A preclinical pharmacokinetic/pharmacodynamic approach to determine a dose of GnRH, for treatment of ovarian follicular cyst in cattle

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The objective of this study was to explore the value of a preclinical PK/PD approach to determine a gonadotropin-releasing hormone (GnRH) dose in cows using the pituitary LH response as a surrogate endpoint.

Using an indirect effect model with stimulation of the LH entry rate, the *in vivo* basic pharmacodynamic parameters of GnRH were determined. The EC_{50} of GnRH was 51 ± 16 pg/mL, the EC_{50} being the GnRH plasma concentration able to produce 50% of the maximum possible stimulation (S_{max}) of the hypophysis (S_{max} = 48 ± 13). From individual PK/PD parameters, the ED_{50} of GnRH, i.e. the estimated dose of GnRH required to determine half the maximum possible stimulating effect on LH release, was calculated to 62 μg/h per cow. Using the PK/PD model, the GnRH dose required to achieve a selected breakpoint value of 5 ng/mL for maximum LH concentration (surrogate value for LH concentration predicting clinical efficacy for cystic conditions), was 52 ± 18 μg and for a standard GnRH dose of 100 μg, the mean maximum plasma LH concentration predicted by the model was 7.22 ± 0.98 ng/mL.

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INTRODUCTION

Cystic follicles are one of the most common causes of reproductive failure in cattle (review by Peter, 2004). Ovarian cysts are defined as pathologic follicular structures (≥2.5 cm in diameter), which persist for 10 days or longer in the absence of a functional corpus luteum (CL) (Seguin *et al.*, 1976). When a cyst develops, the follicles continue to enlarge to an abnormal size instead of ovulating and transforming into a CL. The presence of ovarian cysts is associated with an abnormal pattern of estrus behavior (nymphomania, anestrus, etc.) and the cow is prevented from having a regular estrus cycle. This impairs reproductive efficiency because cows cannot become pregnant until the cyst regresses spontaneously or responds to luteolytic treatment.

It has been suggested that an important physiologic change in cows with cyst formation is the absence of a preovulatory luteinizing hormone (LH) surge because of a functional abnormality in the feedback regulation of LH secretion by estradiol (Kaneko *et al.*, 2002). It has also been reported that injection of unfractionated anterior pituitary extract is able to re-establish estrous cycles in cows with ovarian cysts (Casida *et al.*, 1944). Moreover, it is recognized that any product with LH activity, such as pregnant mare's serum gonadotropin (PMSG) or human chorionic gonadotropin (HCG), can be used to treat cystic conditions successfully. This exogenous LH activity causes luteinization of the cyst and the natural luteolytic process (or an injection of prostaglandin) will then cause luteal regression followed by a subsequent estrus and ovulation (Seguin *et al.*, 1976). However, PMSG and HCG are not without disadvantages because antibody formation can occur which may impede future re-treatment (because of their large molecular weights). In contrast to HCG and PMSG, administration of the gonadotropin-releasing hormone (GnRH) may produce a LH response similar to the preovulatory LH surge and may initiate estrus cycles in cows with ovarian follicle cysts (Kittok *et al.*, 1973), but is unlikely to stimulate an immune reaction (Peter, 2004).

The GnRH is a deca-peptide produced by GnRH neurons in the basal hypothalamus. It is released by the hypothalamus and travels via a portal circulatory system to the pituitary. Here, it stimulates the synthesis and release of follicle-stimulating hormone (FSH) and of LH from the anterior pituitary. GnRH has been synthesized and several GnRH products, including GnRH hydrochlorides and GnRH diacetate, are used by the i.v. or i.m. route at the recommended dosage regimen of 100 μg per cow. This dose was determined by classical dose-titration studies using a parallel dose design and typically comparing four dose concentrations (e.g. 0, 50, 100 and 200 μg). The use of a parallel design does not permit the establishment of a full dose–effect relationship and only one of the tested doses can be selected for a dose confirmation study (Toutain, 2002). Thus, nothing guarantees that this selected

(efficacious) dose is an optimal dose. The pharmacokinetic/pharmacodynamic (PK/PD) approach provides an alternative to conventional dose-titration (Toutain & Lees, 2004) without such a limitation. By testing a single dose, it is theoretically possible to establish the entire concentration–effect relationship and to estimate the three key PD parameters of a drug, i.e. efficacy, potency and sensitivity (slope of the concentration–effect relationship). Using this approach, a dose–effect relationship can readily be deduced and an optimal dose finally selected.

The objective of this study was to explore the value of a preclinical PK/PD approach to determine a GnRH dose in cows using the pituitary LH response as a surrogate effect. The optimal GnRH dose for treatment of a cystic cow was assumed to be the one, which was able to trigger a LH release similar to that of a natural LH surge.

MATERIAL AND METHODS

Animals

Lactating cows (Prim'Holstein) ($n = 12$) no more than 7 years old, weighing 597 ± 96 kg at the beginning of the study and displaying a normal estrus cycle were included in the trial. Animals were individually identified with numbered ear tags and were fed with a ration consisting of silage, hay and commercial concentrates. In addition, the cows were grazed on meadow during the period between the morning and evening milkings.

Products

Gonadorelin as the diacetate tetrahydrate (Cystoreline, CEVA Santé Animale, Libourne, France) was used. The test product was a solution ready for injection containing 5 mg of GnRH in 100 mL of 0.9% saline (Aguettant, Lyon, France).

