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Amplified androstenedione enzymeimmunoassay for the diagnosis of cryptorchidism in the male horse: comparison with testosterone and estrone sulphate methods☆

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Abstract

An amplified enzymeimmunoassay (EIA) was validated for androstenedione in the serum of male horses. We will use the assay as a tool for the diagnosis of equine cryptorchidism. We will compare androstenedione EIA to the currently used methods (testosterone and estrone sulphate determinations). The study was conducted on 115 horses of pure Spanish and Arabian breeds, that included 30 geldings, 60 bilateral cryptorchids and 25 stallions. Androstenedione standard curve covered a range between 0 and 1 ng per well. Low detection limit was 1.54 pg/ml. Intra- and inter-assay coefficients of variation (CV%) were <8.2 and <9.3, respectively ($n = 10$). Recovery rate of known androstenedione concentrations averaged from 96.62 ± 2.69 to 97.63 ± 1.87%. Androstenedione mean ± S.E. serum concentrations were 10.52 ± 1.36 ng/ml in stallions ($n = 25$), 0.51 ± 0.04 ng/ml in cryptorchids ($n = 60$), and 0.03 ± 0.01 ng/ml in geldings ($n = 30$). Diagnostic validation parameters in basal samples showed for estrone sulphate the lower positive predictive value (0.85) with the higher number of false positives, and lower specificity (0.84). Testosterone showed the higher number of false negatives with a negative predictive value of 0.85, and lower sensitivity (0.85). Among the three hormones evaluated, androstenedione presented the best results with the smaller number of horses diagnosed as false positives (0.93) or negatives (0.91). This technique also resulted in higher sensitivity, specificity and efficiency over the other two methods assayed. We concluded that our amplified EIA is a highly sensitive and specific assay that provides a rapid, simple, and inexpensive alternative to other methods.

Keywords: EIA; Androstenedione; Equine cryptorchidism

1. Introduction

The development of accurate hormone laboratory methods for the diagnosis of equine cryptorchidism is important, specially when exploratory abdominal surgery is considered for confirmatory diagnosis in the case of male horses, which have no visible or palpable testes but display stallion-like behaviour, and may include bilateral cryptorchids, hemicastrates, or geldings (Palme et al. [1], and Searle et al. [2]). The ability of the equine testis to synthesise androgens and oestrogens has been widely used to differentiate geldings from horses with testicular tissue. Currently, the most accepted test to determine the presence of testicular tissue in a horse is the measurement of testosterone concentrations, whether unstimulated or after the stimulation with human chorionic gonadotropin (hCG); based on the ability of Leydig cells for producing androgens (Cox et al. [3], Raeside [4], Setchell and Cox [5], Cox et al. [6], Ryan et al. [7], Muyan et al. [8]). Some authors stated that the determination of other hormones in blood, such as estrone sulphate, could be more accurate and easy to perform than the measurement of testosterone levels (Cox et al. [6], Silberzahn et al. [9], Palme et al. [1], Carneiro et al. [10]). However, Cox et al. [6] indicated that the accuracy of this diagnostic test is restricted to horses older than 3 years. There is little information regarding the possibility of the use of androstenedione for the diagnosis of equine cryptorchidism (Silberzahn et al. [9]), and this hormone seems to show a potential as a diagnostic tool for several reasons. Thus, the interest of androstenedione determinations is supported by the fact that this hormone is the precursor hormone of androgens and estrogens, both in males (testicle) and females (ovary) of mammalian species, it is involved in the regulation of many

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reproductive processes, such as puberty, control of follicular development, male reproductive physiology, and several reproductive disorders (Inoue et al. [11], Muyan et al. [8], Eisenhower et al. [12], Hoffman and Landeck [13]).

The aim of the present study was to validate a highly sensitive, reliable, accurate and direct amplified enzymeimmunoassay (EIA) method to measure androstenedione concentrations, in the serum of male horses. We will use this new assay as a tool for the detection and diagnosis of equine cryptorchidism, and therefore we will also try to compare this EIA to the currently used methods (testosterone and estrone sulphate determinations).

