Preparation, Characterization and Related in Vivo Release, Safety and Toxicity Studies of Long Acting Lanreotide Microspheres

Shuang Wang, a Mingsheng Wu, b Dan Li, c Mingli Jiao, d Lan Wang, e Haifeng Zhang, e HuaiYu Liu, f Daifeng Wang, g and Bing Han*.c

a China Japan Union Hospital, Jilin University, Changchun 130033, China; b School of Medicine, Johns Hopkins University, Baltimore 21231, U.S.A.; c School of Pharmacy, Jilin University; Changchun 130021, China; d School of Public Health, Harbin Medical University; Harbin 150081, China; e School of Pharmaceutical Engineering, Shenyang Pharmaceutical University; Shenyang 110016, China; and f Experiment Animal Center of Jilin University; Changchun 130021, China.

Received December 12, 2011; accepted August 4, 2012; advance publication released online August 27, 2012

The goal of this project was to prepare long-acting lanreotide acetate poly(lactic-co-glycolic acid) (PLGA) microspheres and to analyze the in vivo and in vitro release, safety and toxicology of these prepa-

rates. Long-acting lanreotide acetate PLGA microspheres that exhibited a 5-week slow-release period were prepared by a multiple-emulsion solvent evaporation method. Physical characterization, as well as the analysis of the in vivo and in vitro release, safety, acute toxicity and chronic toxicity of the lanreotide micro-

spheres, were conducted in animal models in rats, guinea pigs, rabbits and beagle dogs. The lanreotide acetate PLGA microspheres prepared by multiple-emulsion solvent evaporation had smooth surfaces, uniform particle size and stable lanreotide loading. In vivo and in vitro experiments showed that the lanreotide acetate PLGA microspheres could continuously release lanreotide for 5 weeks. The safety of these long acting lanreotide microspheres was good in the following animal models: active systemic anaphylaxis test in guinea pigs, passive cutaneous anaphylaxis test in rats, hemolytic test in rabbits, local skin irritation test after sub-

cutaneous administration in rabbits and muscle stimulation test in rabbits. Furthermore, no significant acute toxicity or chronic toxicity was observed after administration of lanreotide acetate PLGA microspheres in beagle dogs at dosages up to 22 mg/kg. The lanreotide acetate PLGA microspheres that were prepared in this study exhibited beneficial characteristics in apparent property and structural stability, as well as in release trends in vivo and in vitro.

Key words  lanreotide; poly(lactic-co-glycolic acid); microsphere; in vivo release; safety; toxicology

Acromegaly is an abnormal proliferative skin and bone disease that is caused by excessive secretion of growth hormone (GH) from a pituitary gland hyperplasia or a tumor and the average life expectancy of patients with acromegaly is shortened by 10 years. Recent studies have shown that the prevalence of acromegaly in the population has increased over time. Daly et al. found that prevalence was up 3.5 to 5 times the original level in the Belgium Patient Population Disease Preva-

lence Survey. Therefore, this disease warrants more attention from the field of clinical treatment. Traditionally, surgical treatment, such as transcranial pituitary tumor resection or transsphenoidal microsurgical operation, is the first-line treat-

ment of acromegaly to inhibit GH secretion. However, surgical treatment is limited by the inability to completely remove a tumor, surgery failures and the patient’s inability to undergo surgery because of poor health. Therefore, other methods of treatment, as well as postoperative adjuvant therapy, such as radiation therapy, dopamine agonist therapy and somatostatin analogs (SSAs) treatment, are needed. However, these treatment options are also limited. Radiation therapy is slow and may lead to hypopituitarism after treatment. Dopamine agonists have no significant effect on reducing tumor size, and several adverse effects that frequently occur after treatment that make these agonists much less popular, such as nausea, vomiting, orthostatic hypotension and neurologic symptoms.

In contrast, SSAs used to treat acromegaly can effectively control GH and insulin-like growth factor-1 (IGF-I) levels, and their side effects are mostly mild symptoms, like gastro-

intestinal reactions. Therefore, application of SSAs has become the treatment of choice for acromegaly, especially in patients whose tumor is large enough to obstruct the optic chiasm and impact surgical operation or in patients who have heart or lung complications, and SSAs are often used in the place of surgery.

Lanreotide is a somatostatin octapeptide analog with a surface disulfide bond and a non-physiological amino acid d-tryptophan in the molecular ring to increase its stability. d-β-Naphthyl alanine outside the molecular ring endows a higher selectivity. Lanreotide exhibits a high affinity for somatostatin receptors 2 and 5, which are highly expressed on the surface of pituitary adenomas, and lanreotide binding inhibits GH secretion by the adenoma, as well as GH cell proliferation, which leads to reduced IGF-I secretion levels after lanreotide binds to these receptors. This is the main mechanism of treatment for acromegaly. By reducing GH secretion, lanreotide treatment reduces adenoma proliferation, thereby inhibiting tumor growth and reducing tumor size. Lanreotide was first developed by the French company, Ipsen Pharmaceuticals, and several formulations have since been introduced for therapy: short-acting, microsphere long-acting slow-release (SR) and lanreotide acetate supersaturated aque-

ous solution (Autogel; ATG).

