Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

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To cite this article: S. Todini, G.M. Terzano & A. Malfatti (2007) Validation of ELISA kits for determination of Inhibin-A and Estradiol-17-beta concentrations in Buffalo plasma, Italian Journal of Animal Science, 6:sup2, 585-588, DOI: 10.4081/ijas.2007.s2.585

To link to this article: https://doi.org/10.4081/ijas.2007.s2.585

Validation of ELISA kits for determination of Inhibin-A and Estradiol-17-beta concentrations in Buffalo plasma

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ABSTRACT: The aim was to evaluate the suitability of two commercial ELISA kits for human serum or plasma, to measure Inhibin-A (In-A) and Estradiol-17-beta (E2) concentrations in buffalo plasma. Blood samples were obtained by jugular venipuncture from buffalo heifers and cows, and plasma samples were stored at – 20°C until assays. Precision of the methods was evaluated by the intra- and inter-assay coefficients of variation (CVs) of buffalo plasma sample replicates, at different concentrations. Accuracy was evaluated calculating the recovery rates of different proportions of the highest standard added to a buffalo plasma sample at low concentration (observed/expected values x 100). Linearity was evaluated by serially diluting one buffalo plasma sample at high concentration with the assay buffer and calculating by regression analysis the parallelism of the resulting line with the standard line. Intra-assay CVs were 11% and 15.1% for In-A and 1.8% and 3.3% for E2. Inter-assay CVs were 13.9% and 7.4% for In-A and E2, respectively. Mean recovery rate was 97.9% and 98.5% for In-A and E2, respectively. Dilution tests gave good parallelism between the lines obtained and the standard lines. It is concluded that the kits tested are suitable and reliable for buffalo plasma samples.

Key words: Buffalo, Inhibin-A, Estradiol-17-beta, Enzyme Immunoassay.

INTRODUCTION - Inhibins are heterodimeric protein hormones secreted by granulosa cells of the ovary in the female and Sertoli cells of the testis in the male, which play an important role in inhibiting follicle stimulating hormone (FSH) secretion (Tilbrook and Clarke, 2001) and also have local autocrine and paracrine actions in the gonads (Campbell and Baird, 2001). The form Inhibin-A (In-A) is produced by large follicles and plasma concentrations are strongly correlated with estradiol-17-beta (E2) levels during the follicular phase of the estrus cycle (Knight et al., 1998). There is an increasing interest to measure blood In-A levels in ruminants, as they are correlated with ovarian follicular dynamics (Medan et al., 2003), and being potential predictor of the responses to the superovulatory treatments (Gonzalez-Bulnes et al., 2002 and 2004). In-A concentrations were previously assayed in buffalo plasma by RIA (Palta et al., 1996; Palta et al., 1997a; Palta et al., 1997b; Singh et al., 2001). The aim of the present trial was to evaluate the suitability of two commercial ELISA kits for human serum or plasma, in order to measure In-A and E2 concentrations in plasma of buffalo heifers and cows.

MATERIAL AND METHODS - Buffalo heifers and cows were treated with exogenous progesterone and gonadotropins for estrus and multiple ovulations induction. At different days of treatments, blood samples were collected by jugular venipuncture with evacuated tubes containing K3 EDTA (Venoject, Terumo Europe NV, Leuven, Belgium), immediately centrifuged (2500 g for 15 min) and the plasma stored at –20°C until assayed. In-A concentrations were determined in the plasma samples by an enzimatically amplified "two-step" sandwich type immunoassay kit for human serum or plasma (Inhibin A DSL-10-28100, Diagnostic Systems Laboratories Inc, Webster, Texas, USA). Standard, controls and unknown samples are incubated in microtitration wells coated with anti-In-A beta subunit antibody. After incubation and washing, another anti-In-A alpha subunit detection antibody labeled with the enzyme horseradish peroxidase is added into the wells. After a second incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine. The resulting absorbance is directly proportional to the concentration of In-A present in the sample. E2 concentrations were measured by a solid phase ELISA kit, based on the principle of competitive binding (Estradiol EIA-2693, DRG Instruments GmbH, Marburg, Germany). The microtiter wells are coated with a polyclonal antibody directed towards a unique antigenic site on the E2 molecule. E2 in standard and unknown samples competes with an E2 horseradish peroxidase conjugate for binding to the coated antibody and after incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate, the intensity of colour developed after addition of the substrate and the resulting absorbance are reverse proportional to the concentration of E2 in the sample. Sets of standards are used to plot standard curves of absorbance versus hormone concentration, from which the hormone concentrations in the unknown samples can be calculated. The absorbances were measured using the photometric microplate reader Multiskan Ascent (Labsystems Oy, Helsinki, Finland). Precision of the methods was evaluated calculating the intra- and inter-assay coefficients of variation (CVs) of buffalo plasma sample replicates, at different concentrations. Accuracy was evaluated calculating the recovery rates of different proportions of the highest standard added to a buffalo plasma sample at low concentration (observed/expected values x 100). Linearity was evaluated by serially diluting one buffalo plasma sample at high concentration with the assay buffer and calculating by regression analysis the parallelism of the resulting line with the standard line.

