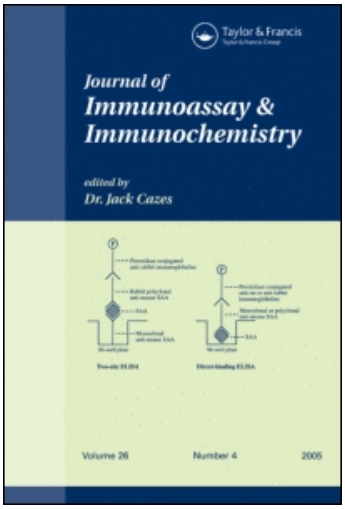

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RADIOIMMUNOASSAY OF GANIRELIX IN PLASMA OR SERUM

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ABSTRACT

A procedure for the radioimmunoassay (RIA) of ganirelix in plasma or serum at concentrations as low as 0.050 ng/ml is described. Antiserum was produced by coupling the N-terminus glycyl analog of ganirelix to BSA by a carbodiimide reaction and immunizing rabbits with this conjugate. The antiserum did not crossreact with LHRH or with various ganirelix peptide fragments. For RIA, ^{125}I labeled ganirelix was used as the tracer and a double antibody procedure was used to separate the free and bound fractions. No purification of the analyte was required prior to RIA. Accuracy of the method was assessed by adding known quantities of ganirelix to ganirelix-free plasma and determining the ratio of measured to added analyte. Linear regression analysis for the concentration range 0.050 - 50.0 ng/ml yielded a regression equation of $y = 0.97x+0.18$, $r = 0.999$, where x is the amount added and y is the amount measured. Additional validation was obtained from an in vivo study in which [^3H]-ganirelix was administered to monkeys and plasma clearance profiles were determined by RIA and an HPLC-radiochemical method. The results were in agreement within experimental error of the two methods. Linear regression analysis of the comparative data gave the equation $y = 0.92x + 33.7$, $r = 0.980$, where x is the amount measured by RIA and y is the amount measured by HPLC-radiochemical analysis.

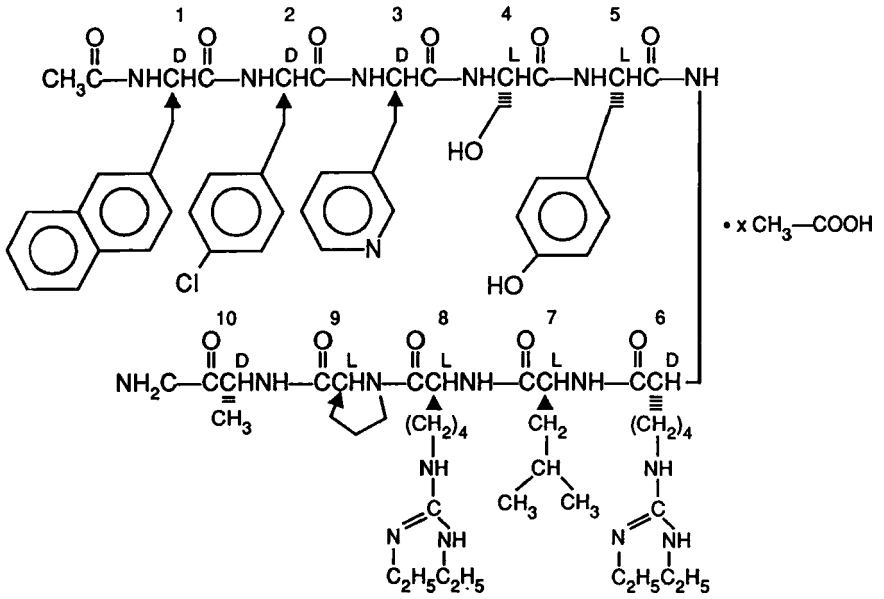

FIGURE 1: Structure of ganirelix. [N-Ac-D-Nal (2)[1], D-p-CL-Phe[2], D-PAL (3)[3], D-hArg(Et_2)[6], L-hArg(Et_2)[8], D-Ala[10]] LHRH.

