

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

DETECTION, QUANTIFICATION AND IDENTIFICATION OF 55 ANABOLIC STEROIDS IN EQUINE PLASMA BY UPLC-MS/MS.

DEVELOPED BY

PA EQUINE TOXICOLOGY & RESEARCH LABORATORY

**220 EAST ROSEDALE AVENUE
WEST CHESTER UNIVERSITY
DEPARTMENT OF CHEMISTRY
WEST CHESTER, PA 19382
Phone: (610) 436-3501
Fax: (610) 436-3504**

Director: Dr. Cornelius Uboh

E-mail: ubohcorn@vet.upenn.edu

cuboh@state.pa.us

Laboratory Manager: Jeffrey Rudy

E-mail: jeffrudy@verizon.net

jrudy@state.pa.us

Method Development: Dr. Fuyu Guan

E-mail: guanf@vet.upenn.edu

Drug Administration:

University of Pennsylvania School of Veterinary Medicine

New Bolton Center

834 West Street Road

Kennet Square, PA 19483

Phone: (610) 925-6265

Contact: Dr. Lawrence R. Soma

E-mail: soma@vet.penn.edu

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

DETECTION, QUANTIFICATION AND IDENTIFICATION OF FIFTY FIVE ANABOLIC STEROIDS IN EQUINE PLASMA BY UPLC-MS/MS

I. INTRODUCTION

Anabolic and androgenic steroids (AAS) are synthetic substances relating to the male sex hormone, testosterone (androgen). AAS promote the growth of skeletal muscle (anabolic effects) and the development of male sexual characteristics (androgenic effects). These compounds are used in treating delayed puberty, select impotence, and wasting of the body caused by some diseases. AAS are abused by bodybuilders, weightlifters and other athletes in human sports and horse racing as well, and are prohibited by the International Olympic Committee. Their use in horse racing has also been prohibited by the Association of Racing Commissioners' International (ARCI), and they are, therefore, included in the list of prohibited substances in the horse by ARCI. GC-MS is the technique commonly used by forensic laboratories for screening and confirmation of AAS. Urine samples from donor athletes are analyzed and the metabolites of AAS are monitored. However, GC-MS methods are limited by insensitivity, tedious procedure for derivatization of the metabolites and complication in characterization and confirmation of the metabolites that are very similar to one another in chemical structure. This SOP describes a high throughput UPLC-MS/MS method for detection, quantification and confirmation of 55 AAS including testosterone, boldenone, nandrolone and stanozolol among others in equine plasma. The 55 AAS include most of the AAS on the 2009 Prohibited List of World Anti-Doping Agency (WADA) except those that are not amenable to LC-MS analysis, in addition to other AAS that are of potential to be abused in horse racing. Those AAS that were selected from the WADA List but are not amenable to analyses by LC-MS have been submitted to our GC/TOFMS for development of an applicable method of analysis.

II. SCOPE

This standard operating procedure (SOP) will be limited to detection, quantification and confirmation of the 55 AAS (Figure 1) in equine plasma samples. This SOP is NOT applicable to analysis of equine urine samples. The scope of this work covers verifiable procedures to be used in quantifying and confirming the presence of the AAS in equine plasma. The limit to reporting a positive finding in a test sample to the Racing Commission is confirmation and quantification of an AAS in the sample.

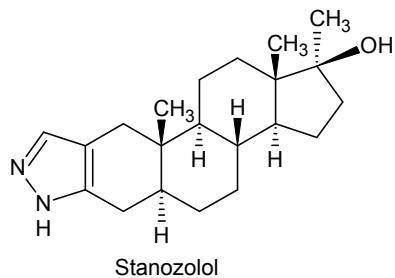
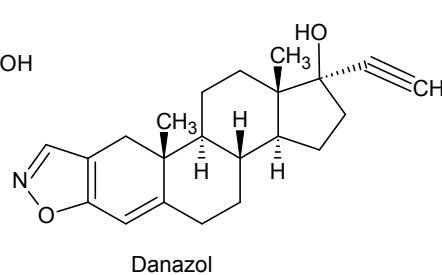
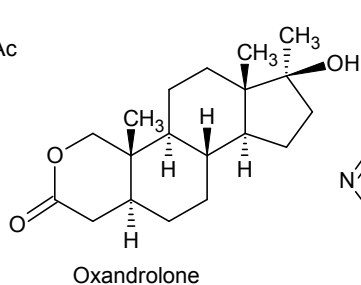
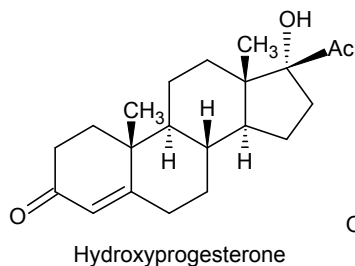
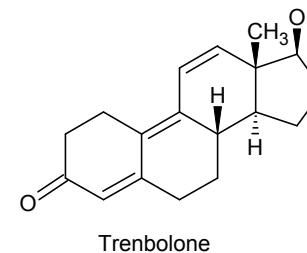
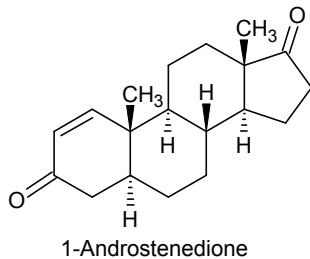
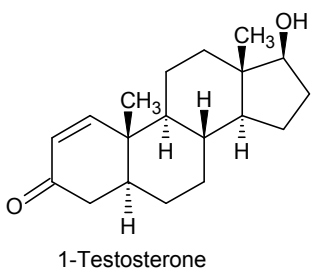
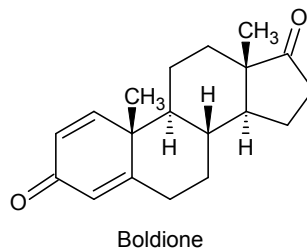
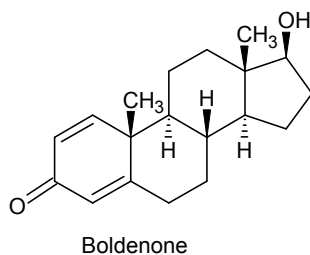
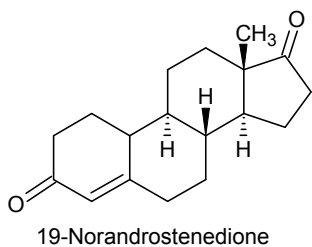
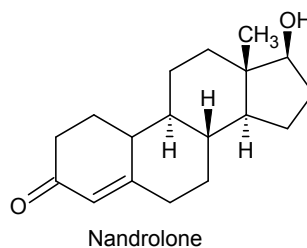
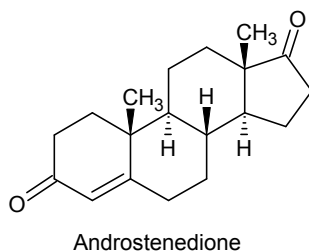
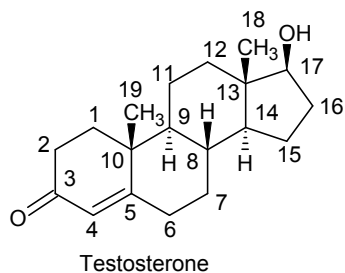
III. PRINCIPLE OF THE METHOD

