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Ex vivo spontaneous generation of 19-norandrostenedione and nandrolone detected in equine plasma and urine

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ABSTRACT

19-Norandrostenedione (NAED) and nandrolone are anabolic-androgenic steroids (AASs). Nandrolone was regarded solely as a synthetic AAS until the 1980s when trace concentrations of apparently endogenous nandrolone were detected in urine samples obtained from intact male horses (stallions). Since then, its endogenous origin has been reported in boars and bulls; endogenous NAED and nandrolone have been identified in plasma and urine samples collected from stallions. More recently, however, it was suggested that NAED and nandrolone detected in urine samples from stallions are primarily artifacts due to the analytical procedure. The present study was undertaken to determine whether NAED and nandrolone detected in plasma and urine samples collected from stallions are truly endogenous or artifacts from sample processing. To answer this question, fresh plasma and urine samples from ≥8 stallions were analyzed for the two AASs, soon after collection, by liquid chromatography hyphenated to tandem mass spectrometry (LC–MS/MS). NAED and nandrolone were not detected in fresh plasma samples but detected in the same samples post storage. Concentrations of both AASs increased with storage time, and the increases were greater at a higher storage temperature (37 °C versus 4 °C, and ambient temperature versus 4 °C). Although NAED was detected in some fresh stallion urine samples, its concentration (<407 pg/mL) was far lower (<0.4%) than that in the same samples post storage (at ambient temperature for 15 days). Nandrolone was not detected in most of fresh urine samples but detected in the same samples post storage. Based on these results, it is concluded that all NAED and nandrolone detected in stored plasma samples of stallions and most of them in the stored urine samples are not from endogenous origins but spontaneously generated during sample storage, most likely from spontaneous decarboxylation of androstenedione-19-oic acid and testosterone-19-oic acid. To our knowledge, it is the first time that all NAED and nandrolone detected in plasma of stallions and most of them detected in the urine have been shown to be spontaneously generated *in vitro* during sample storage. This finding would have significant implications with regard to the regulation of the two steroids in horse racing.

1. Introduction

19-Norandrostenedione (estr-4-ene-3,17-dione, CAS # 734-32-7, NAED) and nandrolone (17β-hydroxy-estr-4-en-3-one, CAS # 434-22-0) are anabolic-androgenic steroids (AASs) related in chemical structure to the primary male hormone, testosterone. AASs possess androgenic, anabolic, and erythropoietic properties [1], and are probably among the most abused drugs in sports. Nandrolone was considered solely a synthetic anabolic-androgenic steroid (AAS) until the 1980s when trace concentrations of endogenous nandrolone were detected in urine samples from intact male horses that were not treated with the AAS [2,3]. Since then, endogenous origin of NAED and nandrolone has been reported; they have been identified in testicular tissue of stallions [4,5]. Nandrolone has been detected in urine [6–8] and testicular tissue sample [9] of boars, urine of bulls [10], and plasma of pregnant women [11]. Epinandrolone (17α-hydroxy-estr-4-en-3-one) has been identified in urine [12,13] and bile of cows [14], urine of sheep [15], goats and mares [16] during pregnancy. In addition, nandrolone was identified in plasma samples of stallions [17,18]. Similarly, the presence of NAED and its sulfate form in yolk-sac fluid of the early equine conceptus was reported in 2008 [19]. Recently, we identified NAED and nandrolone along with androstenedione and testosterone in plasma samples obtained from stallions used for research and actively racing stallions when the samples were analyzed using the LC–MS/MS method for the identification of 55 AASs [20], but they were not detected in plasma

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samples collected from fully castrated male horses (geldings) and adult female horses (mares) (unpublished result). In a recent publication, urinary NAED with 5(10)-estrene-3β,17α-diol was used for identification of cryptorchidism in the horse [21]. In short, on the basis of the publications to date, NAED and nandrolone seem to be accepted as endogenous steroids. Endogenous productions of NAED and nandrolone and their relevance to human doping control have been reviewed [22]. Nonetheless, Houghton et al. recently reported that NAED and nandrolone identified in urine of stallions were primarily artifacts resulting from sample preparation [23,24]. The result of this study led us to the question of whether NAED and nandrolone identified in plasma samples from stallions are endogenous or artifacts caused by the analytical procedure used. The purpose of the present study was to address the issue of origin of NAED and nandrolone detected in plasma and urine of stallions, which has regulatory implications in doping control.

2. Experimental

2.1. Chemicals and reagents

19-Norandrostenedione, nandrolone, androstenedione, and testosterone were purchased from Steraloids (Newport, Rhode Island, USA), and D_3-testosterone was obtained from Sigma (St. Louis, Missouri, USA). Methanol, Water (both Optima grade), and ammonium formate (Certified) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA).

Formate buffer stock solution (pH 6.6) comprising 1.0 mol/mL ammonium formate was prepared from ammonium formate powder without pH adjustment. Formate buffer working solution containing 2 mmol/L ammonium formate (pH 6.6) was prepared by diluting the formate buffer stock solution with water.

A stock solution of each AAS (1.0 mg/mL) was prepared by dissolving weighed dry powder in methanol, and stored at 4 °C. A primary working solution of each AAS at 20 μg/mL in methanol was prepared by diluting the stock solution of each AAS with methanol, and stored at 4 °C. Working solutions of a mixture of four AASs at 250, 100, 50, 25, 10, 5.0 and 2.5 ng/mL were prepared by mixing the primary working solutions of each analyte (20 μg/mL) and diluting with methanol. D_3-testosterone solution (100 ng/mL) was prepared by diluting the primary working solution with methanol.

2.2. Sample collection

Control equine blood and urine samples were collected from drug-free geldings or mares used in research. Plasma was harvested from blood samples. Control plasma and urine samples, determined to be free of the analytes using the LC–MS/MS method previously described [20], were used to prepare calibrators for quantification of the analytes.

Blood samples were collected from ten research stallions in April of 2010, two research Standardbred stallions in May of 2010, and six actively racing Standardbred stallions in June of 2010, using vacuum collection tubes with potassium oxalate as an anti-coagulant and sodium fluoride as an inhibitor of plasma esterases (Kendall, Mansfield, Massachusetts, USA). Urine samples were collected in May of 2010 from a Thoroughbred stallion retired from racing but otherwise in good health, an actively racing Standardbred stallion and six actively racing Standardbred colts (all 3–4 years old) in August of 2010. The blood and urine samples were shipped on ice to our laboratory, or plasma was immediately harvested from blood and then sent on ice to the laboratory within 5 h of collection.

