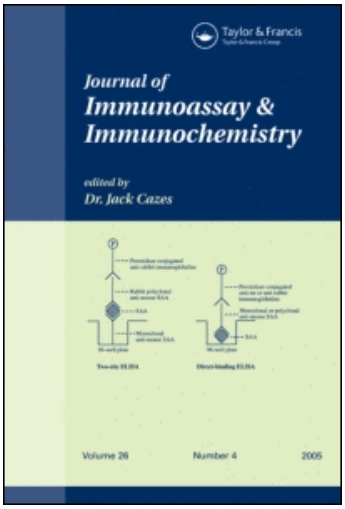

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Development of a High Capacity Radioimmunoassay Procedure: Ovine Follicle Stimulating Hormone Determination in Blood Plasma as a Model

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DEVELOPMENT OF A HIGH CAPACITY RADIOIMMUNOASSAY PROCEDURE: OVINE FOLLICLE STIMULATING HORMONE DETERMINATION IN BLOOD PLASMA AS A MODEL

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ABSTRACT

A sensitive, specific and accurate homologous radioimmunoassay (RIA) for ovine follicle stimulating hormone (oFSH) has been developed, using a [^{125}I]oFSH tracer and a polyclonal rabbit anti-oFSH-serum at a final dilution of 1:224,000. The separation of free and antibody-bound tracer is based on the double antibody solid phase system. The assay was found to be specific for oFSH; cross-reactivity with oLH, oPrl and oGH was lower than 0.2%. The detection limit was 7 pg per tube. Inter- and intra- assay coefficients of variation (CV) were 3.7 - 8.5% and 6.6%, respectively.

The use of a few selfmade appliances in combination with a well-considered time-schedule enabled the processing of three thousand blood plasma samples in triplicate within two weeks. The particular advantage of the method described here is the fast, easy and safe separation of free and antibody-bound tracer with minimal handling of radioactive tubes.

KEY WORDS: (radioimmunoassay) (ovine follicle stimulating hormone)

INTRODUCTION

Endocrinological research often requires the analysis of large numbers of blood samples for several protein hormones. Analysis of all samples of an experiment within a few weeks time has a

number of advantages. Comparability of the results of several groups of samples is optimal because of the use of one (fresh) label for all samples. In addition, the total number of iodinations for different assays is reduced to a minimum. The financial and time benefits of such a procedure are also obvious.

This paper describes the development of a sensitive, high capacity RIA procedure for the assessment of ovine FSH blood plasma levels.

MATERIALS AND METHODS

Materials

Ovine follicle stimulating hormone, highly purified for iodination (NIAMDD-oFSH-I-1), polyclonal rabbit anti ovine follicle stimulating hormone serum (NIAMDD-anti-oFSH-1) and ovine follicle stimulating hormone reference preparation (NIAMDD-oFSH-RP-1), all produced by Dr. A.F. Parlow, were kindly provided by Dr. S. Raiti (National Hormone and Pituitary Program, University of Maryland School of Medicine) through the programs of the National Institute of Diabetes, Digestive, and Kidney Diseases. Ovine prolactin (NIH-P-S-11) and ovine luteinizing hormone (NIH-LH-S15) were a gift from the National Institutes of Health, Bethesda. Hormones and antiserum were stored at -80°C until use.

$Na^{125}I$ (IMS-30, 1 mCi per vial) in alkaline solution was obtained from the Radiochemical Centre, Amersham, England; secondary sodium phosphate, sodium azide, hydrochloric acid, sodium chloride, chloramine-T and potassium iodide (all analytical grade)

from Merck AG, Darmstadt, Germany; bovine serum albumin (BSA, fraction V) and sodium metabisulphite (analytical grade) from Fluka AG, Buchs, Switzerland; Sac-Cel (donkey-anti-rabbit solid phase) from Wellcome Diagnostics, England and Sephadex (G-50-coarse and G-100) from Pharmacia Fine Chemicals, Uppsala, Sweden.