Experimental design

Each cow was allocated to a treatment sequence (A, B, C, or D) according to an equilibrated orthogonal Latin square design for four periods (Jones & Kenward, 1998). The starting date of the animal phase (day 0) was the day of a behavioral estrus as detected by a veterinary clinical examination. Each subsequent cycle corresponded to a period of the orthogonal Latin square, day 0 of a new period corresponding to a new estrus.

Drug administration

The doses to be tested were administered as a single i.m. injection in the neck area, at day 13 (i.e. during the luteal phase). The i.m. injections were administered using needles (30 mm length × 1.0 mm diameter with an appropriate individual syringe of 2 or 5 mL according to the dose). One milliliter of the test product was injected for a dose of 50 μg per animal (treatment A), 2 mL for 100 μg (treatment B) and 4 mL for 200 μg (treatment C). A placebo group (treatment D) received 4 mL of saline.

Sampling procedure

Blood samples were collected from a jugular vein into heparinized tubes (Venoject, heparinate de lithium). Sampling times were 0 h (immediately before GnRH dose) and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 h after the GnRH dose. Plasma samples were collected, frozen and stored (cryotubes steriles Nalgenes) at approximately −80 °C.

Analytical assays

The GnRH concentrations were measured by radioimmuno assay (RIA) in duplicate aliquots after methanol extraction using the BDS antibody as previously described (Caraty *et al.*, 1995). Briefly, 500 μL of plasma were mixed with 2500 μL of methanol. The mixtures were left 1 h at 4 °C and after centrifugation (30 min, 3200 ***g***), the supernatant was poured off in 5 mL glass tubes and dried using a Speed Vac Savant concentrator (Savant Instruments, NY, USA). The pellet was then diluted in 1 mL of RIA buffer (0.01 M phosphate buffer containing NaCl 9 g/L, 0.1% of gelatin and 1% of sodium azoture) and kept frozen at −18 °C until assay. The working range was 4–4000 pg/mL. The recovery rate was 70–80%. The concentration of quantification was 2.1 pg/mL and the inter- and intra-day precision was <13%.

The LH concentration was measured by RIA as described by Pelletier *et al.* (1968). The concentration of quantification was 0.3 ng/mL and the inter- and intra-day precision was <13%.

Pharmacokinetic analyses

GnRH pharmacokinetics

Pharmacokinetic analyses of plasma GnRH concentrations were performed using a program for nonlinear regression analysis (WINNONLIN version 3.2, PHARSIGHT, Mountain View, CA, USA). GnRH concentrations in plasma were fitted to the following general polyexponential equation (Eqn 1):

$$C(t) = \sum_{i=1}^{n} Y_i \exp(-\lambda_i t) \quad (1)$$

In Eqn 1, $C(t)$ is the GnRH concentration (ng/mL) in plasma at time t (h), Y_i is the intercept of the ith exponential term and λ_i is the ith exponential term. Initial estimates were determined using the method of residuals (Gibaldi & Perrier, 1982). These initial estimates were refined by nonlinear regression. The data points were weighted according to Eqn 2:

$$W_i = 1/\hat{Y}_i \quad (2)$$

where, W_i is the weight of the ith observation and $\hat{Y}_i$ is the fitted value of the ith observation.

The number of exponents needed for each data set was determined by application of the Akaike's information criterion (Yamaoka *et al.*, 1978). The data collected from the three GnRH administrations were fitted together in order to determine a single set of PK parameters per animal.

A bi-exponential equation describing a one-compartment open model with first-order elimination from the central compartment was selected (Eqn 3):

$$C(t) = \frac{Ka_1 D \times K_{10}}{V_c(Ka_1 + Ka_{2i} - K_{10})}[\exp(-K_{10}(t)) - \exp-(Ka_1 + Ka_{2i})(t)] \quad (3)$$

In Eqn 3, Ka_1 is the first-order rate constant of absorption (estimated at the same value for the three dose concentrations), Ka_{2i} with $i = 1$, 2 or 3 for dose 50, 100 and 200 μg, is the rate constant expressing incomplete bioavailability (Fig. 1). D is the administered dose, V_c the volume of the central compartment and K_{10} the first-order rate constant of elimination. The relative bioavailabilities of the three dose concentrations are expressed by Eqn 4:

$$F(0 \text{ to } 1) = \frac{Ka_1}{Ka_1 + Ka_{2i}} \quad (4)$$

with Ka_1 and Ka_{2i} as defined above. It should be noted that Ka_1,Ka_{2i} and V_c are not separately identifiable and Eqn 4 does not give an absolute bioavailability but only allows the three curves to be scaled according to their relative bioavailabilities. Thus, individual V_c/F, the apparent volume of distribution was determined by Eqn 5:

$$\frac{V_c}{F} = \frac{V_c \times (Ka_1 + Ka_{2i})}{Ka_1} \quad (5)$$

The apparent plasma clearance ($Cl_{\text{GnRH,model}}/F$) was determined by Eqn 6

