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Sustained Activity and Release of Leuprolide Acetate from an In Situ Forming Polymeric Implant

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ABSTRACT The primary objective of this study was to evaluate the effect of drug loading on the release of leuprolide acetate from an injectable polymeric implant, formed in situ, and efficacy of the released drug in suppressing serum testosterone levels in dogs for at least 90 days. An additional objective was to compare the optimum implant formulation with a commercial microsphere product. Evaluated implant formulations contained 45% w/w 75/25 poly (DL-lactide-co-glycolide) polymer having an intrinsic viscosity of 0.20 dL/g, dissolved in N-methyl-2-pyrrolidone. Irradiated polymer solution was mixed with leuprolide at different drug loads (3%, 4.5%, and 6% w/w) prior to subcutaneous administration to dogs. Dog serum was analyzed for testosterone (RIA) and leuprolide (LC/MS/MS) levels and comparisons within the three implant formulation groups were made. Varying the drug load did not significantly affect the release of leuprolide or efficacy of the implant formulation. Thus, the 6% w/w formulation with the smaller injection volume was selected for comparison with the commercial LUPRON® Depot product, which was administered intramuscularly at a similar dosage. These comparisons of serum testosterone and leuprolide levels showed no significant difference in the pharmacologic efficacy even though drug levels were different at a number of points. This was mainly due to associated high standard deviations. Based on these studies, the 6% w/w leuprolide implant formulation was considered to be a suitable candidate for further development. Additional benefits of this system include its simple manufacturing and lower costs.

KEYWORDS: Polymeric Implant, Leuprolide Acetate, Dog, Sustained Release, Testosterone Suppression

INTRODUCTION

Leuprolide acetate (leuprorelin, D-Leu^{6}- (des-Gly^{10}-NH_2)-LH-RH ethylamide) is a potent luteinizing hormone-releasing hormone (LH-RH) agonist analog that is useful in the palliative treatment of hormonal related prostate and mammary cancers, endometriosis, and precocious puberty (1-5). Sustained leuprolide levels cause desensitization and down-regulation of pituitary-gonadal axis, leading to suppressed levels of luteinizing and sex hormones. Polymer-based microsphere systems that provide sustained drug levels over a period of 1 to 4 months are available commercially (6,7). Compared to daily subcutaneous injections of the analog solution, these forms reduced the needed drug dose to 1/4 to 1/8 and increased patients' compliance and convenience due to less frequent injections (8). However, an expensive and complex manufacturing process, and the inability to retrieve the microcapsules in case of drug adverse reaction are perceived limitations with these systems. A polymeric system (ATRIGEL®), as described by Dunn et al. (9,10) does not have these limitations and can be equally efficient in a variety of drug delivery applications.

The ATRIGEL® system is prepared by dissolving a water-insoluble and biodegradable polymer in a biocompatible organic solvent such as N-methyl-2-pyrrolidone (11). When the polymer solution is injected into the body, the organic solvent dissipates into the surrounding tissue as the water permeates into the implant. This process leads to phase separation and subsequent coagulation of the polymer to form an implant in situ. Active drugs are added

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to the polymer solution to produce a ready-to-use homogeneous solution or dispersion depending upon the solubility of the drug. However, if product stability is an issue due to interaction between the components--drug, polymer, and solvent--the product can be prepared as a two-part system ("A/B"). In this system, one syringe contains an appropriate amount of the polymer solution, whereas the other syringe contains the drug as a dry powder. Before administration to the patient, the two syringes are coupled and the contents mixed with a number of back-and-forth cycles between the two syringes. A sterile product can be made by aseptic manufacturing or exposure to γ -irradiation. Once the drug-containing implant is formed in vivo, drug release is controlled primarily by the properties of the polymer, solvent, and drug employed.

Using the ATRIGEL® technology, an A/B system of leuprolide acetate at a 3% w/w drug load was recently developed (12,13). This formulation showed reproducible efficacy in suppressing and maintaining serum testosterone levels in the desired range of 0.5 ng/ml (6) in rats and dogs for a period of at least 90 days. To deliver the desired 22.5 mg of leuprolide with a drug load of 3% w/w, 750 mg of formulation would have to be injected in humans. An increase in the drug load would reduce the total amount of formulation to be injected and lead to greater patient comfort while simultaneously being more economical. However, the effects of increased drug load on the in vivo drug release, and the pharmacologic efficacy of the ATRIGEL® system with leuprolide were not known.

