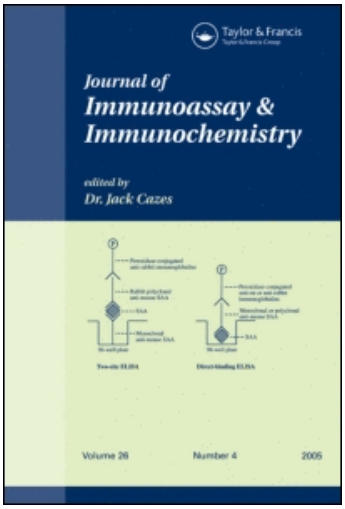

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ENZYME IMMUNOASSAY (EIA) FOR EQUINE CHORIONIC GONADOTROPIN / PREGNANT MARE SERUM GONADOTROPIN (eCG/PMSG)

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ABSTRACT

A simple, accurate, sensitive enzyme immunoassay (EIA) has been developed that permits the measurement of equine Chorionic Gonadotropin activity in pregnant mare plasmas or serums as well as in commercial and highly-purified preparations. This assay is specific for eCG and eLH which share the same polypeptide structure but differ in their oligosaccharidic chains. The more important result is that this EIA has been found to be give data in very close agreement with the *in vivo* assay. Therefore this very rapid and convenient assay can be used to measure the activity of eCG/PMSG in pregnant mares serums in in-field conditions as well as in crude or highly-purified preparations.

(KEY WORDS: PMSG , EIA)

INTRODUCTION

High gonadotropic activity is present in the sera of mares between day 40 and 120 of gestation [1]. Because of its dual FSH/LH activity [2-4], pregnant mare serum gonadotropin (PMSG) has been used for a long time in the induction of superovulation as well as in oestrus synchronization in cattle, sheep and goats [5]. Since this hormone is produced by endometrial cups [6], it is now more appropriately named equine Chorionic Gonadotropin (eCG).

The goal of the present work was to set-up a convenient, rapid and exact assay for eCG aimed to have a precise real-time follow-up of the rise and fall of serum concentrations of the hormone in pregnant mares. Such an assay is aimed 1) to select the mares with high levels of eCG so that blood is only taken from them; 2) to allow rapid measurement of eCG in the course of its purification in order to speed up the procedure and then to lower the costs; 3) to check easily the calibration of eCG preparations.

In order to allow the use of this assay in the fields with minimum equipment, we chose to develop an enzyme immunoassay (EIA) using the same antiserum as the one used in our eCG radioimmunoassay (RIA) [7]. In the present paper we describe the methodological aspects of the eCG EIA and we compare its results with those of the *in vivo* bioassay.

MATERIALS AND METHODS

The method developed in this work for the measurement of eCG is a competitive EIA in which standard or unknown concentrations of eCG in solution compete with immobilized eCG for specific antibodies in solution. The concentrations of eCG in solution are deduced from the inhibition of antibody binding to immobilized eCG using peroxidase-conjugated antibodies directed against rabbit IgG.

Materials. The antiserum against eCG was raised in five rabbits using a previously described protocol [7]. The hormone preparation used as the reference is a commercial preparation (Searle) which has been previously shown to behave like native eCG in RIA, RRA and *in vitro* and *in vivo* bioassays in contrast to the international standard IRP2 [7]. In addition, two other samples of a commercial preparation (Chrono-Gest, lot n° 2970; Intervet) previously giving consistent values in these three assays, were used as internal standards.

Coating of wells. In each well of microtiter plates (96 wells) 100 μl eCG (0.25 IU) in 0.1M carbonate-bicarbonate buffer pH 9.6 were introduced. The wells were sealed with an adhesive film covering the plates and after one hour at 37°C and 16 hours at 4°C, the plates can be used immediately or conserved at 4°C until use.

Saturation of wells. Immediately before use, the wells were emptied either by aspiration or more conveniently by inversion and blotting of the plates on filter paper. Then, 200 μl phosphate buffer saline (PBS) containing 2mg/ml bovine serum albumin (PBS-BSA) are added in each well, the wells were closed using an adhesive film covering the plates and an incubation of at least 30 min at 37°C was performed. The wells were emptied just before the introduction of the mixture of samples and antibody (see below).