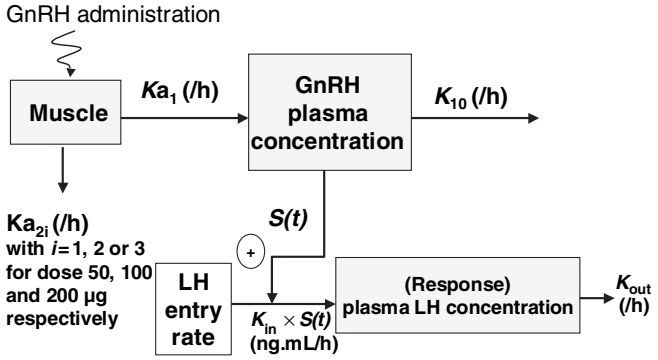

Fig. 1. Pharmacokinetic/pharmacodynamic (PK/PD) model selected to analyze the luteinizing hormone (LH) pituitary response to gonadotropin-releasing hormone (GnRH) administration. Three dose concentrations (50, 100 and 200 μg) of GnRH were injected by the i.m. route. The three corresponding curves were analyzed simultaneously with a common rate constant of absorption (Ka_1) but with an individual Ka_2 (i.e. Ka_{21}, Ka_{22}, and Ka_{23} for the 50, 100 and 200 μg doses respectively) in order to scale the disposition curves according to their relative bioavailabilities. K_{10} is the rate constant of LH elimination. The plasma GnRH concentrations generated by the model were used to simulate throughout an E_{max} function [$S(t)$] the entry rate of LH in plasma. K_{out} is the first-order rate constant of LH elimination.

$$Cl_{\text{GnRH,model}}/F = K_{10} \times V_c/F \quad (6)$$

with V_c/F given by Eqn 5.

Plasma terminal half-life was determined with Eqn 7:

$$t_{1/2} = 0.693/K_{10} \quad (7)$$

with K_{10} as defined above.

The area under the GnRH concentration vs. time curve ($AUC_{\text{GnRH,inf}}$) was computed using the linear trapezoidal rule. Extrapolation to infinity was determined by dividing the last measured GnRH concentration by K_{10}. The apparent GnRH clearance as obtained by trapezoidal rule ($Cl_{\text{GnRH, trapeze}}/F$) was determined with Eqn 8:

$$Cl_{\text{GnRH,trapeze}}/F = \text{Dose}/AUC_{\text{GnRH,inf}} \quad (8)$$

with $AUC_{\text{GnRH,inf}}$ as defined above. Data from an i.v. study were available and the plasma clearance and the absolute bioavailability were calculated for four cows (CEVA, unpublished report).

The GnRH exposure vs. LH response relationship

The LH response to GnRH administrations was assessed by measuring the *AUC* of the plasma LH concentrations from time 0 (time of GnRH dose) to 6 h post-GnRH administration, i.e. AUC_{LH} (0–6 h). No correction was made to account for the basal LH concentration. C_{max} and T_{max} for LH were determined directly from raw data.

The relationship between the GnRH exposure ($AUC_{\text{GnRH,inf}}$) and the LH response (AUC_{LH}, 0–6 h) was explored with a classical E_{max} model (Eqn 9):

$$AUC_{\text{LH}}, (0 - 6\text{h}) = E_0 + \frac{AUC_{\text{LH,max}} \times AUC_{\text{GnRH,inf}}}{AUC_{50}(\text{GnRH}) + AUC_{\text{GnRH,inf}}} \quad (9)$$

where, E_0 is the control *AUC* of LH over 6 h determined after the saline administration, $AUC_{\text{LH,max}}$ is the maximum LH response expressed in terms of *AUC* of LH over the first 6 h following a GnRH administration. AUC_{50}(GnRH) is a measure of the GnRH potency and corresponds to the GnRH exposure required to achieve half the maximum response, i.e. half $AUC_{\text{LH,max}}$; and AUC_{LH},(0–6 h) and $AUC_{\text{GnRH,inf}}$ are as defined above. E_0, $AUC_{\text{LH,max}}$ and AUC_{50}(GnRH) were determined by nonlinear regression.

From the AUC_{50}(GnRH) the total GnRH dose corresponding to the ED_{50} of GnRH, i.e. the GnRH dose required to achieve 50% of the maximum LH response was determined by Eqn 10:

$$ED_{50} = \frac{Cl_{\text{GnRH,trapeze}} \times AUC_{50}(\text{GnRH})}{F} \quad (10)$$

where $Cl_{\text{GnRH,trapeze}}/F$ is as previously determined (Eqn 8) and AUC_{50}(GnRH) as determined with Eqn 9.

The same approach was used to establish the relationship between GnRH exposure and the maximum LH concentration (Eqn 11):

$$C_{max} \text{ observed for LH} = E_0 + \frac{C_{max}, \text{LH} \times AUC_{\text{GnRH,inf}}}{AUC_{50}(\text{GnRH}) + AUC_{\text{GnRH,inf}}} \quad (11)$$

with E_0, the control value of plasma LH concentration (saline administration), and $C_{max,\ LH}$ the maximum possible LH concentration. The other parameters are defined as for Eqn 9. The dose required to achieve a given maximum plasma LH concentration was computed by solving Eqn 11 to estimate the corresponding $AUC_{GnRH,inf}$ and then, the corresponding dose was computed with Eqn 12:

$$\text{Dose to achieve a LH breakpoint} = \frac{Cl_{GnRH,trapeze} \times \text{LH breakpoint}}{F} \quad (12)$$

PK/PD modeling

The estimated GnRH PK parameter sets determined as previously described (Eqn 3) were used as constants in the integrated PK/PD model. To account for the apparent time delay between the observed plasma GnRH concentration profiles and the development of the LH response in time, the PK/PD relationship was described using a model for indirect response as proposed by Dayneka *et al.* (1993). It was assumed that the measured LH response was due not only to an immediate release of LH from a pre-existing available hypophysial pool but, instead, that the binding of GnRH to its hypophyseal receptor, triggers a cascade of events causing LH-containing vesicles to fuse with the plasma membrane for hormone exocytosis (Conn *et al.*, 1987).