Currently, the microsphere long-acting slow-release formulation is the most widely used at two different lanreotide loading doses: 30-mg type (SR LAN30, allows 10 to 14 d of release into the body) and 60-mg type (SR LAN60, allows 21 to 28 d of release into the body). Ambrosio et al. treated 20 acromegaly patients with SR LAN60, which allowed for better...
control of patients’ GH and IGF-1 levels than SR LAN30 and exhibited a longer release period as well as better efficacy. A longer release period will bring patients greater convenience in treatment. In this study, the PLGA microspheres of lanreotide were prepared by multiple-emulsion solvent evaporation exhibited a longer and more stable release period (sustained release for 5 weeks) than lanreotide SR LAN60, and there was only a small difference in release in the body between these two treatments. These results indicate clear advantages of microspheres prepared in this study over current treatment options. In addition to the characterization studies of these microspheres, in vivo and in vitro experiments, comprehensive safety and toxicity were evaluated.

MATERIALS AND METHODS

Materials and Animals Positive allergen ovalbumin was purchased from China Boao Biotechnology Company (Changchun, China); sodium chloride injection was purchased from Changchun Tiancheng Pharmaceutical Co., Ltd. (Changchun, China); lanreotide acetate was purchased from Taizhou Biotechnology Co., Ltd. (Taizhou, China); poly(vinyl alcohol) (average M.W.: 33000) was purchased from Sigma (Warrington, U.S.A.); and dichloromethane was purchased from Tianjin Reagent Factory (Tianjin, China).

The following animals were used in this study: 24 male EWG/B guinea pigs (weight 300 to 400 g), 40 healthy rats (weight 150 to 180 g, 20 male and 20 female), 20 Japanese white rabbits (CBWR/B, weight 2.2 to 2.4 kg, 10 male and 10 female), 48 conventional beagle dogs (24 male and 24 female, weight 8 to 9 kg). Husbandry conditions were quiet, clean, dry and ventilated, and the room temperature and humidity were kept at 20±2°C and 40 to 60%, respectively. All animals were provided by the Animal Experimental Center of Jilin University.

Preparation of Lanreotide Acetate PLGA Microspheres Lanreotide acetate PLGA microspheres were prepared by multiple-emulsion solvent evaporation.13) Lanreotide acetate (1000mg) was accurately weighed and dissolved in water. A high-concentration lanreotide acetate aqueous solution was added to 30% PLGA in dichloromethane, and the subsequent solution was mixed at a high speed (T25 Homogenizer, IKA, Germany) to create a water-in-oil emulsion, and the colostrum was then slowly injected into a 0°C and 0.95% aqueous solution of PVA 4L. In a self-made mixing reactor, a multiple emulsion was stirred to form an oil-in-water multiple emulsion. Then the solution was mixed at a low speed, during which process air was added to the reactor using a vacuum pump and removed from the reactor using fan exhaust. Solid microspheres were produced 2h later. The microspheres were washed 5 times with distilled water and twice with adventitious solvent (1% sodium carboxymethyl cellulose and 7% mannitol), then adventitious solvent (50mL) was added and the solution was freeze-dried to obtain a microsphere powder.14) The product was filtered through a 154-µm sieve and was stored at 4°C.

Determination of the Efficiency of Lanreotide Acetate PLGA Encapsulation After mixing the prepared lanreotide acetate PLGA microspheres to which no adjuvant was added, 30mg was accurately weighed, dissolved into a dichloromethane solution (2mL), placed in a centrifuge tube and sealed at the nozzle. After dissolving, acetate buffer (1mL of 0.1m) was added to the microspheres and the tube was sealed at the nozzle and oscillated for 10min in an oscillator. The solution was centrifuged at 3000rpm for 10min, and the supernatant was transferred to another test tube. This procedure was repeated 6 times, after which the solution was diluted to 6mL with water. The solution (70µL) was analyzed by high performance liquid chromatography against a lanreotide acetate control to calculate the lanreotide content in the test solution by peak area using the external standard method; this measurement was used to determine the average encapsulation efficiency (η), which was an average of 10 measurements. After storing the microspheres under dry conditions at 4°C for 3, 6 and 12 months, the average encapsulation efficiency was measured for comparison.

Apparent Parameter Detection and Characterization Analysis for Lanreotide Acetate PLGA Microspheres After mixing the lanreotide acetate PLGA microsphere preparation to which no adjuvant was added, the average particle size and particle size range were measured using a particle detector (Beckman Coulter LS100, Brea, U.S.A.), and the microspheres were observed under an electron microscope (Hitachi S-520 scanning electron microscope, Tokyo, Japan) to record their shape. Lanreotide acetate PLGA microspheres were dissolved in a solvent composed of mannitol and sodium carboxymethyl cellulose, and the dissolved status and suspension of the microspheres in the solvent were observed; after storage for 3, 6 and 12 months, pH value and water content (DL 32 Mettler-Toledo Karl-Fisher Coulometric moisture tester, Greisensee, Switzerland) of the microspheres were measured. About 10mg each of sample, lanreotide acetate PLGA microspheres to which no adjuvant was added (11.910mg) and PLGA to wrap lanreotide acetate (10.299mg) were prepared and separately placed in a differential thermal scanner (TA instruments Q20, New Castle, U.S.A.) that systematically raised the temperature of the sample to observe the transition from the glass state to the high elastic state to calculate Ƞ′.

