1. Introduction

Recent controversy surrounding the use of anabolic steroids in human and animal athletes has led to widespread investigations and a call for implementing more stringent rules, penalties, and widespread testing procedures in an effort to curtail or eliminate their use.1–4

In the past, equine athletes have routinely been given androgenic and anabolic steroids to promote muscle development, appetite, aggressiveness, or other desirable effects.5 Agents such as testosterone, nandrolone, and boldenone are endogenous to the equine. Additionally, anabolic/androgenic steroids based on testosterone have been synthesized to increase the anabolic pharmacology, such as protein synthesis and accelerated muscle development. These steroids also attempt to decrease the androgenic sexual and aggressive side effects.6

Europe, Asia, and many international regulatory authorities have a >30-yr history of recognizing this class of steroids as agents of significant concern and possible abuse in racing. The International Federation of Horseracing Authorities (IFHA) has long recognized and adopted international thresholds for endogenous versus exogenous steroid presence based on the scientific work of several internationally recognized labs.7–12 These same thresholds have been peer reviewed and tested legally many times over the same time period.

Because of the possible integrity and welfare issues regarding the misuse or overuse of anabolic/androgenic agents, the Iowa regulatory authority established deterrence policies in 1991 to monitor for the presence of exogenous levels of these agents in racing horses. Until recently, Iowa has been the only racing jurisdiction in the United States to implement an anabolic/androgenic steroid testing and penalty policy. The Iowa regulatory agency has used the internationally established urinary thresholds as the base for the determination of negative or positive sample status.

In 2007, the Racing Medication and Testing Consortium in Lexington, Kentucky proposed national model rules for testing and penalties of the four common anabolic steroids. They used urinary threshold concentrations very similar to the internationally accepted IFHA Article 6 levels. However, over the past 18 mo, these proposed policies have faced opposition from some trainers, owners, horse associations, veterinarians, and chemists,
which has resulted in many jurisdictions’ reluctance to start testing.

The purpose of this review is to show that testing for anabolic steroids can be accomplished in the horse racing world and that, at least in one jurisdiction, has proven to be effective in curtailing the use of these products.

2. Materials and Methods

Selection of Test Subjects
All first- and second-place finishers plus other random finishers selected by the stewards or state veterinarian were sent to the detention barn for urine and blood collection after each racing performance at Prairie Meadows Racetrack located in Altoona, Iowa. The samples were identified by bar code and sex, and they were sent by courier in sealed containers to the Racing Chemistry Lab located at Iowa State University after each performance. The subjects tested were racing Thoroughbreds, Quarter Horses, and Standardbreds.

Testing Methods
Limits of detection were based on the IFHA Article 6 recommendations.

Boldenone: 0.015 μg free and conjugated boldenone/ml in urine from male horses (other than geldings).

Estranediol: the mass of free and conjugated 5α-pregnane-3,17β-diol in male horses to the mass of free and conjugated 5α-androstan-3β,17α-diol in urine from male horses (other than geldings) at a ratio of 1.

Testosterone: 0.02 μg free and conjugated testosterone/ml in urine from geldings or 0.055 μg free and conjugated testosterone/ml in urine from fillies and mares (unless in foal).

Drug testing programs routinely use screening tests to determine which samples submitted by the regulatory authority are suspicious; if this happens, all other samples are declared negative, and further work is stopped. The suspicious samples undergo additional investigation using more rigorous analysis to meet legal requirements for forensic evidence.

Anabolic/androgenic steroid screening has been successfully conducted using immunoassay enzyme-linked immunosorbent assay (ELISA) methods. This testing is based on kits that use antibody-antigen specific reactions to detect and estimate concentrations of analyte in the biological sample. Two commercially available test kits are routinely used in the screen-testing detection for nandrolone, boldenone, and testosterone in equine urine and blood serum or plasma: the Boldenone ELISA kit and the Nandrolone ELISA kit. Limits of detection at <1 μg/ml for these anabolic steroids. ELISA screening allows a relatively inexpensive and rapid screening means of differentiating samples that may be positive from samples that are negative and need no further investigation. One disadvantage of such ELISA testing is that the antibody-antigen reaction is not perfectly specific, meaning that the Boldenone ELISA will react with and therefore “hit” on samples containing nandrolone and testosterone also. Similarly, the Nandrolone ELISA kit will detect samples containing boldenone and testosterone. Therefore, the estimated concentration of these agents in the sample will add up and cannot be individually differentiated. Using pure nandrolone to create authentic calibration response samples, Figs. 1 and 2 illustrate the sensitive nature of immunoassay ELISA screening capabilities.