AAS are neutral compounds and can be successfully extracted by liquid-liquid extraction (LLE) from equine plasma samples without any pH adjustment. The dried extracts are reconstituted in LC sample solvent (mobile phase) and the analytes are determined by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) operated in positive ion mode. Selected reaction monitoring (SRM) is used for detection, quantification and confirmation of AAS. Concentrations of AAS are determined by internal

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

calibration using chromatographic peak area ratio. Deuterated testosterone and stanozolol (testosterone-d₃ and stanozolol-d₃) (Figure 1) are used as internal standards. Plasma samples are initially screened for AAS by SRM. If a test sample is presumptively positive for any of the anabolic steroids screened for by this method, it is then confirmed by three ion transitions plus a full product ion spectrum that is complementary for confirmation. An LC mobile phase gradient is used for screening analysis of all 55 AAS. Three fine-tuned shallow mobile phase gradients are employed for confirmation analysis of different AAS. The limit of quantification (LOQ) for AAS in equine plasma by this method is 25 pg/0.5 mL for most of AAS. The limit of confirmation ranges from 25 - 250 pg/0.5 mL.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.



Testosterone subclass

1. Testosterone (CAS #: 58-22-0)
2. Testosterone-d₃ (17 β -Hydroxyandrost-4-en-3-one-16,16,17-d₃, CAS #: 77546-39-5)

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

3. Epitestosterone (17 α -testosterone; CAS #: 481-30-1)
4. Bolasterone (7 α ,17 α -Dimethyltestosterone; CAS #: 1605-89-6)
5. Calusterone (7 β ,17 α -Dimethyltestosterone; CAS #: 17021-26-0)
6. Clostebol (4-Chlorotestosterone; CAS #: 1093-58-9)
7. 6-Dehydrotestosterone (CAS #: 2484-30-2)
8. 9-Dehydrotestosterone (9 (11)-Dehydrotestosterone; CAS #: 2398-99-4)
9. 9-Dehydromethyltestosterone (17 β -Hydroxy-17-methyl-Androsta-4,9(11)-dien-3-one; CAS #: 1039-17-4)
10. Ethinyltestosterone (17 α -ethinyltestosterone; CAS #: 434-03-7)
11. Fluoxymesterone (9-Fluoro-11 β ,17 β -dihydroxy-17-methyl-androst-4-en-3-one; CAS #: 76-43-7)
12. 2 α -Hydroxymethylethisterone (17-Hydroxy-2 α -(hydroxymethyl)-17 α -pregn-4-en-20-yn-3-one; CAS #: 2787-03-3)
13. 15 β -Hydroxytestosterone (CAS #: 39605-73-7)
14. 16 β -Hydroxytestosterone (CAS #: 17528-90-4)
15. Methylclostebol (4-Chloro-17 α -methyltestosterone; CAS #: 5785-58-0)
16. Methyltestosterone (17 α -Methyltestosterone; CAS #: 58-18-4)

Androstenedione subclass

1. Androstenedione (CAS #: 63-05-8)
2. Adrenosterone (Androst-4-ene-3,11,17-trione; CAS #: 382-45-6)
3. 6-Dehydroandrostenedione (CAS #: 633-34-1)
4. 9(11)-Dehydroandrostenedione (4,9(11)-Androstadien-3,17-dione; CAS # 1035-69-4)

