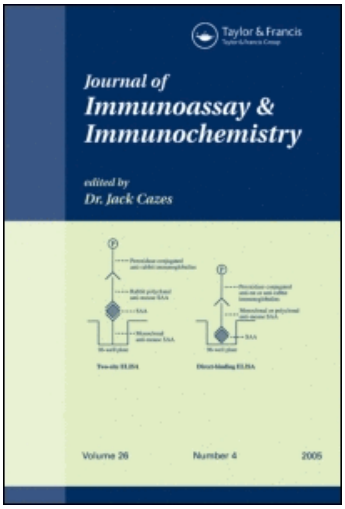

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Radioimmunoassay of Antarelix™, a Luteinizing Hormone Releasing-Hormone Antagonist, in Plasma and Its Application for Pharmacokinetic Study in Dogs

Suzie Sorensen[a]; Jean-Jacques Rondeau[a]; Vincent Lenaerts[b]; François Boutignon[b]; Patrick Wüthrich[b]; Romano Deghenghi[b]; Albert Adam[a]; Huy Ong[a]
[a] Faculté de Pharmacie, Succ, Université de Montréal, Montréal, Québec, Canada [b] Europeptides, Argenteuil, France

To cite this Article Sorensen, Suzie , Rondeau, Jean-Jacques , Lenaerts, Vincent , Boutignon, François , Wüthrich, Patrick , Deghenghi, Romano , Adam, Albert and Ong, Huy(1996) 'Radioimmunoassay of Antarelix™, a Luteinizing Hormone Releasing-Hormone Antagonist, in Plasma and Its Application for Pharmacokinetic Study in Dogs', Journal of Immunoassay and Immunochemistry, 17: 3, 205 — 226
To link to this Article: DOI: 10.1080/01971529608005789
URL: http://dx.doi.org/10.1080/01971529608005789

JOURNAL OF IMMUNOASSAY, 17(3), 205-226 (1996)

RADIOIMMUNOASSAY OF ANTARELIX™, A LUTEINIZING HORMONE RELEASING-HORMONE ANTAGONIST, IN PLASMA AND ITS APPLICATION FOR PHARMACOKINETIC STUDY IN DOGS

Suzie Sorensen[1], Jean-Jacques Rondeau[1], Vincent Lenaerts[2], François Boutignon[2], Patrick Wüthrich[2], Romano Deghenghi[2], Albert Adam[1] and Huy Ong[1]
[1] Université de Montréal, Faculté de Pharmacie, Succ. Centre-Ville, C.P. 6128 Montréal, Québec, Canada,
[2] Europeptides, 95108 Argenteuil, France

ABSTRACT

A procedure for the radioimmunoassay (RIA) of Antarelix™ (teverelix) in plasma has been developed for the pharmacokinetic study of this potent LHRH antagonist in dogs. Antiserum was produced by coupling the deamidated Antarelix analog to bovine serum albumin by a carbodiimide reaction and immunizing rabbits with the conjugate. The crossreactivity of the antiserum with LHRH, LHRH agonist Metereline and LHRH antagonists tested was negligible, except for Antide which displayed a crossreactivity of 33%. No crossreactivity with Antarelix metabolites could be detected. The RIA is suitable for the direct determination of Antarelix in plasma, with a minimum detectable level of 1.12 fmol/assay. The accuracy and precision of the method were assessed with plasma samples spiked with Antarelix at concentrations ranging from 0.4 to 6.4 pmol/ml. The recovery was 104.8% with intra- and interassay CV between 1 and 3.7%. Pharmacokinetic profiles of Antarelix in dogs were established following an IV or a SC dose of 10 μg/kg.

(KEY WORDS: Radioimmunoassay, Antarelix™, Teverelix, LHRH Antagonist, Pharmacokinetics)

INTRODUCTION

It is well established that LHRH analogs are used in the clinical management of steroid dependent neoplasms such as prostatic cancer and endometriosis (1). In order to avoid the initial stimulatory phase of gonadotropins elicited by LHRH agonists prior to the pituitary LHRH receptor down regulation, LHRH antagonists have been developed. In fact, LHRH antagonists have induced an immediate inhibitory effect on gonadotropins secretion which is potentially advantageous in the treatment of hormonally dependent conditions such as prostatic cancer (2). However, most LHRH antagonists display histamine-like side-effects (1). Many efforts have been placed in the design of potent LHRH antagonists with reduced local and systemic allergic responses. A new LHRH antagonist, Antarelix™ (teverelix: AcDNal, DCpa, DPal, Ser, Tyr, D-Hci, Leu, Lys(iPr), Pro, DAlaNH$_2$) has been developed recently, which is characterized by a high potency, a modest histamine-liberating activity and a high water solubility (3).