2. Materials and methods

2.1. Reagents

Horseradish peroxidase (EC 1.11.1.7., RZ = 3, type VI) was obtained from Boehringer Mannheim (Barcelona, Spain). Sephadex G-25 was purchased from Pharmacia fine chemicals (Barcelona, Spain). Source of bovine serum albumin (BSA, Cohn Fraction V) was from Sigma (San Luis, MO, USA) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) was from Pierce Europe B.V. (Oud Beijarland, The Netherlands). All steroids were purchased from Steraloids Inc. (Wilton, NH, USA). The rest of the solvents and reagents were analytical grade and were purchased from Merck (Madrid, Spain) and Panreac (Barcelona, Spain). Ninety-six well flat-bottomed polystyrene microtiter plates (M-29A) and the microplate reader were obtained from Dynatech (Cultek, Madrid, Spain) and BioTeck Instruments Inc. (Winooski, VT, USA), respectively.

2.2. Horses

The new EIA was tested in blood samples collected from a population of male horses of several breeds (pure Spanish breed and Arabian) and ages (3–10 years). A total of 115 horses divided in: 60 bilateral cryptorchids, 30 geldings and 25 stallions were investigated. Some horses included in both gelding and cryptorchid groups, displayed stallion-like behaviour. Testosterone and estrone sulphate determinations were also performed in all samples. Blood samples were collected from jugular vein. Serum was obtained after centrifugation of blood (1500 × g, 20 min at 4 °C), aliquoted and stored at −30 °C until assayed.

In order to evaluate the influence of hCG stimulation test on androstenedione, testosterone and estrone sulphate production by Leydig cells 20 horses were randomly selected among the gelding (10 horses) and cryptorchid (10 horses) groups. An injection of 7500 IU of hCG (Chorulon, Intervet, Salamanca, Spain) were administered i.v. to each horse. Two blood samples were taken, the first just before the injection (pre) and the second 1 h after (post). Serum was obtained and stored as above.

2.3. Polyclonal antiserum and enzyme conjugate

A polyclonal antiserum (C9111) raised in male rabbits against androstenedione-3-carboxymethyloxime was used for development of the assay (Illera et al. [14]). Androstenedione-6-hemisuccinate was labelled with horseradish peroxidase (HRP) by the mixed anhydride method (Munro and Stabenfeldt [15]). Following elution of the conjugate by Sephadex G-25 chromatography in a 0.05 M phosphate buffer pH 7.5, the androstenedione enzyme conjugate was stored freeze-dried until assay. Assessment of the antibody and conjugate was done using the reported methods: titration of antibody, calculation of cross-reactivity with related compounds, calculation of androstenedione:HRP ratio, recovery of enzyme activity after conjugation, and titration of antibody and conjugate in EIA to determine the working solution (Illera et al. [14]).

2.4. Enzymeimmunoassay procedure

The amplified EIA for androstenedione was developed on the basis of the assay reported by Illera et al. [14] for the measurement of androstenedione in culture maturation medium of rabbit oocytes and by Jones and Madej [16] for the measurement of estradiol.

Ninety-six well flat bottomed polystyrene microtiter plates were coated with 100 μl per well of purified antibody solution (C9111, 1/1800 in coating buffer; sodium carbonate, 50 mmol/l; pH 9.6) except for the first well which acted as plate/assay blank, and incubated overnight at 4 °C. Afterwards, non-bound antibodies were removed from the wells by washing plates five times with wash solution (NaCl, 150 mol/l, Tween 20, 0.5 ml/l), inverted and dried.

Standards were solubilized in ethanol, evaporating the solvent and resolubilizing them in assay buffer (sodium phosphate, 100 mmol/l; pH 7.0; with sodium chloride, 8.7 g/l; BSA, 1 g/l). Standard curve covered a range between 0 and 1 ng per well, and was constructed by using 10 standard solutions: 0.1; 0.5; 1; 5; 10; 50; 100; 500 and 1000 pg per well.