General Procedures

The increase of the number of samples that could be analysed by RIA during a certain period of time was based on a well-considered time-schedule combined with the use of a number of selfmade and time-saving appliances. During the first week of a new RIA period an assay, comprising 600 blood plasma samples in triplicate, was started each day. During the second week, following a six day incubation with the first antibody (seven days for the assay started on monday), the second antibody was added and the assay was finished one day later. This way of working has several advantages. Pipetting equipment could be used for five days in the same formation and the long incubation time with the first antibody allowed: a) the reaction to be performed at 4°C instead of room temperature and b) all tubes to reach equilibrium, so that there was no effect of the six hours time difference between the start of the first and the last tube of an assay. This was checked for each assay by comparing two standard curves and two series of control samples that were started at the beginning or at the end of the day.

Reference FSH or sample, tracer and first antiserum solutions and RIA buffer were added simultaneously to each RIA tube by means

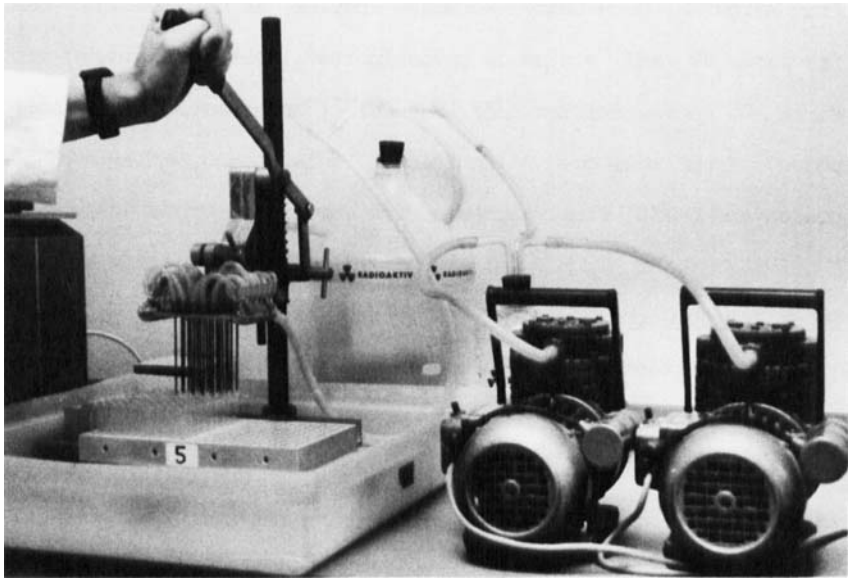


PHOTO 1 - Aspirating device. A downward movement of the arm of the standard drill results in the simultaneous aspiration of the supernatants of twentyfive tubes until a volume of 0.4 ml is left. Removal of more supernatant, with the risk of losing the precipitate, is prevented by blocking the downward movement by means of the adjustment on the standard drill. The tubing of the holder carrying the needles is connected to a vacuum pump via a liquid reservoir and a safety bottle.

of a model 24006 Micromedic automated pipetting station coupled to a model 25006 Micromedic automatic pipette (Micromedic Systems Inc., Horsham, USA). Subsequently the tubes were placed in self-made compact tube holders, consisting of a plastic (pvc) block with two hundred holes, that fitted in the HL-2 rotor of a Sorvall-RC-3 centrifuge (Dupont Company, Newtown, Connecticut, USA). Tubes stayed in these holders during all procedures. In this way