We report here the development and the validation of the radioimmunoassay of Antarelix for the monitoring of plasma levels. This method was used to establish pharmacokinetic profiles of this synthetic peptide in dogs following an intravenous or a subcutaneous administration at the dose of 10 μg/kg.

MATERIALS AND METHODS

Chemicals and Reagents

Antarelix (EP 24332: AcDNal-DCpa-DPal-Ser-Tyr-DHci-Leu-Lys(iPr)-Pro-

D-AlaNH$_2$) and the following analogs (SP 931218: AcDnal-DCpa-DPal-Ser-Tyr-DHci-Leu-Lys(iPr)-Pro-D-Ala, EP 9002, EP 23904) were provided by Europeptides (Argenteuil, France). The LHRH antagonists analogs (34131, 26672) were purchased from Peninsula Laboratories (Belmont, Calif.). Glutaraldehyde 50% in water (code 34085-5) was from Aldrich (Milwaukee, WI). EDC reagent (1-ethyl-3(3dimethylamino propyl) carbodiimide HCl) (code 22980), and trifluoroacetic acid (code 28903) were from Pierce (Rockford, IL). Bovine serum albumin (code A-7030), complete Freund's adjuvant (code F4258), incomplete Freund's adjuvant (code F5506), polyethylene glycol 8000 (code P-2139), and triethylamine (code T-0886) were from Sigma Chemical (St-Louis, Mo). Lysozyme (code 837059) and aprotinin (code 1583794) were supplied by Boehringer Mannheim (Laval, Quebec). The goat antirabbit gamma globulins sera (code PA-3608) were from Immunocorp (Montreal,Quebec). Chloramine-T (code B27670) was purchased from BDH (Toronto, Ontario). [^{125}I]Na (IMS 300) was provided by Amersham (Oakville, Ontario). Unless otherwise specified, all the reagents used were analytical grade and were purchased from Fisher Scientific (Montreal, Quebec).

Radioiodination

EP 24332 was radioiodinated by the Chloramine-T method. Briefly, 15 μ g of EP 24332 was dissolved in 50 μl of 50 mM phosphate buffer, pH 7.5. Then, 1 mCi of [^{125}I] Na and 10 μl of 0.01 % Chloramine-T in water (prepared just prior

to use) were added. Following an incubation period of 20 sec at 25°C, the reaction was terminated by the addition of 1 ml of 50 mM phosphate buffer, pH 7.5. This reaction mixture was immediately loaded on a Sep-Pak light C8 125 Å Cartridge previously activated with acetonitrile (ACN) and reequilibrated with 25 mM TEAP buffer, pH 2.8. The radiolabelled peptide was then eluted with 1 ml of a mixture of ACN/25 mM TEAP (70:30). The eluted fraction was diluted with approximately six volumes of 25 mM TEAP and loaded on a reverse-phase HPLC column (Vydac C18, 4.6 X 250 mm, 5 μm, 300 Å). The mobile phase consisted of 25 mM TEAP in water, pH 2.8 (phase A) and 60% ACN in 25 mM TEAP (phase B). The separation was achieved using a Waters automated gradient controller equipped with M 510 pumps (Waters Assoc., Milford, MA) using a linear gradient of phase B from 40 to 65 % in 50 min (0.5%/min) at a flow rate of 1 ml/min.

The radioiodinated EP 24332, which eluted at 34% (B) was collected, diluted in 0.2 M acetic acid containing 0.2% of lysozyme and kept frozen at -80°C in polypropylene tubes.

Antibody production

One μmole of EP 931218 was diluted in 0.5 ml of distilled water and activated in 0.5 ml of 0.02% of EDC solution in water prepared just prior to use. This peptide solution was slowly added dropwise into 0.5 ml of solution containing

0.1 μmole of bovine serum albumin while stirring. The peptide/protein solution was incubated for 4 hours at room temperature. The conjugation reaction was quenched by addition of 0.5 ml of 0.5 M sodium acetate pH 4.2, followed by an incubation of 1 hour at room temperature. The peptide/protein conjugate was further purified by dialysis against 10.14 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , 137 mM NaCl, 2.68 mM KCl and subsequently lyophilized.

Immunization procedure

Five hundred μg of lyophilized peptide/protein antigen were dissolved in 2 ml of 0.9% NaCl solution, emulsified with 3 ml of complete Freund's adjuvant, and injected subcutaneously, according to the multisite approach, into four New Zealand rabbits (1-1.5 kg) purchased from Cunipur (St. Valerien, Quebec). Six weeks after the primary immunization, the animals were boosted every two weeks by intramuscular injection of the same dose of antigen emulsified in incomplete Freund's adjuvant and serum was collected for antibody titration. After the third boost, the animals were bled and the sera collected, diluted with equal volume of glycerol and stored at -80°C.