INTRODUCTION

Ganirelix, a synthetic LHRH antagonist, is a decapeptide consisting of D and L natural and unnatural amino acids (Fig. 1). LHRH antagonists inhibit the action of endogenous LHRH at the receptor site, in contrast to agonists, which function by down-regulation of pituitary LHRH receptors (1). This mode of action eliminates the initial stimulatory phase observed with agonists, and for steroid-dependant disorders such as prostatic cancer, antagonists offer a therapeutic advantage. Ganirelix has been shown to be a potent inhibitor of gonadotropin secretion and has a lower potential for histamine release than other antagonists.

Preclinical trials of this compound suggest that it has the potential for becoming a clinically useful therapeutic drug for the treatment of gonadal hormone-dependent disorders. The procedure described here was developed to provide analytical support for various clinical, toxicological, formulation and pharmacokinetic studies.

EXPERIMENTAL

Materials and Equipment

All compounds listed in Table 1, with the exception of LHRH, were synthesized at Syntex. Abbreviations used in describing the structures of these analogs are also given in Table 1.

The sources of the following materials are given in parentheses: ^{125}I as NaI, rabbit immunogammaglobulin (ICN Biomedicals, Inc., Costa Mesa, CA); polyethylene glycol 8000, thimerosal, bovine serum albumin (BSA) and EDTA disodium salt (Sigma Chemical Co., St. Louis, MO); LHRH (Peninsula Labs, Belmont, CA); chloramine-T (Aldrich Chemical Co.,Inc., Milwaukee, WI); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide • HCl (Pierce Chemicals, Rockford, IL); Triton X-100 (J.T. Baker Chemical Co., Phillipsburg, NJ); Sephadex QAE-A25 (Pharmacia Fine Chemicals, Piscataway, NJ); Bio-Gel P-4 fine mesh, disposable Econo-columns 1 x 10 cm and 1 x 50 cm (Bio-Rad, Richmond, CA); monobasic and dibasic sodium phosphate (Mallinckrodt, St. Louis, MO); goat anti-rabbit

TABLE 1

CROSSREACTIVITY DATA

Compound	% Cross-Reactivity
[N-Ac-D-Nal(2)^1,D-p-Cl-Phe^2, D-PAL(3)^3, D-hArg(Et$_2$)^6, L-hArg(Et$_2$)^8, D-Ala^{10}][a] LHRH (Ganirelix)	100.0
[N-Ac-D-Nal(2)^1-D-p-Cl-Phe^2] ([1-2] dipeptide of Ganirelix)	<< 0.006
[N-Ac-D-Nal(2)^2-D-p-Cl-Phe^2-D-PAL(3)^3] ([1-3] tripeptide of Ganirelix	<< 0.006
[N-Ac-D-Nal(2)^1-D-p-Cl-Phe^2-D-PAL(3)^3-L-Seryl] ([1-4] tetrapeptide of Ganirelix	<< 0.006
[N-Ac-D-Nal(2)^1-D-p-Cl-Phe^2-D-PAL(3)^3-L-Seryl-L-Tyr] ([1-5] pentapeptide of Ganirelix)	<< 0.006
[N-Ac-D-Nal(2)^1-D-p-Cl-Phe^2-D-PAL(3)^3-L-Seryl-L-Tyr-D-hArg(Et$_2$)^6 ([1-6] hexapeptide of Ganirelix)	<< 0.006
[N-Ac-D-Nal(2)^1-D-p-Cl-Phe^2-D-Pal(3)^3-L-Seryl-L-Tyr-D-hArg(Et$_2$)^6-L-Leu] ([1-7] heptapeptide of Ganirelix)	<< 0.006
[N-Ac-D-Nal(2)^1, D-p-Cl-Phe^2, D-PAL(3), D-hArg(Et$_2$)^6, L-hArg(Et$_2$)^8, D-Ala-OH^{10}] LHRH (Ganirelix free-acid)	1.1
LHRH	<< 0.006

a The abbreviations used are as follows: [D-Nal(2)] is 3-(2-naphthyl)-D-alanine; [D-p-Cl-Phe] is p-chloro-D-phenylalanine; [D-hArg(Et$_2$)] is N^G,N^{G1}-diethyl-D-homoarginine; [D-PAL(3)] is 3-pyridyl-D-alanine.

gammaglobulins (Antibodies Inc., Davis, CA); Spectropor 2 membrane dialysis tubing (Spectrum Medical Industries, Los Angeles, CA); Freunds complete and incomplete adjuvants (Difco Laboratories, Detroit, MI).