Nandrolone subclass

1. Nandrolone (CAS #: 434-22-0)
2. Epinandrolone (17 α -Nandrolone; CAS #: 4409-34-1)
3. 6-Dehydronandrolone (CAS #: 14531-84-1)
4. Gestodene (13-ethyl-17 α -hydroxy-18,19-Dinorpregna-4,15-dien-20-yn-3-one; CAS #: 60282-87-3)
5. Methyldienolone (17 β -Hydroxy-17-methyl-estra-4,9-dien-3-one; CAS #: 14531-89-6)
6. Methylnortestosterone (17-Methyl-19-nortestosterone; CAS #: 514-61-4)
7. Mibolerone (7 α ,17 α -Dimethyl-19-nortestosterone; CAS #: 3704-09-4)
8. Norbolethone (13-Ethyl-17-hydroxy-17 α -18,19-dinorpregn-4-en-3-one; CAS #: 1235-15-0)
9. Norclostebol (4-Chloro-19-nortestosterone; CAS #: 13583-21-6)
10. Norethandrolone (17-Ethyl-19-nortestosterone; CAS #: 52-78-8)
11. Norgestrel (13-ethyl-17 α -hydroxy-18,19-dinorpregn-4-en-20-yn-3-one; CAS #: 6533-00-2)
12. Norlutin (17 α -Ethinylnandrolone; CAS #: 68-22-4)
13. Trestolone (7 α -Methylnandrolone; CAS #: 3764-87-2)

19-Norandrostenedione subclass

1. 19-Norandrostenedione (CAS #: 734-32-7)
2. 6-Dehydro-19-norandrostenedione (CAS #: 13209-45-5)

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

Boldenone subclass

1. Boldenone (CAS #: 846-48-0)
2. Epiboldenone (17 α -Boldenone; CAS #: 27833-18-7)
3. Methylboldenone (1-Dehydro-17 α -methyltestosterone; CAS #: 72-63-9)
4. Turinabol (4-Chloro-1-dehydromethyltestosterone; CAS #: 2446-23-3)

Boldione subclass

1. Boldione (CAS #: 897-06-3)
2. Androstatriendione (1,4,6-Androstatrien-3,17-dione; CAS #: 633-35-2)
3. 11 β -Hydroxyboldione (1,4-Androstadien-11 β -ol-3,17-dione; CAS #: 898-84-0)
4. Δ 1-Adrenosterone (CAS #: 7738-93-4)

1-Testosterone subclass

1. 1-Testosterone (CAS #: 65-06-5)
2. Methenolone (17 β -Hydroxy-1-methyl-5 α -androst-1-en-3-one; CAS #: 153-00-4)
3. Methyl-1-testosterone (17 β -Hydroxy-17-methyl-5 α -androst-1-en-3-one; CAS #: 65-04-3)

1-Androstenedione subclass

- 1-Androstenedione (CAS #: 571-40-4)

Trenbolone subclass

1. Trenbolone (CAS #: 10161-33-8)
2. Altrenogest (17 β -Hydroxy-17-(2-propen-1-yl)-estra-4,9,11-trien-3-one; CAS #: 850-52-2)
3. Gestrinone (13-Ethyl-17-hydroxy-17 α -18,19-Dinorpregna-4,9,11-trien-20-yn-3-one; CAS #: 16320-04-0)
4. Tetrahydrogestrinone (13-Ethyl-17-hydroxy-17 α -18,19-dinorpregna-4,9,11-trien-3-one; CAS #: 618903-56-3)

Hydroxyprogesterone subclass

1. Hydroxyprogesterone (CAS #: 68-96-2)
2. Melengestrol (17-Hydroxy-6-methyl-16-methylene-pregna-4,6-diene-3,20-dione; CAS #: 5633-18-1)

Stanozolol subclass

1. Stanozolol (CAS #: 10418-03-8)
2. Stanozolol-d₃ (CAS #: 88247-87-4)

Figure 1. Chemical Structures of the Anabolic Steroids in this SOP

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

IV. REAGENTS

- A. Methanol, Optima grade (Cat. No. A 454-4, Fisher Scientific).
- B. Water; GC, HPLC and Spectrophotometry grade (Cat. No. 365-4, Burdick & Jackson).
- C. Methyl tert-butyl ether, HPLC grade (Cat. No. E-127-4, ThermoFisher Scientific)
- D. Ammonium Hydroxide, GR (Cat. No. AX1303-3, EMD)
- E. Formic Acid, Suprapur (Cat. No. 11670-1, EMD)

V. SOLUTIONS

A. Formate Buffer Stock Solution (1.0 M formic acid/1.0 M ammonium formate, pH 3.5)

1. Reagents

- a) Concentrated Ammonium Hydroxide (14.8 mol/L)
- b) Formic acid (HCOOH, 98-100%, 26.3 mol/L)
- c) Water, HPLC grade

2. Procedure

- a) Add 13.5 mL of concentrated ammonium hydroxide to 171 mL of water in a 250 mL reagent bottle.
- b) Add 15.2 mL of formic acid (98-100%), cap the bottle, and mix briefly. Adjust pH of the buffer to 3.5 with formic acid and ammonium hydroxide, if necessary.

3. Storage Requirements

- a) Store in a glass container at 4 °C (refrigerator).
- b) Discard five years after preparation date.

B. Formate Buffer Working Solution (2.0 mM formic acid/2.0 mM ammonium formate, pH 3.5) – HPLC mobile Phase

1. Reagents

- a) Formate Buffer Stock Solution (1.0 M formic acid/1.0 M ammonium formate, pH 3.5)
- b) Water, HPLC grade

2. Procedure

- a) Add 1000 mL of water to a liter glass container.
- b) Transfer 2.0 mL of water to waste.
- b) Add 2 mL of stock ammonium formate buffer. Shake briefly.