Radioimmunoassay procedure

Assay procedure. The assay buffer consisted of 10 mM phosphate buffer (pH 7.5) containing: 0.14 M NaCl, 0.02% NaN_3, 25 mM potassium EDTA, and 0.2% lysozyme. The incubation medium consisted of 100 μl [^{125}I] Antarelix (15000

cpm), 100 µl of antiserum (final dilution of 1:400 000), 100 µl of peptide standards ranging from $1x10^{-11}$M to $1x10^{-6}$M, and 25 µl of either Antarelix-free dog plasma or plasma samples. The incubation volume was adjusted to 500 µl with the assay buffer. Following an incubation period of 18 hrs at 4°C, bound and free fractions were separated by adding 500 µl of immunoprecipitation reagent consisting of 0.2% goat antirabbit gamma globulins in 12% polyethylene glycol solution. The tubes were incubated at 4°C for 15 min and centrifuged for 20 min at 3500 rpm. The supernatant was discarded by vacuum aspiration and the radioactivity bound to the pellet was counted with an LKB Wallac 1277 Gammamaster from Fisher Scientific (Montreal, Quebec). All samples were assayed in triplicate.

Standard curve. The standard curve was prepared from serial dilutions of Antarelix at concentrations of: $1x10^{-11}$M, $3x10^{-11}$M, $1x10^{-10}$M, $3x10^{-10}$M, $1x10^{-9}$M, $3x10^{-9}$M, $1x10^{-8}$M, $3x10^{-8}$M, $1x10^{-7}$M, $3x10^{-7}$M, $1x10^{-6}$M. The calibration curves data, expressed by bound radioactivity versus log concentration of Antarelix, were analyzed by RIAFIT program based on the four parameter logistic equation (4).

Validation procedure

Specificity. The crossreactivity of the antibody was tested against LHRH, the LHRH agonist Metereline, and three Antarelix analogs. Crossreactivity was

assessed as the ratio of the concentration of Antarelix, relative to that of LHRH, Metereline, and the three LHRH antagonists, displacing 50% of the maximum tracer binding.

The crossreactivity with metabolites of Antarelix was assessed by documenting HPLC- immunograms of a pool of serum extracts collected from a dog (between 0-8 hrs) following an intravenous dose of 15 μg/kg Antarelix. The pool of plasma (3 ml), was deproteinized with 5 ml of acetonitrile and centrifuged. The resulting supernatant was diluted with 0.1% TFA and loaded on an ODS Vydac HPLC column (5 μm, 4.6 X 250 mm, 300 Å, CSC, Montreal, Quebec). Antarelix and its metabolites were separated using a linear gradient of acetonitrile in 0.1% TFA from 5 to 55% (0.75%/min) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and evaporated to dryness under vacuum (Speed Vac system, Savant Inst. Farmingdale, NY) for the radioimmunoassay determination.

Accuracy. Recovery was assessed by analyzing three control plasma samples spiked at a low (10 fmol/assay), medium (40 fmol/assay), and high (160 fmol/assay) level of Antarelix and by establishing the correlation between theoritical and measured concentrations.

Dilutional parallelism was evaluated by serial dilutions of a plasma dog sample collected between 0.5 to 2 min following the administration of an intravenous dose of Antarelix. The sample was diluted with ligand free dog plasma according to the

following dilution factors; 1:3, 1:10, 1:30, 1:100, 1:300 and analyzed by radioimmunoassay.

Precision, reproducibility and sensitivity of the assay. Twenty replicates of plasma samples containing 10, 40, and 160 fmol/assay of Antarelix were assayed in a single run and the coefficients of variation were calculated. For the inter-assay study, ten determinations of plasma samples containing 10, 40, and 160 fmol/assay of Antarelix in 10 separate assay runs were performed and the coefficient of variations were calculated. The sensitivity of the assay was defined as the value of the mean minus 2 standard deviations of 10 replicates of the maximum binding (B_0).

Ligand-free medium effect. The effect of the plasma volumes on the incubation conditions were documented.

