Plasma concentrations of testosterone and 19-nortestosterone (nandrolone) in the nonracing intact male horse by liquid chromatography-mass spectrometry

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The Commonwealth of Pennsylvania regulates the use of anabolic and androgenic steroids by monitoring plasma samples obtained from equine athletes post competition. Plasma samples were chosen over urine because the pharmacological action of any drug is generally based on plasma concentration of the parent or active metabolite of the compound and not its concentration in urine. Furthermore, the complex excretion pattern of anabolic and androgenic steroids makes urine a more difficult medium to work with. The androgens testosterone and nandrolone are endogenously produced in measurable concentrations in the intact male horse; therefore, the proposed regulation requires that a tolerance threshold be suggested for the intact male horse competing in an official race.

Based on single IM administration, the plasma concentration of anabolic steroids was below the limit of quantification within 30 to 40 days (Soma *et al.*, 2007), Anecdotal information suggests that these agents are typically administered every 2 to 3 weeks, it is suggested, until more information is available that veterinarians and trainers allow a minimum of 120 days withdrawal period following multiple administrations.

Anabolic steroids are synthetic derivatives of the male hormone testosterone that have been modified to promote anabolic rather than androgenic actions. The anabolic effects are considered to be those promoting protein synthesis, muscle growth and erythropoiesis (Mottram & George, 2000). Anabolic steroids can exert strong effects on the body that may be beneficial for athletic performance (Hartgens & Kuipers, 2004).

Androgenic steroids in the intact male horse include androstenedione, dihydrotestosterone, dehydroepiandrosterone, androstanediol, and testosterone, of which testosterone is the dominant steroid (Ganjam *et al.*, 1973). The predominant method of quantification of androgenic steroids in plasma has been radioimmunoassay, which is not as specific as the direct measurement by liquid chromatography-mass spectrometry[a] due to cross reactivity with other steroids (Silberzahn *et al.*, 1988).

Plasma concentrations of testosterone in mature normal males 27 months to 15 years of age measured by radioimmunoassay averaged ~2000 pg/mL (Inoue *et al.*, 1993), concentrations ranging from 65 to 1600 pg/mL have also been reported in the intact male and 15.3 ± 4.9 pg/mL in geldings (Cox *et al.*, 1973). Basal plasma testosterone concentrations showed seasonal variations with a low in January of 200 ± 100 pg/mL to a high in April of 1400 ± 300 pg/mL (Kirkpatrick *et al.*, 1977; Aurich *et al.*, 2003) and diurnal variation was also noted where concentrations were consistently lower at 18 h (Ganjam & Kenney, 1975). Using liquid chromatography-mass spectrometry for analysis, seasonal variation was also observed (Soma *et al.*, 2007). Normal concentrations of testosterone and estrogen in intact male were attained by 16 months of age (Inoue *et al.*, 1993) with plasma concentrations of testosterone increasing with age (Johnson *et al.*, 1991).

Stallions have androgen-to-estrogen conversion capabilities, therefore, intact male horses produce estrogens since testosterone is readily converted to estrogen by the horse testicle (Nyman *et al.*, 1959). Intravenous administration of human chorionic gonadotrophin in horses with testicular tissue will stimulate a rise in testosterone (Cox *et al.*, 1973) and estrogen (Zwain *et al.*, 1989). In contrast, its injection into geldings will not produce the same effect, and castration will result in a rapid drop in both estrogen and testosterone (Ganjam & Kenney, 1975). Intramuscular administration of testosterone hexahydrobenzoate in the intact male produces a rapid rise in estrogen peaking in 24 h and a slower rise in testosterone with a peak concentration in 48 h (Zwain *et al.*, 1989).

Stallions can convert testosterone to estrogens (estrone and 17α-estradiol) by the Leydig cell of the testis that also produces

[a]Triple-stage quadrupole quantum mass spectrometer, Thermo Electron Corporation, San Jose, CA.