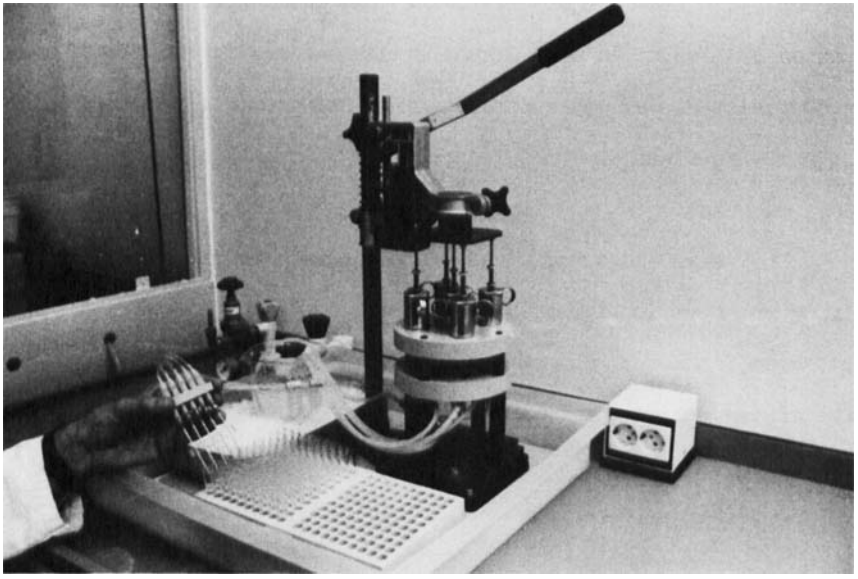


PHOTO 2 - Washing device. Washing buffer is added simultaneously to five tubes by moving the arm of the standard drill downward. During the upward movement the syringes (B-D Cornwall; 0-5 ml) are refilled automatically. The required volume of washing buffer is set by blocking the downward movement at the right level by means of the adjustment on the standard drill.

time consuming and possibly erroneous transposition of tubes was avoided. Second antibody solid phase solution was added to four tubes simultaneously by means of four 1 ml dispensing pumps mounted on the pipetting station. Addition of the solid phase in a volume of 1 ml ensured fast flow through the tubing of the pipetting station and avoided precipitation in the tubing. Supernatants of series of twentyfive tubes were aspirated simultaneously by vacuum by means of a selfmade device, consisting of a standard drill with a holder carrying twentyfive needles that were connected to a

vacuum pump via a liquid reservoir (see photo 1). Precipitates of series of five tubes were washed simultaneously by means of another selfmade device, consisting of a standard drill with a holder carrying five B-D Cornwall syringes (Becton-Dickinson, France SA), each having a capacity of 0-5 ml (see photo 2).

The ease and speed of the sample pipetting step was the most important contribution of the automated pipetting station. Its presence, however, is not an absolute prerequisite for the use of the above-mentioned procedure. In our laboratory pipetting of samples and RIA buffer followed by storage at -20°C until the start of the assays proved to be a very useful alternative.

Iodination and Purification of Labelled oFSH

The procedure of Hunter (1) was used with the following modifications: 4 µg of oFSH-I-1 was iodinated with 1 mCi of $Na^{125}I$ for 30 seconds in the presence of 20 µg of chloramine-T (60 µl volume) ; the reaction was stopped with 240 µg of sodium metabisulphite followed by 2 mg of potassium iodide (360 µl end volume).

Labelled FSH and free ^{125}I were separated on a 15 x 1 cm column of Sephadex G-50-coarse (40 fractions of 0.5 ml). Fractions (10) with the highest amount of protein-associated radioactivity were pooled and transferred to a 90 x 1.6 cm column of Sephadex G-100 to separate immunoreactive labelled FSH from other material (160 fractions of 1 ml). The columns, placed at 4°C, were prepared and eluted with RIA buffer (0.05 M phosphate buffer, 0.15 M sodium chloride, 0.1% sodium azide, pH 7.2) containing 0.5% BSA (added

immediately before use). Fractions with the best immunoreactivity (tested for non-specific and antibody binding in both presence and absence of 0.1 ng unlabelled oFSH-RP-1) were pooled and stored at 4°C until use in the RIA.

Radioimmunoassay Procedures

RIA buffer (0.5% BSA) was used in all dilutions for the first antibody incubation. The following reagents were added simultaneously to a tube: 50 µl containing 20,000 cpm of [^{125}I]FSH; 50 µl of a 1:16000 initial dilution of the antiserum [exept tubes for non-specific binding (NSB)]; 100 µl of reference solution or an appropriate amount of sample; an amount of RIA buffer, necessary to wash the reference or sample quantitatively from the delivery tip, to achieve a total volume of 0.7 ml. Reference solutions: 0.00 (initial binding or B0), 0.01, 0.02, 0.03, 0.05, 0.07, 0.1, 0.15, 0.2, 0.3, 0.5, 0.7, 1, 2 and 5 ng oFSH-RP-1/100 µl. Sample amounts: 200 µl of ram lamb or 50 µl of ewe lamb blood plasma. The tubes were vortexed and covered with parafilm to prevent evaporation during the 6 days incubation time at 4°C, after which 1 ml of water/ice cooled second antibody solid phase solution (1 volume of Sac-Cel anti-rabbit diluted with 7 volumes of 0.1% BSA-RIA buffer) was added. Following a second incubation at 4°C for one night, the tubes were centrifugated for 5 minutes at 1650 g and 4°C, after which the supernatants were aspirated until a volume of 0.4 ml was left. The precipitates were washed twice with 2.2 ml water/ice

cooled RIA buffer. The precipitates were counted for 5 minutes in an automatic gamma system.