Application to pharmacokinetic studies in dogs

IV (Bolus) or SC administration of Antarelix to dogs. The pharmacokinetics of Antarelix following an IV or a SC dose of 10 μg/kg were investigated in a total of 6 dogs (n=3/study). Male and female dogs weighing 20-30 kg were awake throughout the study. The animals were surgically prepared, 48 hours before the study, with vascular access ports to the right external jugular vein. Intravenous administration of the peptide (1 mg/ml in 0.9% NaCl) was performed at the cephalic vein in the left paw while subcutaneous administration (1 mg/ml in 5%

mannitol) was performed at the scruff of the neck (<30 sec). Blood samples (6 ml) were collected prior to injection and at 0.5, 1, 3, 5, 10, 15, 20, 30, 40, 50 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 24, 48 hrs, from the right external jugular vein, after IV injection and at 10, 20, 30, 40, 50 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 24, 48 hrs after SC injection, into tubes containing 371 mM potassium EDTA, and 1 mM aprotinin. The plasma was centrifugated at 2500 rpm for 15 min and stored at -80°C.

Pharmacokinetic analysis. Pharmacokinetic parameters following an IV or a SC administration were analyzed according to a noncompartmental model as described by Gibaldi (5). Cmax and Tmax were determined by inspection of actual data. The area under the curve from time zero to infinity, $AUC_{0-\infty}$, was estimated from the log-linear trapezoidal rule. The intravenous terminal half-life was obtained from linear regression analysis of the log-transformed concentration time data. The clearance, Cl, and the volume of distribution at steady state, Vss, were calculated according to equations 1 and 2

$$Cl = Dose/AUC_{0-\infty} \qquad [1]$$

$$Vss = Dose\ (AUMC)/(AUC)^2 \qquad [2]$$

where AUMC denotes the total area under the first moment of the plasma concentration curves from time zero to infinity (6).

The relative subcutaneous bioavailability (F) in the dog was determined from

$$F = AUC_{SC} \times Dose_{IV}/AUC_{IV} \times Dose_{SC} \quad [3]$$

The pharmacokinetic parameters of the 10 μg/kg dose were used to calculate the relative subcutaneous bioavailability.

RESULTS

Characterization of the antiserum

The final dilution of the antiserum was determined to be 1:400 000 from the antibody titration curve giving a maximum binding (B_0) of 50%. The Scatchard plot analysis of the binding curve gives an affinity constant (Ka) of 43.9 pM with a number of binding sites of 0.0262 pmol/l.

Standard curve

A calibration curve for Antarelix is represented in Figure 1. The analysis of the parameters of 10 standard curves runs in 10 different days, as shown in Table 1, displayed a slope factor of 1.06 ± 0.008 (Means ± SEM), a maximum and a non specific binding of 49.89% ± 0.69% and 2.3 ± 0.047% respectively, and a ED_{50} of 25.84 ± 0.78 fmol/assay. The ED_{50} is the relative dose of unlabelled antigen required to displace 50% of the maximum tracer binding.

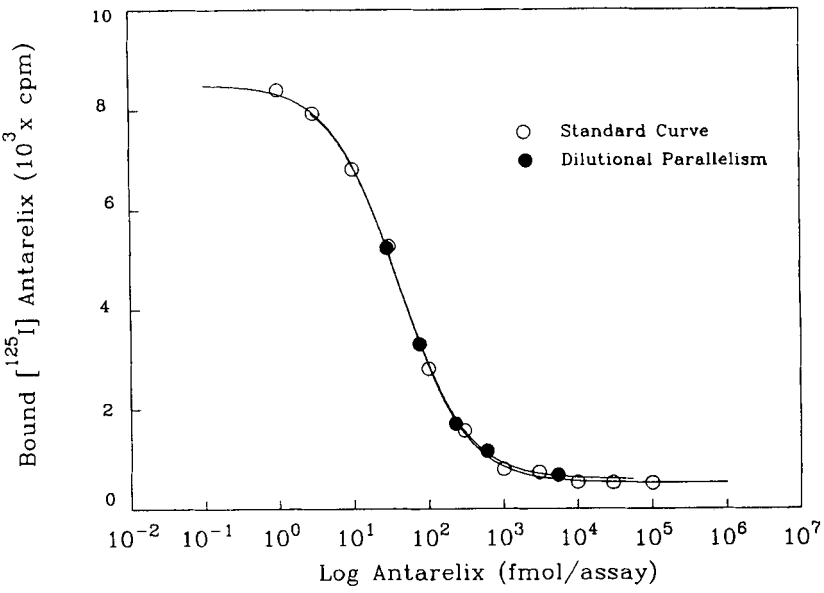

FIGURE 1. Typical RIA standard curve of Antarelix. Displacement curve of Antarelix [^{125}I] with Antarelix (O) is shown, or with the serially diluted plasma samples containing Antarelix (●).