neutral C18-steroids such as 19-nortestosterone (nandrolone) and 19-norandrostendione. The urinary excretion in the intact male of nandrolone (Courtot *et al.*, 1984) and other 19-neutral steroids, including nandrolone have been demonstrated (Bedrak & Samuels, 1969; Dintinger *et al.*, 1989; Dumasia *et al.*, 1989). Subsequent studies using radio-immunoassay for nandrolone supported the endogenous secretion by its presence in both plasma and testis. The assumption by the authors was that at the time of collection the biosynthesis of nandrolone from testosterone was not occurring in all horses, as testosterone was quantified in all these male horses, but nandrolone was not. This also has been suggested by others (Benoit *et al.*, 1985). Nandrolone has been quantified in the plasma of intact nonracing males, but not in nonracing geldings and females (Soma *et al.*, 2007). It has also been suggested that nandrolone detected in the extract of the testis and urine could be an artifact of the chemical procedure (Dumasia *et al.*, 1989; Houghton *et al.*, 2007). In drug surveillance programs the separation of naturally occurring from administered nandrolone in the urine of intact males was based on the urine ratio of measured 5α-esrtane-3β,17α-diol and 5(10)-esrtane-3β,17α-diol or the total quantity of 5(10)-esrtane-3β,17α-diol (Dehennin *et al.*, 2007). The procedure of using urine ratios to predict the administration of commercial nandrolone certainly could be contested. Subsequent studies suggested that urinary nandrolone may be a product of decarboxylation of testosterone and not naturally produced nandrolone (Houghton *et al.*, 2007). This process is unlikely to occur in plasma due to the absence of enzymatic hydrolysis and the more gentle procedure used for the extraction of steroids from plasma (Guan *et al.*, 2005).

Boldenone sulphate that was considered endogenous has been detected in the urine of the intact male horses with urinary concentrations ranging from 0.1 to 1.27 ng/mL (Ho *et al.*, 2004). To date no boldenone has been quantified in the plasma of the intact male horse or are we aware of metabolic pathways suggested for the conversion of testosterone to boldenone in the testis of the horse.

Plasma samples available from on-going behavioral endocrinology projects and clinical service at The University of Pennsylvania were obtained from stallions shown to be free of exogenous administration of anabolic steroids and additional samples collected from stallions at local breeding farms were included in the current analysis. Plasma samples were assayed for testosterone, nandrolone, and boldenone and all samples were screened for other anabolic steroids. These animals included Standardbred, Thoroughbred, warm-bloods and pony breeds. The method of quantification in plasma was as previously described (Guan *et al.*, 2005, 2006).

Plasma samples collected from 144 intact males from 2002 to 2004, 2007, and 2008 during the months of September (20), December (15), January (27), April (10), May (25), June (47), were analyzed. All horses were 2 years or older with the oldest breeding stallion being 17, the mean age was 5.7 ± 4.1 (SD). There was a statistically significant ($P < 0.001$) seasonal variation in the plasma concentration of testosterone (Fig. 1). The plasma concentrations of testosterone samples collected during the breeding months (April, May, June) and nonbreeding months (September, December, January) were 724.9 ± 371.7 and 252.5 ± 187.9 pg/mL, respectively.

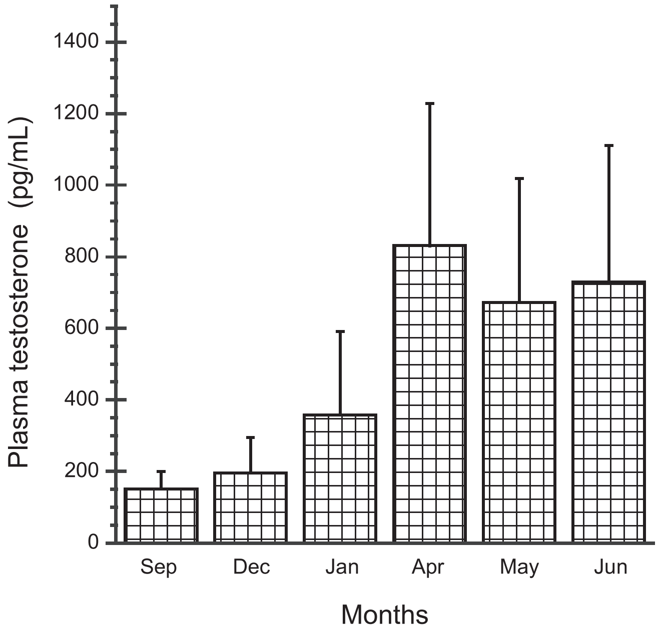

Fig. 1. Seasonal variation (mean and SD) in the plasma concentration of testosterone in the intact nonracing males (n = 144).