3. Storage Requirements

- a) Store at room temperature in a glass bottle.
- b) Discard 12 months after preparation date.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

VI. PRIMARY DRUG AND INTERNAL STANDARD REFERENCE MATERIALS

Table 1. Name, formula and vendors of 55 AAS in this SOP

Name	Formula	M.W.	Vendor	Cat. #
1-androstendione	C ₁₉ H ₂₆ O ₂	286	Steraloids	A4400-000
1-testosterone	C ₁₉ H ₂₈ O ₂	288	Steraloids	A4600-000
11β-Hydroxyboldione	C ₁₉ H ₂₄ O ₃	300	Steraloids	A0170-000
15β-hydroxytestosterone	C ₁₉ H ₂₈ O ₃	304	Steraloids	A5780-000
16β-hydroxytestosterone	C ₁₉ H ₂₈ O ₃	304	Steraloids	A5850-000
19-norandrostenedione	C ₁₈ H ₂₄ O ₂	272	Steraloids	E3350-000
2α-Hydroxymethylethisterone	C ₂₂ H ₃₀ O ₃	342	Cerilliant	NMID 920
6-Dehydro-19-norandrostenedione	C ₁₈ H ₂₂ O ₂	270	Steraloids	E0140-000
6-dehydroandrostenedione	C ₁₉ H ₂₄ O ₂	284	Steraloids	A0420-000
6-dehydronandrolone	C ₁₈ H ₂₄ O ₂	272	Steraloids	E0150-000
6-Dehydrotestosterone	C ₁₉ H ₂₆ O ₂	286	Steraloids	A0450-000
9-Dehydroandrostenedione	C ₁₉ H ₂₄ O ₂	284	Steraloids	A0480-000
9-dehydromethyltestosterone	C ₂₀ H ₂₈ O ₂	300	Steraloids	A0510-000
9-Dehydrotestosterone	C ₁₉ H ₂₆ O ₂	286	Steraloids	A0520-000
Adrenosterone	C ₁₉ H ₂₄ O ₃	300	Steraloids	A7250-000
altrenogest	C ₂₁ H ₂₆ O ₂	310	Steraloids	E3162-000
Androstatriendione	C ₁₉ H ₂₂ O ₂	282	Steraloids	A4100-000
Androstenedione	C ₁₉ H ₂₆ O ₂	286	Steraloids	A6030-000
Bolasterone	C ₂₁ H ₃₂ O ₂	316	Steraloids	A6240-000
Boldenone	C ₁₉ H ₂₆ O ₂	286	Steraloids	A0200-000
Boldione	C ₁₉ H ₂₄ O ₂	284	Steraloids	A0100-000
Calusterone	C ₂₁ H ₃₂ O ₂	316	Steraloids	A6247-000
Clostebol	C ₁₉ H ₂₇ ClO ₂	322.9	Steraloids	A5400-000
Danazol	C ₂₂ H ₂₇ NO ₂	337	Steraloids	A6250-000
Δ1-Adrenosterone	C ₁₉ H ₂₂ O ₃	298	Steraloids	A0230-000
Epiboldenone	C ₁₉ H ₂₆ O ₂	286	Steraloids	A0190-000
Epinandrolone	C ₁₈ H ₂₆ O ₂	274	Steraloids	E4040-000
Epitestosterone	C ₁₉ H ₂₈ O ₂	288	Steraloids	A6900-000
Ethinyltestosterone	C ₂₁ H ₂₈ O ₂	312	Steraloids	A6100-000
Fluoxymesterone	C ₂₀ H ₂₉ FO ₃	336	Steraloids	A6170-000
Gestodene	C ₂₁ H ₂₈ O ₂	310	Steraloids	E0158-000
Gestrinone	C ₂₁ H ₂₄ O ₂	308	Steraloids	E3178-000
Hydroxyprogesterone	C ₂₁ H ₃₀ O ₃	330	Steraloids	Q3360-000
Melengestrol	C ₂₃ H ₃₀ O ₃	354	Cerilliant	NMID 655
Methenolone	C ₂₀ H ₃₀ O ₂	302	Steraloids	A4420-000
Methyl-1-testosterone	C ₂₀ H ₃₀ O ₂	302	Steraloids	A4450-000
Methylboldenone	C ₂₀ H ₂₈ O ₂	300	Steraloids	A0130-000
Methylclostebol	C ₂₀ H ₂₉ ClO ₂	336.9	Steraloids	A5390-000
Methyldienolone	C ₁₉ H ₂₆ O ₂	286	Cerilliant	NMID 916
Methylnortestosterone	C ₁₉ H ₂₆ O ₂	288	Steraloids	E3900-000
Methyltestosterone	C ₂₀ H ₃₀ O ₂	302	Steraloids	A6280-000
Mibolerone	C ₂₀ H ₃₀ O ₂	302	Steraloids	E3250-000
Nandrolone	C ₁₈ H ₂₆ O ₂	274	Steraloids	E4050-000
Norbolethone	C ₂₁ H ₃₂ O ₂	316	Cerilliant	NMID825B

