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# Ultra sensitive steroid radioimmunoassays for the diagnosis of equine gonadal dysfunction

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**Summary**

The normal cycling mare has extremely low concentrations of estradiol and testosterone in peripheral blood compared to other mammalian species. Most radioimmunoassay systems lack the sensitivity required to monitor endocrine ovarian function of the mare. Therefore we have developed new assay systems by modifying existing estradiol and testosterone radioimmunoassay kits. Modifications included extraction of serum samples with organic solvents, reduction of antibody and antigen concentrations, and changes in incubation conditions. The resulting sensitivity for both assays was below 1 pg/ml of blood serum.

**Keywords**: horse, gonadal dysfuction, steroid hormones, radioimmunoassay, reproduction

**Ultrasensitive Radioimmunoassays für die Diagnose gonadaler Dysfunktionen beim Pferd**

Die Konzentrationen von Östradiol und Testosteron im peripheren Blut während des Zyklus der Stute sind im Vergleich zu anderen Säugern extrem niedrig. Die meisten verfügbaren Radioimmunoassays sind nicht empfindlich genug, um die endokrine Ovarialfunktion der Stute zu studieren und um mögliche Störungen zu erfassen. Daher entwickelten wir neue Assaysysteme, die auf kommerziell verfügbaren Assaykits aufbauen. Als wichtigste Modifikationen wurde ein Extraktionsschritt mit organischen Lösungsmitteln eingefügt, sowie Antigen- und Antikörperkonzentrationen verringert und die Inkubationsbedingungen geändert. Es konnte dadurch eine Empfindlichkeit von weniger als 1 pg/ml erzielt werden.

**Schlüsselwörter**: Pferd, Keimdrüsendysfunktion, Steroidhormone, Radioimmunoassay

## Introduction

Steroid hormones are important secretory products of the ovary and testes, and hormone radioimmunoassays (RIA) have long been established to monitor gonadal function. The cycling mare is quite unique amongst other mammals in that her ovarian steroid output is extremely low, particularly that of estradiol. In our attempts to monitor endocrine changes during normal and disturbed follicular development of the mare we have come to the conclusion that the currently available estradiol RIA methods are not sensitive enough for our problems. Furthermore we know from other mammalian species, that some forms of ovarian dysfunction are associated with increased androgen secretion or with elevated androgen/estrogen ratios in peripheral blood. We have developed ultra sensitive radioimmunoassays for estradiol and testosterone by modifying commercially available RIA kits.

## Material and methods

*Animals*

Blood samples of normally cycling mares were provided by Dres. Chr. & J. Aurich, VMU Wien, samples of cycling mares and stallions were provided by Dr. H. Sieme, TiHo, and samples of mares with various suspected ovarian dysfunctions were sent to our laboratory for endocrine diagnosis.

*Estradiol assay*

Estradiol concentrations were measured by a sensitive assay procedure (Ultra sensitive estradiol RIA, DSL-4800, Diagnostic Systems Laboratories, Sinsheim, Germany) that we have modified for use with equine and canine blood plasma, similar to a method described by *Gastal* et al. (1999). Plasma samples (0.1 to 0.3 ml) were extracted for 30 min. with ethyl acetate (3 ml), the aqueous layer was frozen at –200°C and the solvent was decanted and evaporated in a vacuum concentrator (Hetovac VR1, Heto Lab Equipment, Birkerod, Denmark). The dried residues were reconstituted in 0.2 ml of assay buffer. A standard curve was made in assay buffer containing 0.9 to 60 pg estradiol/ 0.2 ml. Standards, controls, and unknown samples were incubated with 50 µl estradiol antiserum from the DSL assay kit for 1h at room temperature. Then 50 µl of the estradiol (I-125) reagent were added and the mixture was incubated for 2h at room temperature. Precipitating agent (1 ml) was then added and vortexed. After another 20 min. incubation all tubes were centrifuged at 4000 x g at 40°C for 20 min. The supernatant was decanted and the precipitate was counted in a gamma counter (Clinigamma, Wallac – Perkin Elmer, Rodgau, Germany). The results were calculated using the counter's RIA-CALC software.