Plasma and urine samples were processed using the following protocol: fresh samples shipped were extracted and analyzed within 5 h of collection; the same samples were stored at different temperatures (4 °C, ambient temperature (21 °C), and 37 °C) for various periods of time in order to monitor the effects of storage temperature and time on concentrations of the analytes.

2.3. Sample preparation

Plasma calibrators containing NAED, nandrolone, androstenedione and testosterone each at 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 pg/mL plus D_3-testosterone as internal standard (IS) at 2000 pg/mL were prepared by spiking control plasma sample aliquots (0.5 mL each) with 10 μL of the working solution of the mixed analytes and 10 μL of the IS solution. The spiked samples were freshly prepared shortly before extraction. Quality control (QC) plasma samples each containing the IS at 2000 pg/mL and the analytes each at 200, 1000, and 5000 pg/mL were similarly prepared. Stallion plasma samples (0.5 mL each) for determination of the 4 analytes were fortified with the IS (10 μL) alone. Urine calibrators (0.5 mL each) were prepared according to the procedure described above for plasma calibrators. Aliquots of urine samples (0.5 mL each) collected from Stallions were similarly prepared as described above for stallion plasma samples.

Plasma and urine samples prepared above were extracted as previously reported [17,20]. Briefly, to each of the plasma or urine samples in test tubes, MTBE (5 mL) was added. The tubes were gently shaken for 5 min on a rotorack device (Thermlyne, Dubuque, Iowa, USA) and centrifuged at ~3000 rpm (~1400 × *g*) for 5 min. The (top) organic layer in each tube was decanted or transferred with a glass Pasteur pipette into a fresh glass tube (16 mm × 100 mm), and dried at 50 °C in a hot block (Techni Dri-Block DB-3, Duxford, Cambridge, UK) under a stream of air or nitrogen gas. The dried sample extract was reconstituted in 100 μL of sample solvent—50% methanol in aqueous formate buffer solution (2 mM, pH 6.6), and a 20-μL aliquot was subjected to LC–MS analysis.

2.4. Instrumentation

All sample analyses were conducted on a system of ultra high-performance liquid chromatography hyphenated to tandem mass spectrometry (UHPLC–MS/MS), consisting of an Accela liquid chromatograph with an Accela autosampler and a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) source (Thermo Fisher Scientific, San Jose, California, USA). The mass spectrometer was operated at unit mass resolution (FWHM set at 0.7 for both Q1 and Q3) in the positive ion mode, and calibrated with polytyrosine-1,3,6 that comprised Tyr, $(Tyr)_3$, and $(Tyr)_6$.

UHPLC separations of the analytes were performed on a Hypersil Gold C_{18} column (50 × 2.1 mm I.D., 1.9-μm particle size; Thermo Fisher Scientific, Waltham, Massachusetts, USA) that was maintained at 50 °C. The separations were conducted with 2 mM aqueous ammonium formate buffer solution at pH 6.6 (mobile phase solvent A) and methanol (mobile phase solvent B). A mobile phase gradient was used for elution of the analytes and programmed as follows: solvent B was increased from 50% (0 min) to 65% (3.5 min), to 90% (4.0 min), held at 90% (4.3 min), decreased to 50% (4.4 min), and held at 50% (5.0 min). The mobile phase flow rate was 500 μL/min.

The H-ESI source parameters optimized for LC flow rate at 500 μL/min (50% solvent A + 50% solvent B) were as follows: spray voltage, 1000 V; sheath gas (nitrogen) setting, 60 arbitrary units; auxiliary gas (nitrogen) setting, 30 arbitrary units; sweep gas (nitrogen) setting, 3 arbitrary units; vaporizer temperature, 350 °C; the ion transfer capillary temperature, 330 °C. Argon gas was used as collision gas for MS/MS experiments, and its pressure was set at 1.5 mTorr in the collision-induced dissociation (CID) cell. Selected-reaction monitoring (SRM) was employed for analyte detection,

Table 1
Parameters for LC–MS/MS analyses of the anabolic-androgenic steroids.

Analyte	Precursor ion, $[M+H]^+$	Collision energy (V)	Product ion A (m/z)	Product ion B (m/z)	Product ion C (m/z)	Retention time (min)
NAED	273.2	26	109.1	197.1	83.1	1.29
Nandrolone	275.2	26	109.0	145.1	135.1	1.65
Androstenedione	287.2	26	97.0	109.0	123.0	1.65
Testosterone	289.2	26	97.0	109.0	123.0	2.03
D_3-testosterone	292.2	26	97.0			2.03

quantification and confirmation. Scan width was set at 0.4 Dalton for each product ion monitored in SRM, and scan time at 75 ms for each SRM transition. Experimental collision energy used and product ions monitored for each analyte are presented in Table 1. For full product ion scan in MS/MS, scan time was set at 150 ms, and scan range was m/z 75–350. Data acquisition and processing were accomplished using Xcalibur software (Version 2.0.7; Thermo Fisher Scientific, San Jose, CA).

2.5. LC–MS/MS method for detection, quantification and identification of AASs

An LC–MS/MS method recently published [20] was slightly modified in this study for detection, quantification and identification of NAED, nandrolone, androstenedione and testosterone in equine plasma and urine samples. The major modification was the change in pH of the buffer solution used as a mobile phase in LC separations; ammonium formate buffer solution (pH 6.6) was used in the present study in place of the formate buffer (pH 3.5) that was previously used. The purpose of this modification was to avoid possible artifactual results in the detection of NAED and nandrolone, because it was reported that the two AASs detected in urine samples from stallions resulted from acid solvolysis [23]. Detection and quantification of the analytes in plasma and urine samples were conducted using a specific precursor-to-product ion (product ion A in Table 1) transition with retention time match (±0.1 min window) for each analyte, and identification using three ion transitions (Table 1) with the retention time match, as described in previous publications [17,20,25]. Quantification of the analytes in plasma samples was performed by internal calibration while that in urine was by external calibration. The limit of quantification (LOQ) was 50 pg/mL for NAED, nandrolone, androstenedione, and testosterone in plasma samples; while LOQ was 100 pg/mL for NAED, 250 pg/mL for nandrolone, 50 pg/mL for androstenedione and testosterone in urine. The method used is specific for identification of each analyte in the present study, as discussed in previous publications [20,26]. Plasma and urine samples were analyzed according to our routine quality assurance (QA) protocol in order to assure that analytical results are reliable. Briefly, the QA protocol requires that quality control (QC) samples, calibrators, and unknown samples be analyzed in the following sequence: QC samples, solvent blank, calibrators, solvent blank, unknown sample A, solvent blank, unknown sample B, solvent blank, repetition of unknown sample and solvent blank pair, replicates of the QC samples.