The manufacturers or suppliers of the following equipment are given in parentheses: model DPR-6000 refrigerated centrifuge with a swing-out head

(IEC/Damon, Needham, MA); Apex Automatic Gamma Counter (Micromedic Systems, Inc., Horsham, PA); model FC-80h fraction collector (Gibson Medical Electronics, Inc., Middleton, WI); model 559 spectrophotometer (Perkin-Elmer, Norwalk, CT); model 2600 Multi-Tube Vortex Mixer (Scientific Manufacturing Ind., Emeryville, CA).

Production of Antiserum

The N-terminus ganirelix protein-hapten conjugate was synthesized by a water soluble carbodiimide method. The coupling reaction was carried out by dissolving 29.4 mg (153.4 μmole) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide • HCl in water (1 ml) and adding it dropwise to 55.8 mg (0.85 μmole) of BSA in 3 ml of water. This solution was stirred for 3 hours at room temperature, and then 24.7 mg (14.0 μmole) of the ganirelix glycyl analog in 1 ml of water was added dropwise and stirring continued for 24 hours at room temperature. Thereafter, the entire reaction mixture was transferred to a dialysis bag and dialyzed exhaustively against 0.9% saline. After dialysis was complete, the entire volume in the dialysis bag (5.1 ml) was apportioned into 0.2 ml aliquots and frozen.

Six New Zealand white rabbits were immunized with the hapten-protein conjugate. The immunization emulsion was prepared by dissolving 1.46 mg of the conjugate in 7.0 ml of water and emulsifying in 8.0 ml of Freunds complete adjuvant. For the booster shots 0.3 mg of the conjugate was used with incomplete

Freunds adjuvant. Typically, 2.0 ml of the emulsion was injected into each animal at four different sites, one in each hind thigh (inside) and two at lateral sites in the intrascapular area. Each site received a cluster of 5 injections of 0.1 ml per injection. Booster shots were given at 4 week intervals and titers were monitored at the same time. After 3-4 months, the antisera were of sufficient quality to be used for methods development.

^{125}I-Labeling

The labeling reaction was carried out in the vial containing 2 mCi of ^{125}I as NaI. To the vial were added, in the following order, 50 µl of phosphate buffer (0.05 M, pH 7.5), 20 µl (2 µg) of aqueous ganirelix, and 30 µl (3 µg) of aqueous chloramine-T. The aqueous ganirelix and chloramine-T solutions (0.1 mg/ml) had been prepared just prior to use. The reaction was allowed to proceed for 60 seconds and then was quenched by the addition of 500 µl of phosphate buffer (0.05 M, pH 8.0, 0.2% BSA, 0.005% thimerosal). This reaction mixture was transferred immediately to a Sephadex QAE-A25 column (1 x 8 cm) and eluted with the same buffer as described above. One-ml fractions were collected in 12 x 75 mm test tubes with a fraction collector, and 10-µl aliquots were used for counting of radioactivity. A column elution profile was plotted and the fractions corresponding to the center portion of the radioactive peak were pooled (3-4 ml). An aliquot (1 - 1.5 ml) of this pool was rechromatographed on a 1 x 30 cm Bio-Gel P-4 column and eluted with 0.1 M sodium acetate buffer, (pH 3.4, 0.2% BSA, 0.001% thimerosal). Fractions (1 ml) were collected in 12 x 75 mm tubes,