Assay. Dilutions of reference, internal standard and unknown samples were performed with PBS-BSA buffer containing 0.1% Tween (v/v) in the range 0.0005 to 1 IU/100 μl. The antiserum was diluted 1/25000 in the same buffer and 200 μl of diluted antiserum were mixed with 200 μl of each standard or unknown dilution. After vortexing, 100 μl-triplicates of each sample were distributed in the coated wells which were emptied just before

(see above). Then the plates were covered with adhesive film and incubated for 1 hour at 37°C.

Detection of bound antibodies. The anti-rabbit IgG γ-globulins conjugated to peroxidase (Pasteur Productions, Paris) were diluted in the PBS-BSA-Tween buffer at a concentration of 2.5 μg/ml. The plates were emptied and washed three times with 150 μl PBS buffer containing 0.1% Tween. After the third washing, 50 μg O-phenylenediamine (OPD; Sigma, St Louis) in 100 μl of 0.1M citrate-citric acid buffer pH 5.6 containing 2% H_2O_2 were added in each well. After 5-10 minutes at room temperature in the dark, 50 μl of 2N H_2SO_4 were added in each well and optical densities at 492 nm were read on a Titertek Multiskan Plus MKII plate reader. A cheap and portable battery-powered plate reader can also be used for outdoor measurements.

Calculations. The optical densities were plotted as a function of log [dose] for the reference preparation, the two internal standards and unknown samples. The curves were analyzed by 3+3 parallel line assay [8]. The data can also be analyzed by non-linear (sigmoid) regression analysis (Graph-PAD; ISI Philadelphia). The assays were rejected when the value for one of the internal standards deviates more than 5% from its nominal value.

In vivo assay The bioactivity of eCG preparations was determined in Wistar (INRA 03) prepubertal female rats using the method of Cole and Erway [9] which is the assay recognized by the International Pharmacopea.

RESULTS

Range of sensitivity. Figure 1 shows the standard dose-response curve obtained using the conditions described under "Materials and

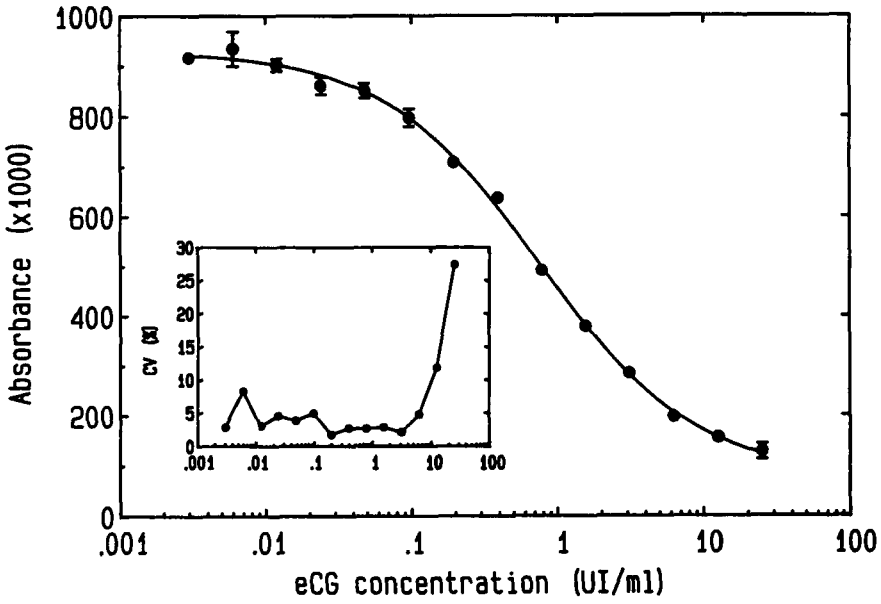

Figure 1
Standard curve of eCG/PMSG in the competitive enzyme immunoassay. The preparation used in this experiment is the commercial preparation (Searle) which is used as the standard for all PMSG activity determinations. The insert shows the coefficient of variation of the determinations as a function of hormone concentration.

Methods". The sensitivity limit as defined by the dose giving a response departing by two standard deviations from the zero value was found to be 20 mIU/ml . When needed this limit can be decreased to 3.7 mIU/ml simply by incubating the mixture of antibody and samples for 3 hours at 37°C before distributing the 100 μl-aliquots to the wells coated with eCG.

Intra- and inter-assay precision. The insert in figure 1 shows the coefficient of variation of eCG determination (n=5) in the EIA as a function of its concentration. Its value is below 6% in a large range of concentrations. The inter-assay precision was determined on the same sample in 5

experiments. The coefficient of variation calculated for the five determinations (each made by the 3+3 parallel line assay) was less than 4.6%.

