group, which means an increased relative bioavailability of LNG compared to the subcutaneous injection of solution. The difference in AUC value between phase sensitive and thermosensitive polymer group was not found statistically significant ($p>0.05$). The average time that LNG drug molecules remains in the body (MRT) after subcutaneous injection of the polymer formulations was significantly prolonged ($p<0.05$); and the apparent total body clearance after subcutaneous administration of the polymer formulations was significantly reduced ($p<0.05$). These findings show that polymer formulations of LNG can increase its systemic residence time and thus enhance the bioavailability compared to the s.c administration of LNG without polymer. Shahiwala and Misra²⁹ reported that LNG with mucoadhesive agents administered nasally in rats is superior for maintaining effective drug concentrations over an extended period of time when compared with the presently available orally administered form. Our study demonstrates prolonged LNG absorption and improved bioavailability after application of gel formulations. Maintenance of an effective drug concentration in the blood for prolonged periods is expected to reduce the dose and/or frequency of drug administration and probably the adverse effect.

To estimate and provide a simple method of assessing the duration of activity *in vivo* of long-acting formulation, the rabbit would appear to be a suitable animal model since it is relatively inexpensive to maintain and can be bled repeatedly. However, the usefulness of this animal as a model depends on if the duration of activity for the formulations in rabbits is correlated with that in human being. According to Nair et al.,³⁰ rabbit model is considered to be a suitable animal model for LNG study, since the pharmacokinetics of LNG in rabbits resembles to that of seen in women. Fotherby et al.³¹ assessed the duration of action of long-acting injectable formulation of gestagens in rabbits. When compared with results obtained in baboons and humans, the rabbit may serve as a suitable animal model for predicting the duration of action of injectable formulations in humans.

***In vivo* absorption of TSN delivery systems:**

A competitive immunoassay for TSN was used to detect and quantify the concentration of TSN in rabbit serum after subcutaneous administration of formulations. The principle of the assay is based on the competitive binding of free TSN and a fixed concentration of alkaline phosphatase-labeled TSN to a mouse monoclonal antibody. Figure 3 shows the calibration curve of TSN *in vivo*. TSN concentration was linear in the range of 0.5–16 ng/mL ($Y = -0.2606\ln X + 1.0063$, $R^2 = 0.9957$). Mean serum TSN levels after subcutaneous application of both solution and gel formulations containing 300 mg of TSN in rabbits are shown in Figure 4. It can be seen from Figure 4A that an immediate increase in TSN concentration after dosing with an average maximum serum concentration of 190.22 ± 60.20 ng/mL occurring at approximately 30 min after subcutaneous injection of TSN solution without polymer. A high initial concentration of TSN followed by a gradual decrease in TSN concentration was observed and then the concentration was gradually decreased over the 90-day period after application of phase sensitive gel formulation of TSN in rabbits (Figure 4B). Similar release profile was found in case of thermosensitive polymer formulation (Figure 4C). Pharmacokinetic parameters such as $C_{\max}$, $T_{\max}$, and area under the curve (AUC) are shown in Table 3. The $C_{\max}$ and $T_{\max}$ were obtained directly from the concentration-time profiles. The AUC value was calculated by the trapezoidal rule method. We found both phase sensitive and thermosensitive gel formulations significantly ($p < 0.05$) increased the AUC value as compared with solution group, which means an increased relative bioavailability of TSN in comparison to the s.c. injection of solution. The difference in AUC value between the phase sensitive and thermosensitive polymer group was not statistically significant ($p > 0.05$).

It has been shown that TSN can have a significant degree of first pass cutaneous metabolism through skin absorption.³²⁻³⁴ In this study, a significant amount of s.c. administered TSN solution (without polymer) seems to be unabsorbed or unaccounted for, which may be due to degradation of TSN at the site of injection. Polymer systems such as poloxamer, PLGA-PEG-PLGA can protect drugs against local enzymes as well as enhance the stability of drugs as long as drug is entrapped in the gel which explains the enhanced bioavailability of TSN from the polymer formulations.^{20, 35}

Xing et al.³⁶ found that the castrated minipigs could be a suitable large animal model for human for studying the potential pharmacokinetics and metabolism of TSN delivered transdermally. In this study, castrated rabbits were used as animal model to exclude the interference from endogenous TSN. Total TSN levels of the male rabbits after castration were found to be lower than TSN levels before castration.³⁷ Yoshida et al.³⁸ reported that the serum TSN levels in castrated rabbits were in the range of 0.1 to 0.7 ng/mL. Kehinde et al.³⁹ also found that the mean TSN level in castrated rabbits was 10.1 nmol/L before castration and 0.8 nmol/L 4 weeks after castration. In this study, blood samples were assayed to confirm the abolition of endogenous TSN. The mean serum TSN level before the castration was found to be about 4 ng/mL while the values in castrated rabbits were below 1 ng/mL. This indicates that the influence of endogenous TSN during the pharmacokinetic studies was limited to a minimum in castrated rabbits. Therefore, rabbit could be a good animal model

for pharmacokinetic analysis of TSN as it is comparable to the situation in hypogonadal men.