Calculations

Evaluation of the decay corrected counting data was performed with a computer program developed by Rodbard and Lewald (2).

Assay Evaluation

Data concerning the specificity of the antiserum, in terms of its reactivity with anterior pituitary hormones other than FSH, were provided by NIAMDD (Technical report number 141 of the Pituitary Hormones and Antisera Center); oLH NIAMDD-23 (2.4 x NIH-LH-S1), oPrl AFP-4328-C (35 IU/mg) and oGH AFP-5285-C (2 IU/mg) showed a relative potency of 0.159, 0.0003 and 0.0202% towards oFSH-RP-1 (100%) respectively, at a final antibody dilution of 1:80,000. These data were confirmed for the hormones available. Parallelity of the standard curve with ram lamb and ewe lamb blood plasma dilution curves was checked. Recovery of several known amounts of reference hormone, added to ovine blood plasma, was determined to test accuracy. From the results of repeated determinations of seven dilutions of an ovine blood plasma control sample the intra- and inter-assay CVs were calculated. Sensitivity of the assay was calculated as the detection limit, defined by Abraham (3) as the antigen concentration corresponding to the lower confidence limit of the number of counts specifically bound in the absence of unlabelled antigen (B0 - 2 sd).

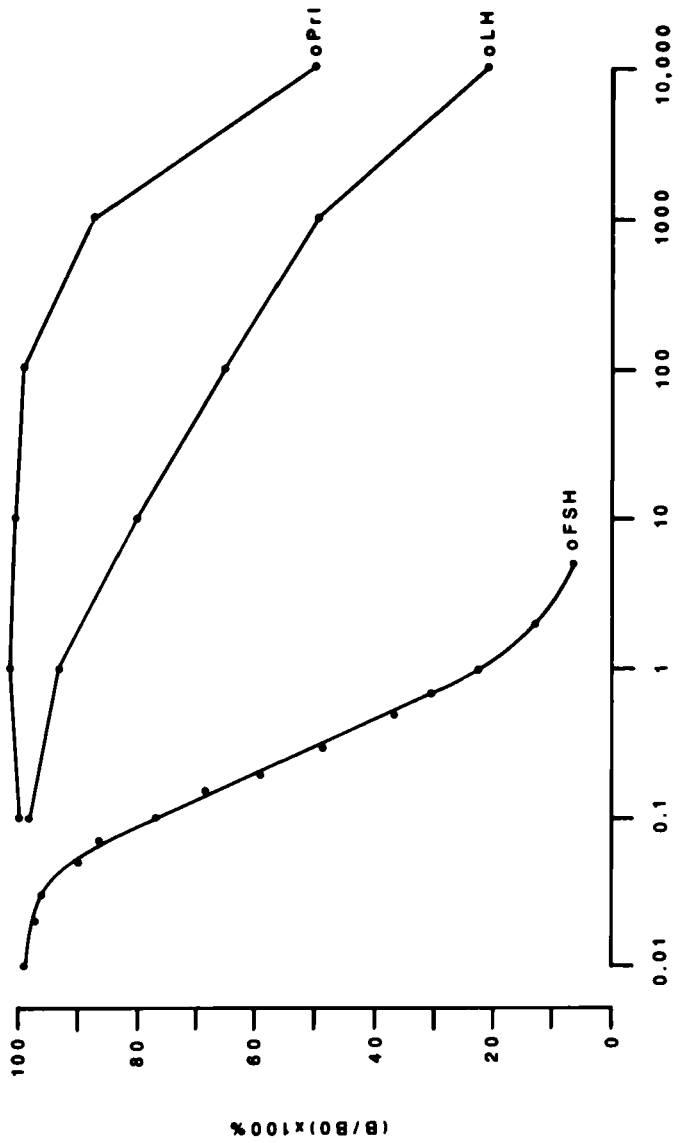
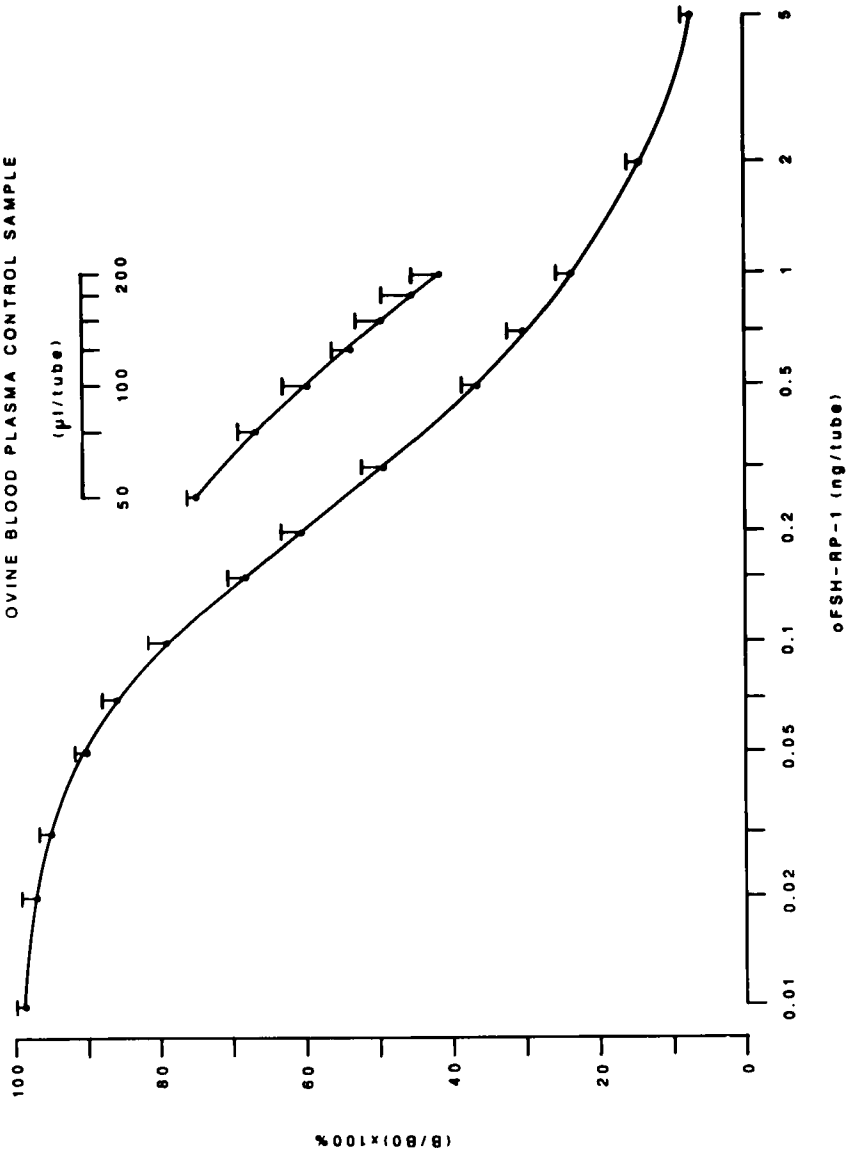


FIGURE 1 - Inhibition curves of oFSH (RP-1), oLH (NIH-LH-S15) and oPrl (NIH-P-S-11) in the oFSH radioimmunoassay at a final antiserum dilution of 1:224,000.



RESULTS

Purification and Immunoreactivity of Labelled oFSH-I-1

The degree of incorporation during four consecutive iodinations ranged between 54 and 86%, corresponding to specific activities of 135 to 214 µCi/µg of oFSH respectively. A single, regularly shaped Sephadex G-100 protein peak was observed with all iodinations. Fractions with the best immunoreactivity were in the upper half of the peak. Freshly prepared tracers showed a B0 of 32 - 40% and were not used longer than about three weeks.