In Vitro Study of Lanreotide Acetate PLGA Microspheres Lanreotide acetate PLGA microspheres (30mg) were placed at the bottom of a 50-mL triangular flask and were shaken and tiled to disperse the particles among each other. Phosphate buffered saline (PBS) (20mL, pH 7.4) was slowly added along the flank wall. The buffer was injected while shaking the bottle gently so that the microspheres were dispersed in the buffer. Sealed at the nozzle and the bottle was placed in a constant temperature shaker at a rotation speed of 60rev/min and a temperature of 37°C. While shaking, the in vitro release process of lanreotide from the microspheres was simulated under the in vitro buffered condition. Samples from the in vitro release system (20µL) were taken at 1, 4 and 24h as well as at 2, 4, 6, 8, 12, 16, 20, 24, 28, 32, 36 and 40d and were analyzed by liquid chromatography to calculate the amount of lanreotide released at each time point using the external standard method. The liquid phase conditions were: column: C18 (GL Sciences, Inertsil ODS-2) (150mm×4.6mm, 5µm); acetonitrile, hydroxide, tetramethylammonium and water as mobile phase gradient elution; detection wave length 220nm; the peak retention time of 11 min. The AUC of lanreotide released in a 40d period was set at 100% since lanreotide microspheres are barely detectable 40d after being injected.
were selected and the 40d total AUC was regarded as the percentage of lanreotide that was released through microspheres during each period. The in vivo release of lanreotide microspheres was analyzed.

**In Vivo Release of Lanreotide Acetate PLGA Microspheres** Six healthy adult beagles, 3 male and 3 female, were selected and were administered microspheres by intramuscular injection in the left anterior limb. To avoid residual drug in syringe wall, an appropriate amount of analytical grade sodium carboxymethyl cellulose solution in solvent was added to the microspheres for complete dissolution in the solvent and to avoid adhering to the syringe wall. The dosage in beagles was 2.67 mg/kg. Consistent with the in vitro sampling time points, blood samples were taken from the vein of the dog at 1, 4, and 24h as well as at 2, 4, 6, 8, 12, 16, 20, 24, 28, 32, 36 and 40d after treatment. LC-MS (API 4000 LC/MS/MS System, Applied Biosystems, U.S.A.) was used to measure the plasma concentration of lanreotide at each time point.

**Safety Analysis for Lanreotide Acetate PLGA Microspheres, Active Systemic Anaphylaxis Test (ASA) after Injection of Lanreotide Acetate PLGA Microspheres** Healthy male EWG/B guinea pigs (24 total) were randomly divided into the following 4 groups of 6: group 1 was the negative control group (blank microspheres were administered at 1190 mg/kg), group 2 was the positive control group (ovalbumin was administered), group 3 was given lanreotide acetate microspheres at 256 mg/kg (containing lanreotide acetate at 18 mg/kg) and group 4 was given lanreotide acetate microspheres at 1280 mg/kg (containing lanreotide acetate at 90 mg/kg). Each group was sensitized by intramuscular injection, and during challenging, each group was administered an intramuscular injection except for the positive control group, which was treated by penile vein injection. All animals were treated with 5 doses of the corresponding test drug (1 mL/kg). Two weeks after the last administration, animals in each group were injected with the test drug (1 mL). Only challenge was performed in the positive control group that was treated with an intravenous administration in the penis. Each animal’s response to the challenge was observed over 60 min after treatment, and times of onset and disappearance of allergic-reaction-related symptoms were recorded, such as restlessness, piloerection, shaking, nose scratching, sneezing, coughing, shortness of breath, urination, defecation, lacrimation, dyspnea, rales, purpura, unsteady gait, jumping, gasping, convulsions, horizontal turn, tidal breathing and death. Because of the slow pharmacokinetic characteristics of lanreotide acetate PLGA microspheres, changes in various symptoms were recorded for each animal daily and continuously for 40d.

**Passive Cutaneous Anaphylaxis (PCA) Test in Rats after Injection of Lanreotide Acetate PLGA Microspheres** Healthy rats (32 total) were randomly divided into the following 4 groups of 8: group 1 was the negative control group (blank microspheres were administered at 595 mg/kg), group 2 was the positive control group (ovalbumin was administered), group 3 was given lanreotide acetate microspheres at 128 mg/kg (containing lanreotide acetate at 9 mg/kg) and group 4 was given lanreotide acetate microspheres at 640 mg/kg (containing lanreotide acetate at 45 mg/kg). Another 8 rats weighing 130 to 150g were divided into the same groups, each group containing 2 rats. Five intramuscular injections were administered once every other day for sensitization, after which blood was collected and centrifuged at 2000rpm for 10min to collect serum that was stored at −20°C for backup. In preshaved areas (3 × 4 cm²) on both sides of the back, anti-serum of the corresponding group was injected subcutaneously (diluted with saline at 1: 2, 1: 8 or 1: 32) at two points for each dilution (0.1 mL per point). After 24h, rats in each group were injected with the corresponding drug, and after 30min, 4 rats from each group were injected with Evans blue dye (0.5%, 1 mL) intravenously. The rats were sacrificed by cervical dislocation 30min later, and the skin at the injection site of the anti-serum was collected by a 1-cm punch, cut into pieces and placed into 5 mL of a saline solution of acetone. Optical density (OD) values were measured 24h later at 590 nm for analysis. After 35d, another 4 rats from each group were injected with Evans blue dye (0.5%, 1 mL) intravenously. The rats were sacrificed by cervical dislocation 30min later, and the skin at the injection site of the anti-serum was obtained by a 1-cm punch, cut into pieces and placed into 5 mL of a saline solution of acetone. OD values were measured 24h later at 590 nm for analysis.