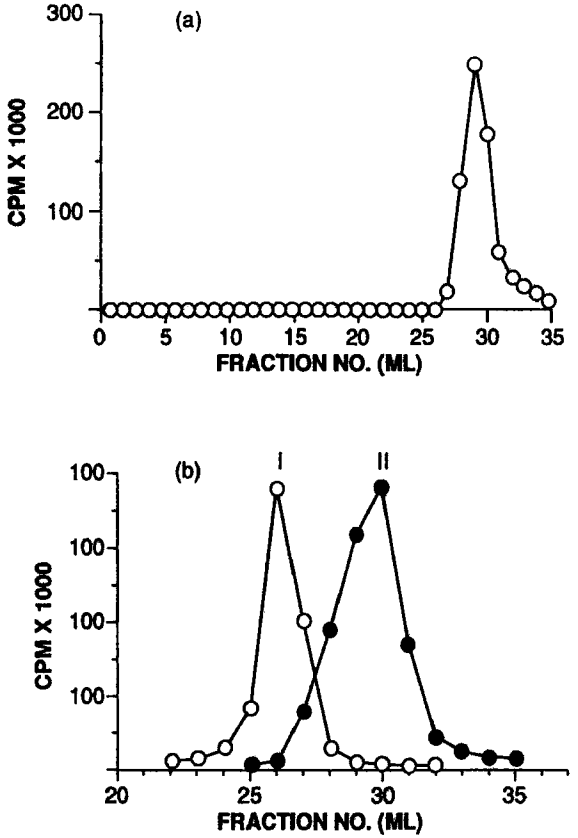

FIGURE 2: Purification of iodinated ganirelix. The major peak in 2a is ^{125}I-ganirelix. Fig. 2b shows the separation of [^{3}H]-ganirelix (I) from ^{125}I-ganirelix (II). Separations were carried out on a 1 x 30 cm Bio-Gel P-4 column eluted with 0.1 M NaAc buffer, pH 3.4, containing 0.2% BSA.

10-µl aliquots were counted and an elution profile was plotted (Fig. 2a). The fractions corresponding to the center portion of the major peak were pooled (5-7 ml) and aliquots of this pool were diluted in RIA buffer for use in the assay. Labeling reactions were normally carried out at intervals of 3-4 weeks.

Radioimmunoassay

Standards for the standard curve were prepared from a primary stock solution containing 0.1 mg/ml of ganirelix in 0.1 N HCl. The exact concentration of this primary standard was determined by UV absorption at 263 nm (absorptivity = 11,000). The primary standard was diluted in RIA buffer (0.1 M phosphate buffer, pH 7.4, 0.9% NaCl, 0.5% BSA, 0.01% thimerosal, 0.01% Triton X-100, 0.0001 M EDTA) to yield a series of standards containing 2, 5, 10, 20, 30, 50 and 100 pg/0.1 ml. These standards were stored at 4°C and were discarded after one week. Antiserum was diluted in RIA buffer such that 40-80% binding of only radiolabeled ganirelix was achieved. This dilution ranged from 1/150,000 to 1/300,000.

The procedure for setting up the assay was as follows: Add 0.1 ml of standards, unknowns, label (3000-4000 cpm), antiserum, etc., to appropriately labeled 12 x 75 mm polypropylene tubes; add RIA buffer to adjust the volume in each tube to 0.6 ml; cover the tubes with parafilm; vortex briefly to wash down the sides of the tubes and then incubate overnight at room temperature. Total counts (TC) and non-specific binding (NSB) tubes which contain radiolabel and buffer only are always included with each assay.

A double antibody procedure was used the following day to separate bound from free radioactivity. To each tube (except TC and NSB) were added 0.1 ml of rabbit immunogammoglobulin (0.5 mg/ml in phosphate buffer, 0.1 M, pH 7.4)

and 0.1 ml of goat anti-rabbit gammaglobulin (diluted 1:2 in water). The rack of tubes was vortexed briefly and then incubated for 1.5 hours at room temperature. After this 0.3 ml of 5% polyethylene glycol was added to each tube and the entire rack of tubes was vortexed and placed in an ice bath for 30 min. The tubes were then centrifuged, the supernatants were aspirated off and each tube was counted for 2 min in a gamma counter. All counters were equipped with direct connect outputs to computers for automatic calculation of RIA results by means of a logistic program (2). Crossreactivities were determined in accordance with the method described by Abraham (3).