3. Results

3.1. Results from fresh plasma and urine samples

Fresh plasma and urine samples were analyzed within five hours of collection to determine if NAED and nandrolone were present. The LC–MS/MS chromatograms depicted in Fig. 1 indicate absence

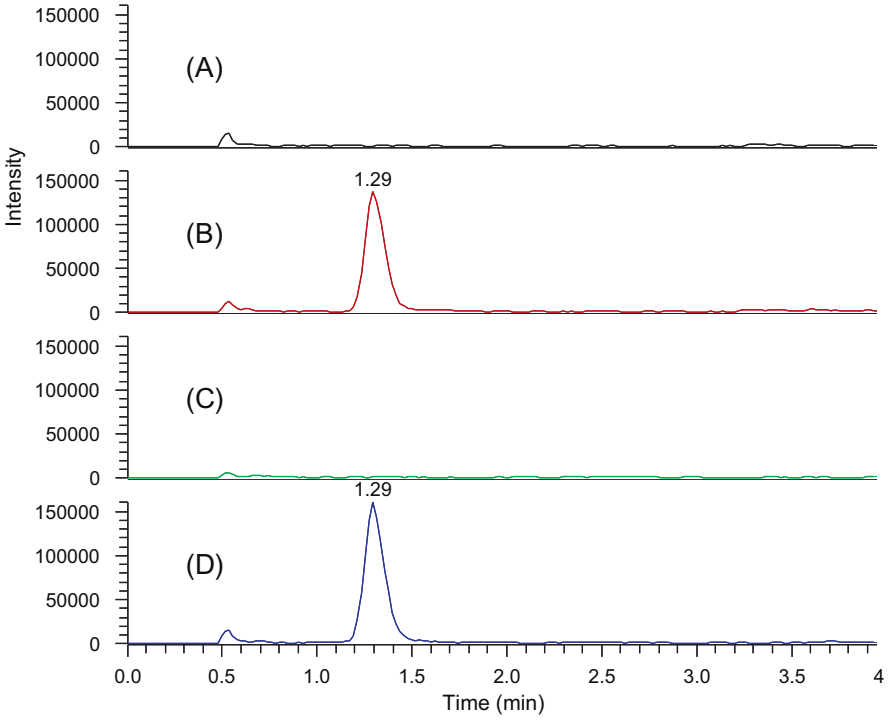


Fig. 1. LC–MS/MS chromatograms of blank and spiked plasma samples, fresh and stored stallion plasma samples. (A) Mare plasma sample, (B) NAED (1000 pg/mL) spiked to mare plasma, (C) a fresh plasma sample analyzed 3 h post collection from a Standardbred stallion, and (D) the same stallion plasma sample stored at ambient temperature for 7 days. The ion transition monitored was m/z 273 → 109.

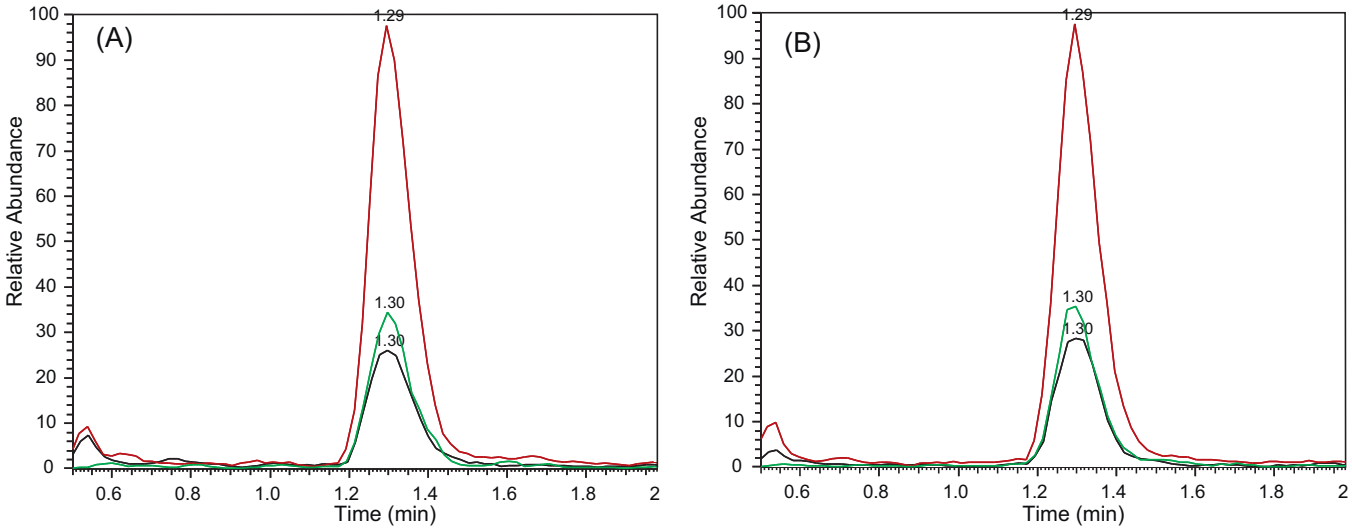


Fig. 2. Comparison of product ion ratios of NAED detected in a stored stallion plasma sample with those of neat NAED standard. (A) NAED (1000 pg/mL) spiked to a blank plasma sample and (B) the stallion plasma sample stored at ambient temperature for 7 days. The three product ions monitored were *m/z* 109 (top trace), 197 (middle trace), and 83 (bottom trace).

of NAED in a fresh plasma sample analyzed within 3 h post collection from a Standardbred stallion and its presence in the same sample stored at ambient temperature for 7 days. The presence of NAED in the stored plasma sample was confirmed by comparing intensity ratios of the three major product ions with those from NAED reference standard spiked to a control plasma sample (Fig. 2). The results for fresh and stored plasma samples from 16 stallions are summarized in Table 2. NAED was not detected in plasma samples 4 h or shorter post collection from all the stallions except one, and nandrolone was not detected in any of these freshly

Table 2
Quantification results of NAED and nandrolone in fresh (≤4 h) and stored (at 4 °C) plasma samples from stallions.

