A Rapid Quantitative Method for Multiple Anabolic Steroids in Equine Serum by Turbulent Flow Chromatography Tandem MS-MS

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Overview:

Purpose: A high-throughput quantitative method for the detection of five anabolic steroids: Testosterone, Nandrolone, Boldenone, Stanozolol and Trenbolone in equine serum.

Methods: 2-Dimensional liquid chromatography utilizing turbulent flow chromatography coupled with tandem mass spectrometry using a new triple quadrupole mass spectrometer.

Results: Limits of Quantitation (LOQ) at 250 pg/mL, linear calibration curves (r^2>0.99), accuracy (% of nominal) and precision (%CV) were experimentally determined. TFC MS/MS allows for rapid analysis with minimal sample preparation.

Introduction:

Anabolic Androgenic Steroids (AAS) are structurally similar androgen (C19) or estrogen (C18) derivatives that are mostly neutral hydrophobic compounds. AAS are abused in both horse racing and in human athletics to promote muscle growth and speed recovery following intensive exercise. The four authorized AAS for administration to horses include: Stanozolol, Nandrolone, Boldenone, and Testosterone. Administration of any non-authorized AAS such as Trenbolone are prohibited in most racing jurisdictions.

Previously, AAS analysis has been accomplished using lengthy liquid-liquid or solid phase extraction of biological fluids followed by derivatization and detection by GC/MS or more recently, LC/MS/MS analysis. These methods are labor intensive and made rapid analysis of large batches of samples problematic. Online sample extraction utilizing a 2-dimensional Turbulent Flow Chromatography (TFC) system coupled with tandem MS-MS was employed to analyze equine serum quickly with minimal sample preparation without sacrificing sensitivity. TFC creates a turbulent environment inside a small diameter extraction column by combining high flow rates with large stationary phase particles, retaining small molecules while allowing large macromolecules to pass to waste. Small molecules are transferred to an analytical column where traditional gradient analysis separates compounds.

Samples were introduced to the mass spectrometer by electrospray ionization and analytes detected using select reaction monitoring (SRM). The TSQ Vantage triple quadrupole mass spectrometer was used to collect data.

Methods:

Chemicals and Reagents:

Boldenone, Nandrolone, Stanozolol, Testosterone, and Trenbolone were purchased from Steraloids and a 1mg/mL standard solution in methanol or acetonitrile was prepared.

Sample Prep:

Serum was collected by jugular venipuncuture and stored at -20°C. Samples were diluted 2:1 with water containing 10 ng/mL D3-Testosterone as an internal standard, vortexed, centrifuged at 3000 rpm for 3 minutes. A 7 point calibration curve from 250-10,000 pg/mL and 3 quality control (QC) levels (6 replicates per level) at 400, 800, and 4,000 pg/mL were spiked in negative control seum.

Mass Spectrometer settings:

Thermo TSQ Vantage Triple Quadrupole Mass Spectrometer

Software: Xcalibur version 2.07 – Quan browser was used for quantitation

SRM mode – 5 transitions per analyte, 3 for ISTD

HESI-II probe – positive mode	Capillary Temperature: 350°C
Spray Voltage: 4000V	Q2 gas pressure: 2.0
Vaporizer Temperature: 200°C	Scan Width: 1.0
Sheath Gas Pressure: 45	Time: 20 milliseconds
Aux Gas Pressure: 30	Q1: 0.7 da
Ion Sweep Pressure: 2	Q2: 0.7 da

Chromatography settings:

Thermo TLX-2 Turbulent Flow Chromatography System

Software: Aria version 1.6.1

Injection Volume: 75 µL

Run Time:12.5 min or 6.5 min/sample with multiplexing

Loop Size/Strength: 100 µL loop with 95:5 B:A mobile phase ratio

Loading Pump	**Eluting Pump**
Column: Cyclone 60µm (0.5x50)	Column: Ace C18 3µm (2.1x100)
Mobile Phase:	Mobile Phase:
A – Water with 0.2% formic acid	A – Water w/ 0.2% formic acid
B – Methanol with 0.2% formic acid	B – Methanol w/ 0.2% formic acid
C – ACN, IPA, Acetone (60/30/10)	