Thus, the first objective of this study was to compare the serum drug levels and efficacy of an ATRIGEL® formulation having different drug loads (3%, 4.5% and 6% w/w). The second objective was to compare the formulations to the commercial 90-day sustained release microsphere product (LUPRON® 90-day Depot) for pharmacologic efficacy and serum drug levels. The dog was used as the animal model for these evaluations.

MATERIALS AND METHODS

Materials

Leuprolide acetate was purchased from Bachem California, Inc. (Torrance, CA). N-methyl-2-pyrrolidone (NMP, Pharmasolve™) was obtained from International Specialty Products (Wayne, NJ). The polymer, 75/25 poly (DL-lactide-co-glycolide) (PLG) with an intrinsic viscosity (I.V.) of 0.20 dL/g, was purchased from Birmingham Polymers, Inc. (Birmingham, AL). I.V. value was determined by the manufacturer using a Cannon-Fenske G6/25 viscometer and 0.5 g/dL chloroform solution of polymer at 30°C. All other reagents used were of high pressure liquid chromatography (HPLC) grade. Commercial LUPRON® 90-day depot product was obtained from TAP Holdings (Deerfield, IL) and stored according to the label guidelines.

Preparation of ATRIGEL® Formulations

Appropriate amounts of 75/25 PLG (I.V. 0.20) polymer and NMP were weighed into glass vials. After initial mixing of the contents, vials were placed on a continuous shaker (Labline® Orbit Shaker, Melrose Park, IL) overnight at room temperature to completely dissolve the polymer. The proper amounts of the polymer solution, based on the body weight of study dogs, were filled into 3-cc male B.D. (Becton Dickinson, Franklin Lines, NJ) syringes. These syringes were double pouched in aluminum foils with a desiccant bag, heat-sealed under nitrogen and sterilized by exposure to γ -irradiation at a dose of approximately 25 kGy (Isomedix, Morton Grove, IL). For the drug part of the system, 3-cc male B.D. syringes were filled with the calculated volumes of non-irradiated leuprolide acetate aqueous solution that was prepared from HPLC grade water with no excipients. These syringes were lyophilized overnight using a Labconco® Freeze Dry System (Kansas City, MO) to form a leuprolide acetate cake. Just before animal injections, syringes containing the irradiated polymer solution and leuprolide acetate were joined with a polypropylene syringe coupler and the contents mixed with 40 back-and-forth mixing cycles. The resulting homogeneous dispersion was then drawn into one of the syringes, a needle was attached and the product injected as a bolus into dogs.

Different drug loads (3%, 4.5%, and 6% w/w, based on the final formulation of polymer, solvent, and drug) were used in preparing the final formulations. The amount of polymer formulation filled into a syringe was adjusted based on the drug load to keep the administered dosage comparable for all the formulations.

Polymer Molecular Weight

Irradiated and non-irradiated polymer solutions were quantitatively dissolved in tetrahydrofuran to yield a polymer concentration of approximately 0.5% w/v. Filtered samples were analyzed by gel permeation chromatography (GPC) to determine the weight-averaged molecular weight of the polymer. Narrow molecular weight polystyrenes in the range of 580-370,000 daltons (Polymer Laboratories, Amherst, MA) were used as standards. The chromatography conditions were: Polymer Laboratories MIXED-D (5 μ m, 30cm × 7.5 mm) column maintained at 40°C, Hewlett Packard (Santa Clara, CA) 1050 series HPLC with isocratic pump, autosampler, 1047A refractive index detector, and 50 μ l injection volume. Tetrahydrofuran at a flow rate of 1mL/min was used as the mobile phase. Polymer Laboratories CALIBER software was employed for GPC calculations.

In Vivo Studies

Adult male beagle dogs (Ridglan Farms, Mt. Horeb, WI) with a baseline weight range of 9.65-18.75 kg (median weight of 15.5 kg) were distributed randomly into 4 study groupsof 5 dogs each (N=20). Dogs were acclimated for at least a month and identified by ear tattoos. Food and tap water were provided ad libitum. The animals were maintained according to AAALAC requirements and were in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Pub. No. (NIH) 78-23, revised).