Under these conditions, the observed delay between the PK of the plasma GnRH concentrations and the time development of the LH response is not of distributional origin but rather reflects the intrinsic temporal responsiveness of the system according to Eqn 13

$$\frac{dLH}{dt} = K_{in} \times S(t) - K_{out}LH(t) \quad (13)$$

where, dLH/dt represents the rate of variation in plasma LH concentration. The model assumes that the observed plasma LH concentration results from an equilibrium between LH secretion rate and LH elimination rate. The LH secretion rate is reflected by K_{in}, an apparent zero-order production rate of the response (here expressed in terms of LH concentration per time unit) and K_{out} is the first-order rate constant for LH elimination (i.e. LH disposition is assumed to be a first-order process). The action of GnRH consists of stimulating K_{in} throughout the function $S(t)$. $S(t)$, the stimulating effect of GnRH, is described by Eqn 14:

$$S(t) = \frac{S_{max} \times C(t)^n}{EC_{50}^n + C(t)^n} \quad (14)$$

where, EC_{50} is the plasma GnRH concentration producing 50% of the maximum possible stimulation (S_{max}), S_{max} being a positive number and $C(t)$, the plasma GnRH concentration vs. time; n is the Hill coefficient giving the slope of the concentration–effect relationship.

Incorporation of the stimulatory function into Eqn 13 gives a stimulatory PD model (Fig. 1), as expressed in Eqn 15:

$$\frac{dLH}{dt} = K_{in}\left(1 + \frac{S_{max} \times C(t)^n}{EC_{50}^n + C(t)^n}\right) - K_{out} \times LH \quad (15)$$

Before GnRH administration, LH concentrations result from initial steady-state conditions and are equal to K_{in}/K_{out}.

The ED_{50} of GnRH, which is the GnRH dose producing half S_{max}, was calculated using Eqn 16.

$$ED_{50} = \frac{Cl_{GnRH,trapeze} \times EC_{50}}{F} \quad (16)$$

where, $Cl_{GnRH,\ trapeze}$ is the apparent body clearance of GnRH as determined by Eqn 8, and EC_{50}. The EC_{50} of GnRH as determined by the PK/PD model.

Statistical analysis

Statistical analysis was performed with STATGRAPHICS PLUS version 4.1, professional computer program (Manugestics, Inc., Rockville, MD, USA).

Results are presented as mean ± SD; dose proportionality for GnRH (linearity of the GnRH disposition) was tested by a two-way ANOVA with the dose concentration and cow as factors. $P < 0.05$ was considered as significant.

RESULTS

Figure 2 presents the semi-logarithmic plot of the plasma GnRH concentration vs. time after the i.m. administration of GnRH diacetate at 50, 100 and 200 μg doses in the 12 cows. GnRH concentration was detected up to 1–2.5 h after administration of the 50 and 100 μg doses and up to 4–5 h post-administration for the 200 μg dose. Visual inspection of the three curves reveals the parallelism of the three terminal slopes. $AUC_{GnRH,inf}$ values were 48 ± 15, 88 ± 33 and 221 ± 51 pg·h/mL. For C_{max}, the corresponding values were 60 ± 35, 124 ± 46 and 267 ± 115 ng/L. In most instances, T_{max} was observed at the first sampling time (0.25 h). After scaling by the administered dose, $AUC_{GnRH,inf}$ and C_{max} were subjected to an ANOVA to test the hypothesis of dose proportionality. There was

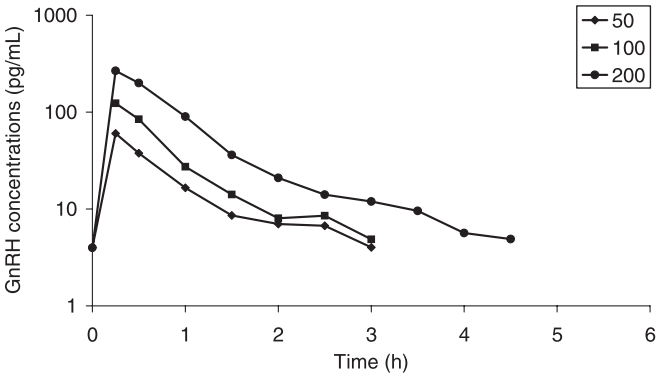

Fig. 2. Semi-logarithmic plot of plasma gonadotropin-releasing hormone (GnRH) concentration vs. time after i.m. administration of GnRH doses of 50, 100 and 200 μg *in toto* in dairy cattle during the luteal phase (SD bars are omitted for clarity).

Table 1. Mean ± SD GnRH pharmacokinetic parameters for 12 cows after i.m. administration of GnRH at doses of 50, 100 and 200 μg

Parameter	Mean ± SD
V_c/F (L/kg)	1.43 ± 0.76
Ka (per h)	22.37 ± 12.9
Ka_1 (per h)	14.0 ± 9.01
K_{10} (per h)	1.71 ± 0.56
$T_{1/2}$ K_{10} (h)	0.46 ± 0.20
Clearance/F (mL/kg/min)	43.18 ± 35.99

Data collected for the three dose concentrations were fitted simultaneously to give a common set of pharmacokinetic parameters.
V_c/F, apparent volume of distribution as given by Eqn 5; Ka, $Ka_1 + Ka_2$ is the overall rate of gonadotropin-releasing hormone (GnRH) removal from the injection site; Ka_1 specifically expresses the rate of GnRH absorption; K_{10}, rate constant of GnRH elimination; $t_{1/2}$ K_{10}, plasma half-life; Clearance/F, apparent clearance determined by $K_{10} \times V_c/F$.