Horse #	Breed	Age of horse (years)	Time post collection	Date of collection	NAED (pg/mL)	Nandrolone (pg/mL)	Androstenedione (pg/mL)	Testosterone (pg/mL)
1	Standardbred	12	4 h	4/13/2010	ND[a]	ND	68	141
			8 days		809	88	76	187
2	Standardbred	3	4 h	4/13/2010	ND	ND	176	1119
			8 days		105	54	194	1381
3	Quarter horse	8	4 h	4/13/2010	Detected[b]	ND	281	836
			8 days		853	134	251	1011
4	Pony	20	4 h	4/13/2010	ND	ND	117	353
			8 days		726	122	182	507
5	Pony	10	4 h	4/13/2010	ND	ND	318	895
			8 days		783	156	448	1068
6	Pony	12	4 h	4/13/2010	ND	ND	235	906
			8 days		953	192	433	1085
7	Pony	11	4 h	4/13/2010	ND	ND	365	1075
			8 days		696	145	395	1329
8	Pony	7	4 h	4/13/2010	ND	ND	245	983
			8 days		624	139	394	1189
9	Pony	11	4 h	4/13/2010	ND	ND	543	1524
			8 days		461	117	475	1661
10	Connemara	10	4 h	4/13/2010	ND	ND	148	882
			8 days		360	117	156	1001
1	Standardbred	12	1.5 h	5/6/2010	ND	ND	124	246
			13 days		1138	129	101	212
2	Standardbred	3	1.5 h	5/6/2010	ND	ND	81	532
			13 days		157	Detected	60	455
11	Standardbred	3	3 h	6/9/2010	ND	ND	71	254
			6 days		487	Detected	75	238
12	Standardbred	3	2 h	6/9/2010	ND	ND	60	279
			6 days		376	Detected	67	258
13	Standardbred	4	3 h	6/9/2010	ND	ND	Detected	161
			6 days		258	ND	Detected	161
14	Standardbred	4	3 h	6/9/2010	ND	ND	437	1218
			6 days		219	Detected	420	1096
15	Standardbred	4	3 h	6/9/2010	ND	ND	66	176
			6 days		531	Detected	67	176
16	Standardbred	4	2.5 h	6/9/2010	ND	ND	323	671
			6 days		595	72	321	608

[a] ND: not detected.
[b] Detected: the AAS was detected, but its concentration was below the limit of quantification (50 pg/mL).

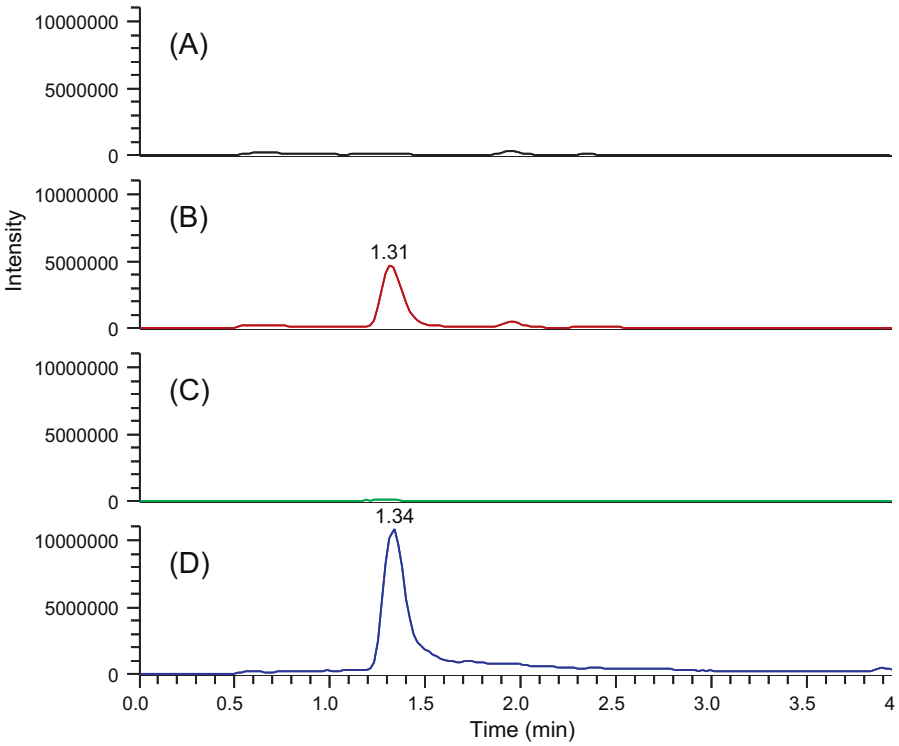


Fig. 3. LC–MS/MS chromatograms of blank and spiked urine samples, fresh and stored stallion urine samples. (A) A mare urine sample, (B) NAED (10,000 pg/mL) spiked to mare urine, (C) a fresh urine sample analyzed 2 h post collection from a Standardbred stallion, and (D) the same stallion urine stored at ambient temperature for 22 days. The ion transition monitored was m/z 273 → 109.

analyzed samples. However, NAED was identified in all the plasma samples stored at 4 °C for 6–13 days, and nandrolone in most of the samples (Table 2). Additionally, two endogenous androgenic steroids, androstenedione and testosterone, were also monitored and detected in all the plasma samples at different time points post collection; concentrations of androstenedione and testosterone quantified in each plasma sample did not significantly change with sample-storage time (Table 2). These two endogenous steroids can be regarded as internal references, and the consistency in their concentrations quantified further validates the quantification results for NAED and nandrolone in all the plasma samples (Table 2). In short, NAED and nandrolone were not detected in fresh plasma samples from stallions but were identified only after plasma samples were stored at 4 °C for 6–13 days. Thus, it is concluded that NAED and nandrolone are not detected in blood of stallions, based on the limit of detection (LOD) of the LC–MS method used, and that the two AASs detected in stored plasma samples are spontaneously generated.