**Hemolysis Test after Injection of Lanreotide Acetate PLGA Microspheres** Using Japanese white rabbits as the animal model, a 2% erythrocyte suspension was first prepared using the following protocol: about 10 mL of blood was collected from the rabbit, placed in a triangular flask with glass beads and was shaken for 10min; fibrinogen was removed to obtain blood without fibrinogen; sodium chloride was added at about 10 times the volume of the blood, shaken well, transferred into centrifuge tube and centrifuged at 2500rpm for 5min, and the supernatant was removed. The precipitation of red blood cells was then mixed with the sodium chloride injection again and centrifuged, and this was repeated several times until the supernatant was colorless and transparent for the assay. According to the volume of red blood cells, a 2% suspension was made with the sodium chloride injection for testing.

After preparing the red blood cell suspension, 7 tubes were numbered and tubes 1 to 5 were filled with different amounts of the test solution and the 2% erythrocyte suspension in order. Tube 6 contained blank microspheres, instead of a test solution, and the 2% erythrocyte suspension as a control. Tube 7 contained distilled water, instead of a test solution, and the 2% erythrocyte suspension as the hemolysis positive control. After gently shaking, the tubes were placed into a 37°C water bath. The results were recorded at 30min as well as at 1, 2, 3 and 4h. The results were interpreted qualitatively as follows: transparent and red solution indicated hemolysis, brown or reddish flocculent precipitate in the solution indicated erythrocyte agglutination and sinking red cells leaving a colorless and clear supernatant indicated that no hemolysis had taken place.

**Local Skin Irritation Test after Subcutaneous Administration of Lanreotide Acetate PLGA Microspheres in Rabbits** Japanese white rabbits were used as the animal model, and 8 rabbits (4 female, not pregnant, and 4 male) were selected. After the chest wall skin on both sides of each rabbit was disinfected with povidone iodine, 1 mL of lanreotide acetate microspheres was injected subcutaneously into the left chest wall at a concentration of 1 g/mL, and the right chest wall was injected with an equal volume of blank microspheres.
in the same way. Four animals were sacrificed by air injection into the ear vein 72h and 20d after treatment. Changes in skin, subcutaneous tissues and vessels at the injection site were observed macroscopically. The skin and subcutaneous tissues at the injection site were collected and fixed in 10% formaldehyde for pathological examination. Samples were visualized under a microscope for infiltration of inflammatory cells, proliferation and necrosis.

The Muscle Stimulation Test after Intramuscular Administration of Lanreotide Acetate PLGA Microspheres in Rabbits Japanese white rabbits were used as the animal model, and 8 rabbits (4 female, not pregnant, and 4 male) were selected. After the left hind limb of each rabbit was disinfected with povidone iodine, 1 mL of lanreotide acetate microspheres was administered in the quadriceps, and the right quadriceps were injected with an equal volume of blank microspheres in the same way. Four animals were sacrificed by air injection into the ear vein 72h and 20d after treatment. The quadriceps were dissected to examine changes, which were translated into a corresponding reaction grade. Then the tissue was fixed with 10% formaldehyde for pathological examination. The quadriceps were observed under a microscope for inflammatory cell infiltration, proliferation, and necrosis. The response grade of the muscle stimulation test was recorded.

Lanreotide Acetate PLGA Microsphere Toxicity Test. Acute Toxicity Test Beagle dogs were randomly divided into the following 3 groups of 6 (3 male and 3 female): blank control group, blank microsphere group and lanreotide acetate microsphere group. Using the maximum administration method, a maximum dose of 22 mg/kg (calculated for lanreotide acetate) was injected into the body (equivalent to about 8.5 times the normal clinical dose for humans in terms of body surface area). For the blank microsphere group, an equivalent volume of adjuvant was given, and for blank control group, an equivalent volume of normal saline was given. Each animal was injected one time in the right hind limb deep biceps femoris muscle and was observed for 45 consecutive days for characteristics such as activity status, eating situation and weight. The dogs were sacrificed at the end of the observation period, and their digestive organs (stomach, intestine, gallbladder, etc.), in which the side effects of lanreotide are more frequent, were dissected. Paraffin sections were also prepared for histopathologic examination.