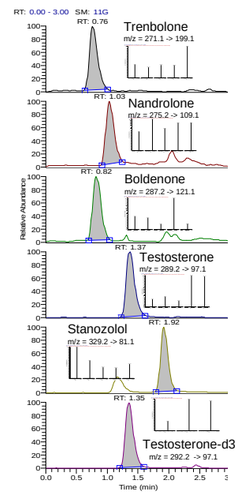


Figure1. 5000 pg/mL Calibrator

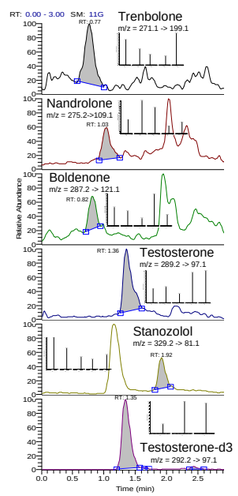


Figure 2. 500 pg/mL Calibrator

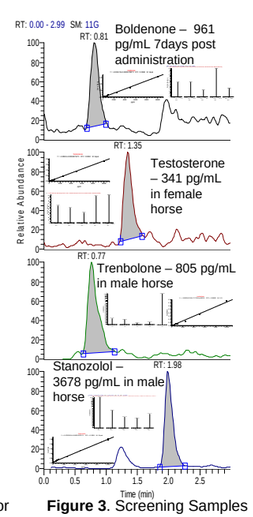


Figure 3. Screening Samples

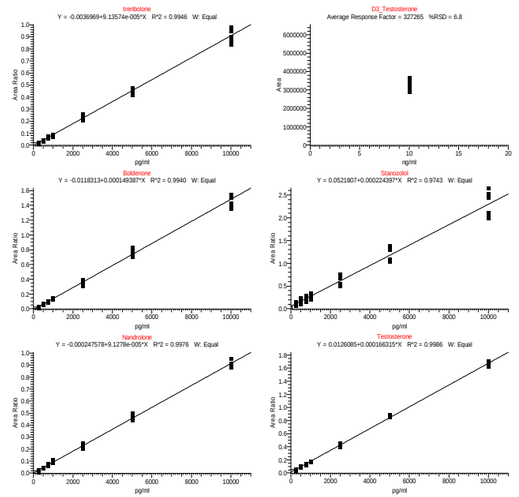


Figure 4. Calibration curves – 8 replicates/level over 2 days

Inter-Assay Accuracy (n=24 samples over 2 days)

Analyte	400 pg/mL	% of nominal	800 pg/mL	% of nominal	4000 pg/mL	% of nominal	Average % of nominal
Trenbolone	348.8	87.2	773.2	96.7	3486.3	87.2	90.3
Nandrolone	386.5	96.6	821.1	102.6	4095.4	102.4	100.5
Boldenone	347.0	86.8	755.8	94.5	4086.8	102.2	94.5
Testosterone	371.3	92.8	789.3	98.7	4080.6	102.0	97.8
Stanozolol	357.3	89.3	786.1	98.3	4087.8	102.2	96.6

Inter-Assay Precision (n=24 samples over 2 days)

Analyte	400 pg/ml (%CV)	800 pg/ml (%CV)	4000 pg/ml (%CV)	Average (%CV)
Trenbolone	21.3	12.0	12.9	15.4
Nandrolone	18.0	14.9	5.7	12.9
Boldenone	14.2	6.1	4.7	8.3
Testosterone	13.6	7.8	4.6	8.7
Stanozolol	13.2	7.8	5.7	8.9

Intra-Assay Accuracy (n=6 samples)

Analyte	400 pg/mL	% of nominal	800 pg/mL	% of nominal	4000 pg/mL	% of nominal	Average % of nominal
Trenbolone	354.9	88.7	843.4	105.4	3192.4	79.8	91.3
Nandrolone	328.3	82.1	721.9	90.2	4092.5	102.3	91.5
Boldenone	350.4	87.6	747.5	93.4	4083.5	102.1	94.4
Testosterone	359.8	89.9	731.5	91.4	4043.6	101.1	94.1
Stanozolol	328.9	82.2	756.5	94.5	4098.4	102.5	93.1

Intra-Assay Accuracy (n=6 samples)

Analyte	400 pg/ml (%CV)	800 pg/ml (%CV)	4000 pg/ml (%CV)	Average (%CV)
Trenbolone	10.2	11.7	14.3	12.1
Nandrolone	16.2	6.2	3.4	8.6
Boldenone	15.0	6.8	3.1	8.3
Testosterone	4.6	8.2	3.2	5.4
Stanozolol	13.1	4.6	4.8	7.5

Table1. Intra-Assay and Inter-Assay Accuracy and Precision

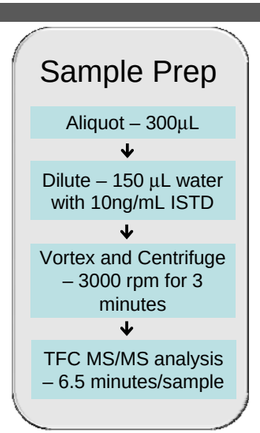
Analyte Recovery (n=4)

