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Pulmonary bioavailability of leuprolide acetate following multiple dosing to beagle dogs: some pharmacokinetic and preclinical issues

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Abstract

Localized delivery of drugs to the lung has long been known as an effective and rapid modality for treating various pulmonary diseases. Because of its large surface area, the lung serves also as a port of drug entry to the systemic circulation. However, lung deposition of pharmaceutical aerosols is generally less than 100% of the nominal dose. This is due largely to complex biophysical factors associated with filtration mechanisms of the respiratory system (Byron et al., *Pharm. Res.*, 3 (1989) 225–229) and patient factors. The incomplete absorption of pharmaceutical aerosols in the lung may be due in part to mechanical losses of drug during drug administration. For example, some drug is retained usually on the mouth adapter (actuator) of the inhaler during use, and often there is significant loss in the throat as a result of inertial impaction (Ganderton and Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood, 1987; Byron et al., *Pharm. Res.*, 3 (1989) 225–229). Inefficient delivery of drugs to the airways may be the single largest cause for low drug absorption via lung. For peptides, physicochemical characteristics of the drug, stability to metabolizing enzymes, molecular weight, permeability to lung mucosa, and stimulation of the alveolar macrophage clearance mechanism (Forrest, In Morén et al. (Eds), *Aerosols in Medicine, Principles, Diagnostics and Therapy*, Elsevier, 1985) may be more significant in decreasing the efficiency of absorption from the airways. In a previous study (Adjei et al., *Int. J. Pharm.*, 61 (1990) 135–144), male and female beagle dogs were administered an inhalation solution aerosol formulation of leuprolide acetate at daily dosages of 0, 0.5, 1, and 2 mg for 14 consecutive days. The results demonstrated: (a) significant plasma levels following administration of leuprolide to the lung compared to a placebo aerosol formulation as control; (b) a linear dose-dependent increase in pulmonary bioavailability of leuprolide in the dose range of 0.5–2.0 mg/dog per day; (c) no significant differences in pulmonary absorption between male and female dogs; (d) a corresponding decrease in plasma gonadotropins with sequential increases in plasma leuprolide concentrations; and (e) approx. 50% lower bioavailability on day 14 compared with day 1 of the study. The present study clarifies phenomenologic and pharmacokinetic issues associated with lung delivery of leuprolide acetate. For this study, male and female beagle dogs were administered leuprolide using a suspension aerosol formulation instead of a cosolvent based solution aerosol formulation of the drug (Adjei et al., *Int. J. Pharm.*, 61 (1990) 135–144). The results demonstrated: (a) linear dose-dependent increases in plasma AUC of leuprolide in the dose range of 1.5–9 mg/dog per day; (b) no change in bioavailability with multiple dosage of the

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aerosol up to 14 days; (c) no toxicologic findings over the 14 day dosing period; and (d) the no-toxic effect level of leuprolide was 9 mg/dog per day. This paper also attempts to explain differences between in vivo performance of the alcohol-based solution aerosol (Adjei et al., *Int. J. Pharm.*, 61 (1990) 135–144) compared with a suspension aerosol formulation of the drug.

Key words: Peptide; LH-RH analog; Peptide drug delivery; Inhalation; Aerosol formulation; Leuprolide aerosol; Dose linearity; Preclinical safety

1. Introduction

Most peptide drugs are not orally bioavailable (Banerjee et al., 1991) and therefore are administered parenterally. Recent events in biotechnology and in the fields of protein and peptide engineering have increased our knowledge regarding how and where response modifiers function within the body. As a result, several classes of biochemically and biologically active compounds have been identified. Most of these are peptides. Yet, peptide drug delivery by non-parenteral routes of administration is fraught with formidable challenges. These include interactions with biologic interfaces such as biocompatibility, local metabolism, and immunological consequences that often are too complex for the pharmaceutical scientist to accommodate during drug formulation studies. Many of these newer compounds are therapeutically most useful when administered in pulsatile mode. Thus, the non-injectable routes (i.e., nasal, rectal, transdermal, buccal, pulmonary) may be clinically advantageous for several candidate compounds now in clinical development (Lee, 1991). A significant fraction of these may be peptide and protein drugs.

