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Validation of an EIA technique for the determination of salivary cortisol in cattle

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Abstract

This work involves the development and validation of an enzyme immunoassay technique (EIA) for the measurement of the cortisol concentration in cattle saliva. Saliva samples present several advantages over plasma samples in animal welfare studies. Saliva collection avoids venipuncture as a stress factor. Also, saliva components do not affect EIA as plasma components do. At present, there is no validated commercial method for saliva cortisol determination in cattle. Commercially available radioimmunoassay kits for human plasma (detection range: 10-100 ng ml^{-1}) are not sensitive enough for animals with low concentrations of salivary cortisol (< 4 ng ml^{-1}). Thus, EIA is the method of choice in cattle. Sensitivity, specificity, precision and accuracy EIA tests showed this method to be suitable and reliable. The detection limit was found to be 0.024 ng ml^{-1}, representing an improvement on previously described techniques. Intra-assay and inter-assay variation coefficients were 1.47-7.30% and 2.40-9.78%, respectively. The recovery rates for cortisol added to saliva samples were 91.36-126.5%. Parallelism tests showed that saliva cortisol levels can be determined in cattle samples without extraction. The correlation between saliva and plasma cortisol was positive (r = 0.75) and the saliva/plasma cortisol ratio was around 10%. Therefore, saliva samples are a suitable alternative to plasma samples in bovine HPA (hypothalamic-pituitary-adrenal) axis evaluation.

Key words: immunoassay, HPA axis, cow, stress.

Resumen

Validación de una técnica de EIA para la determinación de cortisol en saliva de ganado bovino

En este trabajo se desarrolla y valida una técnica de enzimoinmunoensayo (EIA) para la determinación directa de cortisol en muestras de saliva de ganado vacuno. La saliva como muestra tiene gran importancia en los estudios de bienestar animal, ya que presenta ventajas respecto al plasma, como son el menor estrés al que se somete a los animales para su obtención y la menor interferencia que producen los componentes de la saliva en la determinación directa en el EIA. Hoy en día no existe en el mercado ningún método validado para la determinación de cortisol en saliva en la especie bovina. Las técnicas de radioinmunoensayo comercializadas para plasma de la especie humana (rango de detección de 10 a 100 ng ml^{-1}) no son suficientemente sensibles para el ganado bovino, que presenta concentraciones de cortisol salival muy reducidas (< 4 ng ml^{-1}), por ello no es posible su adaptación para esta especie, siendo los métodos de EIA los de elección. El límite de detección de la técnica EIA fue de 0,024 ng ml^{-1}, mejorando la sensibilidad de técnicas descritas previamente; los coeficientes de variación intra e interensayo fueron de 1,47-7,30% y 2,40-9,78%, respectivamente. La recuperación de cortisol añadido a muestras de saliva fue de 91,36-126,5%. Las pruebas de paralelismo demostraron que es posible la determinación directa de muestras de saliva sin necesidad de extracción previa. La correlación existente entre las concentraciones de cortisol salival y plasmático fue alta (r = 0,75) siendo la relación cortisol saliva/plasma en torno al 10%. Por ello, en la valoración del eje corticotropo de la especie bovina es posible el uso de muestras de saliva en sustitución a las de plasma.

Palabras clave: inmunoensayo, eje corticotropo, vacuno, estrés.

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Introduction

Cortisol is a glucocorticoid hormone synthesized by the adrenal cortex. Its production, regulated by the corticotropic axis can be altered in different circumstances. Cortisol concentrations rise in situations of stress and this parameter is considered to be an indicator of animal welfare (Cook *et al*., 1996; Ekkel *et al*., 1997).

Although traditionally cortisol concentrations have been determined in plasma or serum, their determination in other fluids or organic tissues (saliva, milk, muscle, urine, faeces and hair) can be of interest in animal welfare studies of different species (Cooper *et al*., 1989; Fritsche and Steinhart, 1998; Verkerk *et al*., 1998; Cirimele *et al*., 1999; Fritsche *et al*., 1999; Antignac *et al*., 2000; Morrow, 2000; Palme *et al*., 2000).