Chronic Toxicity Test Beagle dogs were randomly divided into the following 5 groups of 6 (3 male and 3 female): blank control group, blank microsphere group and lanreotide acetate PLGA microspheres low (5.5 mg/kg), medium (11 mg/kg) and high (22 mg/kg) dose groups, respectively, which corresponded to 8.5 times, 4.3 times and 2.1 times the dose used in clinical practice. The amount of microspheres administered in the blank microsphere group was equivalent to the amount administered in high dose group. The blank control group was administered with the same volume of saline. Doses were given as a deep muscle injection every 5 weeks continuously for 45 weeks. Several characteristics were observed and recorded for each dog throughout the test period such as condition, eating situation and weight. Gross observations and histological observations were performed at necropsy, 45 weeks after treatment, and at the end of the two-month recovery period.

RESULTS

Encapsulation Efficiency for Lanreotide Acetate PLGA Microspheres The average encapsulation efficiency of lanreotide acetate PLGA microspheres was 71.3±0.5%, and the average encapsulation efficiencies were 71.1±0.5%, 70.9±0.6%, and 70.8±0.8%, respectively after storage at 4°C for 3, 6, and 12 months, respectively. Encapsulation efficiency was relatively stable over 9 months, and there was no significant difference (p>0.05) in the encapsulation efficiency between each period, suggesting that the encapsulation efficiency of the microspheres was not related to the duration of storage over 9 months.

Apparent Parameter Detection and Characterization Analysis for Lanreotide Acetate PLGA Microspheres Scanning electron microscopy revealed that the prepared microspheres were an average particle size of 55μm with a range of 35 to 70μm and exhibited apparent regularity and regular spherical physical appearance (Fig. 1). After standing for 5min, by shaking, the lanreotide suspension appeared bottle-like with the microspheres remaining the suspended state, which indicated that they had good suspension property. The pH value of lanreotide microspheres was 6.832, 6.841 and 6.839 (average of 3 measurements) after 3, 6 and 12 months, respectively, with an average of 6.837. Therefore, the sample remained neutral in pH. After sealed storage for 3, 6 and 12 months the water contents were 0.38%, 0.41% and 0.37%, respectively. The water content did not increase with time, and each measured value was less than 1.0%, which demonstrated that water absorption deterioration did not occur in lanreotide microspheres that are well sealed. The Tg values of the lanreotide acetate microspheres and PLGA were 35.72°C and 39.82°C, respectively, and hot melt change values before and after the phase transition (Delta Cp) were 0.823 J/g·°C and 1.195 J/g·°C, respectively. The Tg value reflects the stability of the physical structure of a polymer, and the Tg reaction of the polymer that is determined by the DSC method is the dynamic changes of the polymer hot melt. The measured values differed slightly with different sample particle sizes and degrees of filling. There was no significant difference in Tg between the microspheres and PLGA examined in this experiment, and the bias was considered likely to be caused by systemic errors.
Therefore, the physical structure stability of PLGA does not change before and after preparation of the microspheres.

**In Vitro Release Test for Lanreotide Acetate PLGA Microspheres** The *in vitro* release curve (Fig. 2) was produced under conditions of *in vivo* release that were simulated by *in vitro* release. In this curve, lanreotide acetate PLGA microspheres were relatively stable in early stages of *in vitro* release. The entire release process was maintained for about 40 d, thereby suggesting that the release of lanreotide from microspheres in the body could be maintained for about 5 weeks.

**In Vivo Release Test for Lanreotide Acetate PLGA Microspheres** The *in vivo* release curve (Fig. 3) was produced from *in vivo* release data, which was similar to the *in vitro* release curve. Release at early stages was relatively stable but accelerated in the intermediate stage. Compared with the *in vitro* release, *in vivo* release was slightly faster, possibly due to the presence of proteins such as enzymes in the body. Microspheres in the body were completely released after 36 d, which indicates that treatment once every 5 weeks would be possible for long-term medication using this formulation.

**Safety Tests for Lanreotide Acetate PLGA Microspheres. Active Systemic Anaphylaxis Test Using Lanreotide Acetate PLGA Microspheres** Two weeks after administration, all 18 guinea pigs from the blank microsphere group and the two lanreotide acetate microsphere dose groups did not develop any symptoms of allergic reaction and did not die. Forty days after administration, these 18 guinea pigs did not develop any allergic reaction. All 6 guinea pigs in the ovalbumin positive control group exhibited different degrees of standing hair, shivering, sneezing, cough, dyspnea, wheezing, convulsions, incontinence and other obvious symptoms of allergic reactions, and two guinea pigs from this group died.

**Passive Cutaneous Anaphylaxis Test in Rat after the Injection of Lanreotide Acetate PLGA Microspheres** OD values of dissolved skin collected from rats at day 1 and day 35 of passive cutaneous anaphylaxis test are listed in Tables 1 and 2, respectively. OD values in the ovalbumin group were significantly higher (*p*<0.05) than those in the blank microsphere group, and there was no significant difference between OD values from the two lanreotide acetate PLGA microsphere dose groups (*p* > 0.05). Neither lanreotide acetate PLGA microsphere dose groups exhibited passive cutaneous anaphylaxis at either time point tested, indicating that there was no passive cutaneous anaphylaxis to lanreotide acetate PLGA microspheres in rats.