Androgen replacement therapy with TSN has been proposed to be a safe and effective treatment for TSN deficiency. In particular, TSN therapy in hypogonadal men may offer benefit, but long-term studies on its efficacy and safety are still lacking.⁴⁰ The need for a sustained TSN plasma level with a concentration which is above 3 ng/mL for men has been defined, requiring continuous TSN input.⁴¹ Different long-acting delivery systems have been investigated to deliver TSN.^{38,42} Complex high axial ratio microstructures were evaluated for delivery of TSN in rats.⁴³ When injected into rats, TSN was released with biphasic kinetics consisting of a burst followed by a much slower phase. It was reported that 200 mg TSN pellet implant release TSN at a steady rate of 1.3–1.5 mg/day in androgen-deficient men.^{44–45} In our previous studies, both phase sensitive and thermosensitive injectable polymer solutions provided a controlled release of TSN in vitro for longer duration.^{21–22} Biodegradability and injectability of these systems provided additional advantage for delivery of TSN over extended duration.

CONCLUSIONS

This study provides evidence that the thermosensitive and phase sensitive polymers based delivery systems can control the release of LNG and TSN for extended period in rabbits. Both polymers are appropriate for developing long-acting controlled release injectable solution delivery systems for steroidal hormones.

ACKNOWLEDGMENTS

We acknowledge the financial support from NIH grant # HD46483 to JS and presidential doctoral fellowship to SC.

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Figure 1.
Typical MS chromatographs for *in vivo* study of LNG: A) Blank; B) LNG; C) Oxandrolone

Figure 2.

In vivo absorption of LNG in rabbits after s.c. administration of LNG Formulations A) control solution (12 mg/mL LNG in DMSO); B) Phase sensitive polymer formulations (12 mg/mL LNG, 5% w/v PLA, and 85/15 v/v BB/BA); C) Thermosensitive polymer formulations (12 mg/mL LNG, copolymer PLGA-PEG-PLGA, 30% w/v). All the data points are mean $\pm$ s.d. (n=4)

Figure 3.
Calibration curve for TSN *in vivo*

Figure 4.

In vivo absorption of TSN in rabbits after s.c. administration of TSN formulations (A) Control solution (300 mg/mL TSN in DMSO); (B) Phase sensitive polymer formulations (300 mg/mL TSN, 5% w/v PLA, and 85/15 v/v BB/BA); (C) Thermosensitive polymer formulations (300 mg/mL TSN, copolymer PLGA-PEG-PLGA, 30% w/v)

Table 1

Experimental conditions for the HPLC-MS-MS

HPLC conditions	
Instrument	HP 1100 series system
Column	Aquasil C18 (50 X 2.1m, 5um)
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in methanol/water (80/20)
Flow rate	0.1min, 10% B, 0.5ml/min; 0.25min, 100% B, 0.5ml/min; 2.2min, 100% B, 0.5ml/min; 3min, 10% B, 0.5ml/min.
Oven temperature	25°C
Sampling volume	75µl
Mass spectrometer conditions	
Instrument	PE Sciex API 3000
Ionization	APCI, positive ion mode
Scanning mode	MRM
Transitions	m/z 313/245 (levonorgestrel) m/z 307/289 (Oxandralone)
Dwell time	200ms

Table 2

Pharmacokinetic parameters after administration of LNG in rabbits (n=4)

Groups	Cmax (ng/mL)	Tmax (h)	AUC (0-∞) (ng* h/mL)	AUMC (0-∞) (ng* h ² /mL)	MRT (h)	CL (L/h)
Control solution	68±14	0.5±0.0	1002±566	35577±3492	31±12	15±7.7
Thermosensitive	---	---	3074±1306 ^a	1574309±687410 ^a	480±148 ^a	3.9±0.8 ^a
Phase sensitive	---	---	3492±1250 ^a	403013±167219 ^a	114±35 ^a	3.5±0.4 ^a

^aSignificantly different from AUC of control solution without polymer group

Table 3

Pharmacokinetic parameters after administration of TSN in rabbits (n=4)

Groups	TSN (mg)	Cmax (ng/mL)	Tmax (h)	AUC (0-∞) (ng* h/mL)
Control solution	300	190.22±60.2	0.50±0.0	703.27±178.28
Thermosensitive	300	---	---	26945±7926 ^a
Phase sensitive	300	---	---	23420±6199 ^a

^aSignificantly different from AUC of control solution without polymer group