Precision and Accuracy

Precision and accuracy were assessed by determining the recovery of different quantities of ganirelix that had been added to plasma. In addition, a comparative study of the RIA and an HPLC-radiochemical (HPLC-RC) assay was carried out with specimens collected serially from cynomolgus monkeys following intravenous administration of [^{3}H]-ganirelix.

Assay Optimization

The effect of Triton X-100 on the assay was determined by adding varying concentrations of the surfactant to the RIA buffer and then measuring the change in the non-specific binding, in the total binding and in adsorptive losses in the assay tubes.

The separation of bound from free radioactivity using a second antibody procedure was optimized by determining the total binding obtained when varying amounts of rabbit immunogammaglobulin (IgG) or normal rabbit serum (NRS) were added as carrier to varying amounts of goat anti-rabbit gammaglobulin (GARGG). A three dimensional plot of the data was constructed in which the percent bound was plotted on the Z-axis and the GARGG and IgG or NRS were plotted on the X and Y axes, respectively.

RESULTS AND DISCUSSION

Ganirelix lacks a free amino or carboxyl group, which is required for conjugation to BSA if a water soluble carbodiimide is used as the coupling reagent. A suitable analog was synthesized by substituting glycine in the 1 position of ganirelix, thus providing a free amino group for the coupling reaction. The antiserum binding constant, determined by Scatchard (4) analysis, was 2.95 x 10^{11} liters/mole, and the antibody concentration was 0.96 mg/ml in the neat rabbit antiserum.

The purification procedure described here for the ^{125}I-labeled ganirelix is a two step procedure. A rapid preliminary purification by means of the short Sephadex QAE-A25 column provides a convenient means for the elimination of unreacted ^{125}I and side products that can cause chemical degradation of the iodinated ganirelix. The partially purified ganirelix was stable for 3-4 weeks and

served as a convenient source of starting material for the second purification step, which separates nonlabeled from iodinated ganirelix (Fig.2b). The iodinated ganirelix obtained after the second purification was suitable for use in the assay for 2-3 weeks. The specific activity, determined by self-displacement plots (5), was 1438 μCi/μg. The theoretical maximum for mono-iodinated ganirelix calculated according to Roth (6) was 1383 μCi/μg. The agreement with the theoretical value indicates that the mono-iodo analog was the predominant molecular species.

Accuracy of the overall procedure was assessed by means of experiments on the recovery of analyte that had been added to plasma (Table 2). The average intraassay coefficient of variation calculated from the results of four separate experiments for the concentration range 0.050 to 50.0 ng/ml varied from 0.89 to 16.2%. The interassay coefficients of variation calculated for the group mean values ranged from 3.9 to 12.1% over this concentration range. The following linear regression equation was calculated from the means of all the experiments: $y = 0.97\ x + 0.18$, $r = 0.999$, where y is the ganirelix concentration determined by RIA and x is the concentration added.

Determinations of ganirelix in plasma or serum were carried out with no preliminary purification of the analyte. It was necessary, however, to add an equivalent aliquot of ganirelix-free plasma (either diluted or undiluted as

TABLE 2

ACCURACY AND PRECISION DATA FOR THE CONCENTRATION RANGE 0.05 - 50.0 ng/ml[a]

Amount Measured (ng/ml)[b]

Amount Added (ng/ml)	Expt 1 Mean (% CV)[c]	Expt 2 Mean (% CV)[c]	Expt 3 Mean (% CV)[c]	Expt 4 Mean (% CV)[c]	Group Mean (% CV)[d]	Ratio of Measured to Added
0.05	0.051 (10.4)	0.059 (14.8)	0.051 (8.0)	0.047 (7.5)	0.052 (9.7)	1.04
0.20	0.218 (9.4)	0.200 (7.2)	0.182 (4.3)	0.235 (10.2)	0.209 (10.9)	1.05
0.50	0.521 (10.0)	0.501 (7.2)	0.552 (1.5)	0.570 (4.6)	0.536 (5.8)	1.07
2.00	2.058 (8.3)	1.99 (3.9)	1.58 (15.2)	2.07 (12.0)	1.92 (12.1)	0.96
5.00	4.92 (0.89)	5.29 (7.6)	5.35 (7.5)	6.45 (3.2)	5.50 (12.0)	1.10
20.0	21.0 (5.0)	18.7 (12.4)	20.9 (8.3)	19.5 (5.9)	20.0 (5.6)	1.00
50.0	48.6 (16.2)	48.8 (8.9)	46.1 (4.6)	50.7 (6.4)	48.6 (3.9)	0.97