On the start day of the study (day 0), dogs were weighed and given a single bolus subcutaneous injection of the ATRIGEL® formulations between the shoulder blades using a 21-gauge needle. As the total contents of syringes were injected as a bolus, syringes were weighed before and after the injections to determine the injected amount of formulation and subsequently leuprolide acetate, based on the theoretic drug load**.** Based on literature reports the target dose was set at 25.6 μ g/kg/day [14]). The commercial product was administered intramuscularly at a similar dosage following the label instructions to mimic the current clinical treatment regimen. On days 0 (pre-dose), 1, 2, 3, 4, 7, 14, 21, 28, 35, 49, 63, 71, 81, 91, 105, 120, 134 and 150, approximately 8 mL of blood were collected from the anterior jugular vein for testosterone and leuprolide analyses. The dogs were observed for overt toxicity and any adverse conditions at the injection site throughout the study period.

Serum Testosterone Assay

After allowing the blood to clot for 30 minutes on ice, serum was harvested by centrifugation at 3500 rpm for 10 minutes and frozen at -20°C for later analysis by solid-phase radioimmunoassay (RIA). Standard commercial RIA kits (Diagnostic Products, Los Angeles, CA) were used and the mean ± SD of testosterone levels (ng/mL) were recorded. Samples, standards, and controls were analyzed in duplicate as an assay validation. The average values that were reported as nil or that fell below 0.1 ng/mL, the lower detection limit for the assay, were considered to be equal to 0.1 ng/mL. The average of pre-dose serum testosterone values from all the study animals (N=20) was used as the baseline value.

Serum Leuprolide Assay

Leuprolide levels were determined by a proprietary LC/MS/MS method developed by Primedica Corporation, Worcester, MA. In general, this method involved the use of an internal LH-RH peptide standard, protein precipitation with acetonitrile, HPLC separation of the extract and mass spectrophotometric assay of the peptide.

Statistical Analysis

A repeated-measures analysis of testosterone and leuprolide data using an α -significance level of 0.05 was performed to determine the effect due to groups over time. In addition, a by-day analysis of variance was performed (α =0.05) to compare various groups at each time point. Single degree of freedom contrasts were performed to compare various groups. In particular, contrasts of interest were those concerning groups 1 to 3 (ATRIGEL® with 3%, 4.5%, and 6% w/w drug), and group 3 (ATRIGEL® 6% w/w drug) vs. group 4 (LUPRON® Depot).

RESULTS

Earlier studies in rats and dogs showed that an ATRIGEL® implant formulation with a composition of 75/25 PLG polymer (I.V. 0.20 dL/g) and NMP was efficacious in quickly (by 14 days) suppressing and then maintaining serum testosterone levels in the human castrate range (0.5 ng/mL) for at least 90 days (12,13). This formulation had a polymer/solvent ratio of 45:55 w/w and contained leuprolide acetate at 3% w/w drug loading. The purpose of this study was to evaluate the effect of increased drug load (from 3% to 4.5% and 6%) on the formulation efficacy, using the same polymer/NMP composition. An additional objective was to compare, for the first time, the efficacy and serum drug levels obtained after administering ATRIGEL® implant formulations and the commercial 90-day depot microsphere product (LUPRON®).

Polymer solutions used in preparing the Atrigel® formulations were filled in 3-cc syringes and γ - irradiated at 23.2-24.6 kGy. Pre-irradiation, the weight-averaged molecular weight of the polymer, as determined by GPC, was 16,950 daltons and after irradiation it was 15,094 daltons. This slight loss was expected, as irradiation causes scission of polymer chains leading to a lower molecular weight (15). Due to the wide acceptance of this method for terminal sterilization of biodegradable polymer systems such as marketed microspheres and medical devices, no further evaluation of the irradiated product as to possible polymer degradants was carried out. For these studies, the drug was used as supplied by the manufacturer without any sterilization. However, for future development, drug sterilization will be carried out either by sterile filtration of the peptide solution before lyophilization or by irradiation of the lyophilized solid. If irradiation is used, the product will be fully characterized for any degradants.

For this study, all the ATRIGEL® formulations were prepared using the two-part A/B system. All the ATRIGEL® formulations used in this study had essentially the same composition except for the drug load. In the case of higher drug load, a lesser amount of formulation was injected to administer a similar per-body weight drug dose. All the formulations were easy to mix and yielded visually homogeneous suspensions, which were injected immediately after mixing to avoid possible stability concerns. These formulations were injected into dogs using 21-gauge needles with only slight difficulty. In the case of LUPRON®, label instructions were followed and the intramuscular injections with 23-gauge needles presented no difficulties.