Leuprolide acetate is a synthetic nonapeptide analog of naturally occurring GnRH, having greater biologic potency than the natural hormone. It is chemically defined as:

5-Oxo-Pro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-ProNHEt · xCH$_3$COOH

When introduced into the systemic circulation leuprolide induces the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Schally et al., 1971; Belchetz et al., 1978). Leuprolide, like most LH-RH analogs, possesses a long biologic half-life in plasma. Therefore, in contrast to acute dosing, chronic and long-term administration paradoxically desensitizes the pituitary resulting in a reversible biochemical castration. It is effective therapy for many hormonally sensitive diseases, including prostatic cancer (Leuprolide Study Group, 1984) and endometriosis (Meldrum et al., 1982; Lemay et al., 1984). Like most LH-RH analogs, leuprolide acetate causes regression of DMBA-induced mammary tumors, reduced size of sex organs, and reduced gonadotropin and sex steroid levels in both males and females (Karten and Rivier, 1986). The drug has been approved in the United States for daily subcutaneous administration (1 mg) or monthly single intramuscular (7.5 and 3.75 mg) depot injection for palliative treatment of advanced cancer of the prostate and endometriosis. Because leuprolide acetate is not orally active, several non-conventional routes of administration including the pulmonary route were investigated (Adjei et al., 1987).

The lung, with its progressive subdivision distally from the trachea to the alveoli, is a complex organ with high enzymatic activity. This complicates estimation of absolute lung drug bioavailability. Feasibility for systemic delivery of peptides via the lung was first demonstrated by Adjei et al. (1987). In their work (Adjei and Garren, 1990; Adjei et al., 1992), these authors showed that significant lung absorption of the nonapeptide leuprolide acetate is achievable in both laboratory animals and humans. Pivotal preclinical studies were conducted during which multiple doses of a solution-based inhalation aerosol formulation of leuprolide acetate were administered to beagle dogs (Adjei et al., 1990). Results from these studies unexpectedly demonstrated a decrease in lung bioavailability of this LH-RH analog following chronic administration for 14 days.

There was higher variability also in the estimated bioavailability values for day 14 compared with day 1 results. This finding could not be explained by device and impaction losses alone. Therefore, it was assumed that minor microscopic changes in the environment within the lung following chronic administration of the alcohol based solution aerosol might be responsible in part for the observed results. If the assumption is valid, then perhaps formulation changes could resolve the problem. The subject study, therefore, was designed to evaluate functional performance, in vivo absorption kinetics, and preclinical safety of leuprolide acetate when administered to beagle dogs as a suspension aerosol formulation.

2. Experimental

2.1. Formulations, devices, and methods

Micronized drug was used to prepare a leuprolide slurry in the formulation. The drug was presented as a metered dose inhaler (MDI). The actuator for delivery of the formulation was adapted in order to fit with the dog traceostomy model without compromising dosimetry. Methods used in the study are summarized in the following section.

2.2. Aerosol formulation

A suspension aerosol formulation containing leuprolide acetate, lot 35-912-AR, was prepared and used as the active product lot in all dose groups. Shams were used for placebo controls. Generic composition of the formulation is described in Table 1.

2.3. Aerosol devices

A 50 μl metering valve (DF10, sourced from Valois of America) was used to deliver the aerosol formulation described in Table 1. An actuator device (Micron-4, from TAP Pharmaceuticals, Division of Abbott Laboratories) was modified by the removal of the main plastic body. It was then fitted with an extension tube designed for the tracheostomy and used to aerosolize drug to the lungs of the dogs. The formulation was packaged in 20 ml aluminum cans lined with epoxy phenolic coating (from Safet Embamet, France).

2.4. Leuprolide acetate assay

Chemical analysis of leuprolide acetate in the formulation was determined using a previously described HPLC method (Sutherland and Menon, 1987).