Cortisol levels in saliva correspond to the free fraction of cortisol in plasma, which is the only biologically active fraction in the organism, owing to it being able to bind to cell receptors (Vining *et al*., 1983; Lac, 1998). Increased cortisol secretion by the adrenal cortex can saturate plasma cortisol binding proteins increasing the ratio of free/total cortisol (Riad Fahmy *et al*., 1981), and protein-bound cortisol acts as a reserve and can be converted to free cortisol when production is reduced (Rijnberk and Mol, 1989). Therefore, salivary cortisol is a better indicator of the possible effects of the corticotropic axis on the animal organism than plasma cortisol. On the other hand, blood extraction always produces stress in the animal that can cause cortisol levels to rise while the animals are hardly affected by saliva sample collection (Fell *et al*., 1985; Cooper *et al*., 1989).

The analytical technique most used to determine cortisol levels was traditionally radioimmunoassay (Yalow and Berson, 1959, 1960). Nowadays, the use of other non-radioactive markers are becoming increasingly popular. This avoids the complications of using radioisotopes especially those relating to public health risks and the infrastructure required for the distribution, use and elimination of radioactive substances (Munro and Lasley, 1988; Cooper *et al*., 1989; Silvan, 1991; Bertholf and Bowman, 1996).

Since there are no validated commercial kits on the market for the determination of salivary cortisol levels in cattle, it would be useful to develop techniques for this purpose. Moreover, there are also very few reference data about basal salivary levels of cortisol (Fell *et al.*, 1986; Cooper *et al.*, 1989; Schrama *et al.*, 1996) and their relation with plasma cortisol in these species (Coteliouglu *et al.*, 1998).

We hypothesized that saliva could replace plasma samples in studies on the corticotropic axis. Therefore, the aim of this work was to develop and validate an EIA technique for cortisol determination in cattle saliva, calculating basal salivary cortisol for cattle in relation to animal age, the correlation between plasma and salivary cortisol levels and the ratio of salivary to plasmatic cortisol.

Material and Methods

To prepare the EIA technique, the anticortisol specific antibody (Ab) was obtained by immunizing three male New Zealand rabbits with 3CMO-BSA cortisol (Q-3889, Steraloids Inc, Wilton, USA).

Conjugation of horseradish peroxidase enzyme (HRP) (EC 1.11.1.1. Type VI, RZ:3,2, Boehringer, Mannheim) to the cortisol-acetate-3CMO molecule (Q3885, Steraloids Inc, Wilton, USA) was done using the anhydrous mixture method (Erlanger *et al*., 1957).

Development of the technique

The technique consisted in covering the 96-well plate (Dinatech M29AR, Dekenford, Germany) with 100 µl Ab at a dilution of 1/2000 in a 0.05 M bicarbonate-carbonate solution, pH 9.6. After incubation for 24 h at 4ºC, the plate was washed five times in an automatic washer (Wellwash 4MK2, Denley Instruments, UK) with a washing solution of 0.15 M NaCl, 0.05% Tween 20.

The plate was coated with the standards or the samples. The standard solutions were kept dissolved in ethanol at –20°C. After evaporating the ethanol in a nitrogen flow, the cortisol was reconstituted with the 1/160000 dilution of cortisol-HRP conjugate in an assay solution (EIA) (0.1M Na_2HPO_4; 0.1M NaH_2PO_4; 0.15 M NaCl with 0.1% BSA). The standard curve was drawn up with a total of 11 concentrations: 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 pg.

The final samples were prepared by mixing 50 µl of saliva with 250 µl of the conjugate dilution. The cover was made by adding 50 µl of EIA solution in reference wells and 40 µl in the sample wells. Then, 50 µl of standard or 60 µl of each sample was added. Both the samples and the standards were made in duplicate.

After incubating for 2 h at room temperature, the plate was washed again and 100 µl of K-blue substrate solution was added to each well (Neogen Corporation, Lexington, USA). After 20 min, the reaction was stopped by adding 100 µl of a 10% solution of H_2SO_4 followed by reading in an automatic EIA reader (Multiskan RC, v 2.6, Labsystem) with a 450 nm filter, connected to a computer that processes the data using the Genesis Lite computer programme (Labsystem, Finland).

Validation of the technique

Validation tests consisted in calculating the sensitivity, specificity, precision, accuracy and parallelisms.