Detection of NAED in a urine sample collected from a Standardbred stallion and stored at ambient temperature for 22 days is depicted in Fig. 3; the presence of the steroid in the sample was confirmed by product ion spectrum comparison (Fig. 4). The results for NAED and nandrolone in fresh (<5 h) and stored urine samples from eight stallions (Table 3) indicated that the two AASs were either not detected (Horses # 25 and 27) or at very low concentrations (<407 pg/mL) in fresh urine (<5 h). However, their concentrations significantly increased with storage time; the respective increase was more noticeable at higher storage temperature (37 °C and 21 °C versus 4 °C). Even in the case of NAED being detected and quantified in a fresh urine sample (Horse # 20), its concentration was only 0.4% (407 pg/mL compared with 1.01×10^5 pg/mL) of that in the same sample stored at ambient temperature (21 °C) for 15 days. Nandrolone was not detected in fresh urine samples from six out of eight stallions/colts. Even though nandrolone was detected in the fresh urine samples from a stallion and a colt, its initial concentration was below the LOQ (250 pg/mL), negligibly low compared with that determined for the same urine samples stored at ambient temperature (21 °C) for 22 days. From these results, it is concluded that NAED and nandrolone may be originally present in urine of Stallions or Colts but at extremely low concentrations, and that sample storage results in spontaneous generation of the two AASs, which accounts for most of them detected in stored urine samples of stallions.

It is interesting that androstenedione concentration (Table 3) in some urine samples remarkably increased with storage time (Horses # 24 and 25). This unexpected increase might result from possible conversion of testosterone glucuronide present in urine [27] to androstenedione [28]. Testosterone concentration in all the urine samples was close to LOQ and did not noticeably change with storage time (data not shown).

3.2. Time course of NAED and nandrolone concentrations in plasma and urine samples post collection

Plasma samples from 8 stallions were stored under different temperature conditions (4 °C, 21 °C, and 37 °C) for different periods of time for the purpose of examining changes in concentrations of NAED and nandrolone with time. As shown in Table 4, concentrations of the two AASs remarkably increased with storage time, and the respective increase in the AAS concentration with time was more pronounced at a higher storage temperature (37 °C versus 4 °C, and 21 °C (ambient temperature) versus 4 °C). Similar increases in NAED and nandrolone concentrations with storage time also were observed for urine samples (Table 3). These results confirm that NAED and nandrolone detected in plasma and urine of stallions result from sample storage. Thus, equine plasma and urine samples should be stored at a low temperature such as 4 °C or lower, soon after collection, for the purpose of minimizing

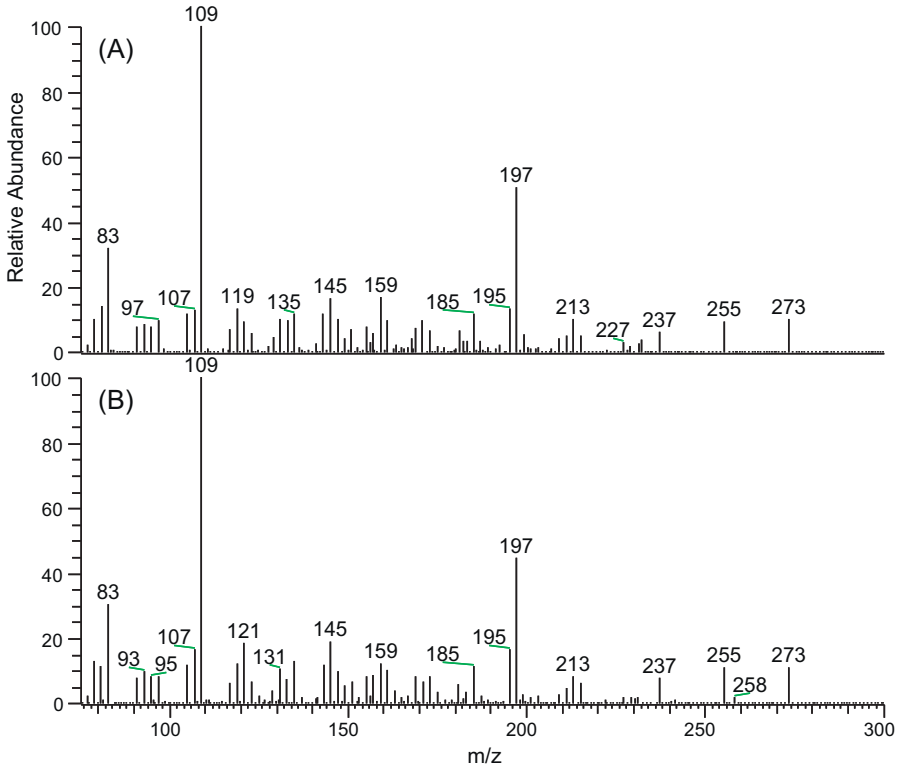


Fig. 4. Product ion spectra of NAED detected in a stored stallion urine sample and NAED standard. (A) NAED (10,000 pg/mL) spiked to mare urine and (B) the stallion urine stored at ambient temperature for 22 days.

spontaneous generation of NAED and nandrolone before sample analysis.

3.3. Differences between whole blood and plasma in concentrations of NAED and nandrolone

Plasma and whole blood samples collected from stallions were compared regarding changes in concentrations of NAED and nandrolone with storage time. As shown in Table 5, the concentration of NAED in plasma samples of Standardbred stallions (Horse # 11-16) stored at ambient temperature (21 °C) for 2–7 days was slightly higher than that in whole blood samples. The reason for the slight difference in NAED concentration between plasma and whole blood samples is unknown. The slightly higher concentration of NAED in plasma samples from another pair of Standardbred stallions (Horse # 1 and 2) compared with that in the whole blood samples may be due to systematic errors, because the concentration of testosterone in plasma samples of the same two horses was also slightly higher than that in the whole blood samples.