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

Norclostebol	$C_{18}H_{25}ClO_2$	308.8	Steraloids	E3670-000
Norethandrolone	$C_{20}H_{30}O_2$	302	Steraloids	E3500-000
Norgestrel	$C_{21}H_{28}O_2$	312	Steraloids	E3525-000
Norlutin	$C_{20}H_{26}O_2$	298	Steraloids	E3650-000
Oxandrolone	$C_{19}H_{30}O_3$	306	Steraloids	A2732-000
Stanozolol	$C_{21}H_{32}N_2O$	328	Steraloids	A2120-000
Stanozolol-d ₃	$C_{21}D_3H_{29}N_2O$	331	Cerilliant	S-903
Testosterone	$C_{19}H_{28}O_2$	288	Steraloids	A6950-000
Testosterone-d ₃	$C_{19}D_3H_{25}O_2$	291	Sigma	T2655-10MG
Tetrahydrogestrinone	$C_{21}H_{26}O_2$	312	Cerilliant	NMID872C
Trenbolone	$C_{18}H_{22}O_2$	270	Steraloids	E3170-000
Trestolone	$C_{19}H_{28}O_2$	288	Steraloids	E3870-000
Turinabol	$C_{20}H_{27}ClO_2$	334	Cerilliant	NMID 613

Obtain these materials from the QAO. Record accession of these materials on the pharmacy log sheet.

VII. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

A. Boldenone

1. Materials
 - a) Boldenone, (Cat. No. A0200-000, Steraloids, Rhode Island).
 - b) Methanol (HPLC grade)
2. Procedure
 - a) Weigh between 3 and 5 mg (X.xx mg) of boldenone into a 7 mL amber glass vial.
 - b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until boldenone is completely dissolved in methanol.
 - c) The resulting concentration of boldenone is 1.00 mg/mL.
3. Storage Requirements
 - a) Store at approximately 4 °C (refrigerator).
 - b) Discard 24 months after preparation.

B. Nandrolone

1. Materials
 - a) Nandrolone, (Cat. No. E4050-000, Steraloids, Rhode Island).
 - b) Methanol (HPLC grade).
2. Procedure
 - a) Weigh between 3 and 5 mg (X.xx mg) of nandrolone into a 7 mL amber glass vial.
 - b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until nandrolone is completely dissolved in methanol.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

- c) The resulting concentration of nandrolone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after preparation.

C. Stanozolol

1. Materials

- a) Stanozolol, (Cat. No. A2120-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of stanozolol into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until stanozolol is completely dissolved in methanol.
- c) The resulting concentration of stanozolol is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after preparation.

D. Testosterone

1. Materials

- a) Testosterone, (Cat. No. A6950-000, Steraloids, Rhode Island).
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of testosterone into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the vial. Cap and mix until testosterone is completely dissolved in methanol.
- c) The resulting concentration of testosterone is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after preparation.

E. Other AAS

Use the same procedure as described above to prepare stock solutions (1.0 mg/mL) of other AAS in methanol.

F. Testosterone-d₃

1. Materials

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

- a) Testosterone-d₃, internal standard, FW: 291.44 (Cat. No. T2655-10MG, Sigma, St. Louis, MO)
- b) Methanol (HPLC grade)

2. Procedure

- a) Weigh between 3 and 5 mg (X.xx mg) of testosterone-d₃ into a 7 mL amber glass vial.
- b) Add certain volume (Volume Y.yy = X.xx in mL) of methanol to the weighed drug in the vial. Cap and mix until testosterone-d₃ is completely dissolved in methanol.
- c) The resulting concentration of testosterone-d₃ is 1.00 mg/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after preparation.

Complete the Balance Use Log and QA Primary Reference Standard Log for this process.

Label the primary reface stock solutions with QA Primary Reference Log SR# (i.e. SR# xxx) and Primary Reference Powder Designation (i.e. R-NAND-1).

VIII. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS AND WORKING SOLUTIONS

A. Working Standard Solution of Mixture of 52 AAS

1. Materials

- a) 1.00 mg/mL of boldenone stock solution.
- b) 1.00 mg/mL of nandrolone stock solution.
- c) 1.00 mg/mL of stanozolol stock solution.
- d) 1.00 mg/mL of testosterone stock solution.
- e) 1.00 mg/mL of stock solution of other individual AAS.
- f) Methanol (HPLC grade).

2. Procedure

- a) Prepare secondary stock solutions of each anabolic steroid according to Table 2.
- b) Prepare working solutions of mixture of the 52 AAS listed in Table 1 except Methyltestosterone, Mibolerone and Trestolone of different concentrations according to Table 3.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after preparation.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

Table 2. Preparation of Secondary Stock Solution of Each Anabolic Steroid

Target Con., $\mu\text{g/mL}$	Stock Solution used, $\mu\text{g/mL}$	Stock Solution Vol. Added, μL	Vol. of MeOH, μL
50	1000	250	4750

Table 3. Preparation of Working Solution of the Mixture of 52 AAS

Target concn. of 52 AAS ng/mL	Total volume mL	Solvent	Stock Soln of each AAS $\mu\text{g/mL}$	Vol. of each stock soln summed μL	Stock Soln of AAS mixture ng/mL	Vol. of AAS mixture μL	Vol. of MeOH mL
500	10	Methanol	50	5200			4.8
250	10	Methanol			500	5000	5.0
100	10	Methanol			500	2000	8.0
50	10	Methanol			500	1000	9.0
25	10	Methanol			250	1000	9.0
10	10	Methanol			100	1000	9.0
5	10	Methanol			50	1000	9.0
2.5	10	Methanol			25	1000	9.0