2.5. Functional performance testing

The formulation (lot 35-912-AR) was characterized for dosimetry (unit spray content and shot weight), spray integrity (plume characteristics), size distribution (i.e., mass median aerodynamic diameter, MMAD, and its associated geometric standard deviation, σ_d, and respirable fraction. The MMAD and σ_d were generated from particle size analysis in the formulation using light scattering (Adjei and Garren, 1990). Cumulative percentiles of particles under specific cut-off-di-

Table 1
Composition and functional performance of suspension aerosol of leuprolide acetate, lot 35-912-AR

Formula composition		Functional performance tests	
Leuprolide acetate	500 μg/spray	leuprolide acetate	96.8% LC
Sorbitan trioleate	250 μg/spray	unit spray content	484 μg/spray
Freon 11	14 μl/spray	MMAD [a]	2.6 μm $\pm$ 1.6
Freon 12	36 μl/spray	shot weight	69.5 mg/spray
Nitrogen, NF	q.s.	respirable fraction	52.4% LC

[a] Mass median aerodynamic diameter obtained by light scattering method (Malvern). Result with the impaction method (cascade impactor) was 3.4 μm $\pm$ 2.8.

ameters (ECD) in the size distribution profile were calculated and plotted as a function of ECD on generate log-probability paper. Values for MMAD were calculated from ECD values corresponding to 50% of the total mass of particles in the respective probability plots. The associated geometric standard deviation, σ_d, was determined as follows:

$\sigma_d = 84.13\%$ diameter/50% diameter

$= 50\%$ diameter/15.87% diameter

At least 3 months formulation stability data to ensure product integrity and compliance with specifications were used to support these studies.

2.6. Laboratory animals

Female and male purebred beagle dogs approx. 10 kg in weight (Marshall Research, North Rose, NY) were obtained from a colony of surgically tracheostomized dogs and allowed to recover over an acclimation period of at least 7 days. Following recovery from surgery, these tracheostomized dogs were fitted with intratracheal tubes during a minimum 3-day pre-treatment period to ensure easy entry into the tracheal stoma. All dogs were housed in stainless-steel double-decked cages equipped with feeders and automatic waterers. Temperature and humidity conditions were monitored and maintained constant throughout the study period. Dogs were fed daily with canine diet (Ralston Purina Co., St. Louis, MO) and water was allowed ad libitum. The intratracheal tubes were removed at least 1 week before drug treatment was initiated.

2.7. Drug adminstration

The tracheostomized beagle dogs were administered a daily dose of the aerosol for 14 days. The drug was aerosolized to the bifurcation of the lung during the inspiration cycle by synchronizing dosing to the dog with activation of the valve. In all cases, the head of the dog was gently raised to approx. 45°, and the delivery device gently inserted into the tracheal stoma so that the spray jet was approx. 1–3 inches posterior to the epiglottis. In this configuration, the aerosol was directly deposited at or near the bifurcation of the lung which also resulted in minimal exhalation of aerosolized drug during expiration.

Table 2
Dose ranging protocol with suspension aerosol of leuprolide acetate

Treatment group	Number of sprays (50 μl/spray) [a]	No. per group	
		Males	Females
Placebo (T0)	0 spray	1	1
1.5 mg/day (T1)	3 sprays	1	1
3.0 mg/day (T2)	6 sprays	1	1
4.5 mg/day (T3)	9 sprays or	1	1
9.0 mg/day (T3a) [b]	18 sprays		

[a] Administered as a suspension aerosol, each spray 500 μg leuprolide acetate/spray.
[b] The two dogs in group T3 were dosed 9 mg instead of 4.5 mg on day 8 to 14.

2.8. 2-week dose ranging pharmacokinetic study

The study protocol used in the subject investigation together with the drug administration schedule is summarized in Table 2. Four groups of two dogs each, one male and one female per group, were used. Each group was administered a daily dose of leuprolide aerosol (0, 1.5, 3.0, or 4.5 mg) for 14, 14, 14, and 7 days, respectively. These were designated groups T0—T3, respectively. The daily dose for group T3 dogs was raised from 4.5 to 9.0 mg on day 8 to day 14, and on this dose regimen the two dogs were re-identified as group T3a. Venous blood was obtained from the jugular vein at approx. 0, 0.33, 0.67, 1.0, 2.0, 3.0, 6.0, and 8 h post dosing, on days 1, 7, and 14 of the study. The blood samples were heparinized, centrifuged and the plasma frozen until needed for leuprolide acetate assay.