The wells of the first and last rows were called B_0 (maximum binding of enzyme conjugate to the antibody), and 100 μl of assay buffer were added to the wells. Standards and serum samples were analysed in triplicates. For standard curve 50 μl of assay buffer followed by 50 μl of each standard were pipetted into the wells from rows 2 and 6. For serum samples 50 μl of assay buffer followed by 50 μl of the sample were pipetted into the wells of 7–11 rows. Plates were covered and incubated overnight at 4 °C.

A cold (4 °C) working solution (1:60,000) of the androstenedione–HRP conjugate, 50 μl per well, was added to the entire plate and then incubated for 2 h at 4 °C. Bound/free separation was achieved by emptying plates by inversion and washing them, five times with wash solution. To evaluate the amount of labelled androstenedione bound to the antibody, 100 μl of substrate solution (3,3′,5,5′-tetramethylbenzidine dihydrochloride, pH 5.0) were added to all wells and

incubated for 15 min at room temperature, this reaction was stopped by the addition of 100 μl of 1 M phosphoric acid. Absorbance was read at 450 nm in an automatic microplate reader. Androstenedione concentrations were calculated by means of software developed for this technique (ELISA AID, Eurogenetics, Belgium). Standard dose–response curve was constructed by plotting the binding percentage ($(B/B_0) \times 100$) against androstenedione standard concentrations added. Androstenedione concentrations were expressed in nanograms per millilitre.

2.5. Androstenedione RIA

Androstenedione was assayed by a commercial RIA (Androstenedione J125, DA) from DRG Diagnostics (Marburg, Germany). The first antibody used in this assay was the same used in our EIA system.

2.6. Testosterone and estrone sulphate assays

Serum samples were assayed by two EIA systems routinely used in our laboratory for these purposes (Carneiro et al. [10]).

2.7. Statistics

Intra- and inter-assay coefficients of variation (CV%) were calculated by Rodbard's method [17]. All values were expressed as mean ± S.E. Statistical differences of hormone levels among groups were determined by Catmod procedure of statistical analysis system (SAS/STAT [18]). Only $P < 0.05$ were considered significant. The threshold for the diagnoses of each category of horses (gelding, cryptorchid or stallions) was established by using the criteria of Wulff [19], in which a hormone concentration >2S.D. above the mean was considered in the next category. Diagnostic parameter values (sensitivity, specificity, diagnostic efficiency, and predictive values of the EIA for androstenedione in serum) were calculated according to the method of Wulff [19] and Smiley and Peterson [20]. The sensitivity of a test expresses the probability that a horse with cryptorchidism will give a positive test. Sensitivity is determined in a population of horses known to have cryptorchidism. Specificity of a test is the probability that a horse will give a negative test in the absence of cryptorchidism. Specificity is measured in a population of horses known to be free of cryptorchidism (stallions or geldings). Efficiency of a test is the percentage of correct results regardless of whether they are positive or negative. Efficiency expresses the combined positive and negative accuracy of a test. Since the diagnostic probabilities are relevant to the clinic diagnostic they have been also calculated. Predictive values predict the probability of the presence (positive predictive value (PPV)) or absence (negative predictive value (NPV)) of cryptorchidism, given a positive or a negative test, and are measured in a population of subjects that contains horses with and without cryptorchidism. Diagnostic probabilities are dependent on the sensitivity and specificity.

3. Results

3.1. Polyclonal antibody and conjugate assessment

Androstenedione antisera reached the required titer for purification after 5 months of immunization. After purification, androstenedione antibodies (C9111) showed a titer of 1:80,000 (by EIA). Specificity of antibodies, expressed as the percentage of cross-reactivity showed for testosterone 3.16% and for estrone sulphate <0.01%, and for other related steroid values less than 5%.

The assessment of the conjugate showed the following results: androstenedione-6-hemisuccinate:HRP ratio was 1.3:1 moles. The recovery of the enzyme activity after conjugation was more than 85%.