Soln = solution

B. Testosterone-d₃ Working Solution (IS, 100 ng/mL)

1. Materials

- a) 1.0 mg/mL of testosterone-d₃ stock solution
- b) Methanol

2. Procedure

- a) Transfer 5.0 mL of methanol into a 7-mL amber glass vial.
- b) Take out 50 μL of methanol.
- c) Add 50 μL of testosterone-d₃ stock solution (1.0 mg/mL). Mix.
- d) The concentration of testosterone-d₃ is 10 $\mu\text{g/mL}$.
- e) Transfer 10.0 mL of methanol into a 15-mL amber glass vial.
- f) Take out 100 μL of methanol.
- g) Add 100 μL of 10 $\mu\text{g/mL}$ of testosterone-d₃ standard solution.
- h) The resulting concentration of testosterone-d₃ is 100 ng/mL.

3. Storage Requirements

- a) Store at approximately 4 °C (refrigerator).
- b) Discard 24 months after the initial date of preparation.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

C. Stanozolol-d₃ Working Solution (IS, 100 ng/mL)

1. Materials
 - a) 100 µg/mL of stanozolol-d₃ stock solution (Cerilliant, Cat. # S-909)
 - b) Methanol

2. Procedure
 - a) Transfer 5.0 mL of methanol into a 7-mL amber glass vial.
 - b) Take out 500 uL of methanol.
 - c) Add 500 uL of stanozolol-d₃ stock solution (100 µg/mL). Mix.
 - d) The concentration of stanozolol-d₃ is 10 µg/mL.
 - e) Transfer 10.0 mL of methanol into a 15-mL amber glass vial.
 - f) Take out 100 µL of methanol.
 - g) Add 100 µL of 10 µg/mL of stanozolol-d₃ standard solution.
 - h) The resulting concentration of stanozolol-d₃ is 100 ng/mL.

3. Storage Requirements
 - a) Store at approximately 4 °C (refrigerator).
 - b) Discard 24 months after the initial date of preparation.

IX. QC WORKING SOLUTIONS

AAS QC Working Solution

1. Materials
 - a) 1.00 mg/mL of boldenone stock solution.
 - b) 1.00 mg/mL of nandrolone stock solution.
 - c) 1.00 mg/mL of stanozolol stock solution.
 - d) 1.00 mg/mL of testosterone stock solution.
 - e) 1.00 mg/mL of other AAS stock solutions.
 - f) Methanol (HPLC grade).

2. Procedure
 - a) Prepare secondary stock solutions of each AAS according to Table 2.
 - b) Prepare working solutions of mixture of the 52 AAS of different concentrations according to Table 3.

3. Storage Requirements

Store at approximately 4 °C (refrigerator)
Discard 24 months after preparation.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

X. MATERIALS

- A. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)
- B. 16 × 100 mm test tubes.
- C. 16 × 125 mm screw-top test tubes.
- D. Screw caps
- E. Test tube rack
- F. Pipettes and tips.
- G. Vortex mixer (Scientific Industries, Inc.)
- H. Rotorack (Speci-Mix, Thermolyne)
- I. IEC HN-SII Centrifuge (International Equipment Company)
- E. Sample Concentrator (Dri-Block DB-3, Techne, New Jersey; Phone: 1-800-225-9243)
- F. 2 mL autosampler vials
- G. 250 uL Insert (Target PP Polyspring, National Scientific Company; ordered from Fisher Scientific)
- H. Eye protection and lab coat
- I. Gloves

XI. MATRIX

Equine Plasma.

XII. VOLUME OF MATRIX FOR ANALYSIS

0.5 mL .

XIII. CONTROL SAMPLES

A. Negative Control Sample

- 1. Equine plasma samples previously demonstrated by LC-MS to be negative for the presence of detectable anabolic steroids.
- 2. Store control samples at approximately -20 °C

Positive Control Sample

Equine plasma samples freshly supplemented with 52 AAS at final concentrations of 50, 500 and 1000 pg/0.5 mL.

XIV. SAMPLE REQUIREMENTS FOR ANALYSIS

A. Calibrators

- 1. Prepare a set of calibrators for analysis of plasma samples.
- 2. Calibrator concentrations as designated in Table 4.
- 3. Prepare plasma calibrators using negative (control) plasma and AAS working mixture solutions as described in Section VIII.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

B. Negative (control) sample

1. Designate **plasma NC or plasma blank.**
2. Prepare negative (control) sample from negative (control) plasma.

C. Positive control samples

1. Designate **plasma QC (or PC)1.....QC (or PC)n.**
2. Prepare positive control samples as described in Table 5.

D. Mobile phase blank

1. Designate **MB1.....MBn**

E. Test samples are designated to use the date of which the sample is analyzed and raw data files are designated to use sequential number.