TABLE 1

Standard Curve Parameters and Statistics

Std Curve #	Slope	ED50	% Binding	NSB (%)	Minimal Dose
1	1.06	26.9	51.5	2.4	0.94
2	1.08	23.8	46.6	2.2	0.88
3	1.03	24.4	48.7	2.3	0.99
4	1.06	28.2	51.6	2.2	1.46
5	1.03	25.1	53.0	2.6	0.59
6	1.01	21.5	45.9	2.3	0.57
7	1.08	27.9	51.0	2.5	1.71
8	1.08	28.8	50.0	2.2	1.95
9	1.07	23.1	49.3	2.2	0.78
10	1.10	28.7	51.3	2.1	1.37
Mean	1.06	25.84	49.89	2.3	1.124
S.e.m.	0.008	0.78	0.69	0.047	0.142
C.V. (%)	0.80	3.02	1.37	2.04	12.65

Radioimmunoassay validation

Specificity of the assay. As shown in Table 2, no crossreactivity with either LHRH, Metereline, or LHRH antagonists tested was detected, except for Antide which displayed a crossreactivity of 33%.

For the assessement of specificity of the antiserum towards endogenous metabolites of Antarelix, HPLC-immunograms were obtained from extracts of dog plasma spiked with Antarelix, and of pooled plasma samples from a dog receiving Antarelix. As shown in Figure 2, the unique immunoreactive peak detected in both extracts corresponds to the elution volume of Antarelix.

Accuracy. The recovery of Antarelix in plasma spiked at concentrations of 10, 40, and 160 fmole/assay was 104.86%. The correlation between theoritical concentrations of Antarelix spiked in dog plasma samples and the measured concentrations was described by the linear regression equation $y = 1.049x - 0.037$, $r^2 = 0.99$, where x is the theoretical concentration value, and y is the measured concentration value.

The slope of the curve for the dilutional parallelism study was found to be 1.03 and was not significantly different from that of the calibration curve.

Precision and Sensitivity. As shown in Table 3, the coefficients of variation of the intra-assay precision ranged between 0.89 and 1.45%, while as shown in

TABLE 2

Crossreactivity of Antarelix Analogs with the C-terminal Antibody

Name	Structure	% cross reactivity
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	<0.03
Metereline (EP23904)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	0.06
Antide (EP9002)	AcDNal-DCpa-DPal-Ser-Lys(Nic)-DLys(Nic)-Leu-Lys(Ipr)-Pro-DAlaNH$_2$	33
(D-Phe^2,Pro^3,D-Phe^6)-LH-RH (34131)	pGlu-DPhe-Pro-Ser-Tyr-DPhe-Leu-Arg-Pro-Gly-NH_2	<0.03
(D-pGlu^1,D-Phe^2,D-Trp^{3,6})-LHRH (26672)	DpGlu-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	<0.03
Antarelix (EP24332)	AcDNal-DCpa-DPal-Ser-Tyr-DHci-Leu-Lys(iPr)-Pro-D-AlaNH$_2$	100

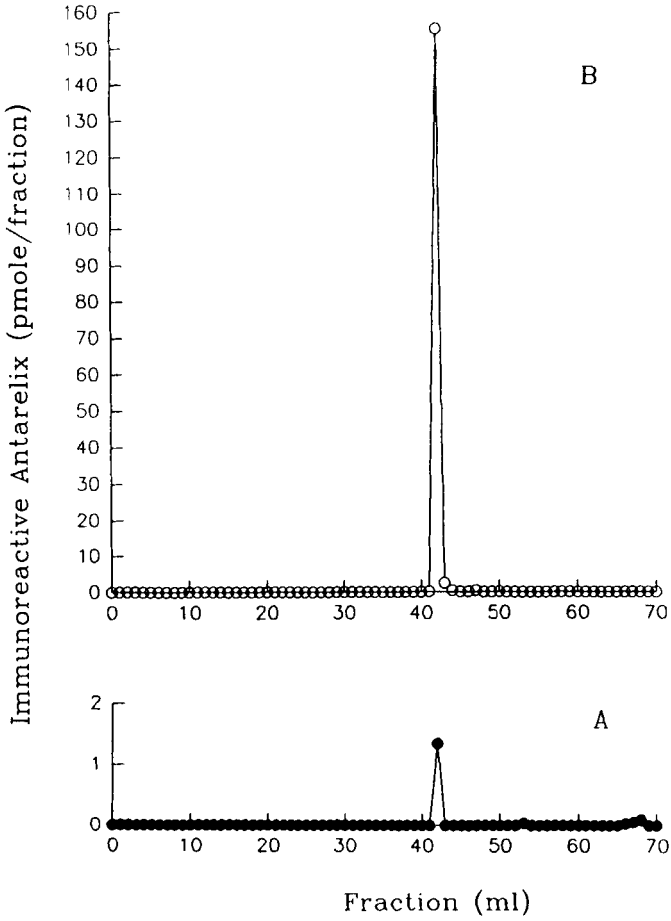

FIGURE 2. Reverse-phase HPLC elution profile of plasma extracts. Blank dog plasma spiked with 50 fmol/assay Antarelix (A) and pooled plasma samples from dog receiving Antarelix (B) were deproteinized with acetonitrile. The clear supernatant diluted with 0.1% TFA was injected into the HPLC. The eluted fractions (1 ml) were evaporated under vacuum. The immunoreactivity was quantified by radioimmunoassay.