The dosage as determined by the literature was set at 25.6 μ g/kg/day 14). The actual dosages that were administered were as follows: 27.8 ± 1.35 μ g/kg/day for 3% formulation, 27.4 ± 0.77 μ g/kg/day for 4.5% formulation, 31.4 ± 1.1 μ g/kg/day for 6% formulation, and 25.3 ± 0.58 μ g/kg/day for LUPRON®; and were close to the target dosage of 25.6 μ g/kg/day. Comparatively, 6% ATRIGEL® formulation received higher dosage than desired. The formulation loss on A/B mixing and injection was estimated from a series of in vitro experiments. However, this estimated loss was found to be slightly variable between in vitro and in vivo conditions, as well as due to unavoidable variation in mixing and injecting the contents. This possibly explains the reason for higher dosage with 6% formulation.

Figures 1 and **2** show the serum testosterone levels that were obtained after administering ATRIGEL® and LUPRON® formulations. **Figure 1** compares the efficacy obtained with the ATRIGEL® formulations having three different drug loads. Leuprolide acetate being a LH-RH agonist analog caused a transient increase in testosterone levels during the early period of the study as expected. The peak levels were noticed at approximately 2 to 3 days after drug administration and were well above the baseline. However, by day 4 the levels started declining to lower than baseline value, and by day 14 the levels were below the targeted level of 0.5 ng/mL. This was noticed with all three formulations and possibly signified the down regulation of the pituitary-gonadal axis. From day 14 onwards, the suppressed levels were maintained well below the castration level of 0.5 ng/mL, until about 105 days. The standard deviation for the five data points was narrow and statistically (α =0.05) there was no significant effect due to the "group over time". Similarly, no significant differences between groups (1 to 3) were observed at any time point during the study.

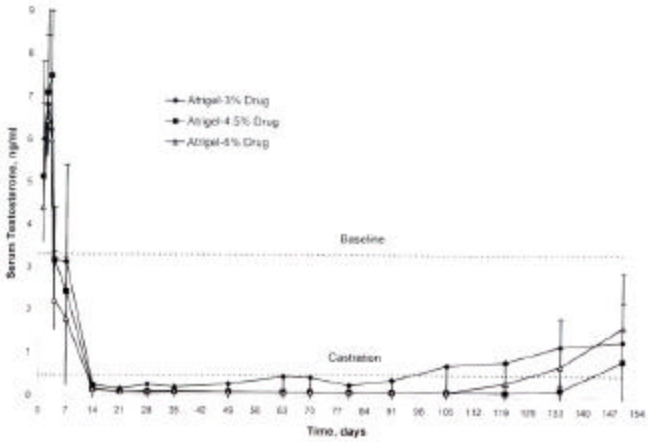

Figure 1. Testosterone suppression in dogs with ATRIGEL® formulations having different leuprolide acetate loads.

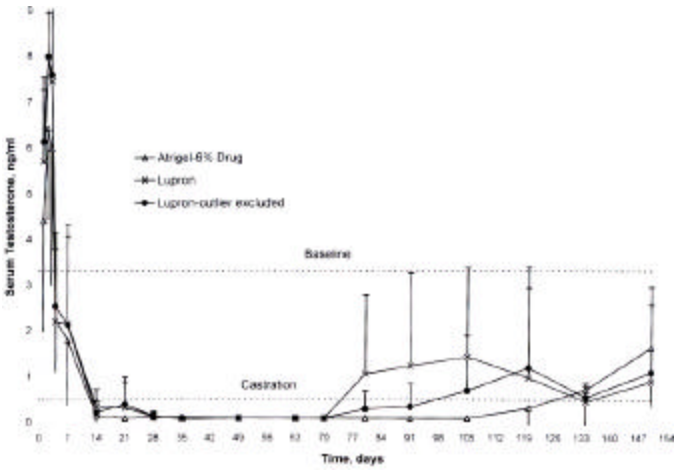

Figure 2. Testosterone suppression in dogs with ATRIGEL® and LUPRON® formulations.

The original plan was to terminate the study at 105 days. However, based on the results that showed continued testosterone suppression at 105 days, it was decided to continue the study with additional sampling time points on 120, 134, and 150 days. This was done to investigate the time at which the cessation of suppression, represented by the elevated testosterone levels, occurred. Mean testosterone levels were seen to steadily elevate from day 91 in case of the 3% formulation, 133 days in case of the 4.5% formulation, and around 119 days for the 6% formulation. The reason for this response between the three formulation groups was not clear. The possibility of longer residence of drug in implants from higher drug loaded formulations leading to longer suppression of testosterone levels could not be verified as the residual implants were not retrieved for analysis of drug content.