Specificity. Since equine Luteinizing Hormone (eLH) differs from eCG only by its oligosaccharide moieties [10], it was interesting to check its cross-reactivity in the eCG EIA. Moreover all pituitary and placental glycoprotein hormones share a common α-subunit; it is thus important to check their cross-reactivity in the assay.

Figure 2 shows the cross-reactivity of the equine pituitary gonadotropins eLH and eFSH and figure 3 shows the behavior of eCGα- and ß-subunits in the assay. On a weight basis, equine LH was 67% as active as eCG in the assay while eFSH exhibited 6% cross-reactivity. The eCGα- and ß-subunits exhibited 3% and 11% cross-reactivity respectively . On a molar basis, the cross-reactivities of eLH, eFSH, eCGα and eCGß were calculated to be 51%, 5%, 1% and 7% respectively assuming molecular weights of 44000 for eCG, 34000 for eLH and eFSH, 15000 for eCGα and 29000 for eCGß .

Comparison with *in vivo* bioassay In the Pharmacopea, the only recognized bioassay for eCG (PMSG) is the *in vivo* assay proposed by Cole and Erway in 1941 [9]. It is thus important to compare the EIA data with those obtained in this *in vivo* method in order to ascertain that there is good correlation between the two methods whatever the origin and method of purification of the hormone.

Sixteen different eCG commercial preparations as well as one highly purified eCG preparation from our laboratory were assayed in both the eCG EIA and the *in vivo* bioassay using the same reference preparation (Searle). Figure 4 shows the correlation of the two assays. Linear regression

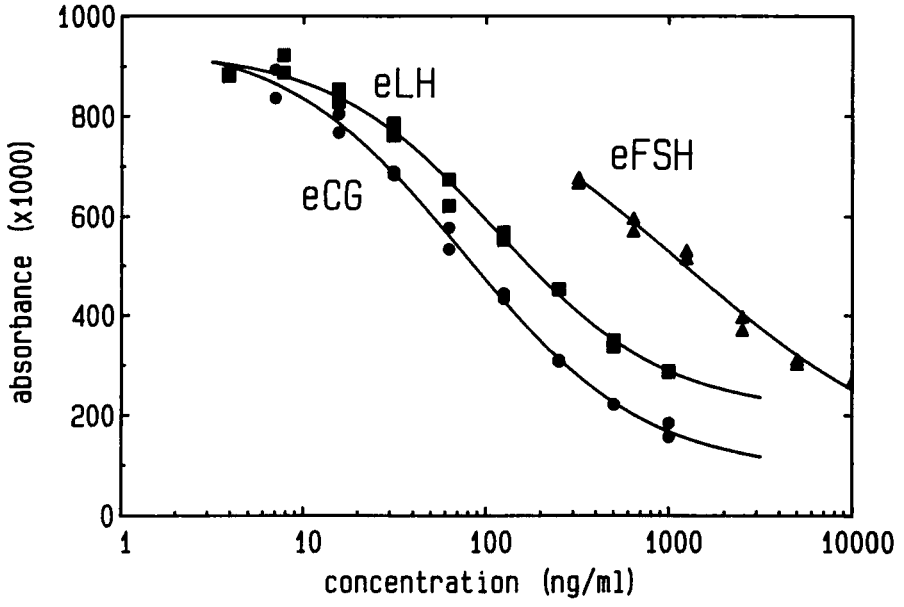

Figure 2
Cross-reactivity of the pituitary equine gonadotropins eLH and eFSH in the eCG/PMSG competitive enzyme immunoassay.