4. Discussion

4.1. Origin of NAED and nandrolone in stored plasma and urine samples of stallions

It has been reported that androstenedione is metabolized by porcine granulosa cells to androstenedione-19-oic acid (3,17-dioxo-androst-4-en-19-oic acid, CAS # 4757-95-3), nandrolone, and NAED among other metabolites, and that NAED is artifactually produced from androstenedione-19-oic acid as a result of degradation in storage and during sample preparation [29]. Similarly, Houghton et al. reported that androstenedione-19-oic acid was detected and identified in urine of stallions and that NAED and nandrolone detected in the urine sample resulted primarily from respective decarboxylation of androstenedione-19-oic acid and testosterone-19-oic acid caused by acid solvolysis [23,30]. McKinney et al. synthesized testosterone-19-oic acid and observed that it converted easily in acidic aqueous solution to nandrolone (complete conversion required 48 h at pH 1 and twice as long at pH 3), but slowly under neutral and basic pH conditions (over a period of weeks rather than days) [31]. In addition, the presence of the 19-oic acid was confirmed in urine of stallions. Based on the above reports, the two 19-oic acids of androstenedione and testosterone are probably generated *in vivo*. Thus, we propose that NAED and nandrolone identified in stored plasma and urine samples are a result of *in vitro*, spontaneous decarboxylation of endogenous androstenedione-19-oic acid and testosterone-19-oic acid during storage of the samples (Scheme 1). In support of the above hypothesis, it is well known that some carboxylic acids, especially β-keto acids, can readily undergo decarboxylation reactions even at 25 °C [32]. As a result of conjugation of the 3-carbonyl group with the 4-double bond (Scheme 2), androstenedione-19-oic acid and testosterone-19-oic acid may exhibit some behaviors of β-keto acids and thus, undergo the facile decarboxylation reaction of the latter even under mild conditions. A similar decarboxylation mechanism was proposed by Dumasia et al. [4] and Houghton et al. [23]. If the above hypothesis is true, the next relevant question would be why the 19-oic acids do not undergo the decarboxylation reaction *in vivo* but do so *in vitro*. A possible explanation might be that they are non-covalently bound *in vivo* to some active biological macromolecule(s) such as a protein(s) so that the decarboxylation reaction is blocked. We propose that NAED and nandrolone are not the intended *in vivo* products of the two relevant 19-oic acids. In other words, the two 19-oic acids are destined *in vivo* for the production of other C18 steroids such as estrogens [4].

It was reported that most of, and possibly all, the nandrolone detected in urine of stallion was not from nandrolone sulfate

Table 3
NAED and nandrolone detected and quantified in urine samples from stallions at different time points post collection of samples.[a]

Horse #	Time	NAED (pg/mL)			Nandrolone (pg/mL)			Androstenedione (pg/mL)	
		4 °C	21 °C	37 °C	4 °C	21 °C	37 °C	4 °C	21 °C
20	1 h	407			Detected[b]			Detected	
	3 h	448	458	608	Detected	Detected	Detected	Detected	ND[c]
	5 h	435	492	884	Detected	Detected	Detected	Detected	Detected
	7 h	410	575	1245	Detected	Detected	Detected	Detected	Detected
	24 h	627	1153	5957	Detected	256	576	94	143
	48 h	683	1971	10,262	Detected	280	797	69	105
	15 days	6509	10,1425		653	4176			
21	2.5 h	Detected			ND			Detected	
	1 day	Detected	Detected		ND	ND		Detected	Detected
	2 days	Detected	55		ND	ND		Detected	Detected
	5 days	Detected	208		ND	ND		ND	53
	6 days	Detected	262		ND	ND		Detected	62
	9 days	Detected	438		ND	Detected		Detected	80
	16 days	Detected	690		ND	407		Detected	Detected
	22 days	188	1625		ND	229		51	ND
22	2.5 h	Detected			ND			Detected	
	1 day	Detected	Detected		ND	ND		Detected	50
	2 days	Detected	Detected		ND	ND		Detected	Detected
	5 days	Detected	327		ND	ND		68	55
	6 days	Detected	357		ND	ND		Detected	Detected
	9 days	101	139		ND	ND		Detected	93
	16 days	Detected	540		ND	ND		Detected	Detected
	22 days	142	1309		ND	ND		77	304
23	5 h	Detected			Detected			Detected	
	1 day	Detected	373		Detected	Detected		107	107
	2 days	119	519		Detected	Detected		92	71
	5 days	313	1482		Detected	299		105	117
	6 days	471	1839		Detected	Detected		122	106
	9 days	981	3004		Detected	652		109	167
	16 days	945	6525		Detected	3054		134	204
	22 days	1440	9023		Detected	3002		148	426
24	2.5 h	Detected			ND			Detected	
	1 day	Detected	ND		ND	ND		78	Detected
	2 days	132	479		ND	Detected		61	54
	5 days	710	4577		Detected	983		85	250
	6 days	436	6965		Detected	1307		70	394
	9 days	1195	8227		Detected	1691		81	731
	16 days	1247	18,769		365	4003		111	1700
	22 days	3393	25,981		407	1846		167	2614
25	2 h	ND			ND			Detected	
	1 day	Detected	211		ND	ND		Detected	Detected
	2 days	105	476		ND	ND		51	77
	5 days	235	2026		ND	341		65	767
	6 days	336	4048		ND	327		58	634
	9 days	386	5550		ND	275		64	842
	16 days	652	16,005		ND	Detected		107	1296
	22 days	1082	20,884		ND	Detected		230	1611
26	2 h	Detected			ND			Detected	
	1 day	Detected	544		ND	ND		61	53
	2 days	263	915		ND	Detected		Detected	71
	5 days	542	6179		ND	585		62	97
	6 days	531	8696		ND	787		51	87
	9 days	1015	6462		Detected	5389		79	121
	16 days	1911	9157		289	12,811		94	150
	22 days	6312	23,759		677	13,246		141	326
27	1.5 h	ND			ND			Detected	
	1 day	Detected	Detected		ND	ND		73	88
	2 days	107	595		ND	ND		56	Detected
	5 days	464	3102		ND	397		87	78
	6 days	833	4493		ND	419		72	81
	9 days	1077	9869		ND	754		84	140
	16 days	1126	25,667		Detected	1158		85	263
	22 days	1578	35,935		ND	541		107	253