*Testosterone assay*

Testosterone concentrations were measured by a sensitive assay procedure (Testosterone RIA, DSL-4100, Diagnostic Systems Laboratories, Sinsheim, Germany) that we have modified for use with equine and canine blood plasma. Plasma samples (100 µl for mares and geldings, 50 µl for stallions) were extracted for 30 min. with ethyl acetate (1.5 ml), the aqueous layer was frozen at –200°C and the solvent was decanted and evaporated in a vacuum concentrator (Hetovac VR1, Heto Lab Equipment, Birkerod, Denmark). The dried residues were reconstituted in 50 µl of assay buffer. A standard curve was made in assay buffer containing 1.5 to 200 pg testosterone/50 µl. Standards, controls, and unknown samples were incubated with 250 µl testosterone (I-125) reagent and 50 µl of the testosterone antiserum from the DSL assay kit for 1h at 37°C, then for another 2.5h at room temperature. Precipitating agent (1 ml) was then added and vortexed. After another 20 min. incubation all tubes were centrifuged at 4000 x g at 40°C for 20 min. The supernatant was decanted and the precipitate was counted in a gamma counter (Clinigamma, Wallac – Perkin Elmer, Rodgau, Germany). The results were calculated using the counter's RIA-CALC software.

*Progesterone assay*

Progesterone concentrations were analysed in an automated chemiluminescence immunoassay system that had been validated for equine and canine samples (Immulite, DPC)

*Reference assays*

All samples were analysed for estradiol and testosterone by conventional H-3 RIA procedures established in our laboratory as described earlier (*Behrens* et al. 1993, *Günzel-Apel* et al. 1990).

## Results and discussion

*Assay performance*

Estradiol assay

The sensitivity of the method, as calculated by the interpolation of the mean minus 2 standard deviations of 10 replicates of the 0 pg/ml standard, is 0.3 pg/ml. The intra- and inter-assay coefficient of variation of a sample containing 3.1 pg/ml was 9.8% and 12.5%, respectively. Recovery was calculated by analysing gelding serum that had been spiked with 20 pg/ml of estradiol and ranged between 90% and 96%. According to the manufacturer, the estradiol antiserum has low cross-reactivity with equilenin (3.4%), estrone (2.4%), and equilin (0.34%). As steroid conjugates remain in the aqueous phase during the extraction procedure, we expect the cross-reactivity of estrone sulphate or estradiol 3-sulphate to be <0.1%. The method was compared with our existing H3-RIA reference method (*Behrens* et al. 1993). Estradiol concentrations from 47 blood plasma samples ranged from 0.93 to 180 pg/ml (Fig. 1). Linear regression analysis resulted in the following statistics: [E2-DSL] = 0.963 x [E2-H3] + 16.1 (pg/ml), R = 0.876, P < 0.01.