3.2. Enzymeimmunoassay validation

The validation of the amplified EIA for equine serum samples was based on the results of accuracy, precision, sensitivity, parallelism and correlation with RIA.

The accuracy of the EIA was tested by determining the recovery rates of known amounts of androstenedione added to a pool of serum samples from stallion, cryptorchids and gelding horses. The recovery rates for geldings ranged from 89.65 to 102.80% (mean ± S.E. = 96.62 ± 2.69%), for cryptorchids ranged from 92.36 to 100.89% (mean ± S.E. = 97.63 ± 1.87%), and for stallions ranged from 94.82 to 101.21% (mean ± S.E. = 97.53 ± 1.35%), and they are shown in Table 1.

Precision of the amplified androstenedione EIA was determined by calculating the intra- and inter-assay coefficients of variation (CV%). The intra-assay CV (%) was calculated by replicate measurements of three standard concentrations of androstenedione added to pooled gelding serum, and also in 10 cryptorchid and 10 stallion serum samples. Each sample was tested in duplicate, 10 times within an assay. Inter-assay

Table 1
Androstenedione concentrations (pg/ml) and recovery rate (%) after addition of androstenedione to different pools of gelding ($n = 6$), cryptorchid ($n = 6$) and stallions ($n = 6$) serum samples

Androstenedione added (pg)	Gelding	Cryptorchid	Stallion
0	27.05	486.82	9145.41
50	69.07 (89.65)	495.81 (92.36)	8873.57 (96.50)
100	130.60 (102.80)	584.12 (99.54)	8766.50 (94.82)
500	509.76 (96.72)	995.60 (100.89)	9762.12 (101.21)
1000	999.42 (97.31)	1453.51 (97.76)	9903.95 (97.62)
Average ± S.E.	96.62 ± 2.69	97.63 ± 1.87	97.53 ± 1.35

Table 2
Intra- and inter-assay CV (%) for androstenedione EIA added to gelding horse serum samples, and for cryptorchid ($n = 10$) and stallion ($n = 10$) serum samples

	Intra-assay (%)	Inter-assay (%)
30 pg/ml	3.2	5.3
300 pg/ml	4.8	7.4
3000 pg/ml	2.9	6.5
Cryptorchid	6.7	9.1
Stallions	8.2	9.3

CV (%) was calculated by the replicate measurements of the above mentioned samples in 10 consecutive assays. Both intra- and inter-assay CV (%) are summarised in Table 2.

The sensitivity of the amplified EIA was tested in two ways: by means of low detection limit, as defined by Abraham [21] and calculated from $B_0 - 2$S.D. in 10 consecutive assays, and it was: 0.154 pg per well (or pg/100 μl), and by means of the amount of androstenedione that caused a 50% reduction of B_0 in the standard curve which was 12.6 pg per well. In order to determine the effects of male horse serum in the androstenedione standard curve, serially diluted pools of serum samples (cryptorchid and stallion) were run in parallel with the standard dose–response curve diluted either in EIA buffer and gelding serum. There was a good degree of parallelism between the serial dilutions and the standard curves (Fig. 1).

The comparison with RIA showed that EIA values were in close agreement ($n = 90$, $r = 0.95$, $P < 0.001$).

Table 3
Androstenedione (ng/ml), testosterone (pg/ml) and estrone sulphate (ng/ml) concentrations (mean ± S.E.) in horse serum samples

Horse (n)	Androstenedione	Testosterone	Estrone sulphate
Gelding (30)	0.03 ± 0.01	35.48 ± 10.89	0.07 ± 0.01
Cryptorchid (60)	0.51 ± 0.04	500.04 ± 64.90	58.53 ± 12.45
Stallions (25)	10.52 ± 1.36	>1000	175.45 ± 28.34

Table 4
Results of hCG stimulation test on hormone concentrations in cryptorchid ($n = 10$) and gelding horses ($n = 10$)

	Cryptorchid	Gelding
Testosterone (pg/ml)		
Pre	297.83 ± 135.12	18.25 ± 1.26
Post	586.96 ± 112.96	19.35 ± 2.31
Estrone sulphate (ng/ml)		
Pre	33.49 ± 6.85	0.02 ± 0.01
Post	32.48 ± 10.22	0.03 ± 0.01
Androstenedione (ng/ml)		
Pre	0.39 ± 0.04	0.36 ± 0.08
Post	0.41 ± 0.05	0.38 ± 0.10

3.3. Hormone concentrations in male horses

Assay of serum samples yielded the results expressed in Table 3.