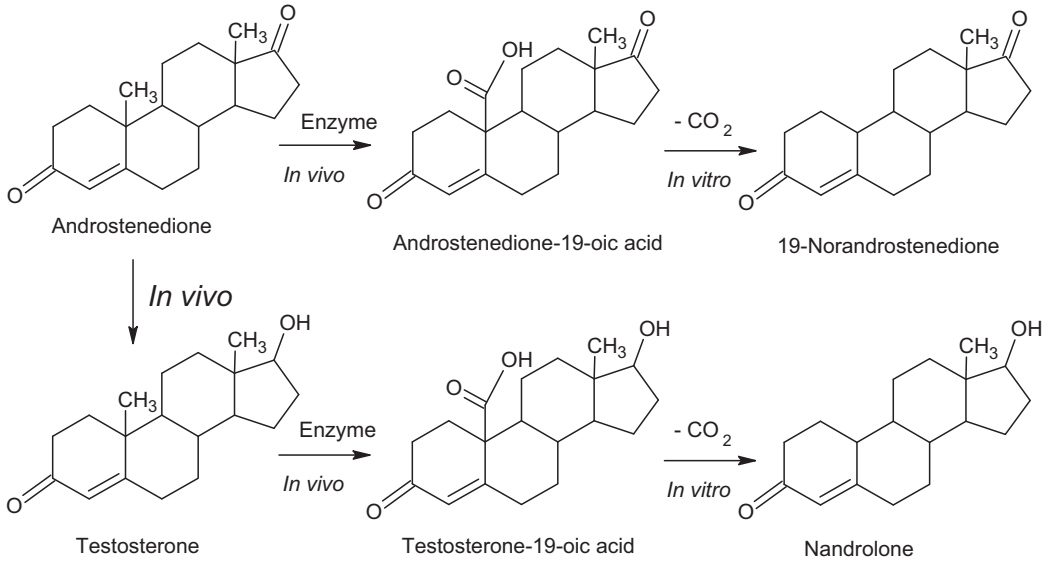
[a] Horse # 20 is a Thoroughbred stallion retired from racing. Horse # 21 is a Standardbred stallion actively racing, and Horses # 22–27 are 3 years old (colts), actively racing Standardbred.
[b] Detected: below the limit of quantification (100 pg/mL for NAED, 250 pg/mL for nandrolone, and 50 pg/mL for androstenedione).
[c] ND: not detected.

[24]. Under the LC–MS/MS conditions employed in the present study, nandrolone and 17α-19-Nortestosterone (epinandrolone) were well resolved chromatographically (better than baseline separated) [20]; the latter was not detected in any of all the fresh stallion plasma and urine samples (data not shown). Thus, such a possibility can be ruled out that nandrolone detected in stored plasma and urine samples is derived from epinandrolone or nandrolone sulfate.

Table 4
Time course of NAED and nandrolone concentrations in plasma samples stored under different temperature conditions.[a]

Horse #	Time post collection	NAED (pg/mL)		Nandrolone (pg/mL)		Androstenedione (pg/mL)		Testosterone (pg/mL)	
		4 °C	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
1	5 h	ND[b]	520	ND	Detected[c]	79	96	233	262
	7 h	Detected	843	ND	65	80	98	238	274
	24 h	90	1665	ND	154	97	101	221	257
	3 days	448		42		96		244	
	7 days	841		88		99		236	
	13 days	1138		129		101		212	
2	5 h	ND	100	ND	Detected	57	52	522	567
	7 h	ND	112	ND	Detected	54	48	516	551
	24 h	ND	252	ND	Detected	67	50	511	528
	3 days	68		ND		66		502	
	7 days	115		Detected		68		507	
	13 days	157		Detected		60		455	
		4 °C	21 °C	4 °C	21 °C	4 °C	21 °C	4 °C	21 °C
11	1 day	Detected	340	ND	Detected	56	53	224	233
	2 days	118	826	ND	89	69	82	231	222
	5 days	439	1513	ND	165	75	84	239	245
	6 days	487	1416	42	207	75	78	238	234
12	1 day	Detected	267	ND	Detected	47	60	261	246
	2 days	87	636	ND	Detected	55	74	275	253
	5 days	307	1135	ND	112	62	74	285	264
	6 days	376	1101	32	128	67	81	258	244
13	1 day	ND	187	ND	Detected	Detected	Detected	167	163
	2 days	52	546	ND	Detected	64	80	161	163
	5 days	205	888	ND	82	48	Detected	158	154
	6 days	258	878	ND	120	Detected	Detected	161	168
14	1 day	ND	142	ND	ND	373	387	1141	1217
	2 days	Detected	381	ND	49	441	465	1099	1163
	5 days	187	690	ND	144	413	432	1164	1240
	6 days	219	711	28	147	420	382	1096	1150
15	1 day	Detected	385	ND	Detected	50	46	168	180
	2 days	122	1017	ND	70	65	68	162	172
	5 days	496	1875	ND	171	72	69	170	169
	6 days	531	1847	43	229	67	69	176	192
16	1 day	66	496	ND	49	280	295	654	649
	2 days	196	1194	ND	134	407	387	607	609
	5 days	533	1967	43	256	355	321	714	624
	6 days	595	1791	72	257	321	306	608	602

[a] Information on horse breed and age is shown in Table 2. Plasma samples from Horses # 1 and 2 were collected on May 6, 2010, and those from Horses # 11–16 were obtained on June 6, 2010.
[b] ND: not detected.
[c] Detected: the AAS was detected, but its concentration was below the limit of quantification (50 pg/mL).

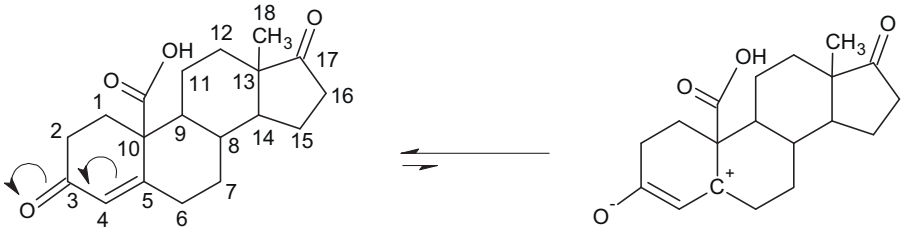


Scheme 1. Proposed *in vitro* spontaneous generation of 19-norandrostenedione and nandrolone from the relevant 19-oic acids.

Table 5
Comparison of concentrations of NAED and nandrolone in plasma and whole blood samples from stallions and following storage at different temperature for varying time periods.[a]