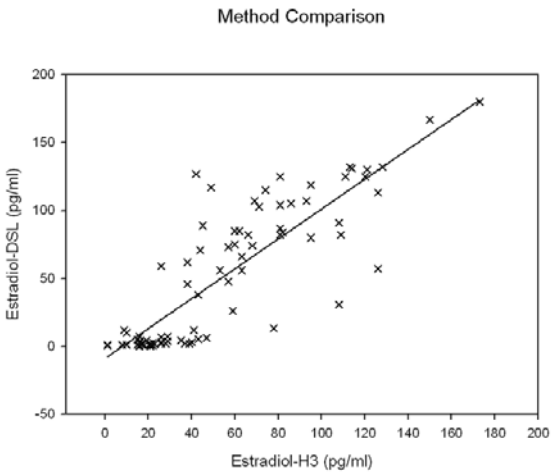


**Fig 1** Method comparison of the estradiol assays.
*Methodenvergleich des Östradiolassays.*

Testosterone assay

The sensitivity of the method, as calculated by the interpolation of the mean minus 2 standard deviations of 10 replicates of the 0 pg/ml standard, is 0.6 pg/ml. The intra- and inter-assay coefficient of variation of a sample containing 184 pg/ml was 11.7% and 13.5%, respectively. Recovery was calculated by analysing gelding serum that had been spiked with 100 pg/ml of testosterone and ranged between 97% and 1005%. According to the manufacturer, the testosterone antiserum has a relatively low cross-reactivity with 5?-dihydrotestosterone (6.6%), androstenedione (0.9%), and dehydroepiandrosterone (<0.1%). The method was compared with our existing H3-RIA reference method (*Günzel-Apel* et al. 1990). Testosterone concentrations from 85 blood plasma samples ranged from 3.3 to 1836 pg/ml (Fig. 2). Linear regression analysis resulted in the following statistics: [Testosterone-DSL] = 1.346 x [Testosterone-H3] - 37 (pg/ml), R = 0.833, P <0.001.

The sensitivity of the assay can be increased by further dilution of both testosterone (I-125) reagent and testosterone antiserum.

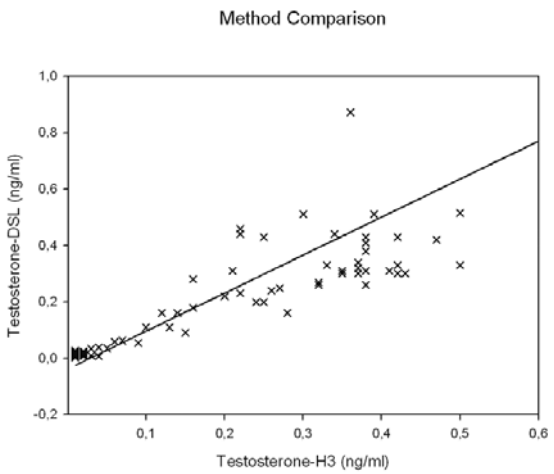


**Fig 2** Method comparison of the testosterone assay
*Methodenvergleich des Testosteronassays*

**Table 1** Hormone concentrations in mares with ovarian dysfunction. GCT-conf: confirmed granulosa cell tumour. GCT-susp: suspected granulosa cell tumour.
*Hormonkonzentrationen bei Stuten mit gestörter Ovarfunktion. GCT-conf: Bestätigter Granulosazelltumor. GCT-susp: Vermuteter Granulosazelltumor.*

| Mare no | E2-H3 (pg/ml) | E2-DSL (pg/ml) | P (ng/ml) | T-H3 (ng/ml) | T-DSL (pg/ml) | |
|---|---|---|---|---|---|---|
| 1 | 35 | 4,4 | 0,14 | 0,07 | 62,7 | GCT-conf |
| 2 | 28 | 2,3 | 0,05 | 0,06 | 58,1 | GCT-conf |
| 3 | 41 | 12,0 | 24,80 | 0,09 | 54,6 | GCT-conf |
| 4 | 29 | 3,9 | 0,05 | 0,04 | 39,6 | GCT-susp |
| 5 | 23 | 1,9 | 3,80 | 0,05 | 35,6 | GCT-susp |
| 6 | 21 | 1,0 | 3,50 | 0,03 | 34,0 | GCT-conf |
| 7 | 10 | 1,1 | 0,05 | 0,01 | 26,3 | GCT-conf |
| 8 | 18 | 0,7 | 5,60 | 0,02 | 24,3 | GCT-susp |
| 9 | 16 | 7,2 | 0,05 | 0,01 | 21,9 | GCT-susp |
| 10 | 26 | 2,3 | 11,80 | 0,02 | 20,7 | GCT-susp |
| 11 | 8 | 1,2 | 2,60 | 0,02 | 20,7 | |
| 12 | 21 | 2,0 | 2,20 | 0,01 | 19,5 | |
| 13 | 29 | 7,5 | 0,05 | 0,02 | 16,8 | |
| 14 | 26 | 6,8 | 4,10 | 0,01 | 13,8 | |
| 15 | 40 | 3,2 | 13,20 | 0,02 | 12,4 | |
| 16 | 16 | 3,3 | 6,10 | 0,02 | 12,4 | |
| 17 | 17 | 1,9 | 7,90 | 0,02 | 12,2 | |
| 18 | 17 | 1,0 | 0,05 | 0,01 | 11,7 | |
| 19 | 15 | 4,8 | 0,05 | 0,01 | 11,2 | |
| 20 | 16 | 0,6 | 3,20 | 0,01 | 9,7 | |
| 21 | 15 | 1,3 | 4,20 | 0,01 | 9,4 | |
| 22 | 26 | 3,1 | 4,50 | 0,03 | 9,2 | |
| 23 | 39 | 2,0 | 0,10 | 0,01 | 8,8 | |
| 24 | 37 | 2,0 | 0,22 | 0,02 | 8,1 | |
| 25 | 22 | 2,7 | 0,57 | 0,04 | 7,9 | |
| 26 | 22 | 0,8 | 1,40 | 0,01 | 7,5 | |
| 27 | 19 | 4,6 | 0,30 | 0,01 | 7,1 | |
| 28 | 16 | 2,6 | 5,50 | 0,02 | 6,3 | |