a Known amounts of ganirelix were added to ganirelix-free plasma and assayed by this method.
b Values are the means of RIA quadruplicates, numbers in parentheses are the CVs for the means.
c Intraassay.
d Interassay.

appropriate) to all standard curve tubes in order to minimize differences between standards and unknowns in the RIA incubation media.

The data on crossreactivity are summarized in Table 1. From this data we conclude that there should be very little interference in the assay from ganirelix

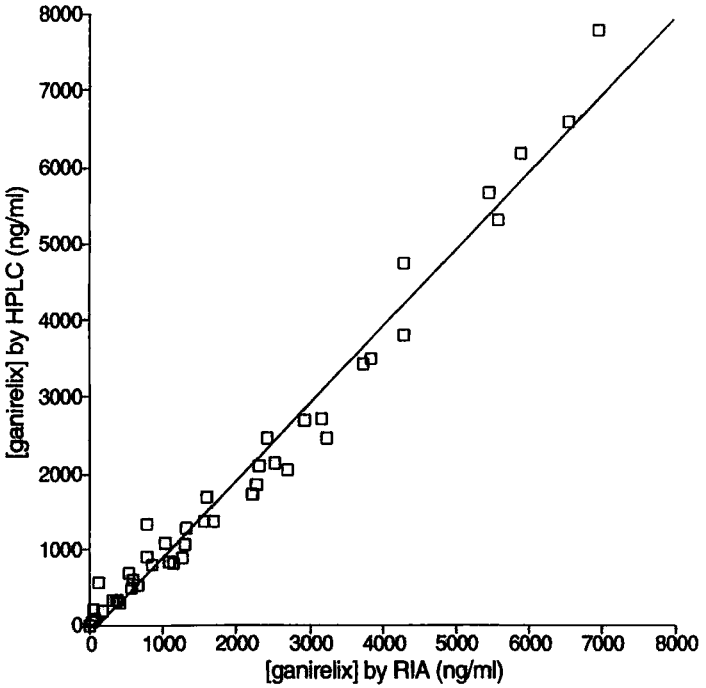

FIGURE 3: Comparison of HPLC-RC assays with RIA for the determination of ganirelix in plasma. Four monkeys were each given a single intravenous dose of [^{3}H]-ganirelix and then serial time blood samples were taken over a 24 hr period and assayed for ganirelix by the two methods.

metabolites. None of the various peptide fragments crossreacted, and ganirelix free acid crossreacted only to the extent of 1%. The free acid has not been identified as a metabolite in animals. Independent evidence of the validity of the RIA was obtained from a comparative study in which plasma samples collected from cynomolgus monkeys that had been given [^{3}H]-ganirelix by intravenous injection were analyzed by both RIA and by an HPLC-RC method (7). The results of this study are plotted in Fig. 3. Linear regression analysis of the data yielded

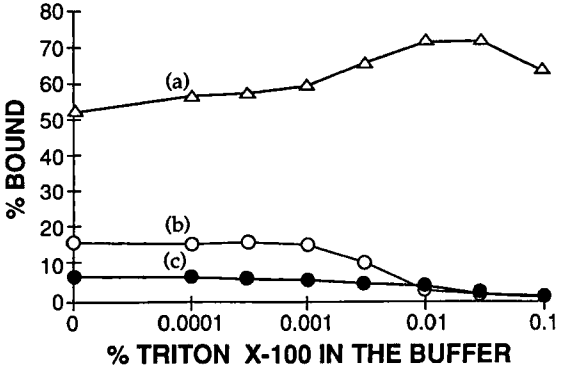

FIGURE 4: The effect of Triton X-100 on the total binding in buffer of analyte to antibody (a) and on the non-specific binding in buffer only (b) and in buffer containing an aliquot of ganirelix-free plasma (c).

the equation $y = 0.92\ x + 33.7$, $r = 0.990$, where x is the RIA value and y is the HPLC-RC value. The reasonably good agreement between the two methods further confirms that there is little metabolite interference in the RIA.