TABLE 3

Intra-Assay Variance and Percent Recoveries of the Antarelix RIA in Low, Medium, and High Concentration Ranges

Expected (fmole/assay)	N	Mean	CV(%)
10	20	10.3	1.24
40	20	40.4	1.45
160	20	146.0	0.89

TABLE 4

Inter-Assay Variance of the Antarelix RIA in Low, Medium, and High Concentration Ranges

Std Curve #	Slope	ED50	10 fmole/assay	40 fmole/assay	160 fmole/assay
1	1.06	26.9	9.9	45.1	164
2	1.08	23.8	10.6	42.0	159
3	1.03	24.4	10.3	40.4	146
4	1.06	28.2	10.6	46.1	186
5	1.03	25.1	10.7	44.7	173
6	1.01	21.5	8.1	35.0	158
7	1.08	27.9	12.0	43.2	168
8	1.08	28.8	12.0	44.3	182
9	1.07	23.1	8.7	35.3	154
10	1.10	28.7	11.5	43.3	188
Mean	1.06	25.84	10.44	41.94	167.8
S.e.m.	0.008	0.78	0.388	1.177	4.268
C.V. (%)	0.8	3.02	3.72	2.80	2.54

Table 4, the coefficents of variation of the inter-assay precision ranged between 2.54 and 3.72%. The sensitivity of the assay, defined as the minimal detectable dose, corresponded to 1.12 ± 0.142 (X ± SEM) fmol/assay (Table 1).

Ligand-Free Media Effect. A volume of 25 µl of plasma was used for the direct assay of Antarelix. However the presence of 25 or 50 µl did not affect significantly the characteristics of the standard curve.

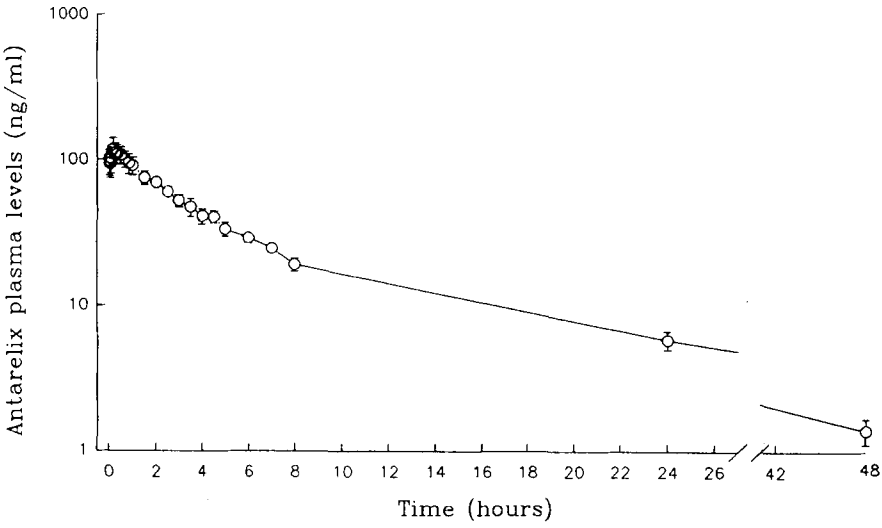

FIGURE 3. Mean plasma levels of Antarelix (MEANS ± SEM) in dogs (n=3) after a bolus intravenous dose of 10 μg/kg in saline.

In vivo data

Pharmacokinetic studies for Antarelix were performed in male and female dogs following an IV or a SC dose of 10 μg/kg. The data were analyzed using noncompartmental and two-compartmental models and the results obtained from the two models were not statistically different. Figure 3 illustrates the mean plasma concentration-time profiles following intravenous administration. As shown, 20 minutes following the administration of the drug, the maximum plasma levels at 123.2 ng/ml gradually declined to 5.8 ng/ml within 24 hours. Pharmacokinetic parameters estimated from the fitted data are presented in Table 5. The terminal half-life of the peptide was determined to be 10.4 hrs. The

TABLE 5

Pharmacokinetic Parameters of Antarelix Following a 10 µg/kg Bolus IV Administration to Dogs *

t 1/2	626.4 ± 69.2 min
AUC	42959 ± 46647 ng.min/ml
Cl	0.2416 ± 0.030 ml/min/kg
Vss	0.1455 ± 0.010 l/kg
Cmax	123.2 ± 21.5 ng/ml

* Abbreviations of mean (+/- SEM) pharmacokinetic parameters: t 1/2, half-life of the terminal phase; AUC, area under the curve from time zero to infinity; Cl, clearance; Vss, volume of distribution at steady-state; Cmax, maximal concentration.

apparent volume of distribution at steady state (V_{SS}) and clearance values were 0.146 l/kg and 0,242 ml/min/kg respectively.

