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Human nutritional supplements in the horse: comparative effects of 19-norandrostenedione and 19-norandrostenediol on the 19-norsteroid profile and consequences for doping control

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Abstract

The dietary supplements 19-norandrostenedione and 19-norandrostenediol are potential metabolic precursors of nandrolone. They are considered by law in the United States as prohormones without proven therapeutic, curative or diagnostic properties, and therefore available as over-the-counter drugs. Oral dosages of 0.1–1 mg/kg body weight were readily absorbed in the equine intestinal tract and thereby led to urinary excretion of drastically increased 5α-estrane-3β,17α-diol conjugates, which are known to be final metabolites of nandrolone. The actual rules for detection of illicit nandrolone administration to the horse have been found applicable for the detection of surreptitious oral 19-norandrostenedione and 19-norandrostenediol supplementation. Secondary markers of these administrations were high-level excretions of conjugated nandrolone, epinandrolone, 19-noretiocholanolone and 19-norepiandrosterone. No significant increase of circulating, biologically active nandrolone could be firmly evidenced, and it is therefore unclear to what extent continuous long-term administrations may have anabolic action.

Keywords: 19-Norandrostenedione; 19-Norandrostenediol; 19-Norsteroids

1. Introduction

In the United States since 1994, the Dietary Supplement Health and Education Act regulates free marketing of dietary or nutritional preparations, among which some are advertised as safe alternatives for hormone enhancement, such as dehydroepiandrosterone and androstenedione, which have been investigated recently for equine supplementation [1]. Two nandrolone (NT) prohormones, 19-norandrostenedione (NAD) and 19-norandrostenediol (NADL), have been a matter of concern for some male and female athletes who were tested positive for urinary NT metabolites, consequent to the ingestion of dietary supplements contaminated with one of these NT precursors [2]. These over-the-counter drugs are made easily available in Europe by purchase orders transmitted via the Internet, and it thus has become feasible to orally supplement human and equine individuals with variable and continuous doses, in order to attain hypothetical enhancement of athletic performance, claimed in misleading advertisements issued by the manufacturers.

Pioneering work has been performed by Houghton et al. [3,5] and by Bonnaire [4] on equine metabo-

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lism of NT, which is an endogenous steroid produced in the gonads of male and female [6]. Their conclusions have led International Racing Authorities to set up an official criterion for illicit NT administration to colts and stallions: the ratio of total conjugate levels of 5α-estrane-3β,17α-diol (EAD, the principal NT metabolite in the equine) over 5(10)-estrene-3β,17α-diol (EED, a reference 19-norsteroid unrelated to NT metabolism), which must remain below 1, otherwise positive NT testing is ascertained. The same ratio was also applicable in females and geldings, but a modification of the rules is yet accepted by Racing Authorities, in the sense that qualitative detection of EAD, by means of its full mass spectrum (electron impact or MS–MS), will constitute a definitive proof of exogenous NT and related 19-norsteroids in the latter two genders. This amendment has been proposed to circumvent the difficulty of estimating very low concentrations of EED, which are often encountered in females and geldings, and in males upon down-regulation of endogenous production by long-term administration of NT or its precursors.

This study reports on characteristic phase I and phase II metabolites in urine and plasma after oral supplementation of horses with either NAD or NADL, and this in comparison with a classical i.m. injection of NT ester.

2. Experimental

2.1. Administration protocol

Five thoroughbred horses were engaged in these excretion studies which were approved by the local Ethical Committee for Animal Studies. One entire male (age 12 y, weight 530 kg), two females (age 9–11 y, weight 575–475 kg) and two geldings (age 4–8 y, weight 485–465 kg) were selected. Nandrolone decanoate (NTD, Deca-Durabolin, Organon SA, Puteaux, France) was injected i.m. to the male (1 mg NTD or 0.73 mg NT/kg body weight). Oral administrations of NAD (Steraloids, Wilton, NH, USA) and NADL (prepared by borohydride reduction of NT and subsequent chromatographic isolation) contained in 1 or 2 capsules, at 2 different dosages (0.1 and 1 mg/kg), were performed in 2 females and 2 geldings. Basal androgen levels were assessed in urine and blood collections made at the time of drug administration and 2 days before. Post-administration times of sampling were at 1, 2, 4, 7, 9, 12, 18, 24, 36 and 48 h for oral administrations, and for i.m. injection every 2 days for 30 days. Twenty ml volumes of blood were drawn in heparinised tubes, centrifuged and the resulting plasma was frozen immediately. Urine volumes, in the 100–250 ml range, were stored frozen until analysis.