The sensitivity corresponded to the detection limit (Abraham, 1975) and sensitivity at 50% (Van Weemen and Shuurs, 1975). The specificity of the technique was estimated by calculating the percentage cross-reaction with different steroids supplied by Steraloids Inc (Wilton, USA). The precision of the technique was calculated by intraassay or interassay coefficients of variation of plasma and saliva samples with high and low cortisol levels. To evaluate accuracy, the percentage recovery of cortisol concentrations added to saliva samples without steroids was calculated. The accuracy of the technique was also studied by correlating the results obtained with our technique and another taken as a reference; in this case with an EIA kit to determine cortisol in human saliva (Salimetrics, Pennsylvania, USA).

To eliminate possible interference of the sample components, parallelism of the reference curve with curves to which 10 µl of plasma or saliva had been added was studied and with curves made using a standard concentration of cortisol in plasma, saliva or EIA solution. Similarly, the serial dilution effect on the cortisol concentration was also estimated. Salivary sample dilutions of 1:1 to 1:20 were made up.

Determination of baseline salivary cortisol levels in cattle

To determine baseline cortisol levels in plasma and saliva of cattle, samples from a total of 63 animals of both sexes and 4 different breeds were used: Brown Swiss, Pirenaica, Holstein and Blonde D'Aquitaine. These animals were divided into two age groups: 40 animals under 1 year-old and 23 animals over 1 year-old.

All samples were collected at the same time of day, in the evening when cortisol levels are lower. Blood and saliva were collected from the calves by holding the animals in the feeding shed. Adult animals were led to an alley for blood extraction and then taken to the feeding shed for collection of saliva samples. Since the animals were accustomed to being moved from the alley to the feeding room there was only a 10 min interval between blood extraction and saliva collection.

Saliva samples were collected in the presence of food to stimulate salivation. Samples were obtained by introducing cotton-wool balls in the parotid and sublingual duct openings. The cotton-wool was then centrifuged at 1500 g for 30 min. Blood samples were collected by puncturing the caudal vein. Plasma were obtained by centrifuging at 1500 g for 15 min. Plasma and saliva samples were kept in Eppendorf tubes at –30ºC until analysis.

Plasma cortisol determination was done using EIA. In the validation tests, the intra and interassay coefficients of variation were 3.47-6.3% and 3.92-9.93%, respectively; the recovery of added cortisol ranged from 92.60 to 103.96% and Pearson's correlation with a commercial RIA technique was $r = 0.973$, $p < 0.001$, $n = 50$.

Results

The standard curve (Fig. 1) was linear from 1 to 100 pg/well (0.1-10 ng ml^{-1}).

Validation of the EIA technique

The EIA technique used to determine cortisol concentration had a detection limit of 0.237 pg/well (0.024 ng ml^{-1}), with a sensitivity at 50% binding of 13.55 pg/well.

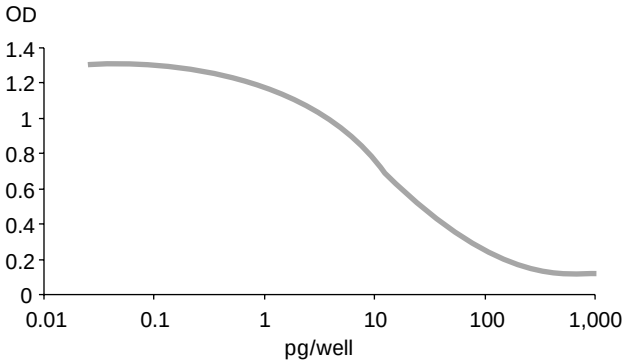

Figure 1. Standard curve for cortisol determination by EIA. OD: optical density.

Table 1. Percentage cross-reaction of the EIA technique for determination of cortisol with other corticosteroids

Steroid	% cross-reaction
Cortisol	100
Prednisolone	157.1
Prednisone	18.9
Cortisone	10.8
Corticosterone	6.4
11 Deoxycortisol	40.31
21 Deoxycortisol	5.31
Progesterone	< 0.1
17 OH progesterone	< 0.1
Dehydroepiandrosterone (DHEA)	< 0.1
Dexamethasone	< 0.1

Cross-reactions with other glucocorticoids are observed in Table 1.

The intraassay variation coefficients were 1.47% and 7.30% and interassay coefficients were 2.40% and 9.78% at high and low cortisol concentrations, respectively (Table 2).

Assessment of the accuracy of the technique revealed recovery values for the different concentrations of added cortisol ranging from 91.36 to 126.50% (Table 3).

Comparing the results obtained with our technique with those obtained with an EIA kit to determine cortisol concentrations in human saliva, a Pearson's correlation of $r = 0.968$, $p < 0.001$, $n = 34$ (Fig. 2) was obtained.