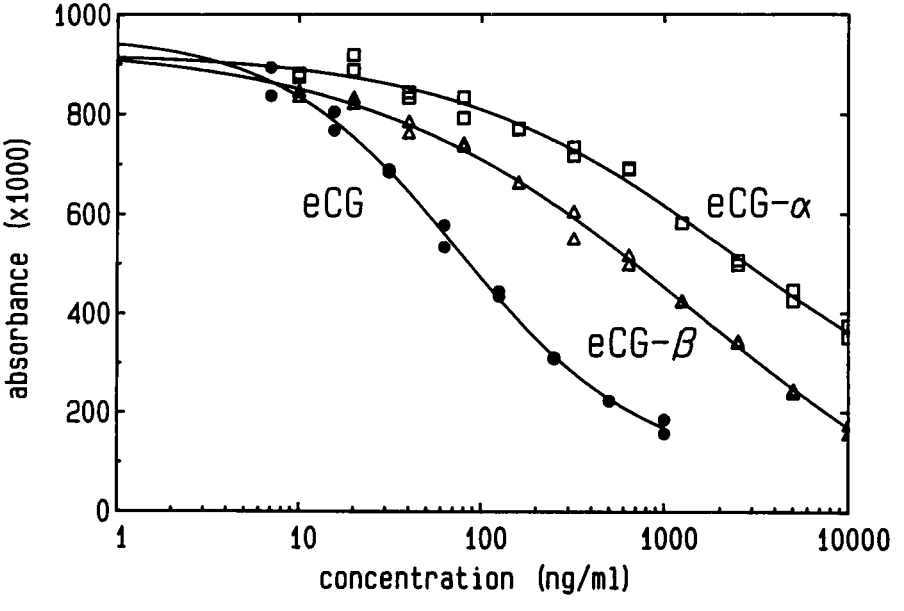

Figure 3
Cross-reactivity of the α- and ß-subunits of eCG/PMSG in the eCG/PMSG competitive enzyme immunoassay.

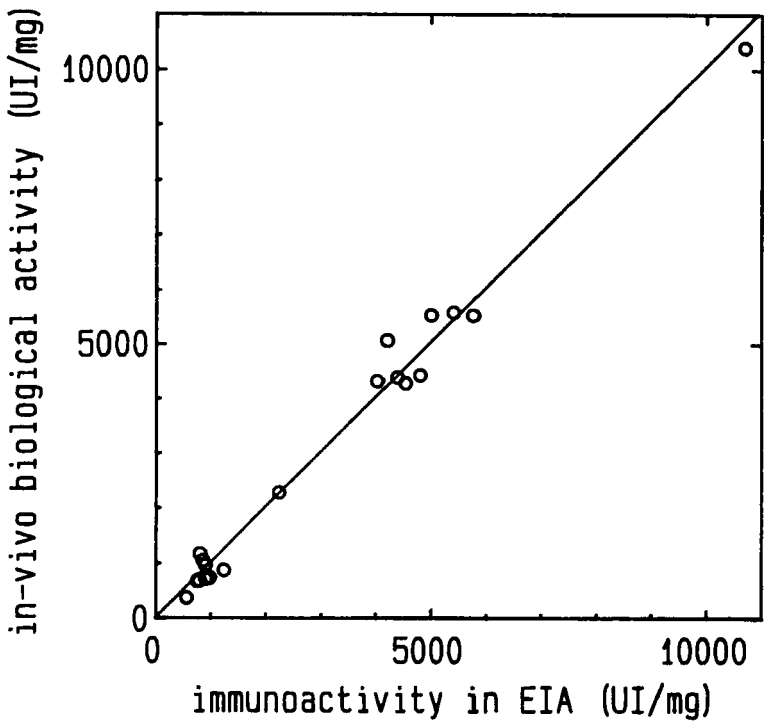

Figure 4
Correlation of the eCG/PMSG enzyme immunoassay with the in vivo biological assay of the International Pharmacopea.

analysis of the data gave a slope of 1.006, an Y intercept of 15.9 and an R^2 value of 0.984. The mean value ($\pm$ S.D.) of the B/I activity ratios for the 17 samples was found to be 1.01 $\pm$ 0.22.

DISCUSSION

Equine Chorionic Gonadotropin is largelly used in several domestic species for oestrus synchronization and/or superovulation. It is thus of utmost importance that the *in vivo* biological activity of eCG can be

determined. However, the *in vivo* assay is very time-consuming and cannot be undertaken in in-field conditions for the selection of mares at the time of blood collection. For these reasons, we have undertaken the setting-up of a rapid, cheap, convenient and accurate immunoassay that permits the measurement of eCG concentrations during its purification and for control purposes.

The assay is specific for eCG and eLH which possess the same amino-acid sequences in both their α- and ß-subunits [10]. Equine FSH which only shares its α-subunit polypeptide chain with eCG and eLH cross-reacts ten time less in the EIA than eLH. In agreement with the lower activity of eFSH in the EIA, the common α-subunit of eCG was found to be less potent than the specific eCGß-subunit. For the determination of eCG during gestation, the cross-reaction with eLH does not matter as this latter hormone is present in very low amounts compared to eCG. In fact, we are currently trying to increase the sensitivity of the EIA described in this paper in order to set-up a eLH EIA that would be valid in the absence of eCG. This assay is also being currently tested as a pregnancy test in the mare (Lecompte, Duchamp, Palmer, Combarnous in preparation).