Radioimmunoassay

Specificity tests with oLH (NIH-LH-S15) and oPrl (NIH-P-S-11) showed relative potencies (calculated at 1/2 B0) of 0.03 and 0.003% towards oFSH-RP-1 respectively (fig. 1). Reproducibility of the standard curve and the control sample dilution curve is shown in fig. 2. No differences were found between the triplicates of standard or control sample dilution curves started at the beginning or at the end of each day. Non-specific binding for tubes containing 0, 50 or 200 µl of blood plasma was 1.7 +/- 0.4% (mean and sd, n = 7), 1.4 +/- 0.5% (n = 6) and 1.5 +/- 0.4% (n = 5), respectively. Addition of 0.1, 0.2, 0.4 and 0.8 ng of oFSH-RP-1 to

FIGURE 2 - Mean standard curve (n=7) and control sample dilution curve (n=6) over a period of 10 months during which 4 iodinations were performed. Vertical bars show positive standard deviations. Coefficients of variation (from left to right) of the standard points are 1.2, 2.2, 1.8, 1.7, 2.4, 3.1, 3.4, 4.8, 6.5, 5.9, 6.9, 8.7, 10.7 and 16.0% respectively, and of the control sample points are 1.7, 3.8, 5.9, 5.0, 6.8, 9.3 and 9.2% respectively.

50 µl ovine blood plasma (containing 0.124 ng FSH) resulted in a recovery of 102, 114, 108 and 92% respectively.

Statistical analysis regarding parallelity of the standard curve with ram and ewe lamb blood plasma dilution curves (see also fig. 3) yielded the following results. Although repeated radioactivity measurements on the same tube were following a Poisson distribution, measurements on replicates were clearly overdispersed. Since the exact nature of the overdispersion was not known it seemed convenient to describe the variance as a constant multiple ϕ of the mean μ. The scale parameter ϕ was estimated from the pooled within concentration variance of the five minutes counts after using the square root transformation. The following relationship between the mean μ of a count and the corresponding concentration x was assumed:

$$\mu = \frac{\mu_0}{1 + \exp[-\alpha - \beta \log(x)]},$$

where μ_0 is the maximum expected count for concentration 0. This is equivalent to a linear relationship between the logit of μ / μ_0 and the log-concentration with intercept α and slope β. For the standard curve the fit of the model was acceptable except for the extreme amounts (0.01, 0.02, 0.03, 0.05, 0.07, 2 and 5 ng) which were dropped from the analysis. The model was fitted with the statistical package Genstat (4). The parameters were estimated by maximum-quasi-likelihood (5). The standard curve and blood plasma dilution curves were compared with respect to the parameters μ_0 and β with the maximum-quasi-likelihood ratio test (6). Since log(x) is only known up to an unknown constant the parameter α is