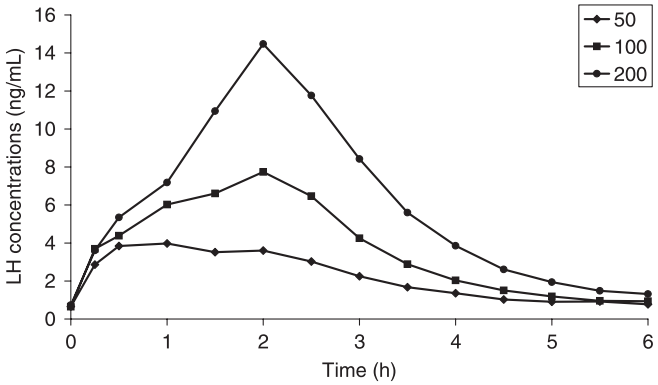

Fig. 3. Arithmetic plot of plasma luteinizing hormone (LH) concentration vs. time after i.m. administration of gonadotropin-releasing hormone (GnRH) doses of 0 (saline), 50, 100 and 200 μg *in toto*, in 12 dairy cows during the luteal phase (SD bars are omitted for clarity).

no significant effect of the dose ($P > 0.05$) indicating that the disposition of GnRH was linear.

Table 1 presents the values of the GnRH PK parameters as determined by the simultaneous fitting of the plasma GnRH concentration obtained for the three dose concentrations of GnRH. The terminal half-life was 0.46 ± 0.20 h and the apparent GnRH clearance ($Cl_{GnRH,\ model}/F$) was 43 ± 36 mL/kg/min.

For the four cows for which the i.v. data were available the plasma clearance was 24.84 ± 5.97 mL/kg/min (i.e. 1053.5 ± 253.5 L/h) and the absolute bioavailability was 89 ± 25%.

Table 2 presents PK parameters determined by the noncompartmental approach for each dose. The apparent clearance ($Cl_{GnRH,\ trapeze}/F$) was 35 ± 18 mL/kg/min, i.e. a value not significantly different from that determined by the modelling approach.

Figure 3 presents the arithmetic plots of the mean plasma LH concentration following i.m. dose of 50, 100 and 200 μg of GnRH in 12 cows. Visual inspection of Fig. 3 shows that the LH response increases with the administered GnRH dose. For the 100 and 200 μg GnRH doses, a peak plasma LH concentration was clearly identified approximately 2 h post-GnRH dose and, after a delay of 6 h, the plasma LH concentrations had returned to control values for all three GnRH doses. The AUC_{LH},(0–6 h) values were 5.21 ± 2.14, 16 ± 4.6, 25 ± 8.3 and 40 ± 10.3 ng/h/mL for the 0, 50, 100 and 200 μg GnRH doses, respectively. The control LH concentrations were 0.869 ± 0.358 ng/mL and the maximum concentrations achieved after the 50, 100 and 200 μg GnRH doses were 4.6 ± 1.2, 8.9 ± 4.7 and 16.0 ± 6.3 ng/mL, respectively. These C_{max} were determined at 1.4 ± 0.87, 1.9 ± 0.6 and 1.9 ± 0.3 h for the 50, 100 and 200 μg GnRH doses, respectively.

Table 2. Mean ± SD GnRH pharmacokinetic parameters for 12 cows after i.m. administration of GnRH at doses of 50, 100 and 200 μg

	Doses (μg *in toto*)		
Parameters	50	100	200
AUC_{inf} (ng/h/L)	48 ± 15	88 ± 33	221 ± 51
C_{max} (ng/L)	60 ± 35	124 ± 46	267 ± 115
T_{max} (h)	0.27 ± 0.07	0.25 ± 0.0	0.29 ± 0.10
Clearance/ F (mL/kg/min)	33.82 ± 18.00	44.53 ± 43.96	27.30 ± 8.54

The parameters were determined using a model-independent approach.
AUC_{inf}, total area under the gonadotropin-releasing hormone (GnRH) concentration vs. time curve calculated by the trapezoidal rule; C_{max}, maximum plasma GnRH concentration; T_{max}, time of maximum plasma GnRH concentration; Clearance/F, apparent GnRH clearance.

Figure 4 represents the dose–effect and the exposure–effect relationships between the GnRH dose or the GnRH exposure vs. AUC_{LH},(0–6 h) following the GnRH administrations. Visual inspection of Fig. 4 indicates the existence of both dose–effect and exposure–effect relationships. When the dose–effect relationship was analyzed by an ANOVA with dose as the main factor followed by a Bonferroni test for pairwise mean comparisons, the dose of 100 μg gave a significantly greater response than the dose of 50 μg but the doses of 100 and 200 μg were not significantly different ($P > 0.05$). This suggests that the appropriate dose for GnRH is 100 μg (see Discussion). On the contrary, using the E_{max} model (Eqn 9), the estimated maximum possible response to GnRH administration over the subsequent 6 h was 57.15 ng·h/mL (corresponding to a mean increase in plasma LH concentration of 9.52 ng/mL) and the estimated GnRH potency, calculated from the exposure required to achieve half the maximum LH effect was 167 pg·h/mL. Using Eqn 10 and the mean apparent GnRH clearance determined by the trapezoidal rule in the 12 cows (1213 L/h), the ED_{50} for GnRH to achieve half the maximum effect was 203 μg *in toto*.