Adsorptive losses of the analyte in any assay involving the determination of picogram quantities of the analyte are always of primary concern. It became apparent during the development of this method that ganirelix would be problematic, and that the use of relatively high concentrations of BSA (up to 1%) in the RIA buffer would not fully protect against significant losses. We found that the presence of Triton X-100 in the RIA buffer was essential for acceptable assay performance. However, the amount of Triton X-100 added was critical because high concentrations of the surfactant severely inhibited the binding of ganirelix to

TABLE 3

THE EFFECT OF TRITON X-100 IN THE RIA BUFFER ON THE ADSORPTIVE LOSSES OF THE ANALYTE ON THE WALLS OF THE ASSAY TUBES

% Triton X-100 in the Buffer	% ^{125}I-Ganirelix Adsorbed on Assay Tubes
0.00	21.7
0.0001	15.4
0.001	0.58
0.01	1.4
0.10	1.4
1.00	2.9

the antibody. It was, therefore, important to determine the optimum concentration required. The results in Fig. 4 show that a Triton X-100 concentration between 0.01 and 0.03% results in the highest total binding and the lowest non-specific binding. At a concentration of 0.1% the surfactant is already beginning to inhibit the total binding. Furthermore, the data in Table 3 show that a concentration of 0.01% Triton X-100 is in the optimum range for minimizing adsorptive losses of ganirelix on the walls of the assay tubes.

The results of the optimization of the separation of bound from free radioactivity with the second antibody precipitation procedure are summarized in the three dimensional plot in Fig. 5. When rabbit IgG was used as the carrier rather than NRS a flatter and more uniform plateau was obtained. The optimum concentrations for IgG and GARGG were derived from this plot. The plateau for

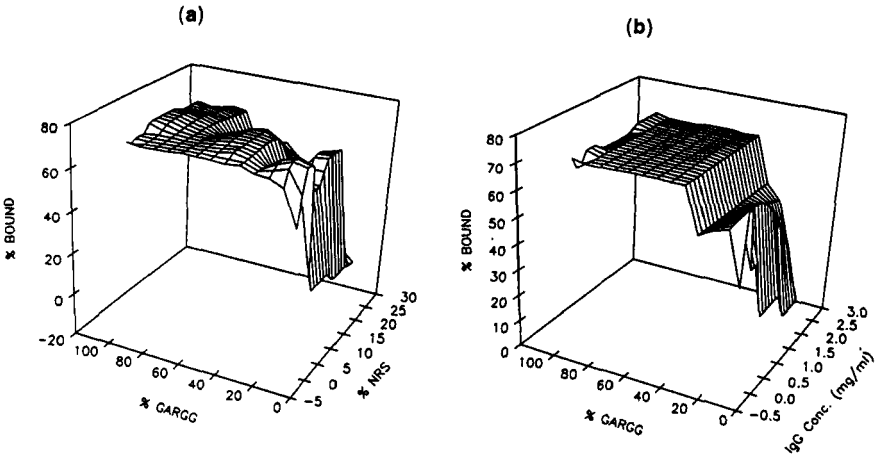

FIGURE 5: Three dimensional plot summarizing optimization of the second antibody precipitation. The total binding was plotted as the % bound (Z-axis) for varying concentrations of GARGG (X-axis) and varying concentrations of NRS (5a) or IgG (5b) on the Y-axis.

NRS (Fig. 5a) was not as smooth as for IgG (Fig. 5b) and the interpretation of this was that a greater degree of variability in the precision of the replicates would be obtained. Assays in which some of the data was derived from NRS as the carrier and some from IgG showed that for the most part, IgG gave less variable and more accurate results. This was apparent in spiked quality controls which were run with each assay during the course of a study.

The RIA method described here has been applied to pharmacokinetic, toxicological, formulation and bioavailability studies.

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