The results of parallelism tests between the standard curves and those drawn up with plasma, saliva or assay solution (EIA) can be observed in Figure 3. The standard curve was parallel to curves drawn up with saliva or EIA, but curves drawn up with plasma did not maintain this parallelism.

Table 2. Intra-assay and inter-assay variation of the EIA technique in determination of salivary cortisol at high and low concentrations expressed as percentages

	Intra-assay			Inter-assay		
	X (ng ml^{-1})	SD	CV	X (ng ml^{-1})	SD	CV
High concentration	5.745	0.085	1.47	6.366	0.157	2.40
Low concentration	0.337	0.025	7.30	0.134	0.013	9.78

X: arithmetic mean. SD: standard deviation. CV: coefficient of variation.

Table 3. Percentage recovery of cortisol added to saliva samples determined by EIA

Endogenous cortisol (pg/well)	Added cortisol (pg/well)	Concentration observed (pg/well)	% recovery
2.256	1	3.521	126.5
2.305	10	11.441	91.36
2.123	100	119.490	117.36

Similarly, serial dilutions with saliva maintained linearity in the concentrations obtained up to a dilution of 1:20, with concentrations of 96.97 to 110.71% of the concentration of the undiluted sample (Table 4).

Basal cortisol levels in cattle

Plasma and salivary cortisol concentrations obtained by EIA are showed in Table 5.

The correlation between plasma and salivary cortisol was highly significant (Pearson's $r = 0.746$, $p < 0.001$, $n = 63$) (Fig. 4).

Discussion

The validation tests for sensitivity, specificity, accuracy, precision and parallelisms showed that the technique is valid and reliable for cortisol determination in saliva in cattle species.

In the specificity tests an important cross reaction is observed against prednisolone (157.17%) and to a

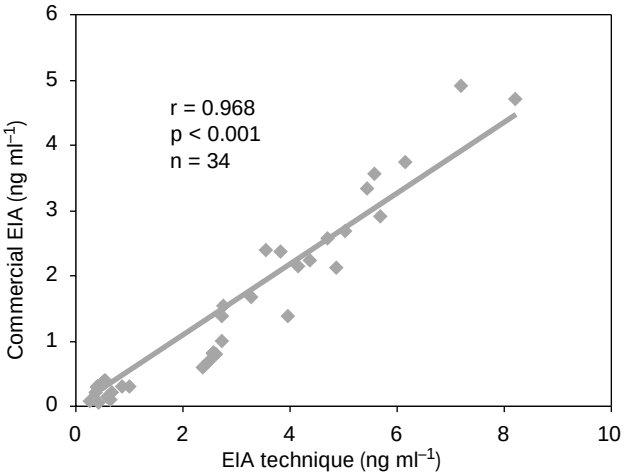

Figure 2. Pearson's correlation coefficient for the cortisol concentration in cattle saliva samples determined by the EIA technique and by an EIA marketed for human use.

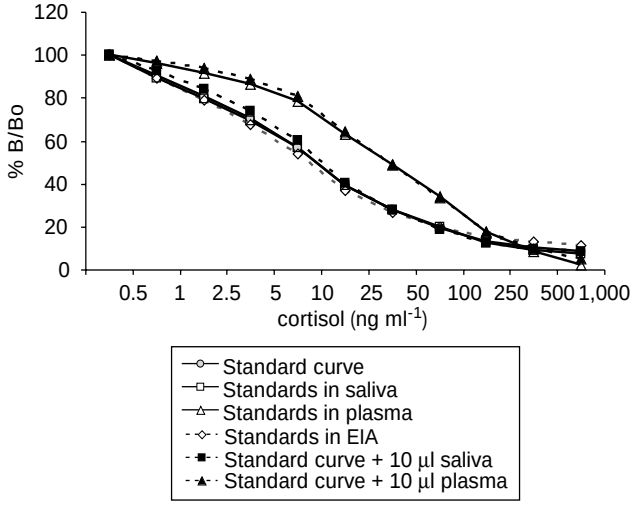

Figure 3. Parallelism tests for the cortisol standard curve for the EIA technique and curves drawn up using cattle plasma and saliva samples. B/Bo: binding of antibody conjugate.

lesser extent against prednisone (18.3%). This technique is, therefore, counter-indicated for determinations in animal samples that are being or have recently been treated with this type of drug.