2.2. Materials

Reference steroids were obtained from Steraloids (Wilton, NH, USA) or Sigma (St Louis, MO, USA): NT (19-nortestosterone, 17β-hydroxy-4-estren-3-one), epinandrolone (eNT, 17α-hydroxy-4-estren-3-one), NAD (4-estrene-3,17-dione) and NADL (4-estrene-3β,17β-diol), 19-norepiandrosterone (eNA, 3β-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (NE, 3α-hydroxy-5β-estran-17-one). EAD and EED were gifts from Organon Scientific Group, Oss, Holland). Deuterium-labelled analogs of nandrolone (NTd_3) and 19-noretiocholanolone (NEd_3) were obtained from Radian (Austin, TX, USA). Heterologous labelled internal standards for EAD and EED quantifications were respectively 5α-[16,16,17-$^2H_3$]estrane-3β,17β-diol ($EADd_3$, kindly donated by Doctor S. Westwood from NARL, Pymble, Australia) and 5β-[16,16,17-$^2H_3$]androstane-3α,17β-diol (ADd_3), prepared according to procedures outlined previously [7].

DEAE-Sephadex A25 (chloride form) and Sephadex LH-20 were liquid chromatography supports, produced by Pharmacia-Upjohn (Uppsala, Sweden). Solid-phase extraction cartridges loaded with 500 mg Bond-Elut C_{18}-HF (octadecyl silica) were obtained from Varian Associates (Harbor City, CA, USA). β-Glucuronidase (EC 3.2.1.31) from *Escherichia coli* (200 I.U./ml) was supplied by Roche Diagnostics (Mannheim, Germany). Trimethyliodosilane (TMIS) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) came from Fluka (Buchs, Switzerland). Organic solvents and other usual chemicals were of analytical grade and obtained from common suppliers.

2.3. Gas chromatography–mass spectrometry

GC–MS was carried out using a Hewlett–Packard 6890 gas chromatograph (HP Analytical Division, Waldbronn, Germany), equipped with a HP 7673 autosampler and coupled to a HP 5973 mass selective detector. Instrument control and data processing were performed with a HP Vectra XA Computer and ChemStation software. GC separation was achieved on a HP-5 Trace (5% phenyl-methyl-polysiloxane) fused-silica column (30 m×0.25 mm I.D., 0.25 μm film thickness) operated with a helium inlet pressure of 108 kPa and temperature programming: 130°C for 0.5 min, ramped at 5°C/min to 260°C, ramped at 20°C/min to 310°C and held for 3 min (run time=32 min). Injections of 2 μl samples were made at 275°C in the splitless mode (0.75 min) into a split–splitless injection port with an inner silanised glass liner containing silanised glass wool, and the transfer line was heated at 280°C. The ion source was operated in the electron impact mode with 70 eV electron energy and the electron multiplier was set to 400 V above the automatic tune voltage.

2.4. Analytical method

2.4.1. Sampling, internal standard addition and extraction

Urine sample sizes were variable, depending on the expected concentration range: 5 ml volumes for baseline and late-time collections, 2 ml volumes for early- and middle-time collections. Plasma sample volume was 4 ml. Internal standard additions were within the expected concentration ranges of native compounds. Solid-phase extraction was performed with reversed-phase cartridges mounted on a Benchmate Workstation (Zymark, Hopkinton, MA, USA). Conditioning, loading and elution were done as follows: 4 ml of methanol, 4 ml of water, sample aspiration, rinsing with 5 ml of water and elution with 6 ml of methanol. Eluates were evaporated at 60°C with a TurboVap VL Evaporator (Zymark).

2.4.2. Nonconjugate fraction in plasma

Solid-phase extracts were dissolved in 1 ml phosphate buffer (0.1 *M*, pH 6.5) and extracted with 3 ml of a mixture of *n*-hexane–diethyl ether (1:1, v/v).

2.4.3. Conjugate fractionation by ion-exchange chromatography

Small disposable DEAE-Sephadex columns (20× 5 mm) were packed in 20% aqueous methanol (methanol–water, 8:2, v/v). Glucuronides (together with minute amounts of nonconjugated androgens, in the case of urine) were eluted with 3 ml 0.03 *M* LiCl in 20% aqueous methanol, sulfates were recovered with 2.5 ml 0.3 *M* LiCl in 20% aqueous methanol. Eluates were evaporated at 60°C with TurboVap VL Evaporator.