2.9. Radioimmunoassay (RIA) for leuprolide acetate

Plasma leuprolide concentrations were determined using a modified radioimmunoassay (RIA) procedure (Yamazaki and Okada, 1980). Leuprolide was labeled with ^{125}I via chloramine-T oxidation and purified by ion exchange chromatogra-

phy (Yamazaki and Okada, 1980). Approx. 15 000 cpm of ^{125}I-[Tyr^5] leuprolide was utilized as a tracer. An antibody capable of recognizing the tripeptide antigenic determinant *x*-Leu-Arg-Pro-NHEt was utilized. The EC_{50} and limit of detection for the assay were approx. 100 pg and 10 pg/sample, respectively. A standard curve with sample concentrations within 5–25 000 pg per tube was used in the assay. Blood samples (approx. 1 ml) were drawn by venipuncture into 3 ml tuberculin syringes prewetted with 15% Na_3 EDTA. The areas under the blood level vs time curves were calculated and used to estimate absorption differences between the respective treatment groups. Coefficient of variation of the assay was approx. 10% at concentrations of ≈ 10 pg/sample. The intra-assay coefficient of variation was $\approx 6\%$. Samples were assayed in triplicate.

2.10. Clinical pathology

Venous blood samples were withdrawn in the morning during the baseline period and also near the end of the 14 day dosing period. Samples were taken after an overnight fast and monitored for basic hematology and clinical chemistry parameters. After the 14 day drug dosing period, all dogs were fasted overnight and exsanguinated under pentobarbital (Nembutal®) anesthesia. Necropsies were performed and certain critical organ samples were fixed in 10% neutral buffered formalin. Histology was performed on samples taken from the gonads (testis and ovary), heart, liver, lung (including the major bronchi), thoracic lymph node, and trachea.

2.11. Analysis of results

Plasma concentrations of leuprolide in samples from each treatment group were plotted as a function of sampling time after drug administration. Areas under these plasma leuprolide concentration vs time-course curves (AUC) were estimated using trapezoidal approximation. Effects of dosage and period on AUC values were tested for significance using repeated measures analysis of variance (Winer, 1971). Doses in group T3 were included in the analysis despite the increase in dose for day 14 data. Pairwise comparisons between dosage groups were made by *t*-tests on the average AUCs over days 1, 7, and 14.

3. Results and discussion

3.1. Functional performance of delivery device

A summary of the functional performance of the formulation used in the study is presented in Table 1. Leuprolide unit spray content assays at both the beginning and end of the dosing period were well within acceptable limits ($100 \pm 4.0\%$) of label claim. Particle size distribution data and cumulative percentiles at certain preset effective cut-off diameters (ECD) were plotted on log-probability paper. From this plot, the mass median aerodynamic diameters (MMAD) for drug particles in the emitted drug spray were calculated. A summary of the results and the associated geometric standard deviation, σ_g, are reported in Table 1. The geometric standard deviation, σ_g, was calculated using a ratio of the diam-

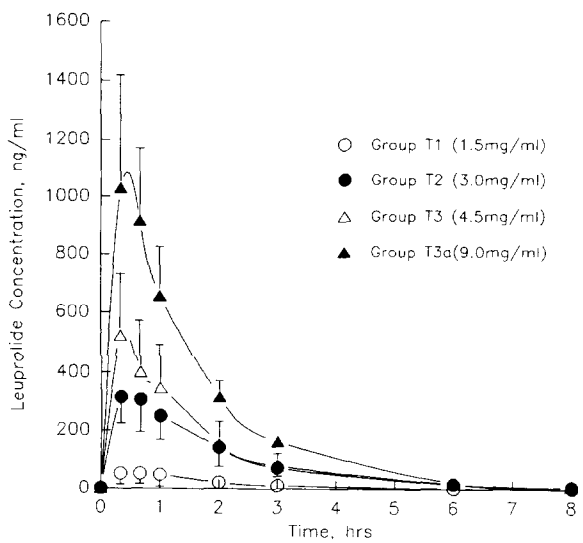

Fig. 1. Plasma profiles of leuprolide following multiple inhalation dosing to beagle dogs. Solid lines represent mean plasma concentrations on three days, respectively days 1, 7, and 14 following chronic administration of leuprolide acetate for 14 days.