Results from hCG stimulation test are reflected on Table 4. After 1 h of the injection, only testosterone concentrations were significantly higher in cryptorchid horses ($P < 0.05$)

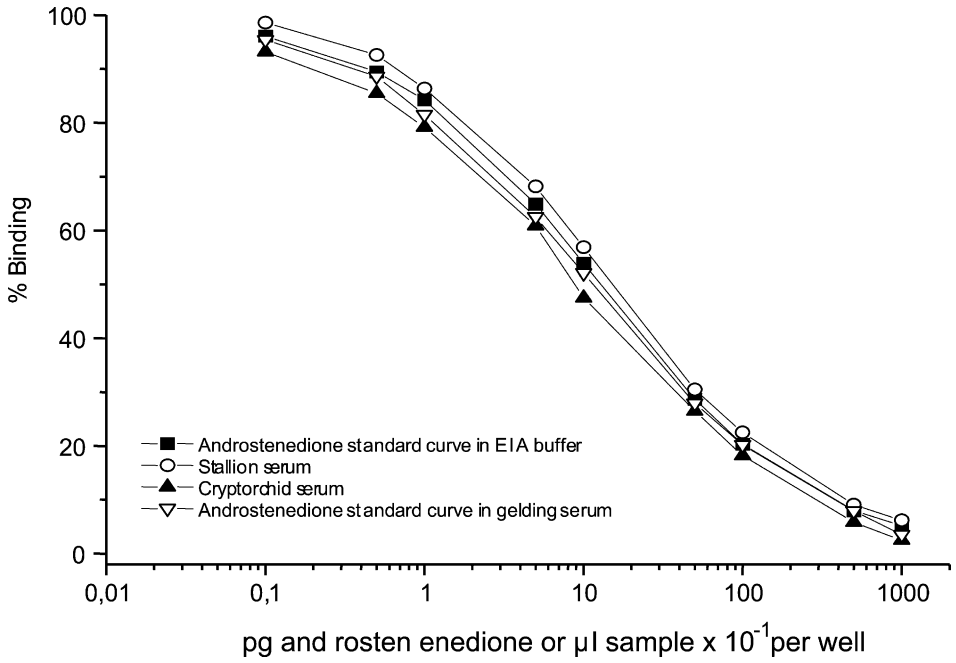

Fig. 1. Androstenedione standard dose–response curve diluted either in gelding serum (▽), or EIA buffer (■) are parallel to serial dilutions of cryptorchid (▲) and stallion serum (○).

Table 5
Results of diagnostic tests for serum samples in a population of horses

	Positive	Negative	Total
Testosterone			
Cryptorchid	51	9	60
Geldings and stallions	5	50	55
Total	56	59	115
Estrone sulphate			
Cryptorchid	53	7	60
Geldings and stallions	9	46	55
Total	62	53	115
Androstenedione			
Cryptorchid	55	5	60
Geldings and stallions	4	51	55
Total	59	56	115

but not in geldings ($P > 0.05$). Estrone sulphate and androstenedione concentrations were unaffected by hCG in both groups of horses ($P > 0.05$).

The clinical diagnostic validation parameters were calculated from the data on Table 5. For testosterone assay results were as follows: sensitivity, 0.85; specificity, 0.91; efficiency, 0.88; PPV, 0.91; and NPV, 0.85. For estrone sulphate results of diagnostic parameters were: sensitivity, 0.88; specificity, 0.84; efficiency, 0.86; PPV, 0.85; and NPV, 0.87. Finally, sensitivity of androstenedione EIA was 0.92, specificity was 0.93, and efficiency was 0.92. The diagnostic accuracy measured was as follows: positive predictive value was 0.93 and negative predictive value was 0.91, respectively.