Figure 5 represents the exposure–effect relationship between GnRH exposure and the observed maximum plasma LH concentration. Using the E_{max} model (Eqn 11), the estimated maximum plasma LH concentration that can be produced by GnRH was 26 ng/mL and the GnRH potency for this end-point was 194 pg·h/mL. Using Eqs 11 and 12, the estimated GnRH doses required to achieve the selected plasma LH concentration breakpoints of 5 and 10 ng/mL used as the surrogate to predict

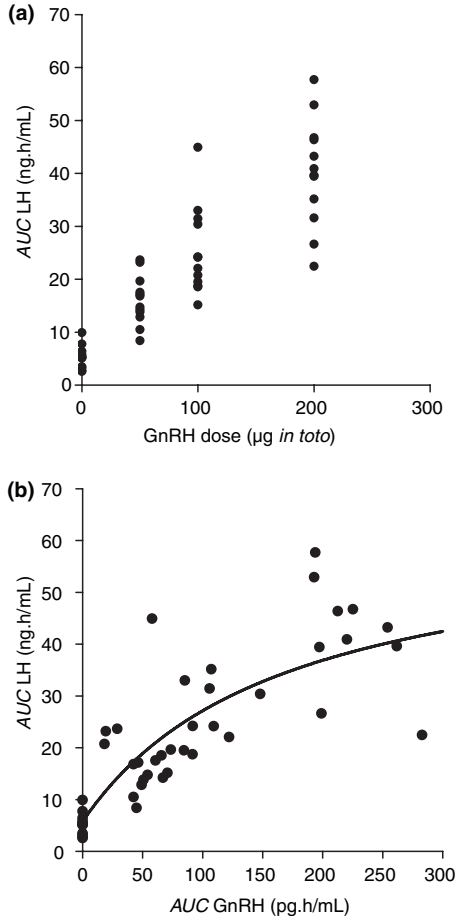

Fig. 4. Dose or exposure–effect relationship. Top panel (a): the classical dose–effect relationship is plotted with the gonadotropin-releasing hormone (GnRH) dose (0, 50, 100 or 200 µg *in toto*) as the independent variable. When analyzed as a parallel design (i.e. ignoring that each cow was tested four times), this design is unable to provide information on the shape of the individual dose–response curves and the effective dose is imposed by the statistical analysis, i.e. by testing the null hypothesis with an ANOVA. Bottom panel (b): the dose, as explicative variable, has been replaced by the individual exposure which allows better characterization of the mean response curve and computing of the maximum effect and drug potency. This approach is a 'naive pooled data approach' and does not guarantee that the individual concentration–effect relationship has a similar shape to the mean shape.

clinical efficacy of GnRH in cystic ovarian disease (see Discussion) were 44.3 and 126 µg, respectively.

All 12 cows were successfully analyzed using the PK/PD modeling of GnRH effect on LH secretion. Figure 6b shows the plasma LH concentration time-course (observed and fitted) and the time course of plasma GnRH concentration for a representative cow. The maximum stimulating effect of GnRH (S_{max}) was 47.6 ± 13.0 (a scalar). The control LH entry rate (K_{in}, see Eqn 13) was 0.416 ± 0.150 µg/L/h. K_{out}, i.e. the elimination rate of LH was 0.784 ± 0.269/h, corresponding to a mean half-life for LH of approximately 0.89 h. The EC_{50} of GnRH was 51.0 ± 15.6 pg/mL. The shape of the concentration–effect relationship (Hill coefficient) was 1.27 ± 0.57. Individual cow parameters are given in Table 3.

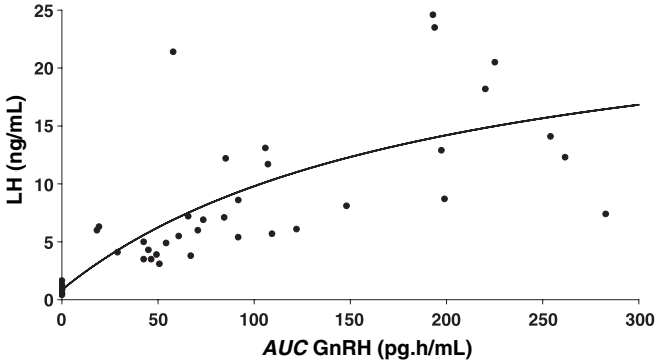

Fig. 5. Exposure–effect relationship between gonadotropin-releasing hormone (GnRH) area under the curve (*AUC*) and the maximum plasma luteinizing hormone (LH) concentration; using an E_{max} model, the estimated maximum possible LH value is 26 ng/mL.