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Note that the response curve is a reverse correlation polynomial fit. This means that the lower the analyte concentration, shown here on the x axis, the higher the kit reading will be, shown here on the y axis. Kit testing limit of sensitivity is ~150–200 pg/ml, but this is dependent on sample non-specific background that can contribute to the reading.

Forensic confirmation is accomplished using analyte extracted from the urine sample followed by gas chromatography/mass spectrometry of the extract by one of two distinct processes, either the Hydroxylamine-HCl (in pyridine)-(N-methyl-N-(t-butyldiemethylsilyl trifluoroacetamide) (OX-TBDMS) process or the Methoxyamine-HCl solution (in pyridine)-(N-methyl-N-(trimethylsilyl trifluoroacetamid) (MOX-TMS) process.

Extraction involves enzymatic hydrolysis and/or solvolysis of the urine to convert all anabolic agents in the sample from conjugated metabolites to the free agents. These are then extracted from the mixture using solid-phase extraction (SPE) with C18 Sep Pak Waters SPE extraction cartridges. This yields a concentrated and cleaner extract ready for mass-spectral instrumental analysis.

Figure 3 summarizes the extraction process.

Derivatization
The OX-TBDMS gas chromatography/mass spectrometric process involves forming an oxime-silylated
derivative of the anabolic steroids in the sample with hydroxyamine hydrochloride and N-methyl-N-t-butylidemethylsilyl trifluoroacetamide. These OX-TBDMS derivatives of the anabolic steroids in the sample yield very sensitive detection limits for the various individual anabolic steroids down to 1 \( \mu \text{g/ml} \) and less and also allows monitoring of other naturally occurring hormones and related compounds of interest. Figures 4 and 5 illustrate the sensitivity of gas chromatography/mass spectrometry for the forensic detection and identification of Nandrolone in urine as the OX-TBDMS derivative.

Nandrolone in Equine Urine by Gas Chromatography/Mass Spectrometry (GCMS)

The MOX-TMS gas chromatography/mass spectrometric process involves forming an oxime-silylated derivative of the anabolic steroids in the sample with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl trifluoroacetamide. These MOX-TMS derivatives of the anabolic steroids in the sample yield slightly less sensitive detection limits than does the OX-TBDMS process, but the mass spectral data are much more specific and allow much more confident identification of the various anabolic steroids for legal purposes.

3. Results

In 1991, the Iowa commission instituted an anabolic steroid deterrence policy, and the stated intent was to give 3-mo notice of the upcoming anabolic steroid policy. No withdrawal times were recommended before implementing this policy (Tables 1 and 2; Fig. 6).

4. Discussion

The data presented shows a long-standing and effective monitoring and deterrence policy based on well-established international standards. This is especially apparent compared with recently collected data from two other U.S. jurisdictions sampled using these same screening and confirmatory methods where no deterrence policy has been adopted.

Table 1 reveals a static number of positive tests over the 16-yr period. Certain year spikes could be attributed to an influx of new veterinarians and trainers who perhaps tested the recommended withdrawal times. The testing procedures were explained to all veterinarians and trainers in pre-season meetings. The penalties issued were as follows:

- First Offense = $300 fine to trainer
- Second Offense = $500 fine to trainer
- Third Offense = $1000 fine to trainer/loss of purse/suspension; only one trainer ever met the conditions of the third offense.