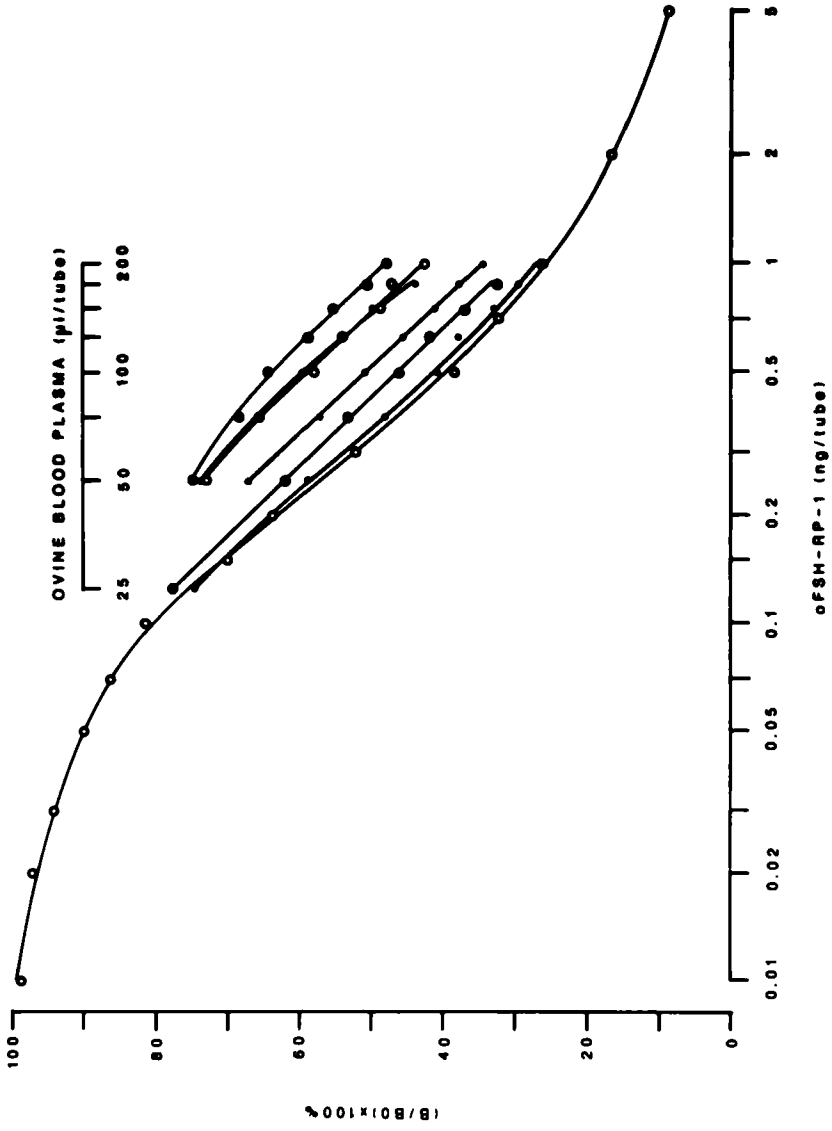


FIGURE 3 - Parallelity of ram (upper 3 lines) and ewe lamb blood plasma inhibition curves (lower 3 lines) with the standard curve.

not estimable and therefore of no use for a comparison. The non-linear model was fitted to the sums of the triplicate five minutes counts per concentration. A significant difference ($p<0.005$) between the curves was found, resulting in a slight decrease of the determined blood plasma FSH values with increasing amounts of sample. For the ram and ewe lamb blood plasma dilution curves (going from right to left) in fig. 3 the FSH concentration per ml of plasma for the highest amount of sample was 0.78, 0.87, 0.99, 0.93, 0.97 and 0.98 times the value for the lowest amount of sample.

Ovine blood plasma control sample amounts of 50, 75, 100, 125, 150, 175 and 200 µl, that were analysed during 6 assays (fig. 2), yielded mean concentrations and inter-assay CVs of 2.38 ng/ml and 7.1%, 2.29 ng/ml and 6.8%, 2.27 ng/ml and 7.4%, 2.28 ng/ml and 3.7%, 2.21 ng/ml and 6.5%, 2.23 ng/ml and 8.5% and 2.24 ng/ml and 7.6%, respectively. The intra-assay CV for twelve replicates of the 50 µl amount during these assays was 6.6 +/- 2.0% (mean and sd). The detection limit of the assay was 7 pg per tube, corresponding to 0.14 or 0.035 ng/ml if 50 or 200 µl of blood plasma was taken for analysis.

DISCUSSION

The validity of the assay has been carefully evaluated. The iodination and tracer purification procedure yielded tracers with excellent binding characteristics, resulting in a decreased first antiserum concentration needed and an increased sensitivity of the

standard curve, both by a factor three as compared to data from NIAMDD (Technical report number 141 of the Pituitary Hormones and Antisera Center). Data regarding reproducibility of the assay are comparable to those reported for other assays using the same NIAMDD ovine FSH RIA materials (7,8,9). Data from NIAMDD, demonstrating the high specificity of the antiserum, were confirmed. Statistical analysis regarding parallelity of standard and blood plasma dilution curves revealed significant differences, however resulting only in minor differences in the results of a wide range of sample volumes.

The outlined RIA procedure, based on known methods, has enabled us to gather highly comparable oFSH data of large numbers of samples within a minimum of time. The long incubation time with the first antibody limits the use of the assay as a rapid procedure. Although building of the appliances, using commercially available basic components, is not a difficult task for an experienced technician, provision by a commercial firm would insure availability for every laboratory. The procedure has proven to be applicable for all protein radioimmunoassays performed in our laboratory.

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