2.4.4. Enzyme hydrolysis of glucuroconjugates

Extraction residues were dissolved in 1 ml phosphate buffer (0.1 *M*, pH 6.5) and incubated with 12 I.U. of β-glucuronidase during 1 h at 55°C. The unconjugated and deconjugated steroids were extracted with 3 ml of a mixture of *n*-hexane–diethyl ether (1:1, v/v). The organic phase was dried with anhydrous sodium sulfate and the solvent was evaporated under a nitrogen stream at 60°C.

2.4.5. Methanolysis of sulfoconjugates

Extraction residues were dissolved in 1 ml methanolysis mixture (1 *M* HCl and 1 *M* LiCl in methanol) and heated for 1 h at 55°C. Neutralisation was then performed with saturated aqueous $NaHCO_3$ and methanol was evaporated in TurboVap VL Evaporator. Solvent extraction was as described for the glucuronide fraction.

2.4.6. Group fractionation by partition chromatography

Reusable columns (200×5 mm) were packed with Sephadex LH-20, swollen in a mixture of dichloromethane–methanol (95:5, v/v). After deposition of the extraction residue dissolved in 0.25 ml of dichloromethane–methanol mixture on top of the column, the first 3 ml of eluent were discarded. Diketones and monohydroxy-ketones, with or without a double bound, were recovered in the next 2.5 ml. Diols were eluted in the next 4 ml.

2.4.7. Derivatisation and selected ion monitoring

Trimethylsilyl-enol-trimethylsilyl-ethers were made by dissolving final dry residues, which had been transferred into appropriate injection vials, in 30 μl derivatization reagent (MSTFA containing 0.2%

TMIS, v/v) and by subsequent heating at 70°C for 30 min. Molecular ions used for quantification were: *m/z* 416 (NAD), 418 (NT and eNT) and 421 (NTd_3). Fragment ions used for quantification were: *m/z* 405 (NE and eNA), 408 (NEd_3), 332 (EAD), 335 ($EADd_3$), 330 (EED) and 349 (ADd_3).

2.4.8. Quantification

Response factors of the mass selective detector for standard mixtures containing equal amounts of analyte and internal standard ([421/418] for NT and eNT, [421/416] for NAD, [408/405] for NE and eNA, [335/332] for EAD and [349/330] for EED) were measured. Ion abundance ratios of analyte versus internal standard ([418/421] for NT and eNT, [416/421] for NAD, [405/408] for NE and eNA, [332/335] for EAD and [330/349] for EED) were in the range 0.2–2.5, and had a linear relationship with corresponding concentration ratios. Concentrations were calculated as outlined previously [8]. Briefly, the ratio of peak intergrations of unlabelled to labelled analyte in the urine sample was multiplied by the corresponding response factor and the concentration in ng/ml of added labelled analog. Intra- and inter-assay RSD ($n=3$, with measurements performed either in a single series, or in 3 series on different days) for urine and plasma determinations, in the concentration ranges of respectively 10–100 ng/ml and 0.05–1 ng/ml, were comprised between 4 and 9%. Concentrations were expressed in nanograms of free steroid/ml. Accuracy was evaluated by standard additions of all analytes (range 5–50 ng/ml) to a urine pool, and in a plasma pool spiked with NT and EAD (range 0.05–0.5 ng/ml). Equations relating added to found analyte had slopes not significantly different from 1. Detection limits for quantification (peak heights corresponding to 3 times baseline variability) were in the range 0.2–1 ng/ml for urinary analytes, and 0.03 ng/ml for plasma estimations of NT and EAD.

3. Results and discussion

3.1. Urine

Sulfoconjugates constitute the majority of urinary steroids in the equine species; stereochemistry and position of secondary alcoholic groups are determinant factors for conjugating enzyme affinity. Glucuronoconjugates may be preponderant in some cases, e.g. 17α-hydroxy-metabolites. Sulfates and glucuronides were analysed separately, because of the deconjugation strategy which involved a specific procedure for each group; consecutive extracts were more appropriate for subsequent GC–MS quantification. Analytical data are however presented as total conjugate concentrations (i.e. glucuronides + sulfates + minute amounts of nonconjugated steroid), because this approach is in accordance with the rules established by horse racing authorities for doping control purposes.