A cross-reaction is also observed with 11 deoxycortisol (40.31%). This 11 deoxycortisol and 21 deoxycortisol are direct precursors of cortisol, 11 deoxycortisol is found in plasma at a concentration 100 times lower than cortisol and does not have a glucocorticoid effect and hardly any mineral corticoid effect (López-Calderón, 1999). This cross-reaction would only be a drawback in patients with congenital adrenal hyperplasia, a rare disease in human medicine that has not been described in domestic animal species yet (Feldman and Nelson, 1996).

The baseline cortisol concentration in cattle saliva was 1.22 ± 1.23 ng ml^{-1} in young animals and 1.32 ± 0.85 ng ml^{-1} in adult animals, and in all cases was lower than 4 ng ml^{-1}. Cooper *et al*. (1989) obtained, using the EIA technique in 8 adult females, a higher concentration than this (9.15 ± 1.35 ng ml^{-1}), as also observed by Fell *et al.* (1986) in 4-11 week old calves (3.03 ± 0.27 ng ml^{-1}). However, Schrama *et al.* (1996) described lower values (0.56 to 1.43 ng ml^{-1}) in calves a few days old. It is important to bear in mind the large variability between individuals, within the same breed and age group. Therefore, when designing an experimental study it is recommendable to obtain baseline cortisol levels of the animals that form part of the experimental protocol to later use this as a reference value.

Table 4. Linearity of salivary concentrations of serial dilutions of saliva samples

Dilution	Expected concentration (ng ml^{-1})	Observed concentration (ng ml^{-1})	% cortisol in the undiluted sample
1:1	2.243	2.243	
1:2	1.122	1.088	96.97
1:3	0.748	0.728	97.33
1:4	0.561	0.573	102.14
1:5	0.449	0.482	107.35
1:15	0.149	0.164	110.07
1:20	0.112	0.124	110.71

Table 5. Basaline cortisol concentration in cattle plasma and saliva samples by the EIA technique

	N	Mean (ng ml^{-1})	SD	Confidence interval
Animals < 1 year				
Plasma	40	8.911	11.158	0-31.23
Saliva	40	1.220	1.227	0- 3.674
sv-pl	40	13.69%		
Animals > 1 year				
Plasma	23	14.174	7.916	0-30.52
Saliva	23	1.324	0.852	0- 3.028
sv-pl	23	10.31%		

N: number of animals. SD: standard deviation. sv-pl: proportion of salivary cortisol to plasmatic cortisol concentrations.

There is a high correlation between plasma and salivary cortisol concentrations (Fig. 4), salivary cortisol concentration can, therefore, be used instead of plasma concentrations. The correlation obtained in this work (r = 0.746) was higher than that described pre-

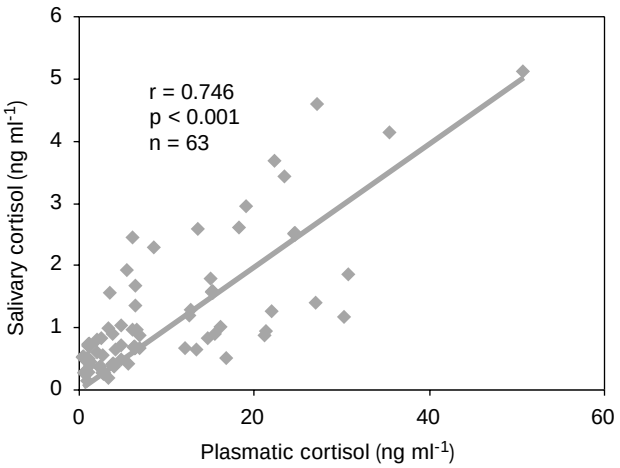

Figure 4. Pearson's correlation coefficient between plasma and saliva cortisol determined by EIA.

viously by other authors (Cotelioglu *et al*., 1997; Steinhardt and Thielscher, 2000, 2001).

The salivary cortisol concentration was around 10% of the plasma cortisol concentration (10.31 and 13.69% in older animals and animals under 1 year-old, respectively). These results agree with those recorded in human beings (Vinnig *et al*., 1983; Rijnberk and Mol, 1989; Lac, 1998), and are slightly higher than those described by Cotelioglu *et al.* (1997) for cattle (6.1-8.5 %).