Table 4. Preparation of Plasma Calibrators for 52 AAS

Target conc. of each AAS (pg/0.5 mL)	Working mixture solution (ng/mL)	Vol. of working mixture solution (µL)	Volume of negative plasma (mL)	Vol. of 100 ng/mL of testosterone-d ₃ (µL)	Vol. of 100 ng/mL of stanozolol-d ₃ (µL)
25	2.5	10	0.5	10	10
50	5.0	10	0.5	10	10
100	10	10	0.5	10	10
250	25	10	0.5	10	10
500	50	10	0.5	10	10
1000	100	10	0.5	10	10
2500	250	10	0.5	10	10

Table 5. Preparation of Plasma Positive Control (QC) Samples for 52 AAS

Target conc. of each AAS (pg/0.5 mL)	Working QC solution of the mixture (ng/mL)	Vol. of working QC solution (µL)	Volume of plasma (mL)	Vol. of 100 ng/mL of testosterone-d ₃ (µL)	Vol. of 100 ng/mL of stanozolol-d ₃ (µL)
50	5.0	10	0.5	10	10
50	5.0	10	0.5	10	10
500	50	10	0.5	10	10
500	50	10	0.5	10	10

XV. PLASMA CALIBRATOR AND SAMPLE PREPARATION

A. Calibrator and Sample Preparation for the Anabolic Steroids.

1. Label 16 × 125 mm test tubes.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

2. All samples are prepared in labeled tubes as per Table 6.
3. Vortex for 5-10 seconds to mix the contents of each tube.

Table 6. Preparation of Plasma Samples for the Anabolic Steroid Analysis

Items	Blank	Blank+IS	Sample
Blank plasma (mL)	0.5	0.5	N/A
Testosterone-d ₃ (IS) solution (μL)	0	10	10
Stanozolol-d ₃ (IS) solution (μL)	0	10	10
Plasma samples (mL)	N/A	N/A	0.5

B. Sample Extraction/Preparation by Liquid-Liquid Extraction

Safety Requirements: eye protection

1. Prepare each sample in an individual screw-top test tube, according to Table 6.
2. Add 5 mL of methyl tert-butyl ether (MTBE) into each tube, cap all screw-top tubes tightly with screw caps, and mix on a rotorack for 5 minutes.
3. Centrifuge at 2,500 ~ 3,000 rpm (839 ~ 1,409 g) for 5 minutes.
4. Decant the (top) organic layer into a labeled fresh test tube for each sample.
5. Bring the extracts in test tubes to dryness in a fume hood, by heating in a hot block (Techni-Dri-Block DB-3 Duxford, Cambridge, UK) at 50 °C under a stream of compressed air (or nitrogen).
6. Remove test tubes from the hot block, place in a rack, and allow to cool to room temperature.
7. Reconstitute the residue in each test tube with 100 μL of 50% methanol in formate buffer (2 mM, pH 3.5)
8. Transfer the above solution into a 250 μL insert in an autosampler vial. All the samples are now ready for LC/MS/MS analysis.

XVI. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND QUANTITATION OF ANABOLIC STEROIDS

A. Instrumentation

1. Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer with Xcalibur V2.0.7 for system control and data acquisition and processing (Thermo Fisher Scientific).
2. Accela quaternary UPLC pump, autosampler, column compartment and on-line degasser (Thermo Fisher Scientific).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

B. HPLC conditions

1. HPLC Column
 - a) Type: Hypersil GOLD C₁₈ Analytical Column (Part No. 25002-052130, Thermo, Phone: 1-800-532-4752).
 - b) Dimension: 2.1× 50 mm
 - c) Particle size: 1.9 μm
 - d) Temperature: 50 °C
2. Mobile Phase
 - a) Mobile phase A: 2.0 mM formate buffer solution (pH 3.5)
 - b) Mobile phase B: Methanol
 - c) Flow rate: 500 μL/min
3. Injection Volume: 20 μL.
4. Mobile phase gradients for anabolic steroid screening and confirmation are shown in Tables 7, 8 and 9.

Table 7. Mobile phase gradient 1 for screening analysis

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)
0	50	50
2.4	40	60
4.0	10	90
4.3	10	90
4.4	50	50
5.0	50	50

^a Ammonium formate, 2 mM, pH 3.4.

Table 8. Mobile phase gradient 2 for confirmation analysis of select AAS

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)
0	60	40
3.5	45	55
4.0	10	90
4.3	10	90
4.4	60	40
5.0	60	40

^a Ammonium formate, 2 mM, pH 3.4.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

Table 9. Mobile phase gradient 3 for confirmation analysis of select AAS

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)
0	50	50
3.5	35	65
4.0	10	90
4.3	10	90
4.4	50	50
5.0	50	50

^a Ammonium formate, 2 mM, pH 3.4.

Table 10. Mobile phase gradient 4 for confirmation analysis of select AAS

LC Run time (min)	Formate buffer ^a (%)	Methanol (%)
0	45	55
4.0	25	75
4.3	10	90
4.4	45	55
5.0	45	55

^a Ammonium formate, 2 mM, pH 3.4.

C. Mass Spectrometric Conditions

1. Ionization mode
 - a) Heated Electrospray ionization (H-ESI)
 - b) Positive ion mode
2. ESI source settings
 - a) Spray voltage: 1000 v (volts)
 - b) Sheath gas (nitrogen) pressure: 60 (units)
 - c) Auxiliary gas flow: 35.
 - d) Sweep gas: 3.
 - e) Vaporizer temperature: 350 °C.
 - f) Ion Transfer Capillary Temperature: 330 °C.
3. Mass spectrometer settings for SRM experiments
 - a) Peak Width relating to mass resolution (FWHM): 0.7 for both Q1 and Q3
 - b) Collision gas: Argon

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

- c) Collision gas pressure: 1.5 mTorr.
- d) Scan width for SRM: 0.4 Dalton.
- e) Scan range for full product ion scan: 70 – 360 Dalton.
- f) Scan time: 30 - 50 ms for each SRM transition in screening analysis, while 50 ms for each SRM and 200 ms for full product ion scan in confirmation analysis.
- g) Three time segments are set for SRM acquisition in screening analysis: 0 – 1.4 min., 1.4 – 2.45 min., and 2.45 – 5.0 min. For any of those AAS of which retention time is close to the border of a time segment, the same SRM acquisition is conducted in two neighboring segments that would cover the AAS. This will prevent the chromatographic peak profile of that AAS from incompleteness.