Table 3
Mean plasma T_{max} following multiple dosing of suspension aerosol of leuprolide acetate to beagle dogs

Sampling protocol (treatment group)	Mean plasma $T_{max} \pm$ SD (h)			
	Day 1	Day 7	Day 14	Mean
1.5 mg/day (T1)	0.33 ± 0.00	0.50 ± 0.15	0.67 ± 0.00	0.54 ± 0.24
3.0 mg/day (T2)	0.50 ± 0.15	0.67 ± 0.00	0.33 ± 0.00	0.50 ± 0.00
4.5 mg/day (T3)	0.33 ± 0.00	0.33 ± 0.00	ND	0.33 ± 0.00
9.0 mg/day (T3a)	ND	ND	0.33 ± 0.00	0.33 ± 0.00

SD, standard deviation of mean values.

eters of particles represented by 15.87, 50, and 84.13% of the total spray, namely:

σ_d = 84.13% diameter/50% diameter

= 50% diameter/15.87% diameter

In general, the results demonstrated a nearly monodispersed distribution with respirable fraction (fractional dose estimated to deposit in the lung) being approx. 52%. There was no difference between particle size distribution results obtained before and after the 14 day dosing period. Valve performance as measured by the shot weight was consistent during the duration of the study.

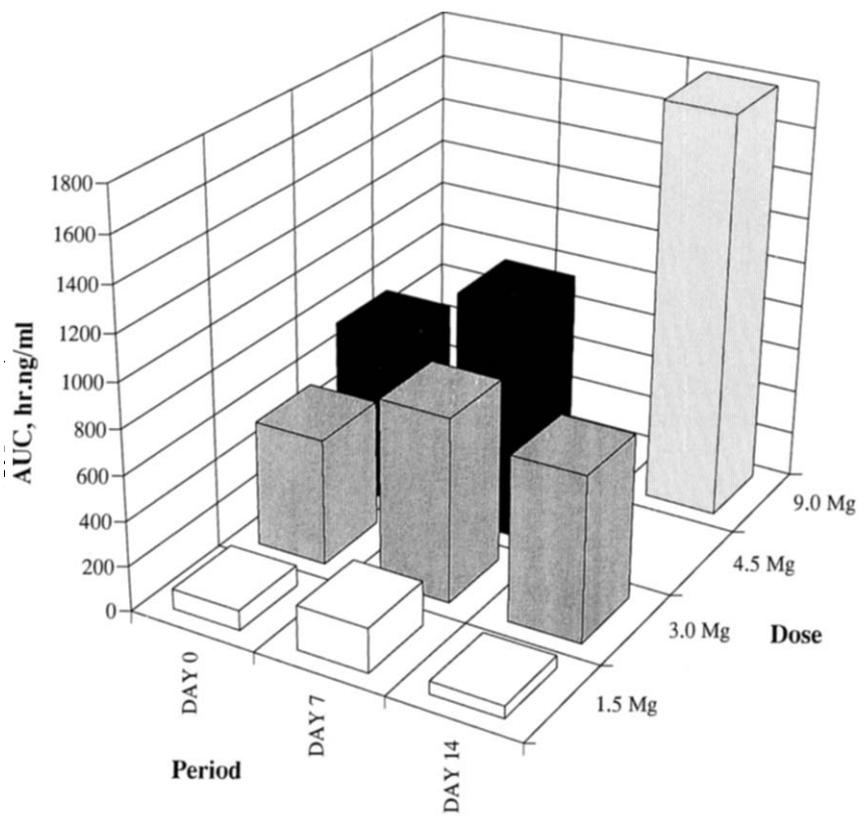

Fig. 2. Mean plasma AUC following multiple dosing of leuprolide aerosol to beagle dogs.

3.2. Plasma concentrations of leuprolide acetate

Plasma concentration time profiles for leuprolide acetate following inhalation delivery to the beagle dogs are summarized in Fig. 1. Results represent mean plasma concentrations pooled for all dogs (males and females together) on the basis of dose administered on days 1, 7, and 14. This was justified, firstly, because examination of the data showed neither meaningful sex differences nor period effects in the extent of absorption of leuprolide acetate from the lung. Secondly, the number of animals per group in this dose ranging study was small (i.e., two dogs per treatment group, each with three data points). Furthermore, the independence of absorption of leuprolide acetate from the airways on gender had been shown in a previous report (Adjei et al., 1990).