For non-denatured intact eCG preparations, the enzyme immunoassay described in this paper gave values that were in perfect agreement with the values determined by the *in vivo* bioassay of the Pharmacopea. By contrast, when it is not the case this is indicative of modifications in the eCG molecules. As previously shown [7], it is the case for the International Reference Preparation (IRP2 from WHO) which contains a large amount of denatured molecules that are inactive in the *in vivo* bioassay but that are active in the *in vitro* assays. With this reference preparation, completely different values are obtained for native intact eCG

preparations in *in vivo* and *in vitro* assays respectively [7]. It thus absolutely necessary to abandon the IRP2 reference preparation to correctly characterize eCG preparations.

Divergent biological/immunological (B/I) activity ratios are often observed for gonadotropins. These hormones are highly polymorphic because of their carbohydrate heterogeneity and their different isoforms exhibit different B/I ratios. Similarly, the *in vitro* bioassays such as radioreceptor assays or cell culture stimulation assays sometimes give different values than *in vivo* bioassays [7]. This is most probably because of differences in the half-life of different preparations. In the case of eCG, its desialylation by neuraminidase drastically diminishes its *in vivo* activity while it increases its activity in both LH and FSH RRAs [11] as well as in cell culture assays. Therefore, the determination of B/I ratios and of *in vivo/in vitro* activity ratios is a good way to ascertain that a eCG/PMSG preparation is devoid of denatured forms.

This eCG EIA has been used for more than three years for the rapid in-field detection of mares with high levels of hormone. This has permitted the commercial preparation of the hormone starting from large batches of sera that were two to three-fold more active than when the mares were selected on the basis of their gestational age (H. & M.R. Danigno; personnal communication). Moreover, the eCG EIA has been routinely used to rapidly monitor the hormone activity during its purification permitting to speed-up the sequence of steps .

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This paper is dedicated to the memory of our friend, **Dr Horacio Dagnino** (Syntex, Buenos-Aires, Argentina) who took a great part in our motivation to develop this assay.

REFERENCES

1. COLE H.H. & HART G.H. 1930. The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am. J. Physiol. 93, 57-62

2. STEWART F., ALLEN W.R., MOOR R., 1976. Pregnant mare serum gonadotrophin: ratio of Follicle-Stimulating Hormone and Luteinizing Hormone activities measured by radioreceptor assay. J. Endocr., 71, 371-82.

3. COMBARNOUS Y., HENNEN G. & KETELSLEGERS J.M. 1978. Pregnant Mare Serum Gonadotropin exhibits higher affinity for Lutropin- than for Follitropin receptors of porcine testis. FEBS Lett. **90**, 65-8

4. MOORE W.T. & WARD D.N. 1980. Pregnant Mare Serum Gonadotropin. An in-vitro biological characterization of the Lutropin-Follitropin dual Activity. J. Biol. Chem. **255**, 6930-6

5. MURPHY B.D. & MARTINUK S.D. 1991. Equine Chorionic Gonadotropin. Endocrine Rev. 12, 27-44

6. ALLEN W.R. and MOOR R.M. 1972. The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. J. Reprod. Fertil. 29, 313-9

7. CAHOREAU C., COMBARNOUS Y. 1987. Comparison of two reference preparations for horse chorionic gonadotrophin in four in-vivo and in-vitro assays. J. Reprod. Fertil. 79, 281-7

8. COLQUHOUN D (1971) Lectures on Biostatistics. An introduction to statistics with applications in biology and medicine. Clarendon Press, Oxford.

9. COLE HH & ERWAY J. 1941. 48-hour assay test for equine gonadotrophin with result expressed in international units. Endocrinology 29, 514-9

10. SUGINO H., BOUSFIELD G.R., MOORE WT & WARD D.N. 1987. Structural studies on equine Glycoprotein hormones. Amino acid sequence of equine chorionic gonadotropin ß-subunit. J. Biol. Chem. 262, 8603-9

11. COMBARNOUS Y., GUILLOU F, MARTINAT N. & CAHOREAU C. 1984. Origine de la double activité FSH+LH de la Choriogonadotropine équine (eCG/PMSG). Ann. Endocrinol. 45, 261-8