The parallelism between the reference curve and those drawn up for saliva, indicate that saliva components do not produce interference in the assay, while the plasma components do, with lost of parallelism between the standard curves and those drawn up with plasma (Fig. 3). This finding, together with the greater stress associated with blood sample collection and the presence in the saliva of the biologically active cortisol fraction, make the use of saliva a very interesting alternative to plasma samples.

RIA techniques available on the market for cortisol determination in plasma or serum in humans have a detection interval of 10 to 100 ng ml^{-1} (Coat a Count, Diagnostic Products Corporation, Los Angeles, USA; ICN Biomedical, Costa Mesa, USA). It is, therefore, not possible to validate these for salivary cortisol determinations in cattle since these concentrations are much lower (0-4 ng ml^{-1}).

The technique prepared in our laboratory had a detection limit below those described previously for salivary cortisol determination in cattle: 0.237 pg/well *vs* 1 pg/well (Cooper *et al.*, 1989) and 0.024 ng ml^{-1} *vs* 0.05 ng ml^{-1} (Schrama *et al.*, 1996). It also had a higher sensitivity than the EIA technique marketed by Salimetrics (USA) for human use (< 0.07 ng ml^{-1}).

In conclusion, the EIA technique developed can be used for salivary cortisol determinations in cattle, with a better sensitivity than techniques used previously. This is especially important taking into account the low cortisol concentrations present in this species. Therefore, salivary cortisol determinations can be used instead of plasma cortisol determinations in studies of the cattle corticotropic axis.

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References

ABRAHAM G.E., 1975. Radioimmunoassay of steroids in biological fluids. J Steroid Biochem 6, 261-270.

ANTIGNAC J.P., LE BIZEC B., MONTEAU F., POULAIN F., ANDRÉ F., 2000. Collision induced dissociation of corticosteroids in electrospray tandem mass spectrometry and development of a screening method by high performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 14, 33-39.

BERTHOLF R.L, BOWMAN M.A., 1996. Microbeads, magnets and magic: The enchanting science of immunochemistry. Ann Clin Lab Sci 26(5), 377-388.

CIRIMELE V., KINTZ P., DUMESTRE V., GOULLÉ J.P., LUDES B., 1999. Identification of ten corticosteroids in human hair by liquid chromatography-ionspray mass spectrometry. Forensic Sci Internat 107(1-3), 381-388.

COOK N.J., SCHAEFER A.L., LEPAGE A., MORGAN JONES S., 1996. Salivary vs. serum cortisol for the assessment of adrenal activity in swine. Can J Anim Sci 76, 329-335.

COOPER T.R., TRUNKFIELD H.R., ZANELLA A.J., BOOTH W.D., 1989. An enzyme-linked immunosorbent assay for cortisol in the saliva of man and domestic farm animals. J Endocr 123, R-13, R-16.

COTELIOGLU U., ARSLAN M., MATUR E., OZCAN M., 1997. The comparison of plasma and saliva steroid hormone levels during pregnancy and delivery in heifers. Vet Fakultesi Dergisi Istambul 23(2), 331-343.

EKKEL E.D., SAVENIJE B., SCHOUTEN W.G.P., WIEGANT V.M, TIELEN M.J.M., 1997. The effects of mixing on behavior and circadian parameters of salivary cortisol in pigs. Physiol Behav 62(1),181-184.

ERLANGER B.K., BOREK F., BEISER S.M., LIEBERMAN S., 1957. Steroid-protein conjugates I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. J Biol Chem 228, 713-727.

FELDMAN E.C., NELSON R.W., 1996. Adrenal Gland. In: Canine and feline endocrinology and reproduction. 2nd ed. W.B. Saunder Company, Philadelphia, 186 pp.

FELL L.R., SHUTT D.A., BENTLEY C.J., 1985. Development of a salivary cortisol method for detecting changes in plasmas «free» cortisol arising from acute stress in sheep. Aust Vet J 62(12), 403-406.

FELL L.R., WELLS R., SHUTT A., 1986. Stress in calves castrated surgically or by the application of rubber rings. Aust Vet J 63(1), 16-18.

FRITSCHE S., SCHMIDT G., STEINHART H., 1999. Gas chromatographic-mass spectrometric determination of natural profiles of androgens, progestogens and glucocorticoids in muscle tissue of males cattle. Eur Food Res Technol 209, 393-399.