The time course of urinary EAD concentrations and EAD/EED ratios, after different types of NT and precursor administrations, are displayed in Fig. 1. It is clear that EAD is a common final metabolite, and thus a unique marker of all these 19-norsteroids, whatever the administration route. It was confirmed that injectable NTD has long-acting properties, since even 30 days after administration, testing was still found positive according to the EAD/EED ratio. In comparison with NTD injection, even low-dosage (0.1 mg/kg) oral administrations of NAD and NADL to mare and gelding, afforded higher maximum excretions of EAD for short times (approx. 24 h) with detectability, based on EAD/EED ratio, lasting for up to 3 days. This however demands the measurement of EED concentrations at the sub-nanogram/ml level, which required highly purified urinary extracts, and which in some cases may be difficult to attain. The lower right panel of Fig. 1 illustrates the quasi-proportional relationship between the areas under the curve for high (1 mg/kg) and low (0.1 mg/kg) dosage ingestion of NADL, and thereby the proportionality of intestinal absorption for such loadings.

Excretion data of other relevant NT metabolites (Table 1) led to peak levels, after oral administrations, which were lower than those of EAD (except for NAD), but they had, in general, longer delays for return to baseline excretion values, in particular for eNT, NE and eNA. These metabolites may be considered as complementary markers of NT or congener administration. NAD (supposed to be excreted unconjugated, since no definitive proof of conjugation has yet been reported), although being very abundant in entire male urine, was still further