Based on the equivalency of efficacy observed from these formulations it was decided to select and characterize further the 6% drug loaded formulation, as a higher drug formulation would mean a lower injection volume. For example, compared to a 3% formulation, only half the volume of formulation is needed with a 6% formulation for the same kind of efficacy. **Figure 2** compares the efficacy profile obtained from LUPRON® to that of the ATRIGEL® 6% leuprolide acetate formulation. Until about day 70, there were no significant differences between the profiles. However, at the data points 80, 91, and 105 days the mean levels with LUPRON® were high and well above the castration levels of 0.5 ng/mL, even though not statistically different (α =0.05) due to associated high standard deviation. One of the dogs in the LUPRON® group (group 4) showed very high values at these time points, leading to elevated mean values. As these values were greater than at least 2 times the standard deviation from the mean testosterone value obtained from the other four animals, this dog was deemed as an outlier. The mean data from the other four dogs were plotted and are shown in **Figure 2.** Even when the data from this particular dog were excluded for calculating mean values, ATRIGEL® 6% leuprolide acetate formulation was seen to be equal to or better than the LUPRON® formulation at providing the pharmacologic efficacy. ATRIGEL® 6% formulation was, however, given at a slightly higher dose compared to LUPRON®. It should also be noted that the pharmacologic efficacy obtained with 3% and 4.5% formulations dosed comparatively, was not statistically different from the efficacy obtained with LUPRON® or ATRIGEL® 6% formulation.

Thus, the efficacy obtained with the ATRIGEL® formulation not only met the objective of quickly obtaining and maintaining suppressed testosterone levels for at least 90 days, but also appeared to have exceeded the desired duration of testosterone suppression by at least 45 days. Complete suppression of pituitary-gonadal axis however, could not be verified, as the animals were not challenged with

leuprolide solution. In a repeat study that was carried out in dogs under GLP conditions, challenging the dogs with a second injection of formulations on day 91 showed no testosterone flare-up (acute-on-chronic effect) (data not shown). This unreported data confirmed the formulations to be effective until at least 91 days post dosage administration.

Figures 3 and **4** depict the serum leuprolide levels after administering the ATRIGEL® and LUPRON® formulations. The leuprolide analysis was carried out at only selected time points. For the 6% ATRIGEL® formulation and LUPRON® all the samples (n=5) at the selected time points were analyzed individually and mean and standard deviation were determined from this data. However, for the 3% and 4.5% formulations, the serum at the selected time point was pooled for analysis so as to minimize the analytical costs. Thus, only mean values but not the standard deviation were obtained, and accordingly, no statistical comparisons involving these groups could be made, which inhibited the ability to interpret the data comprehensively.

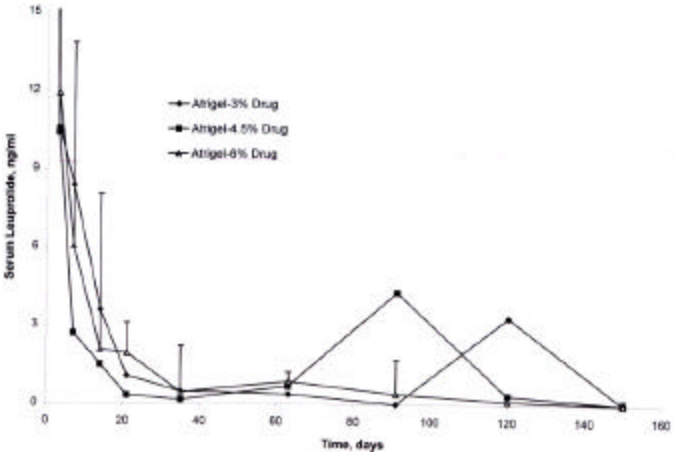

Figure 3. Serum leuprolide levels in dogs after administering ATRIGEL® formulations having different drug loads. (day 1 leuprolide levels were 45.6 ng/mL for 3% formulation, 32.4 ng/mL for 4.5% formulation, and 108.98 ± 101.16 ng/mL for 6% formulation).