*Normal ovarian cycles*

The mean hormone concentrations of 6 cycling mares are shown in figure 3. Estradiol concentrations are very low, much lower than values we have published in the past, using our H3-E2-RIA (*Behrens* et al. 1993, *Hoppen* 1995). However, the above data agree with recent reports from the literature (*Gastal* et al. 1999, *Ginther* et al. 2005, *Ginther* et al. 2007). One possible explanation for these discrepancies may be the higher cross reactivities of the H3-E2-RIA with estrone, equilin, and equilenin.

*Mares with ovarian dysfunction*

Hormone concentrations of some mares with various suspected or confirmed ovarian dysfunctions are listed in table 1. Again, estradiol concentrations read lower with our new assay as compared to the H3-E2-RIA. However the new testosterone method appears to be better suited in detecting ovarian problems and particularly ovarian tumours of the mare than does the old H3-testosterone RIA. Granulosa cell tumours (GCT) are the most common ovarian tumours in the mare.

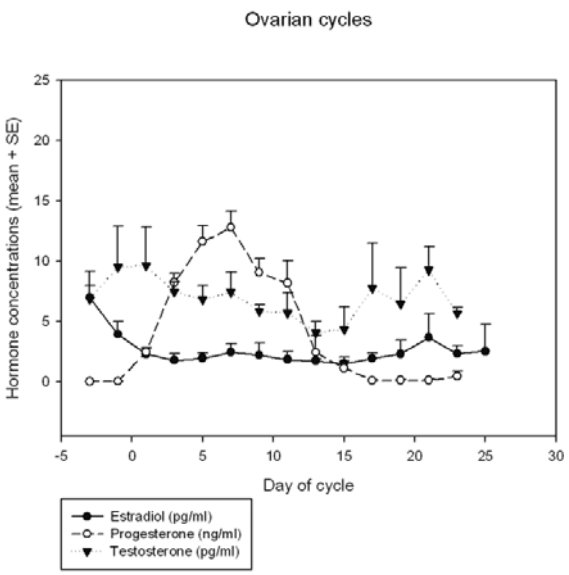


**Fig 3** Normal ovarian cycles of mares (n = 6, mean + S.E.)
*Normale ovarielle Zyklen von 6 Stuten (Mittelwerte +S.E.)*

They are hormonally active, and therefore diagnostic assays rely on the measurement of testosterone, progesterone, or inhibin (*Troedsson* et al. 2003). Testosterone in peripheral blood is reported to be elevated in 50-60% of cases with GCT (*Troedsson* et al. 2003). From the above data one could speculate that through increased sensitivity of the testosterone assay the diagnostic sensitivity could be improved for this condition.

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