---

**Table 1. OD Values at Day 1 of the Passive Cutaneous Anaphylaxis Test in Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>1:2 dilution OD value</th>
<th>1:8 dilution OD value</th>
<th>1:32 dilution OD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank microsphere group</td>
<td>0.0071±0.0022</td>
<td>0.0071±0.0023</td>
<td>0.0069±0.0025</td>
</tr>
<tr>
<td>Ovalbumin group</td>
<td>0.0169±0.0096*</td>
<td>0.0159±0.0081*</td>
<td>0.0158±0.0092*</td>
</tr>
<tr>
<td>Lanreotide microsphere group (128mg/kg)</td>
<td>0.0060±0.0022</td>
<td>0.0062±0.0022</td>
<td>0.0069±0.0024</td>
</tr>
<tr>
<td>Lanreotide microsphere group (640mg/kg)</td>
<td>0.0079±0.0022</td>
<td>0.0080±0.0023</td>
<td>0.0083±0.0028</td>
</tr>
</tbody>
</table>

* *p*<0.05 compared to the blank microsphere group.

**Table 2. OD Values at Day 35 of the Passive Cutaneous Anaphylaxis Test in Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>1:2 dilution OD value</th>
<th>1:8 dilution OD value</th>
<th>1:32 dilution OD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank microsphere group</td>
<td>0.0071±0.0027</td>
<td>0.0072±0.0033</td>
<td>0.0067±0.0021</td>
</tr>
<tr>
<td>Ovalbumin group</td>
<td>0.0169±0.0078*</td>
<td>0.0157±0.0073*</td>
<td>0.0168±0.0069*</td>
</tr>
<tr>
<td>Lanreotide microsphere group (128mg/kg)</td>
<td>0.0061±0.0050</td>
<td>0.0059±0.0033</td>
<td>0.0073±0.0036</td>
</tr>
<tr>
<td>Lanreotide microsphere group (640mg/kg)</td>
<td>0.0084±0.0025</td>
<td>0.0072±0.0031</td>
<td>0.0079±0.0043</td>
</tr>
</tbody>
</table>

* *p*<0.05 compared to the blank microsphere group.
Hemolysis Test after Injection of Lanreotide Acetate PLGA Microspheres  

In vitro hemolysis test results showed hemolysis at each test time point (30 min, 1, 2, 3, 4 h) for distilled water (tube 7), but no hemolysis at any experimental observation time point (30 min as well as 1, 2, 3, 4 h) for lanreotide acetate microsphere injections (tube 1 to 5) or blank microsphere injections (tube 6). Furthermore, red blood cell agglutination was not observed, and no changes were found after storage for 24 h at room temperature. Under the in vitro experimental conditions, no significant hemolysis or agglutination was observed in the presence of lanreotide acetate PLGA microspheres over 4 h after administration. Therefore, lanreotide acetate microspheres administered by intramuscular injection at 1 g/mL do not induce hemolysis.

Local Skin Irritation Test after Subcutaneous Administration of Lanreotide Acetate PLGA Microspheres  

Macroscopically, no congestion, swelling or purple color in the subcutaneous tissues and peripheral vessels of the injection sites was observed at 72 h and 20 d after treatment in either the control or treatment group. Microscopic pathology at 72 h after treatment revealed infiltration of inflammatory cells in rabbits from both the control and treatment groups, but no proliferation or necrosis was observed. At 20 d after treatment, inflammatory cell infiltration and fibrous tissue proliferation were observed in rabbits in both the control and treatment groups, but no necrosis was observed.

Muscle Stimulation Test in Rabbit after Injection with Lanreotide Acetate PLGA Microspheres  

Lanreotide acetate microspheres were injected into the rabbit quadriceps area. At both 72 h and 20 d after treatment, the sum of the response grades for 4 quadriceps from 4 rabbits was 0 (<10). At 72 h after treatment, pathological section observation revealed the infiltration of inflammatory cells in both the control and treatment groups, but no adverse conditions such as proliferation and necrosis were observed (Figs. 4a, b). Comparing the pathologies from several samples suggests that inflammatory cell infiltration might have been caused by physical injury from injection. At 20 d after treatment, inflammatory cell infiltration and fibrous tissue proliferation were observed in both the control group and treatment groups, but no necrosis was observed. Lanreotide acetate PLGA microspheres did not induce significant stimulation to injection site, and the muscle stimulation test demonstrated that the microspheres could be used for intramuscular administration without causing permanent damage to the muscle.

Toxicity Test for Lanreotide Acetate PLGA Microspheres  

Throughout the treatment period, no abnormal manifestations or toxicity signs were seen in any groups. Before and after treatment, food intake was normal in all groups, and all surviving animals could finish eating immediately after feeding, indicating that lanreotide acetate microspheres had no effect on food intake in animals. Body weight maintained a normal increasing trend in both the treatment and control groups, and there were no statistically significant differences (p>0.05) between any groups, indicating that lanreotide acetate microspheres had no effect on animal weight gain. Digestive organ autopsy showed no gross pathological change, and gallstones were not found in the gallbladder. Compared with the control group, no tissue abnormality was seen in any sections of treatment groups.