As can be seen from **Figure 3**, the mean leuprolide profiles with the ATRIGEL® formulations were very similar. Relatively high levels of leuprolide during the initial time points were observed. Upon injection of the ATRIGEL® formulation, the NMP solvent rapidly dissipates while the physiological fluids permeate the implant. As the organic solvent leaves the system it carries out some of the drug leading to a short initial burst release of drug. Factors such as the solvent employed, drug solubility (solution or suspension in the solvent used), and interaction between the drug and polymer control initial drug release. Except for two time points, day 91 for 4.5% and day 120 for 3% formulation, when there appeared to be a slight burst release of drug, the leuprolide levels were uniform but low. However, these levels appeared sufficient to cause and maintain testosterone suppression. It is reported in the literature that once the gonadotropin LH-RH receptors are downgraded, a minimum amount of leuprolide is needed to sustain the suppression even though the exact levels needed are not known (16).

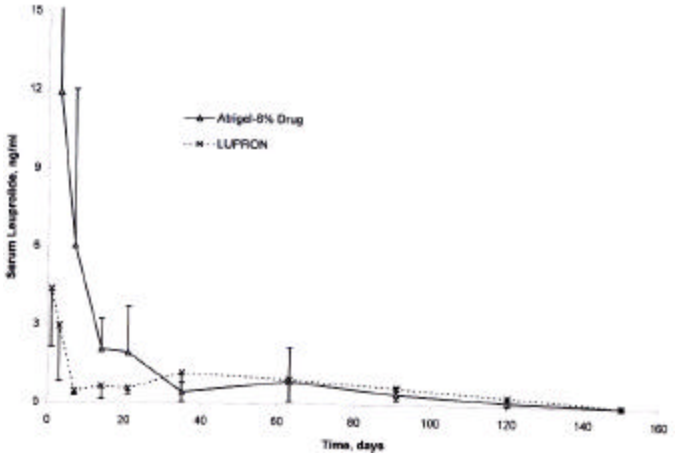

Figure 4. Serum leuprolide levels in dogs after administering ATRIGEL®-6% w/w drug and LUPRON® formulations (at day 1 leuprolide level for ATRIGEL® formulation was 108.98 ± 101.16 ng/mL).

Figure 4 compares the drug levels obtained after administering 6% drug ATRIGEL® and LUPRON® formulations. Both products gave a high initial release of drug, but the levels with the ATRIGEL® formulation were higher than the LUPRON® microspheres until about 30 days when the two became equivalent. The reason for higher standard deviations associated with ATRIGEL® formulations can not be speculated on other than the varied animal response to released leuprolide. Unlike the ATRIGEL® system, only surface associated drug is released initially from microspheres. This is usually followed by a lag period until water permeates the polymer mass to create pores for diffusion of the drug. Later drug release is associated with

polymer erosion (17-19). It appears from these data that the exchange of water and solvent between the ATRIGEL® implant and the body fluids during the initial solidification phase eliminates the lag period and provides a more sustained and elevated release of drug until polymer erosion occurs. The inherent property of high initial release from the ATRIGEL® system can be termed advantageous or disadvantageous only on a case specific (drug and therapeutic window; application and need for a loading dose) basis.

A certain correlation between the drug and testosterone levels from these two formulations, especially during the first 28 days, can also be seen. If only the absolute mean values without the associated standard deviations are considered, the higher mean drug levels seen with the ATRIGEL® formulation gave lower mean testosterone levels. Elevated levels of testosterone in the LUPRON® group during days 80 to 120 were observed even when determined leuprolide levels appeared to be sufficient. As described above, only one dog from this group had high values of testosterone during this period and the mean values were high due to this bias. Thus this unexpected raise in mean testosterone levels was due to an anomalous animal in the group.

CONCLUSION

In conclusion, these studies have shown that the three ATRIGEL® formulations with different drug loads: 3%, 4.5%, and 6% w/w are equally efficacious in obtaining and maintaining suppressed testosterone levels from day 14 to day 91 of the study. Simultaneous evaluation of a commercial product, LUPRON® Depot, with the 6% w/w ATRIGEL® formulation at a slightly higher dosage, has shown both to be comparable in pharmacologic efficacy. Mean leuprolide levels obtained after administering ATRIGEL® formulations were similar in three drug loads, and comparison between levels from the 6% formulation and LUPRON® did not show any significant difference, mainly due to the associated high standard deviation values during the early time period. With a reduced need for injection volume and equal efficacy, the 6% ATRIGEL® formulation appears to be a suitable candidate for further development. The additional benefits offered by this system are the ease of manufacture and administration, and lower costs.

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