4. Discussion

The validation of simple, reliable, and precise methods, highly specific, and able to discriminate between androstenedione among other androgens present in the serum of horses, by diminishing at the same time the incidence of false positives or negatives, is decisive for diagnostic purposes. EIA methods have demonstrated that they unit these features (Munro and Lasley [22]), and this led us to validate a direct, simple and precise amplified androstenedione EIA. Our results allowed excellent discrimination between androstenedione and other androgens based on antibody specificity. The conditions under which EIA has been carried out are based on the perfect statement of the working solutions both antibody and conjugate, and are in agreement with those previously reported (Silván et al. [23], Illera et al. [14], Carneiro et al. [10]). Precision of our direct (without previous extraction of the sample) system is also elevated, and may be due to the amplification step (24 h sample incubation with the solid-phase antibody) and temperature (4 °C in all steps except for the substrate). This simplifies largely the method and reduces the possible errors due to the extraction procedure. Parallelism test confirmed the excellent discrimination of androstenedione in horse serum samples, showing no interferences of the horse serum components in the assay. Comparison with the results obtained by RIA showed elevated correlation coefficient between both techniques (>95%). In this sense, both the EIA intra- and inter-assay CV (%) are within acceptable limits, and are similar to those obtained with a more traditional RIA (Claus et al. [24], Hoppen [25], Raeside and Christie [26]).

Testosterone and estrone sulphate concentrations were determined in the serum of the same animals by the current methods used in our laboratory (Carneiro et al. [10]). These results were consistent to previously reported data (Bono et al. [27]; Cox et al. [6], Silberzahn et al. [9], Claus et al. [24], Raeside and Christie [26], Hoffman and Landeck [13]). EIA values for androstenedione followed a similar pattern observed for testosterone, but there were not affected by hCG stimulation since androstenedione concentrations before and after hCG injection did not show a significant elevation ($P > 0.05$), showing the ability of the amplified androstenedione EIA for the diagnosis of the presence of testicular tissue, the diagnosis of cryptorchidism or the exploration of testicular function in the horse with the advantage that it is not necessary to perform the hCG stimulation test as for testosterone based diagnosis.

Our results in terms of diagnostic validation parameters showed the present EIA has an elevated sensitivity, specificity and efficiency comparable and even higher than other methods reported for steroid hormones in horses (Cox et al. [6], Silberzahn et al. [9], Palme et al. [1]). In basal samples, and among the three hormones investigated, estrone sulphate showed the lower value of PPV (0.85) with the higher number of false positives and lower specificity (0.84) than the other two hormones, this means that 15% of horses diagnosed as cryptorchids could be geldings. Testosterone showed the higher number of false negatives with a negative predictive value of 0.85 and lower sensitivity (0.85) than the other two hormones; in other terms it is possible to find a 15% of horses suffering cryptorchidism diagnosed as geldings. Among the three hormones evaluated, androstenedione presented the best results with the smaller number of horses diagnosed as false positives (0.93) or negatives (0.91). This technique also resulted in higher sensitivity, specificity and efficiency over the other two methods assayed.

In conclusion, the determination of androstenedione in the serum of horses offers an attractive alternative for the diagnosis of cryptorchidism in the horse over the commonly used methods such as testosterone or estrone sulphate determinations. The EIA system presented here is simple, reliable, efficient, specific and precise. It does not require extraction of samples prior to assay, and it is inexpensive with the potential for determining a large number of samples in a short period of time. Also, this EIA shows the advantage that for a highly sensitive, specific and efficient diagnosis of cryptorchidism in this species it is not necessary to perform a previous stimulation with hCG and that means in terms of economic point of view a considerable

reduction in the cost of clinical diagnosis with the possibility of being prescripted by a high number of veterinarians.

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