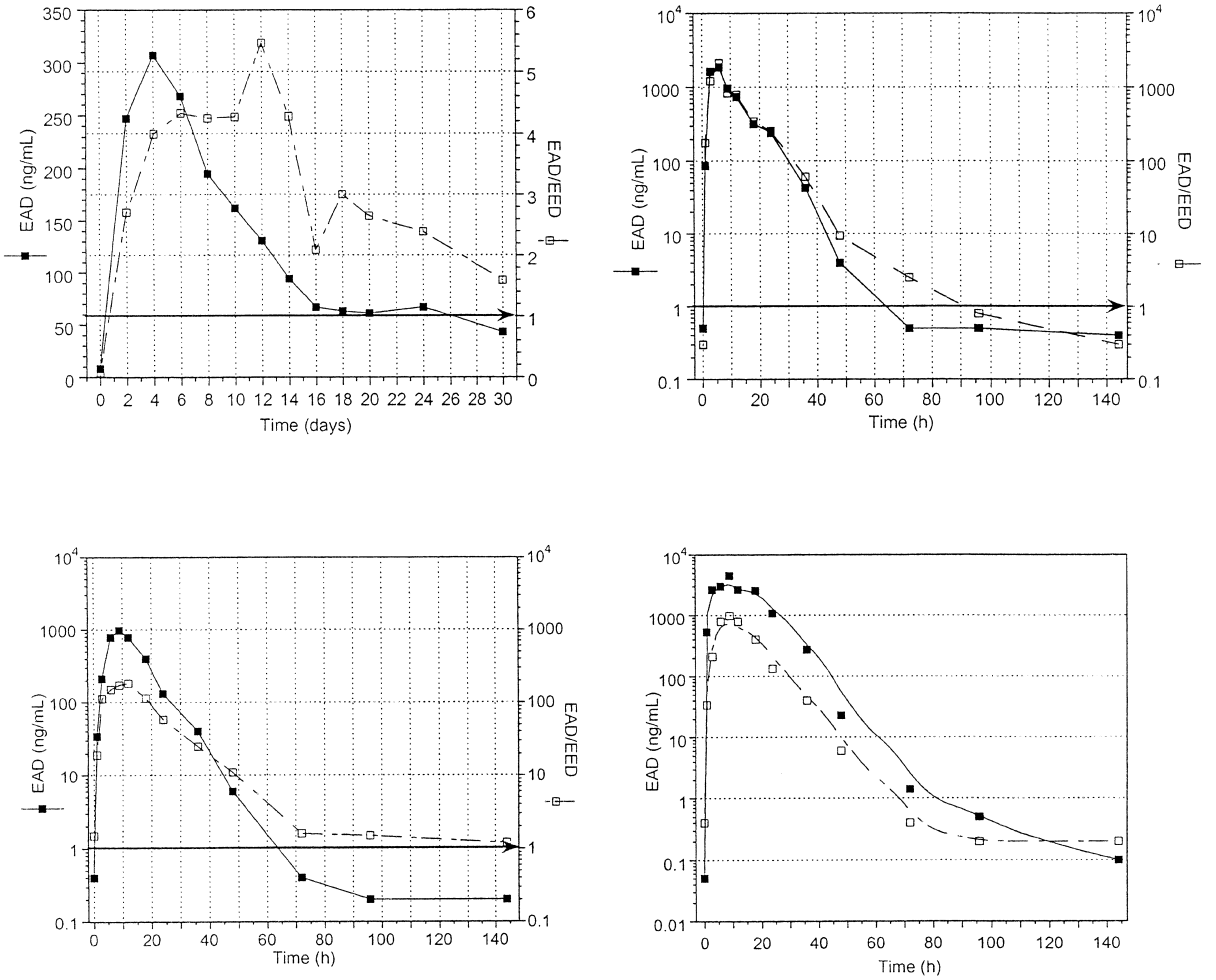

Fig. 1. Urinary concentration ratio (EAD/EED = 5α-estrane-3β,17α-diol/5(10)-estrene-3β,17α-diol) and/or concentration of 5α-estrane-3β,17α-diol (EAD in ng/ml). Upper left panel: Effect of i.m. nandrolone decanoate (1 mg/kg) in an entire male. Upper right panel: Effect of oral 19-norandrostenedione (0.1 mg/kg) in a gelding. Lower left panel: Effect of oral 19-norandrostenediol (0.1 mg/kg) in a female. Lower right panel: Effect of 19-norandrostenediol dosage (□ 0.1 mg/kg in a female, ■ 1 mg/kg in a gelding).

increased in the very early stages after NTD injection, but was thereafter drastically decreased, as a consequence of down-regulation of endogenous production.

Concerning the detection period of biological markers generated by these nutritional supplements, it should be kept in mind that they are intended for continuous oral administration at supra-physiological doses (1–5 mg/kg range). It is plausible that the detection period after continuous daily ingestions may be longer than the one found after a single loading.

3.2. Plasma

The principal objective of these plasma determinations was to demonstrate the presence and the extent of circulating biologically active hormone (i.e. nonconjugated NT), and therefore were analysed only the highest dosage experiments, the lower ones (0.1 mg/kg) affording undetectable levels. NTD injection to the male led to a drastic increase of free NT, with concomitant down-regulation of endogenously produced NAD (Fig. 2, left panel). Free NT increase after oral administrations of NAD or NADL

Table 1
Urinary total conjugates of ketonic nandrolone metabolites: after i.m. nandrolone decanoate in an entire male; after oral 19-norandrostenediol in a gelding (high dosage) and in a mare (low dosage); after oral 19-norandrostenedione in a gelding

	NT	eNT	NAD	NE	eNA
NT decanoate (NT = 0.73 mg/kg)					
Maximum concentration ng/ml at (day)	162 (4)	58 (4)	427 (2)	15 (4)	135 (6)
Time (day) for return to basal excretion	30	30	[a]	30	30
19-Norandrostenediol (1 mg/kg)					
Maximum concentration ng/ml at (h)	620 (6)	270 (6)	25 (3)	1425 (12)	1785 (9)
Time (h) for return to basal excretion	144	144	36	180	180
19-Norandrostenediol (0.1 mg/kg)					
Maximum concentration ng/ml at (h)	53 (3)	26 (3)	2 (3)	350 (12)	300 (6)
Time (h) for return to basal excretion	96	144	9	180	180
19-Norandrostenedione (0.1 mg/kg)					
Maximum concentration ng/ml at (h)	360 (3)	500 (3)	11 (3)	800 (6)	950 (6)
Time (h) for return to basal excretion	96	144	24	144	144

NT = nandrolone, eNT = epinandrolone, NAD = norandrostenedione, NE = noretiocholanolone, eNA = epinorandrosterone.
[a] Endogenous production still downregulated at 30 days post-administration.

was low or inexistent, but a significant simultaneous enhancement of circulating NAD was observed, specially after NAD ingestion (Fig. 2, right panel). Therefore, is it plausible that the latter oral 19-norsteroid supplementations have no anabolic effect by means of circulating NT, but the possibility exists that implementation of plasma NAD may conduct to intracrine conversion to NT in muscle tissue.