Figure 4 illustrates the mean plasma concentration-time profiles following subcutaneous administration. The pharmacokinetic parameters following subcutaneous administration of Antarelix are presented in Table 6. Maximum plasma level, reached at 1.06 hr following administration of the peptide was 23.9 ng/ml. The terminal half-life was estimated to 23.9 hrs. The volume of distribution at steady state and clearance values were 0.715 l/kg and 0.384 ml/min/kg respectively.

DISCUSSION

In order to document the pharmacokinetics of Antarelix, a promising LHRH

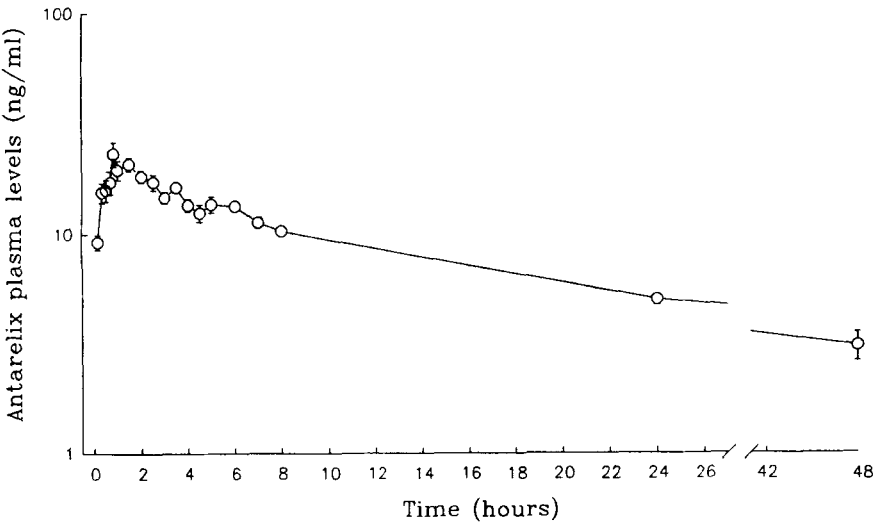

FIGURE 4. Mean plasma levels of Antarelix (MEANS ± SEM) in dogs (n=3) after a subcutaneous dose of 10 µg/kg in 5% mannitol.

TABLE 6

Pharmacokinetic Parameters of Antarelix Following a 10 µg/kg SC Administration to Dogs *

Tmax	63.3 ± 10.9 min
Cmax	23.9 ± 2.4 ng/ml
t 1/2	1439.8 ± 280.1 min
AUC	26694 ± 2301 ng/min.ml
Cl/F	0.6113 ± 0.059 ml/min/kg
Vd/F	0.7147 ± 0.080 l/kg

F, Relative bioavailability (%): 62.8 ± 0.04

* Abbreviations of mean (+/- SEM) pharmacokinetic parameters: t1/2, half-life; AUC, area under the curve from time zero to infinity; Cl, clearance; Vd, apparent volume of distribution.

antagonist, we developed a highly sensitive and specific RIA procedure for the direct determination of this peptide in a minimum volume of 25 µl of plasma.

The antiserum was generated against the deamidated Antarelix which displayed a free carboxyl group activable under its ester active form by carbodiimide for the covalent linking of the peptide to the carrier protein. This antiserum showed a high specificity as no crossreaction was observed with LHRH, LHRH analog Metereline nor with the LHRH antagonists tested. However, there was some crossreactivity with Antide (33%), another LHRH antagonist which differs from Antarelix by N^{ε}-nicotinoyllysine and D-N^{ε}-nicotinoyllysine residues in position 5 and 6 respectively (6). Furthermore, no crossreactivity with any metabolites resulting from the administration of Antarelix could be detected. This was confirmed by obtaining HPLC-immunograms of plasma extracts from a dog receiving this peptide, which displayed a unique immunoreactive peak corresponding to that of Antarelix. The RIA developed has been validated with respect to the accuracy of the method with a recovery of 104% and a precision ranging between 1 and 3.7%. The minimum detectable concentration was 1.12 fmol/assay.