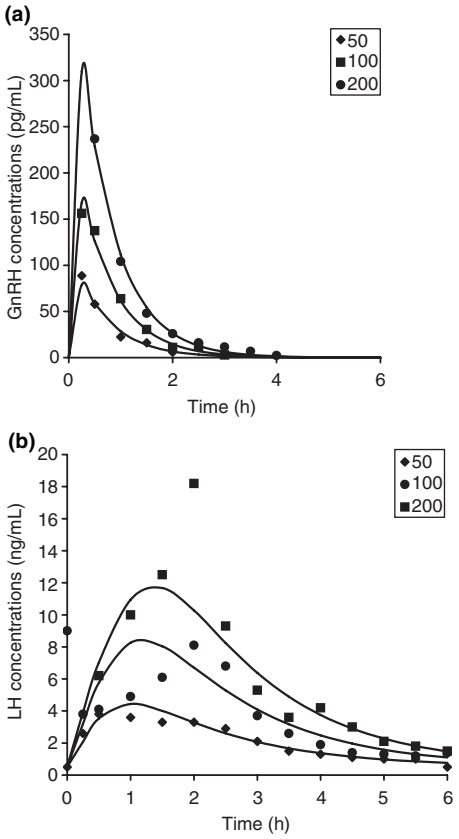

Fig. 6. Observed and fitted values for gonadotropin-releasing hormone (GnRH) (upper panel) and plasma luteinizing hormone (LH) concentration (lower panel) for a representative cow. Pharmacokinetic (GnRH) and pharmacodynamic (LH) data were determined after administration of GnRH at doses of 50, 100 and 200 µg *in toto*. The three dose concentrations were fitted together using an indirect effect of PK/PD model.

From the individual PK/PD parameters, the ED_{50} of GnRH, i.e. the estimated dose of GnRH required to determine half the maximum possible stimulating effect on LH release, was 104 ± 63 ng/h/kg, i.e. a mean value of 62 µg/h per cow.

Table 3. Pharmacodynamic parameters for 12 cows after i.m. administration of GnRH at doses of 50, 100 and 200 μg

Cows	1	2	3	4	5	6	7	8	9	10	11	12	Mean ± SD
EC_{50} (pg/mL)	46.60	73.56	54.46	47.52	38.14	40.45	82.71	22.03	53.42	50.18	52.19	50.3	50.96 ± 15.60
S_{max} (no unit)	51.98	50.19	44.53	31.73	40.65	40.37	57.18	51.91	24.50	42.91	70.72	64.07	47.56 ± 13.00
K_{in} (ng/mL/h)	0.32	0.32	0.36	0.45	0.42	0.34	0.27	0.43	0.62	0.76	0.46	0.23	0.42 ± 0.15
K_{out} (per h)	0.58	0.61	0.81	0.89	0.77	0.63	0.48	1.61	0.85	0.91	0.73	0.55	0.78 ± 0.30
n	0.67	1.82	1.24	1.85	2.4	1.12	1.45	0.62	1.06	0.85	0.56	1.55	1.27 ± 0.57
LH_0 (ng/mL)	0.55	0.52	0.45	0.51	0.55	0.54	0.56	0.27	0.72	0.84	0.63	0.42	0.55 ± 0.14
ED_{50} (ng/h/kg)	117	83	131	108	52	56	159	30	93	96	267	63	104 ± 63

The parameters were determined from the simultaneous fitting of the three dose concentrations of gonadotropin-releasing hormone (GnRH).
EC_{50}, GnRH concentration that produces 50% of the maximum stimulating effect on luteinizing hormone (LH) production rate; S_{max}, maximum stimulating effect on LH production rate attributed to GnRH; K_{in}, apparent zero-order rate constant for the production of LH; K_{out}, first-order rate constant for the elimination of LH; n, Hill constant giving the shape of the concentration–effect relationship; LH_0, baseline LH concentration obtained by the ratio K_{in}/K_{out}; ED_{50}, computed from individual apparent clearance (Cl/F) as determined by the trapezoidal rule and EC_{50}.

By comparing the ED_{50} obtained by the PK/PD approach, i.e. an ED_{50} per hour (62 μg/h) to the ED_{50} for the overall LH response (203 μg), it can be deduced that a 50% maximum stimulation of the hypophysis is equivalent to a 3.27 h secretion of GnRH at a rate of 104 ng/h/kg.

Using the PK/PD model, the GnRH dose required to achieve a selected breakpoint value of 5 ng/mL for maximum LH concentrations (surrogate value for the LH concentration predicting clinical efficacy for the cystic condition, see Discussion) was 52 ± 18.4 μg and for a standard dose of 100 μg, the mean maximum plasma LH concentration predicted by the model was 7.22 ± 0.98 ng/mL.

DISCUSSION

The present experiment demonstrates that a PK/PD approach can be successfully applied to determine *in vivo* the three basic PD parameters of GnRH, i.e. its potency to stimulate LH secretion, the maximum possible stimulation of the hypophysis (efficacy) and the shape of the dose–effect relationship (sensitivity).

The main objective of this trial was to assess the value of titrating the dose–effect relationship of GnRH preclinically in healthy cyclic cows during the luteal phase, using the hypophyseal release of LH as a surrogate end-point and to select a GnRH dose for subsequent clinical confirmation.

The LH pituitary response was selected as a surrogate because the plasma LH concentration offers ideal properties for a surrogate: it is graded, sensitive, continuous and is an objectively measurable end-point. In addition, it has critical pathophysiologic significance, as the primary cause of cystic follicles is a deficiency in the preovulatory surge of LH (Peter, 2004). Our hypothesis was that a dose of GnRH able to trigger a LH response similar to a spontaneous preovulatory LH surge should provide an appropriate starting GnRH dose for clinical confirmation. A prerequisite to this approach is that the pituitary responsiveness to GnRH during the luteal phase is similar in cystic and healthy cows. This is likely to be the case because the magnitude of the LH response observed in the present experiment at a dose of 100 μg was very similar to the one described in cystic cows by Garverick *et al.* (1976).