Chronic Toxicity Test for Lanreotide Acetate PLGA Microspheres  

Before treatment as well as during the entire treatment period and the recovery period, animals in the high, medium and low dose groups had smooth and clean hair, unlimited movement, bright eyes, steady breath, absence of vomiting or salivation and normal stool shape and color. Before treatment, the average weights of animals in the high, medium and low dose groups were similar. During the first 6 months after treatment, no abnormal eating was observed, but 6 months later, one animal in high dose group exhibited a loss of appetite and reduced weight gain but exhibited normal eating in the recovery period. Throughout the experimental period, the average weight of animals in each group showed no statistically significant differences (p>0.05).

On necropsy at 45 weeks after treatment and at the end of recovery period, no visible pathological changes were observed in digestive system organs or tissues in any groups, and no abnormality was found in animal tissue sections from any groups. After treatment in the high dose group there was no change in mucosal thickness, gland volume/number or depth of gastric pits as well as no occurrence of stomach lesions (Fig. 5a), cystic expansion, lymph cell infiltration in the lamina propria or fibrosis in mucous layer compared to pre-treatment. In addition, there was no occurrence of intestinal ulcers or inflammation (Fig. 5b), inflammatory exudate in the intestinal surface, necrotic tissue or fibrosis in the intestinal subsurface or intestinal small artery abnormalities after treatment in the high dose group compared to pre-treatment. In the high dose group, gallbladder tissue structure was normal, no gallstones were found within the animal gallbladder, the gallbladder mucosa showed no congestion or edema and no epithelial cell degeneration, necrosis, or loss occurred (Fig. 5c). No tissue monocyte or lymphocyte infiltration and no fibrosis were observed. Gallstones were not observed in any of the high, medium or low dose groups.
At 45 weeks after treatment, granuloma at the injection site was observed (Fig. 5d), for 100% of the blank microsphere group, 83% of the lanreotide acetate microsphere low dose group and 100% of the middle and high dose groups. However, at the end of the recovery period, no injection site granulomas were found in either adjuvant group or any of the treatment groups. Furthermore, changes in abnormal tissue were not found in pathological sections from the chronic toxicity test after treatment.

DISCUSSION AND CONCLUSION

Lanreotide acetate is SSA, which acts in combination with SSR. Its mechanism of treatment of abnormal GH secretion diseases that are caused by pituitary adenomas is by specific binding with SSR in the adenoma to inhibit hormone secretion. Therefore, lanreotide exhibits better specificity than other chemotherapies for the treatment of these diseases.

Octreotide is a somatostatin analog of lanreotide acetate. Natural somatostatin is a cyclic peptide of 14 amino acids that exhibits an in vivo half-life only 3 min. Its analogs, lanreotide acetate and octreotide, are synthetic octapeptide compounds that exhibit much longer in vivo half-lives than the natural peptide. Therefore, these analogs can have more lasting effects in the body, thereby solving the short lifetime problem of the natural medicine that can lead to disease rebound after discontinuation of the treatment. These treatments exhibit a variety of physiological activities, such as inhibiting secretion of GH, thyroid hormones and gastrointestinal and pancreatic endocrine hormones. These treatments lead to satisfactory results for the treatment of diseases that are caused by pathologically elevated secretion of hormones, such as acromegaly, hepatic portal hypertension and esophageal varices. Octreotide exhibits a stronger affinity for peripheral somatostatin receptors, while lanreotide exhibits a stronger affinity for central somatostatin receptors.

Although SSAs have a longer action compared with that of natural somatostatin, daily medication is still needed for regular formulation. For example, short-acting octreotide must be administered subcutaneously 3 times a day, thereby reducing patient compliance with their medication. Because of this limitation, lanreotide and octreotide are typically manufactured as long-acting formulations to further extend the duration of drug metabolism in the body and to extend the treatment period for greater patient convenience.

Long-acting formulations of lanreotide acetate and octreotide are produced at different strengths. For example, long-acting octreotide microspheres are produced at three strengths (10, 20, 30 mg), and the difference between these three preparations is the dose of the drug that is packed in the microspheres. The dose of drug substance is positively related to the control of the disease, such that if a patient does not respond to an intermediate dose (20 mg), they are switched to a higher dose (30 mg). However, regardless of the dose strength, all long-acting octreotide formulations maintain month-long action in the body, and the drug release period does not increase with the increasing dose. However, lanreotide long-acting microspheres are only produced at doses of 30 and 60 mg, which differ with respect to how long they remain effective in the body: a 30-mg dose remains effective for 10 to 14 d, while a 60-mg dose remains effective for 21 to 28 d. Different strengths of lanreotide and octreotide microspheres exhibit different release time characteristics that depend on the preparation technology and adjuvant properties of lanreotide 30 mg and 60 mg. Because microsphere drug release in the body does not depend on the amount of the drug, its release is related to its preparation technology parameters and the properties of the slow-release materials used in its preparation. In this study, lanreotide acetate microspheres were prepared by multiple-emulsion solvent evaporation. The microspheres that were obtained exhibited regular shape and smooth surface, which should endow a stable release capacity. The adjuvant chosen was a 50:50 type PLGA, which is required for microspheres to maintain a release period of 1 month in the body.