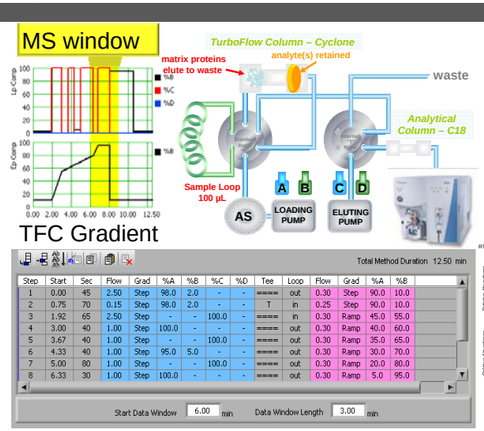
TX injection	Average Area Ratio	%CV	% recovery
Trenbolone	0.0349	1.59	100.1
Nandrolone	0.0230	5.46	102.2
Boldenone	0.0389	2.64	106.2
Testosterone	0.0398	2.97	101.9
Stanozolol	0.1015	4.28	94.6

Ratio = Analyte Peak Area/ISTD Area

LX injection	Average Area Ratio	%CV
Trenbolone	0.0349	1.70
Nandrolone	0.0225	1.77
Boldenone	0.0366	1.40
Testosterone	0.0390	2.54
Stanozolol	0.1073	2.50

Table 2. Analyte Recovery - 150 pg on column





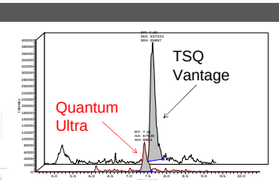


Figure 7. TSQ Vantage vs Quantum Ultra – 1 ng/mL testosterone fortified in serum

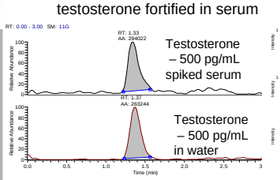


Figure 6. Matrix effects

Analyte	LOD (pg/mL)	LOQ (pg/mL)
Trenbolone	100	250
Nandrolone	175	250
Boldenone	175	250
Testosterone	175	250
Stanozolol	175	250

Table 3. Limits of Detection/Quantitation

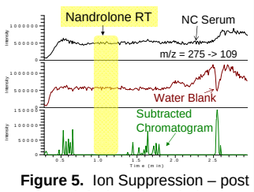


Figure 5. Ion Suppression – post column infusion of nandrolone

Results:

Multiplexed TFC MS/MS allowed for the extraction, separation and detection of 5 anabolic steroids from equine serum in 6.5 minutes per sample. Analytes were separated and introduced to a triple quadrupole mass spectrometer over a 3 minute window. All AAS were well resolved and minimal peak tailing was observed (Figures 1 and 2). SRM (5 product ions per analyte) allowed for both accurate and precise quantitation and qualitative determinations of AAS in equine serum.

The method was evaluated for sensitivity, precision, accuracy, and robustness. Limits of detection and quantitation were experimentally determined by fortifying negative control serum with decreasing amounts of drug (Table 3). Linear calibration curves (r^2>0.99) were observed for each analyte over a 250 – 10,000 pg/mL range. Calibrators and ISTD (%RSD) were plotted over 4 runs in two days showing instrument stability with r^2>0.99 for all analytes except stanozolol r^2>0.97 (Figure 4). Intra- and inter-day accuracy and precision data was evaluated for each compound at 3 QC levels with six replicates per level (Table 1). Analyte recovery was evaluated by averaging four injections of 150 pg on column of each drug with/without turbulent flow chromatography (Table 2). Ion suppression and enhancement were evaluated by post-column infusion (5 µL/minute) of a 1 ng/µL solution of all AAS and comparing ion intensity from negative control serum samples to water blanks (Figure 5). Matrix effects on ionization and extraction efficiency were also evaluated by spiking water and serum at 500, 1000, 5000 pg/mL and comparing peak area ratios (Figure 6).

This method was used to screen several hundred equine serum samples for AAS abuse allowing for rapid turnaround of samples. Several horses were found to contain quantifiable levels of AAS (Figure 3). In addition, serum samples collected following a 500 mg IM administration of boldenone undecylenate (Equipoise®, Fort Dodge) to a female horse were analyzed. This method allowed for detection of boldenone 21 days after administration, with 961 pg/mL detected after 7 days (Figure 3). The Thermo TSQ Vantage was compared to the Thermo Quantum Ultra triple quadrupole mass spectrometer by attaching the TFC system to each and data collected for testosterone, stanozolol and d3-testosterone at 1,000 pg/mL spiked in serum (Figure 7).

Conclusions:

- TSQ Vantage (S-Lens) gave an ~4X increase in signal as compared to the Quantum Ultra allowing for decreased LOQ and a ~5X increase in peak area.
- TFC along with MS-MS detection can rapidly analyze >200 equine serum samples in 24 hours by eliminating the need for time consuming, expensive and labor intensive sample preparation steps.
- Method allows for low limits of quantitation (LOQ=250 pg/mL) with high precision and accuracy over a several day period.
- TFC MS/MS allows for determination of abuse of AAS in performance horses.

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TFC MS/MS system: Thermo Aria TLX-2 – Turbulent Flow Chromatography System and Thermo TSQ Vantage