3.3. Time for peak plasma concentrations to occur, T_{max}

The time for peak plasma leuprolide concentrations to occur, T_{max}, were examined for all dose groups and found to be similar. Data for days 1, 7 and 14, were therefore pooled, and the mean T_{max} levels evaluated for differences among the respective dosage groups. The results are summarized in Table 3. Analysis of variance (ANOVA) on these mean results did not yield statistically significant differences.

3.4. Area under plasma concentration vs time curve, AUC

Fig. 2 summarizes the mean plasma AUC values for the respective dose groups in the study. This graph also summarizes the mean AUC values on days 1, 7, and 14. A summary of the results of repeated measures analysis of variance and pairwise comparisons is given in Table 4. There was no significant period effect, nor was the period by dose interaction statistically significant. Results within each dose group were therefore pooled and the mean AUC value was computed over days 1, 7, and 14 for each dose group. The mean AUC value was statistically significantly different from zero for the 3.0 and 4.5/9.0

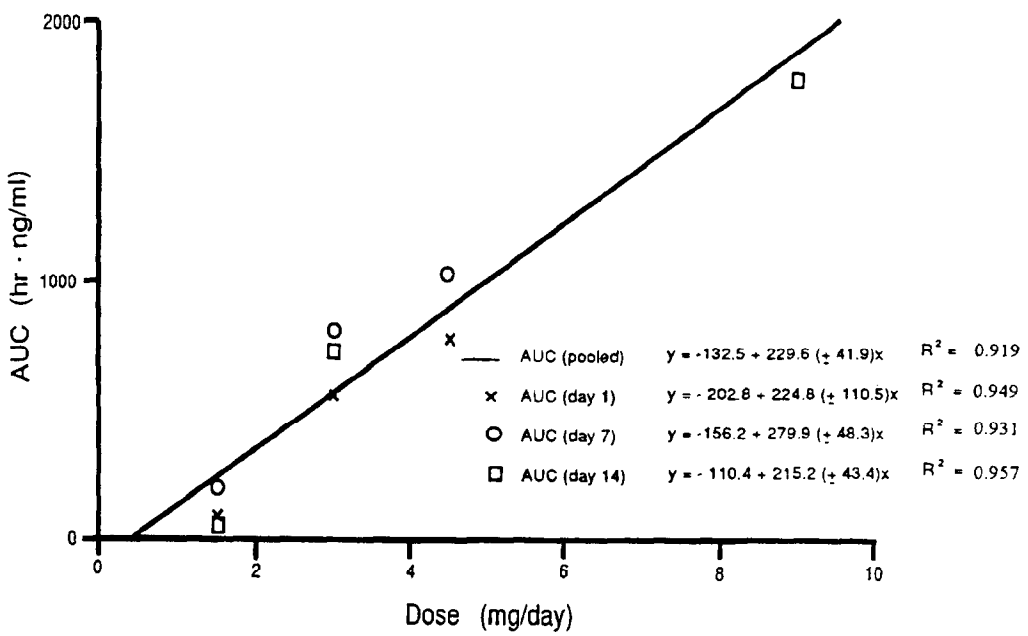

Fig. 3. Pulmonary bioavailability of leuprolide in beagle dogs: regression of AUC on dose. The daily dose for group T3 was raised from 4.5 to 9.0 mg from day 8 to 14. The group was re-identified as group T3a and assigned a mean dose of 6 mg/dog in the statistical analysis.

Table 4
Results of repeated measures analysis of variance on plasma AUC and of pairwise comparisons

Repeated measures analysis of variance			
Source	DF	Mean square	F ratio
Between dogs	5		
Doses	2	1 760 668	21.89
Dogs (doses)	3	80 421	($p = 0.0162$)
Within dogs	12		
Periods	2	224 921	2.45 ($p = 0.2089$)
Periods × doses	4	190 734	2.08 ($p = 0.2612$)
Periods × dogs (doses)	6	91 303	

Pairwise comparisons of AUC (h ng ml^{-1})				
Treatment group	Day 1	Day 7	Day 14	Average
1.5 mg (T1)	89.2	197.7	55.3	114.1
3.0 mg (T2)	561.8	815.2	731.8	702.9 [a,b]
4.5/9.0 mg (T3 and T3a)	763.5	1037.4	1787.4	1196.1 [a,b]

[a] Significantly different from 0 at the 0.05 level.
[b] Significantly different from the 1.5 mg/day (T1) group at the 0.05 level.

mg/day groups. The pairwise comparisons of the mean AUC data showed significant differences between the low dose (1.5 mg/day) group and each of the remaining treatment groups (3.0 and 4.5/9.0 mg/day). The comparison between the medium dose (3.0 mg/day) and high dose (4.5/9.0 mg/day) groups yielded a near statistical significance ($p = 0.0571$).