The RIA method was applied to the monitoring of Antarelix plasma levels in dogs. The parameters of the intravenous pharmacokinetic study, as shown in Table 5, demonstrate a terminal half-life of 10.4 hrs, which is longer than those

reported for Detirelix in monkeys (7.1 hrs for 80 μg/kg) and Ganirelix in monkeys and in rats (5.1 and 1.4 hrs respectively for a dose of 1000 μg/kg). This result confirms that LHRH antagonists, as a class, are likely to be "long acting" *in vivo*. The prolonged residence of intact peptide in the vascular compartment can be explained by several factors such as an increased stability over proteolytic degradation. The volume of distribution at steady-state (V_{SS}) of Antarelix is less than the total body fluid volume. Thus, Antarelix is probably not extensively bound to most of the extravascular sites, as observed with other antagonists. The clearance of Antarelix is less than those reported for Detirelix in monkeys (1.3 ml/min/kg for 80 μg/kg and 1.6 ml/min/kg for 40 μg/kg) and in rats (3.3 ml/min/kg for 300 μg/kg), and for Ganirelix in monkeys and in rats (0.8 ml/min/kg and 2.5 ml/min/kg, respectively for 1000 μg/kg) (7,8).

Subcutaneous pharmacokinetic study, reported in Table 6, indicates that Antarelix is slowly (1.06 hr) and partially absorbed (62%) in dog. The terminal half-life of 24 hrs is longer than those calculated for Detirelix (1.6 hrs for 40 μg/kg) and Ganirelix (3.6 hrs for 1000 μg/ml) in rats. This longer half-life, observed following SC administration, might be explained in part by a depot gel formed at the site of injection, as observed for most of the LHRH antagonists (7,8,9).

In conclusion, we report here a highly sensitive and specific radioimmunoassay procedure for monitoring the plasma levels of Antarelix. As other LHRH

antagonists, the expected doses of Antarelix to be administered in humans would be in the range of 10-100 µg/kg. Therefore, this assay is suitable for pharmacokinetic studies of this LHRH antagonist in preclinical as well as in clinical studies.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Dr Nadeau for providing access to the animal facilities at the Centre de Recherche de l'hôpital Sacré-Coeur, to Dr Marleau for helpful discussions and Ms Bellemare for her technical assistance. This work was supported by a grant from Europeptides (Argenteuil, France).

REFERENCES

(1) Karten, M.J. An overview of GnRH Antagonist Developpement: Two Decades of Progress. In: Crowley, W.F. and Conn P.M., eds. Modes of Action of GnRH and GnRH Analogs. New York: Springer-Verlag, 1992:277-97.

(2) Pavlou, S.N. GnRH antagonists in men. In: Crowley, W.F. and Conn, P.M., eds. Modes of Action of GnRH and GnRH Analogs. New York: Springer-Verlag, 1992:298-309.

(3) Deghenghi, R., Boutignon, F., Wüthrich, P. and Lenaerts, V. Antarelix (EP 24332) a Novel Water Soluble LH-RH Antagonist. *Biomed. Pharmacother.* 1993; 47: 107-10.

(4) De Léan, A., Munson, P.J. and Rodbard, D. Simultaneous Analysis of Families of Sigmoidal Curves: Application to Bioassay, Radioligand Assay, and Physiological Dose-Response Curves. *Am. J.Physiol.* 1978; 235: E97-102.

(5) Gibaldi, M. Biopharmaceutics and clinical pharmacokinetics. Philadelphia: Lea and Febiger, 1991: 14-23.

(6) Ljungqvist, A., Feng D.M., Hook, W., Shen, Z.X., Bowers, C. and Folkers, K.

Antide and Related Antagonists of Luteinizing Hormone Release with Long Action and Oral Activity. *Proc. Natl. Acad. Sci. USA* 1985; 85: 8236-40.

(7) Chan, R.L., Ho, W., Webb, A.S., LaFargue, J. and Nerenberg C.A. Disposition of Detirelix, a Potent Luteinizing Hormone-Releasing Hormone Antagonist, in Rats and Monkeys. *Pharm. Res.* 1988; 5: 335-40.

(8) Chan, R.L., Haroldsen, P.E., Ho, W. and Nestor, J.J. Disposition of RS-26306, a Potent Luteinizing Hormone-Releasing Hormone Antagonist, in Monkey and Rats after Single Intravenous and Subcutaneous Administration. *Drug Metab. Dispos. Biol. Fate Chem.* 1991; 19: 858-64.

(9) Powell, M.F., Sanders, M.L., Rogerson, A. and Si, Vi. Parenteral peptide formulations: Chemical and physical properties of native luteinizing hormone releasing hormone (LHRH) and hydrophobic analogues in aqueous solution. *Pharm. Res.* 1991; 8: 1258-63.