It was realized by the Iowa Racing and Gaming Commission and Board of Stewards that by adopting the above listed penalties, horses with uncertain medication status from other jurisdictions could still race at Prairie Meadows without fear of purse loss or suspension. Although perhaps giving an advantage to those individuals that were only shipping in for select races, there were relatively few positive results from those horses. Many out-of-state trainers con-
Anabolic Steroid Analysis in Equine Urine

Dispense blank, sample(s), or spike(s) urine
Adjust each to pH 6.8 with 1M phosphate buffer

Enzymatic Hydrolysis
-Glucuronidase (E. coli) in Phosphate buffer pH 6.8
Hydrolize overnight at 37°C or for 2 hours at 50°C overnight

Add D-3 androstenediol (internal standard)

Steroid Extraction using Solid Phase Extraction
C18 Sep Pak cartridges
Add sample and:
-wash with hexane
-dry thoroughly

Elute the Glucuronide fraction with diethyl ether.

Elute the SO4 fraction
With EtAc : MeOH : H₂SO₄
Solvolyze at 37°C or for 2 hours at 50°C overnight

Add 2M NaOH and mix
Discard the aqueous phase
Reserve the Organic fraction
Repeat

Add sat’d NaCl and mix
Discard the aqueous phase
Reserve the organic fraction

Dry over anhydrous Na₂SO₄
Conc. to dryness with OFN and heat
Ready for derivatization to either OX-TBDSM or the MOX-TMS

Derivatization

The OX-TBDSM gas chromatography/mass spectrometric process involves forming an oxime-silylated derivative of the anabolic steroids in the sample with hydroxyamine

Fig. 3. Anabolic standard analysis method outline.

<table>
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<tr>
<th></th>
<th>D3</th>
<th>epinandrol</th>
<th>nandrolone</th>
<th>nand/D3</th>
<th>conc nand</th>
<th>nand</th>
<th>conc nand</th>
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Fig. 4. Gas chromatography/mass spectrometry nandrolone in vitro spiked calibration in urine data.
tailed the overuse of anabolic steroids; additionally, it still allows some room for legitimate use by practicing veterinarians.

Over the past 16 yr, the Iowa Racing and Gaming Commission policy has proven an effective deterrent to anabolic steroid abuse by testing equine urine samples using international practices and standards of testing.

There has been a suggestion in the United States that anabolic steroid testing should or must be done in blood. Our experiences illustrate that urine-based testing for the anabolic steroids pursuant to international practice has resulted in effective deterrent compared with jurisdictions that have no testing or deterrence policy in place. Certainly, anabolic steroids can be tested in blood as well. Although both urine and blood offer analytical and policy advantages and disadvantages, these advantages and disadvantages should be considered when a jurisdiction is deciding when and how to adopt testing and deterrent policies for anabolic ste-

![Graph](image-url)

Fig. 5. Gas chromatography/mass spectrometry nandolone in vitro spiked calibration in urine curve.

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**Table 1. Test Results**

<table>
<thead>
<tr>
<th>Year</th>
<th>Urines Tested</th>
<th>Nandrolone</th>
<th>Testosterone</th>
<th>Boldenone</th>
<th>Stanozolol</th>
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<td>Total</td>
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<td>43</td>
<td>28</td>
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*One trainer with four testosterone positives.
†Feed additive attributed to five testosterone positives.

**Table 2.**

<table>
<thead>
<tr>
<th>Jurisdiction</th>
<th>Urines Tested</th>
<th>Nandrolone</th>
<th>Testosterone</th>
<th>Boldenone</th>
<th>Other</th>
<th>Total</th>
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<tr>
<td>3 mo</td>
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<td>23</td>
<td>44</td>
<td>116</td>
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<td>Agency B</td>
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<td>20</td>
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It should be noted, however, that no international thresholds, no national thresholds, and no agency-approved and peer-reviewed thresholds currently exist for blood-serum testing at the time of this review.

The authors wish to acknowledge and thank the staff of the Iowa Racing and Gaming Commission veterinary team and the analysts and staff in the Iowa State Racing Chemistry Laboratory for the many years of work that has yielded the basis for this presentation. Also a huge thanks to the HFL, LTD (formerly the Horseracing Forensic Laboratory, Newmarket, United Kingdom), Dr. Edward Houghton, Dr. Minoo Dumasia, and the HFL research group for the many years of collaboration in this area of testing.

References and Footnotes


*Neogen Corporation, 620 Lesher Place, Lansing MI 48912.
+Waters Corporation, 34 Maple St., Milford, MA 01757.