A distribution plot of all the data (JMP, version 6.0, SAS Institute Inc, Cary, NC) showed a median value of 402.2 pg/mL; the 25% and 75% quartiles were 213.1 and 807.6 pg/mL,

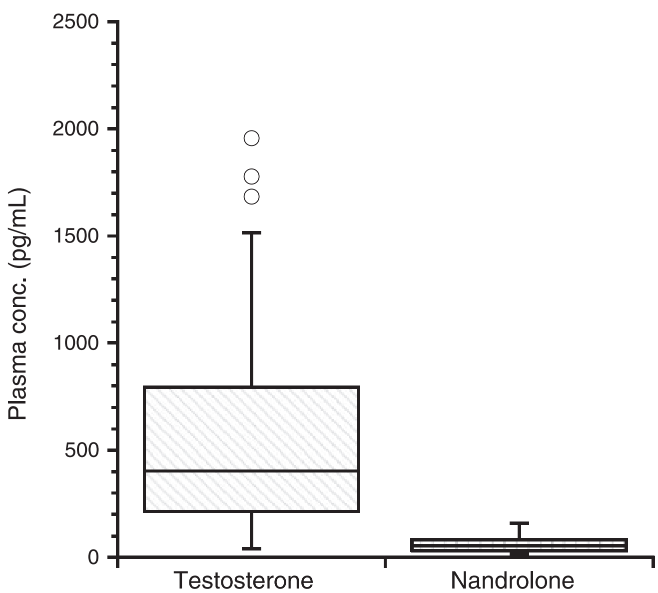

Fig. 2. Box plot of plasma concentration ranges of testosterone and nandrolone. Each box contains 50% of the measured concentrations with the median. The top and bottom of each box mark the 25% and 75% quartiles of the population. The extending lines are the minimum and maximum concentrations. Individual points (open circles) are outliers.

Table 1. Plasma concentrations (pg/mL) of testosterone and nandrolone in nonracing intact male horses (mean and SD) and the calculated upper tolerance concentration (X_{tol})

Hormone	No of samples	Mean (SD)	X_{tol}
Testosterone	144	521.5 ± 385.3	1928.3
Nandrolone	73	62.6 ± 36.5	169.2

respectively, which indicated that 50% of normal intact males were between 213.1 and 807.6 pg/mL (Fig. 2).

Quantifiable concentrations of nandrolone were found in 73 of the 144 samples (50.7%). Seasonal variation was not significant. The median concentration was 54.4 pg/mL, the 25% and 75% quartiles were 31.0 and 84.2 pg/mL (Fig. 2). The Limit of Quantification for testosterone and nandrolone in this study was 25 pg/mL and in 50% of the horses nandrolone was not quantified. Concentrations below this cannot be resolved analytically and therefore, nandrolone was considered not detectable and analysis was on this basis.

Due to the widespread use of anabolic steroids and testosterone in the racehorse population, it was not possible at this time to determine the normal plasma concentrations of testosterone and nandrolone in untreated racing-fit intact male horses in Pennsylvania. The concentrations of testosterone in the racing intact male horse were lower than the nonracing male, possibly by the suppression of endogenous hormone production due to the administration of anabolic steroids, months during which the samples were taken, lack of stimulation and possibly athletic competition (Soma *et al.*, 2007).

The calculation of the upper tolerance limit (X_{tol}) of testosterone and nandrolone was based on the methodology described[b].

$$X_{tol} = m + k * s$$

Where m and s were the mean and standard deviation of the *ln* transformed plasma concentrations and k was the one-sided tolerance limit factor (Owen, 1962). The k factors for samples sizes $n = 100$ (1.927) and $n = 73$ (1.990) for testosterone and nandrolone were selected (Table 1). The testosterone X_{tol} was also calculated for the plasma samples collected during the breeding season (April, May, June). Using these samples the concentrations were higher, the SD (variability) was lower and the k factor was larger based on fewer samples ($n = 82$), resulting in an X_{tol} of 1914.0 pg/mL, compared to 1928.3 pg/mL when all the samples were used. Based on the round-off rule, a suggested upper tolerance plasma concentration for testosterone and nandrolone in the racing intact male would be 2000 and 200 pg/mL, respectively.

[b]The European Agency for the Evaluation of Medicinal Products, Evaluation for Veterinary Use, Committee for Medicinal Products, Note for Guidance for the Determination of Withdrawal Periods for Milk: 7 Westferry Circus, Canary Wharf, London, E144HB, UK.

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