Horse #	Time post collection	NAED (pg/mL)		Nandrolone (pg/mL)		Androstene-dione (pg/mL)		Testosterone (pg/mL)		Ratio of concentrations (pla/blo)			
		Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	NAED	Nandr.	Androst.	Testos.
		37 °C											
1	2 h	223	161	ND[b]	ND	123	101	266	219	1.38		1.23	1.21
	4 h	520	410	Detected[c]	50	96	79	262	200	1.27		1.22	1.31
	6 h	843	628	65	73	98	78	274	214	1.34	0.89	1.26	1.28
	24 h	1665	1416	154	172	101	106	257	201	1.18	0.89	0.96	1.28
2	2 h	Detected	Detected	ND	ND	87	80	583	505	1.09		1.08	1.16
	4 h	100	76	Detected	Detected	52	65	567	530	1.31		0.80	1.07
	6 h	112	97	Detected	Detected	48	59	551	519	1.15		0.81	1.06
	24 h	252	267	Detected	77	50	100	528	500	0.94	0.61	0.50	1.06
		21 °C											
11	1 day	340	232	Detected	Detected	53	Detected	233	226	1.46			1.03
	2 days	826	614	89	114	82	63	222	214	1.35	0.78	1.31	1.04
	5 days	1513	1140	165	196	84	73	245	210	1.33	0.84	1.14	1.16
	6 days	1416	1158	207	213	78	72	234	217	1.22	0.97	1.09	1.08
12	1 day	267	183	Detected	Detected	60	Detected	246	271	1.46			0.91
	2 days	636	448	Detected	Detected	74	60	253	280	1.42		1.22	0.90
	5 days	1135	868	112	114	74	71	264	272	1.31	0.97	1.05	0.97
	6 days	1101	857	128	160	81	63	244	270	1.28	0.80	1.29	0.90
13	1 day	187	119	Detected	ND	Detected	Detected	163	162	1.57			1.00
	2 days	546	339	Detected	Detected	80	69	163	157	1.61		1.15	1.04
	5 days	888	715	82	90	Detected	Detected	154	172	1.24	0.91	1.12	0.89
	6 days	878	709	120	96	Detected	Detected	168	156	1.24	1.25	0.95	1.07
14	1 day	142	84	ND	Detected	387	341	1217	1183	1.69		1.13	1.03
	2 days	381	241	Detected	Detected	465	389	1163	1103	1.58		1.19	1.05
	5 days	690	526	144	125	432	392	1240	1095	1.31	1.15	1.10	1.13
	6 days	711	547	147	145	382	372	1150	1124	1.30	1.01	1.03	1.02
15	1 day	385	192	Detected	Detected	Detected	Detected	180	185	2.00			0.97
	2 days	1017	555	70	74	68	62	172	165	1.83	0.94	1.09	1.04
	5 days	1875	1345	171	203	69	76	169	196	1.39	0.84	0.91	0.87
	6 days	1847	1452	229	205	69	78	192	171	1.27	1.11	0.89	1.12
16	1 day	496	248	49	54	295	263	649	671	2.00	0.91	1.12	0.97
	2 days	1194	750	134	136	387	342	609	600	1.59	0.99	1.13	1.01
	5 days	1967	1563	256	269	321	345	624	636	1.26	0.95	0.93	0.98
	6 days	1791	1551	257	278	306	308	602	595	1.16	0.93	0.99	1.01

[a] Information about horse breed and age is shown in Table 2. Blood samples from Horses # 1 and 2 were collected on May 6, 2010, and those from Horses # 11–16 were on June 6, 2010.
[b] ND: not detected.
[c] Detected: the AAS was detected, but its concentration was below the limit of quantification (50 pg/mL).



Scheme 2. Possible resonance structures of androstenedione-19-oic acid indicating that it may behave like a β-keto carboxylic acid in decarboxylation.

4.2. Previous detections of "endogenous" nandrolone in various animal species

In the past, AAS analysis was usually performed by GC–MS and involved steps of acid solvolysis and/or enzyme hydrolysis at elevated temperatures (or acid hydrolysis). These steps would lead to *ex vivo* generation of NAED and nandrolone by both acid-catalyzed and spontaneous decarboxylation of androstenedione-19-oic acid and testosterone-19-oic acid, as discussed above. Thus, NAED and nandrolone detected in various animal species, as reported in previous publications, may not have been endogenous in origin, but result from the *in vitro* conversion as mentioned above.

4.3. Implications of the results from the present study

Nandrolone has been regarded as an endogenous steroid in horse racing [2,33,34], human sports [22,35], and meat animal surveillance programs [10] for decades, but despite this status, its possible physiological roles have not been defined yet. Now, for the first time, NAED and nandrolone identified in stored equine plasma samples have been shown to be not from endogenous origins but to result from spontaneous conversion during sample storage. This finding may have regulatory implications for doping control of these two AASs in horse racing. In particular, threshold values for the two AASs in plasma of stallions would have to be carefully revisited, with considerations of sample shipment and storage conditions such as temperature and length of storage.

Nandrolone sulfate and boldenone sulfate in the urine have also been reported to be endogenous in origin [24,36]. Given the above results on the *in vitro* generation of NAED and nandrolone, we would now raise the same question: are nandrolone sulfate and boldenone sulfate really endogenous or not? Investigations are currently in progress to address this question.

It was reported that NAED and nandrolone identified in the urine of stallions were primarily artifacts that resulted from the analytical procedures used [23,24]. In the present study, the sample preparation procedure including liquid–liquid extraction of plasma and urine samples without any pH adjustment was mild, and LC separations were conducted at pH 6.6. Consequently, the analytical procedures used in this study did not result in artifacts of NAED and nandrolone, as indicated by the results for fresh plasma samples in which these two AASs were not detected.

The results of the present study are different from those by Houghton et al. [23] in that we have demonstrated that NAED and nandrolone are not detected in fresh plasma samples of stallions and that all of the two AASs detected in the stored plasma samples and most of them detected in the stored urine samples are not from endogenous origins but result from spontaneous chemical conversions during sample storage even under low temperature conditions, such as at 4 °C.

5. Conclusion

NAED and nandrolone were not detected in fresh (≤4 h post collection) plasma samples collected from racing and non-racing stallions but were detected and identified in the same samples after storage for longer time periods. Although NAED was detected in some fresh (≤5 h) urine samples of stallions, its concentrations were significantly lower than those in the same urine samples stored at ambient temperature for 22 days. Similarly, nandrolone was not detected in most of the fresh urine samples but was detected in all of these samples after they had been stored at ambient temperature for periods of time. Consistent with these findings, the concentrations of these two steroids in the post-collection plasma and urine samples increased with storage time, and the increase was greater at higher storage temperatures (37 °C versus 4 °C, and ambient temperature versus 4 °C). Based on these results, it is concluded that all NAED and nandrolone identified in stored plasma samples of stallions and most of them in stored urine samples are not endogenously produced but result from the spontaneous conversion during sample storage. The most likely interpretation is that these two steroids are generated by spontaneous decarboxylation of androstenedione-19-oic acid and testosterone-19-oic acid, respectively, during sample storage. To our knowledge, it is the first time that the two steroids have been shown to result from sample storage rather than endogenous sources. These findings have substantial implications for regulatory control of the two steroids in horse racing.

The LC–MS/MS method used in this study for analyses of plasma and urine samples is sufficiently mild to prevent possible production of artifacts of NAED and nandrolone.

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