Regression analysis of plasma AUC on daily dosage of leuprolide was performed. Group means from fitted regression lines of $AUC_{(0-8\ h)}$ on dose for days 1, 7 and 14 are shown in Fig. 3. The results represent both pooled (over days) and unpooled data. This graph demonstrates a linear dose-response relationship following pulmonary delivery of leuprolide within the dose range of 1.5–9.0 mg/dog per day. As reported in previous work (Adjei et al., 1990), absorption of leuprolide from the lung was rapid with mean peak plasma levels occurring within approx. 30 min after dosing for all dose groups. The regression analysis of the AUC data showed that except for day 1 the regression coefficient of AUC vs dose was significantly different from zero and that the data fitted a simple linear regression model, i.e., the test for lack of fit was not statistically significant. The regression analysis also showed that on days 1, 7, and 14, as well as on the pooled data, the regression coefficient of AUC vs dose was similar. None of the regression coefficients on days 1, 7, and 14 deviated more than 1.5-times the standard error of the regression coefficient obtained from the pooled data. This implies that the absorption kinetics for leuprolide acetate in this formulation did not change during the 14 day dosing period. This finding is significant because it also suggests that neither saturation nor physiologic changes in the absorption barrier were involved in the delivery of leuprolide across the airways to the systemic circulation.

3.5. Clinical pathology

The results showed no histologic, hematologic, or pathologic changes for either of the two high dose groups (group T3, administered 4.5 mg leuprolide acetate via inhalation aerosol daily for 7 days, and group T3a, administered 9 mg of leuprolide acetate via inhalation aerosol daily for 7 days following 7 days of 4.5 mg/day of leuprolide treatment). There were no treatment related changes in clinical signs observed during the study. Body weights, food consumption, and clinical chemistry parameters revealed no treatment-related abnormalities. Tissues were examined for microscopic lesions, but none were found demonstrating no adverse effects associated with the multiple dosing of the leuprolide aerosol suspension through the duration of the study.

4. Conclusions

Results from this investigation are in basic agreement with those in a previous study (Adjei et al., 1990) which utilized a solution aerosol of leuprolide acetate containing approx. 35% ethyl alcohol. However, in this earlier study a decrease in lung leuprolide acetate bioavailability was noticed when day 1 results were compared with day 14 plasma AUC data. It was proposed that the decrease in plasma levels may have been related to a tolerance build-up and/or increased enzy-

matic activity of the lung following chronic administration of the alcohol based solution aerosol formulation. No gross pathologic changes were observed at the end of the 14 day dosing period. However, mild microscopic and inflammatory reactions of the lung tissue were noted in post mortem examinations. Similar observations were noted with the placebo control group (alcohol-based propellant system without drug). Model-dependent changes, i.e., biophysical parameters and their effect on the breathing maneuver during lung deposition of the aerosol, were suggested as a second plausible explanation for the decrease in pulmonary bioavailability of leuprolide acetate from the solution aerosol formulation.

The delivery of a suspension aerosol formulation of leuprolide to beagle dogs resulted in significant plasma levels of the drug up to 8 h following drug administration. Results from the present study evaluated absorption of a suspension aerosol of leuprolide containing no alcohol. The results demonstrated good linearity and dose dependency of plasma AUC in the dose range of 1.5–9 mg/dog per day. Histopathology data after the 14 day treatment period were normal when tissue samples of the drug treated groups were compared with sham treated controls. Decreases in lung bioavailability, as reported in the previous study utilizing a solution aerosol (Adjei et al., 1990) of leuprolide, were not seen, suggesting that the mild microscopic and inflammatory reactions of the lung tissue resulting from the co-solvent (i.e., alcohol) may have affected absorption in the previous study.

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