A linear log–dose relationship was concluded by others who used a conventional GnRH titration design in cycling cows at the mid-luteal stage (Webb *et al.*, 1977). As explained by Toutain (2002) a dose–effect relationship cannot be unequivocally established using a conventional parallel design. The present experiment used the overall GnRH exposure (continuous explicative variable), rather than the dose concentration (discrete explicative variable), to explain the LH response. This approach indicated that the relationship was actually curvilinear and it allowed estimation of both GnRH efficacy (i.e. the maximum possible effect in terms of LH release) and GnRH potency (i.e. the GnRH exposure necessary to achieve half the maximum LH release). The estimated average maximum possible increase in LH concentration, during a period of 6 h, was approximately 10 ng/mL. Expressed in terms of maximum possible plasma LH concentration, the efficacy of GnRH was 26 ng/mL.

In order to select a GnRH dose using the aforementioned relationships, it is necessary to establish some LH breakpoints, i.e. LH response (overall release or maximum plasma LH concentration) that are predictive of GnRH efficacy to treat cystic conditions. Our hypothesis was that the appropriate GnRH dose would be the one able to trigger a LH release mimicking a natural preovulatory LH surge. The duration of the LH surge is usually about 12 h with a maximum plasma LH concentration maintained for approximately 10–12 h. In the conditions of this investigation, it appears that GnRH was able to increase the maximum plasma LH concentration to a level comparable with that determined during a preovulatory surge. Irrespective of dose, however, the LH response lasted for only 6 h, i.e. about half the duration of a natural preovulatory surge. These results are in agreement with those of Lucy and Stevenson (1986) who compared, in the same trial, the spontaneous LH surge in cattle (heifer, dairy cows) and the LH release induced by GnRH (Cysterelin from CEVA, 100 μg). They showed that the duration of the LH surge in GnRH-induced animals (6.1 ± 0.8 vs. 11.0 ± 0.71 h) was signifi-

cantly shorter than the preovulatory LH surge. However, the maximum plasma concentration was slightly higher in GnRH-induced (19.8 ± 4.0 ng/mL) than in control animals (15.2 ± 3.7 ng/mL). Although it is not possible to determine a 12 h surge of LH with administered GnRH, it was shown that ovulation could be induced in 90% of cows treated with GnRH for a maximum plasma LH concentration of 6.6 ± 4.3 ng/mL (Bentley *et al.*, 1998).

Such a maximal LH concentration (from 5 to 10 ng/mL), according to our model (see Eqns 11 and 12) can be determined with average GnRH doses of 44 and 126 μg, respectively. These doses bracket the dose (100 μg) selected as the classical dose.

This approach using the overall (*AUC*) GnRH exposure as an explicative variable to compute a GnRH dose requires the experimental investigation of many animals and also of different dose levels in order to generate a series of GnRH exposures with their associated LH responses that are able to cover the entire range of the exposure–effect relationship. In the present experiment, we used 48 couples of individual GnRH exposures vs. LH responses to build the exposure vs. effect relationship and to estimate a single set of PD parameters. This is rather demanding and this approach is cumbersome for documenting the inter- and intra-animal variability of the response to a GnRH treatment (e.g. to qualify the responsiveness of the GnRH treatment taking account of hormonal status, etc.). A more advanced approach in data analysis allowed us to circumvent this difficulty. It consists of analyzing individual data sets using a PK/PD modeling approach, i.e. recognizing that individual plasma GnRH concentration–time and individual effect–time profiles allow for single-sweep coverage of the entire concentration–effect relationship. Thus, individual sets of PD parameters may be computed for subsequent statistical analysis. Alternatively, all the animals can be analyzed simultaneously using a nonlinear mixed effect model including relevant covariables to explain inter-animal variability. Another advantage of this individual modeling approach is that it takes into account information on the time development of the LH response, allowing time to become a second independent variable. For this reason, the PK/PD analysis enabled us to compute, not an overall GnRH dose, but instead, a GnRH dosage (dose per time unit). For the present experiment, this was not essential because GnRH is a single dose treatment but for drugs requiring multidose treatments, a PK/PD trial provides the most suitable method for simultaneously determining the two main components of a dosage regimen, i.e. dose and dosage interval.

This PK/PD approach can also be a useful tool for mechanistic purposes. For example, the potency of GnRH was evaluated from its EC_{50} to be approximately 50 pg/mL, i.e. 42 pM. This value may be directly compared with those obtained from cultured pituitary cells. For example, an *in vitro* EC_{50} of 210 pM was reported for *in vitro* GnRH stimulation of LH release in sheep (Millar *et al.*, 1989), which is consistent with the *in vivo* values collected presently in cattle. Similarly, the PK/PD model was able to qualify the efficacy of GnRH on LH synthesis/release by estimating a possible 47-fold increase of the LH production rate. These parameters could prove valuable to physiologists for explaining the influence of hormonal status on pituitary function, and on the possibility or not of depleting the pituitary gland.

The ultimate goal of the present experiment was to determine a suitable GnRH dose to treat cystic conditions in cows. The dose selected for clinical confirmation could be the one that guarantees, in most cows, production of a critical maximum plasma LH concentration of 5 ng/mL (Bentley *et al.*, 1998). Using this approach the proposed GnRH dose would be 74 μg *in toto*. Alternatively, a dose of 100 μg guarantees in all cows that a maximum LH concentration higher than 5.86 ng/mL is produced.

In conclusion, the present experiment demonstrates the feasibility of selecting for clinical confirmation a dosage regimen for GnRH in cows using a PK/PD approach with the LH response as the surrogate. In addition, the present approach illustrates the advantages of a more advanced design and data analysis over the conventional dose-titration study.

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