RESULTS AND CONCLUSION - The intra- and inter-assay coefficients of variation of buffalo sample replicates, at different concentrations are shown in Table 1. Recovery rates are shown in Table 2, and the means were 97.9% and 98.5% for In-A and E2, respectively. Figure 1 shows the regression lines for the linearity: the lines resulting from the dilutions displayed a good parallelism with the standard lines (differences between the slopes not significant, $P = 0.20$ and $P = 0.37$ for In-A and E2, respectively). For each sample, In-A and E2 concentrations were higly correlated ($r = 0.43$; $P < 0.01$).

A similar two-site ELISA test was previously validated and successfully utilized for bovine (Bleach et al., 2001; Parker et al., 2003), sheep (Souza et al., 1997; Knight et al., 1998) and goats (Medan et al., 2003; Gonzalez-Bulnes et al., 2004). The present results let us to conclude that the ELISA methods tested here are fully suitable for buffalo plasma samples.

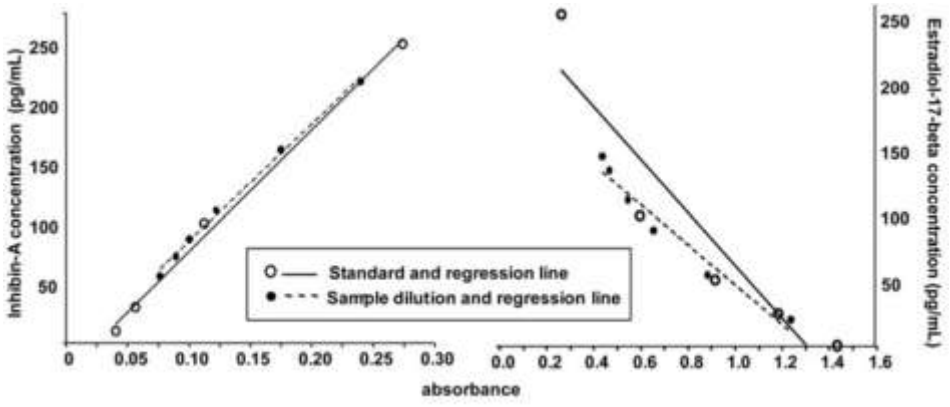
Table 1. Precision: Coefficients of Variations (CV) of different replicates of buffalo plasma samples.

Hormone	n	Concentration mean +/- sd (pg/mL)	Intra-assay CV (%)	Inter-assay CV (%)
Inhibin-A	4	137+/-15	11	
Inhibin-A	6	31+/-5	15.1	
Inhibin-A	4	65+/-9		13.9
Estradiol-17-beta	8	156+/-3	1.8	
Estradiol-17-beta	4	20+/-1	3.3	
Estradiol-17-beta	4	145+/-11		7.4
Estradiol-17-beta	4	20+/-5		23.2

Table 2. Accuracy: Recovery rates (RR) of different buffalo plasma samples mixed with different proportions of the highest standard.

Hormone	observed value	expected value	RR (%)
Inhibin-A	121	125	97
Inhibin-A	293	222	132
Inhibin-A	274	351	78
Inhibin-A	437	514	85
Estradiol-17-beta	126	143	88
Estradiol-17-beta	304	261	117
Estradiol-17-beta	344	379	91

Figure 1. Linearity: parallelism of the lines obtained from serial dilutions of one buffalo plasma samples versus the standard lines.



ACKNOWLEDGMENTS - Work supported by Project "PROBUF", Italian Agricultural Ministry.

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