Fig. 5. (a) Dog Gastric Tissue Pathological Sections Are Shown at 40× from the High Dose Group in the Chronic Toxicity Test for Long Acting Lanreotide Microspheres; (b) Dog Intestinal Tissue Pathological Sections Are Shown at 40× from the High Dose Group in the Chronic Toxicity Test for Long Acting Lanreotide Microspheres; (c) Dog Gallbladder Pathological Sections Are Shown at 40× from the High Dose Group in the Chronic Toxicity Test for Long Acting Lanreotide Microspheres; and (d) Dog Injection Site Pathological Sections Are Shown at 40× from the High Dose Group in the Chronic Toxicity Test for Long Acting Lanreotide Microspheres
The multiple-emulsion solvent evaporation method used in this study for the preparation of microspheres is simple and requires only mild conditions for preparation, thereby not damaging the structure of drug substance. DSC tests before and after the preparation of microspheres also demonstrated that there was no significant change in the vitrification point, i.e., the phase transition temperature for polymer transition from a glass state to a high-elastic state, of the adjuvant high molecular polymer PLGA. The molecular structure of the high molecular polymer under a glassy state is fixed, in which only the atoms constituting the molecule have balanced vibration within a certain space, while the polymer under a high-elastic state possesses molecular segment motion.20) The vitrification point of a high molecular polymer reflects the stability of its physical and chemical structure. Because this parameter was unchanged, the structure and stability of the adjuvant did not seem to be affected by the preparation process. Neither drug substance nor excipients changed during the microspheres preparation, indicating that this preparation method is suitable for the preparation of lanreotide acetate microspheres.

Wang et al.21) evaluated the safety of oxaliplatin microspheres in a muscle stimulation test, hemolysis test and systemic anaphylaxis test. Dian et al.22) demonstrated the safety of erythromycin gelatin microspheres in a systemic allergy test and in vitro hemolysis test. Zhang et al.23) validated the safety criteria for the anticancer drug vinorelbine liposome microspheres in a hypersensitivity test in guinea pigs. These methods common for the evaluation of drug safety, indicating drug safety can be accurately assessed by these methods. In this study, we conducted the following tests to evaluate drug safety: a drug allergy test in guinea pigs and rats, as well as a drug hemolysis test, local irritation test and muscle stimulation test in rabbits. Experiments testing the safety of lanreotide acetate microspheres were performed in a comprehensive manner, and the experimental results demonstrated that lanreotide acetate microspheres exhibit adequate safety without causing adverse side effects.

There are several known adverse effects of clinically used lanreotide, such as flatulence, nausea, diarrhea, abdominal pain, gallstone, bradycardia, arthralgia, anemia, alopecia, injection site mass and glucose homeostasis disorder.24–26) Gastrointestinal adverse effects are the most common of these effects, while the frequencies of the other adverse effects are very low. The main cause of the gastrointestinal adverse effects is that specific action of the SSA and its receptor can change the secretion of digestive enzymes, such as reduced secretion of pancreatic exocrine enzymes and cholecystokinin, which reduce vitality of the intestinal and biliary system. Therefore, continuous action of SSAs can cause gastrointestinal adverse effects.27)

In addition to gastrointestinal adverse effects, gallstones are also more frequent with this treatment. Clinical studies have shown that the incidence of lanreotide-induced gallstone increased by 10 to 63%.28) Lanreotide-induced gallstone result from SSA inhibition of bile flow and gallbladder activity, and studies have shown that lanreotide can inhibit the release of cholecystokinin (CCK). These changes can alter bile secretion and inhibit gallbladder movement, which induce cholestasis, gallbladder and biliary tract dilatation and gallstone formation.28,29) Clinically, 1% of the patients receive a cholecystectomy after SSA treatment. For patients with a history of gallstone-related diseases, the use of lanreotide should be particularly monitored.30)

Clinical use of lanreotide can cause many of the adverse effects mentioned above. A particular concern is whether its toxicity can lead to substantial organ damage and the extent of that injury. One of the advantages of slow-release drugs is delay of the drug release process to maintain homeostasis between drug release and metabolism, which can limit organ damage caused by long-term maintenance of a drug’s plasma concentration at high levels. The maintenance of this balance is closely related to whether the drug has regular appearance, uniform size, smooth release characteristics and other properties. The microspheres prepared in this study have regular appearance, uniform size, and smooth release, which implicate this preparation for slow-release and low toxicity characteristics. In this study, acute and chronic toxicity tests demonstrated that self-made microspheres did not cause adverse digestive (stomach, intestine, and gallbladder) effects that are commonly reported in the clinic system and are mentioned above. Pathological sections also revealed no pathological changes in gastrointestinal tissue before or after the experiment, demonstrating that these self-made microspheres exhibited no gastrointestinal toxicity. These results verify that these microspheres possess good pharmacokinetic characteristics and release characteristics in the body and that a balance between drug release and metabolism was achieved.

Lanreotide acetate microspheres that were prepared in this study possessed beneficial properties with respect to apparent characteristics, structural stability, release trends in the body and drug safety. These results warrant further investigation into using long acting peptides formulation for clinical treatments.

Acknowledgements This project was supported by National Natural Science Foundation of China (70903019), the Science and Technology Planning Project of Jilin Province (20100117) and Basic Science Research Foundation of Jilin University (450060445266).

REFERENCES

8) Tolezano Y, Rot L, Greenman Y, Orlovsky S, Pauker Y, Olchovsky