FRITSCHE S., STEINHART H., 1998. Differences in natural steroids hormone patterns of beef from bulls and steers. J Anim Sci 76, 1612-1625.

LAC G., 1998. Intérêt et champs d'application des dosages salivares. Sci Sport 13, 55-63.

LEWIS J.G., ELDER P.A., 1985. An enzyme-linked immunosorbent assay (ELISA) for plasma cortisol. J Steroid Biochem 22(5), 673-676

LÓPEZ-CALDERÓN A., 1999. Glándulas suprarrenales. In: Fisiología humana, 2nd ed. (J.A.F. Tresguerres, ed.), Mc Graw-Hill Interamericana, Madrid, pp. 931-952.

MORROW C.J., KOLVER E.S., VERKERK G.A., MATTHEWS L.R., 2000. Urinary corticosteroids: an indicator of stress in dairy cattle. Proc. of the New Zealand Society of Animal Production 60, 218-221.

MUNRO C.J., LASLEY B.L., 1988. Non radiometric methods for immunoassay of steroid hormones. In: Non radiometric assays: technology and application in popyleptide and steroid hormones detection (Alan R., ed.) Liss Inc., NY, pp. 289-329.

PALME R., ROBIA C., BAUMGARTNER W., MÖSTL E., 2000. Transport stress in cattle as reflected by a increase in faecal cortisol metabolite concentration. Vet Record 146, 108-109.

RIAD-FAHMY D., READ G.F., JOYCE B.G., WALKER R.F., 1981. Steroid immunoassays in endocrinology. In: Immunoassay for 80's (A. Voller, A. Barrtlett and D. Bidweld, eds). Mtp Press Ltd., Lancaster, UK, pp. 205-261.

RIJNBERK A., MOL J.A., 1989. Adrenocortical function. In: Clinical biochemistry of domestic animal, 4th ed. (J. Kaneko). Academic Press Inc., San Diego, CA, USA, pp. 610-629.

SCHRAMA J.W., HEETKAMP M.J.W., VERSTEGEN M.W.A., SCHOUTEN W.G.P., VAN DER VEEN F., HELMOND F.A., 1996. Responses of young calves on two levels of feeding to transportation. J Anim Sci 63, 79-89.

SCHROEDER H.R., BOGUSLAOKI R.C., CARRICO R.J., BUCKLER R.J., 1978. Monitoring specific protein-binding reactions with chemiluminescence. In: Methods in Enzymology (de Luca M., ed.). Academic Press, NY, pp. 424.

SEREN E., 1973. ACTH and glucocorticoids in cattle. Folia Vet Lat 3, 584-605.

SILVÁN G., 1991. Métodos inmunológicos de determinación de hormonas esteroides. In: Correlación entre el tamaño folicular y niveles de hormonas esteroides en ganado vacuno. Doctoral thesis. Universidad Complutense, Madrid.

STEINHARDT M., THIELSCHER H.H., 2000. Observations on dairy calves infesting liquid feed after ACTH application at different age points before and in the course of rearing with automatic milk feeding. Plasma cortisol, salivary cortisol, haematology, metabolic variables and heart rate. Deutsch Tierarztl Wochenschrift 107(5), 180-187.

STEINHARDT M., THIELSCHER H.H., 2001. Reaction of suckler calves from the mother cow herd to transport and temporary separation at different age periods. Proteins, minerals, metabolic variables, plasma and salivary cortisol. Tierarztl Prax 29(3), 132-140.

VAN WEEMEN B.K., SCHUURS A.H.W.M., 1975. The influence of heterologous combinations of antiserum and enzyme-labelled estrogen on the characteristics of estrogen enzyme-immunoassays. Immunochem 12, 667-670.

VERKERK G.A., PHIPPS A.M., CARRAGHER J.F., MATTHEWS L.R., STELWAGEN K., 1998. Characterization of milk cortisol concentration as a measure of shortterm stress response in lactating dairy cows. Anim Welfare 7, 77-86.

VINING R.F., MCGINLEY R.A., MAKSVYTIS J.J., HOK Y., 1983. Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. Ann Clin Biochem 20, 329-335

YALOW R.S., BERSON S.A., 1959. Assay of plasma insulin in human subjects by immunological methods. Nature 184, 1648-1649.

YALOW R.S., BERSON S.A., 1960. Immunoassay of endogenous plasma insulin in man. J Clin Invest 39, 1157-1175.