Further data on the impact of i.m. and oral administrations on some constituents of the plasma 19-norsteroid profile are listed in Table 2. It is probable that the major part of the observed differences can be ascribed to liver first-pass metabolism of the ingested drugs. Glucuronide and sulfate were the major circulating conjugates of EAD and NT respectively, while eNA circulated to a large extent unconjugated, for an unknown reason. Whether NT sulfate originated exclusively from hepatic metabolism, or from some intracrine conversion of NAD or NADL, was not elucidated.

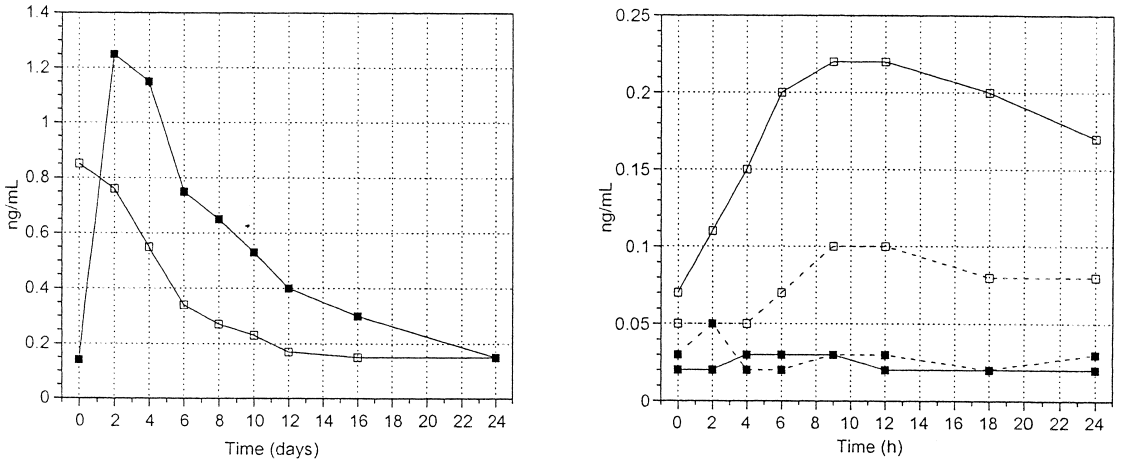

Fig. 2. Plasma levels of nonconjugated nandrolone (■) and 19-norandrostenedione (□). Left panel: After i.m. injection of nandrolone decanoate (1 mg/kg) to an entire male. Right panel: After ingestion of 19-norandrostenedione (1 mg/kg) by a female (—), and 19-norandrostenediol (1 mg/kg) by a gelding (------).

Table 2
Plasma levels of phase I and phase II metabolites of nandrolone: After i.m. nandrolone decanoate in an entire male; after oral 19-norandrostenediol in a gelding; after oral 19-norandrostenedione in a mare

	EAD NC	EAD G	eNA NC	eNA G	NT S
NT decanoate (NT = 0.73 mg/kg)					
Basal concentration ng/ml	0.04	0.08	0.01	0.01	0.02
Maximum concentration ng/ml at (day)	0.06 (4)	0.82 (4)	0.35 (4)	0.20 (4)	0.16 (4)
19-Norandrostenediol (1 mg/kg)					
Basal concentration ng/ml at (h)	0.02	0.03	0.02	0.02	0.02
Maximum concentration ng/ml at (h)	0.80 (2)	82 (2)	11 (2)	10 (2)	6.5 (2)
19-Norandrostenedione (1 mg/kg)					
Basal concentration ng/ml	0.01	0.05	0.01	0.01	0.02
Maximum concentration ng/ml at (h)	1.2 (6)	14 (6)	9.2 (6)	4.4 (6)	0.82 (6)

NT = nandrolone, EAD = 5α-estrane-3β,17α-diol, eNA = epinorandrosterone, NC = nonconjugated, G = glucuronide, S = sulfate.

4. Conclusions

In conclusion, NAD and NADL considered as prohormones without intrinsic anabolic action, and therefore available as noncontrolled over-the-counter drugs, are absorbed by the equine intestinal tract and, thereby, lead to drastic increase of urinary EAD output. The present rules for detection of illicit NT administration to the horse allow also the detection of surreptitious oral NAD and NADL supplementations. To what extent such continuous long-term administration may enhance athletic performance remains to be established.

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