4. SRM acquisition parameters are shown in Table 11.

Table 11. Parameters for SRM acquisition and LC retention time in AAS screening and confirmation analyses*

Name	Precursor Mass	Collision Energy	Product Ion 1	Product Ion 2	Scan Time in Screening	Retention Time in Screening	Retention Time in Confirmation analysis		
	<i>m/z</i>	V	(100%)		ms	min.	LC gradient 2 min.	LC gradient 3 min.	LC gradient 4 min.
15β-hydroxytestosterone	305.2	26	97.1	109	50	0.66	1.17		
Δ1-Adrenosterone	299.2	22	171	223.1	50	0.76	1.48		
Adrenosterone	301.2	26	257.1	121.1	50	0.80	1.58		
1,4-Androstadien-11β-ol-3,17-dione	301.2	20	147	173	50	0.97	1.95		
16β-hydroxytestosterone	305.2	26	97.1	109.1	50	1.10	2.23		
Androstatrienedione	283.2	24	147	171	50	1.15	2.33		
6-Dehydro-19-norandrostenedione	271.2	26	149.1	105.1	50	1.18	2.38		
Boldione	285.2	20	121	147.1	50	1.21	2.5		
19-norandrostendione	273.2	26	109.1	197.1	30	1.35		1.45	
Trenbolone	271.2	30	199	227.1	30	1.49		1.59	
6-dehydroandrostenedione	285.2	30	149.1	105.1	30	1.52		1.59	
2α-Hydroxymethylethisterone	343.2	28	139	121	30	1.53		1.56	
Fluoxymesterone	337.2	34	131.1	181.1	30	1.53		1.64	
4,9(11)-androstadien-3,17-dione	285.2	26	252.1	227.1	30	1.56		1.66	
Boldenone	287.2	20	121.1	135.1	30	1.57		1.7	

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

6-dehydronandrolone	273.2	26	133.1	105.1	30	1.59	1.69
Oxandrolone	307.2	20	229.1	121.1	30	1.62	1.74
Androstenedione	287.2	26	97	109	30	1.71	1.83
Nandrolone	275.2	26	109	145.1	30	1.71	1.83
Norlutin	299.2	26	109.1	231.1	30	1.81	1.94
9-Dehydrotestosterone	287.2	28	147	145.1	30	1.81	
Methyldienolone	287.2	30	159	135	30	1.90	1.89
6-Dehydrotestosterone	287.2	28	133.1	105.1	30	1.91	2.04
Methylboldenone	301.2	20	121.1	149.1	30	1.91	2.05
Gestodene	311.2	24	109.1	201	30	1.94	2.06
Epiboldenone	287.2	20	121.1	135.1	30	2.0	2.09
1-androstendione	287.2	26	185.1	143.1	30	2.03	2.18
Gestrinone	309.2	28	241.1	199.1	30	2.03	2.19
Trestolone	289.2	30	107.1	119.1	30	2.04	2.19
Epinandrolone	275.2	26	109	145.1	30	2.07	2.17
Testosterone	289.2	26	97.1	109	30	2.10	2.25
Ethinyltestosterone	313.2	26	97.1	109.1	30	2.18	2.33
Methylnortestosterone	289.2	26	109.1	213.1	30	2.20	2.32
Hydroxyprogesterone	331.2	26	109	97.1	30	2.21	2.36
9-dehydromethyltestosterone	301.2	28	159.1	147	30	2.22	2.39
Norclostebol	309.2	26	143	213.1	30	2.31	2.43
Melengestrol	355.2	26	279.2	237.1	30	2.43	2.41
Mibolerone	303.2	28	107.1	121.1	40	2.53	2.72
Norgestrel	313.2	28	109.1	135.1	40	2.53	2.68
1-testosterone	289.2	26	187.1	145.1	40	2.55	2.72
Methyltestosterone	303.2	26	109	97.1	40	2.57	2.72
Epitestosterone	289.2	26	109.1	97.1	40	2.60	2.76
Turinabol	335.2	20	155	149.1	40	2.63	2.63
Methenolone	303.2	26	187.1	83.1	40	2.65	2.82
Clostebol	323.2	24	143	131	40	2.70	2.85
Bolasterone	317.2	26	97.1	123	40	2.93	2.18
altrenogest	311.2	26	227.1	251	40	2.98	2.2
1-dehydromethandrostenolone	303.2	26	201.1	145.1	40	3.10	2.33
Norethandrolone	303.2	28	109.1	135.1	40	3.14	2.36
Methylclostebol	337.2	26	143	131	40	3.15	2.35
Calusterone	317.2	26	97.1	123	40	3.19	2.42
Tetrahydrogestrinone	313.2	28	241	159	40	3.27	2.48
Danazol	338.2	32	148	120	40	3.53	2.83
Norbolethone	317.2	26	109	245.1	40	3.58	2.80
Stanozolol	329.3	42	329.2	107.1	40	3.59	2.91

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

* For screening analysis, one ion transition from precursor ion to product ion 1 (100% in relative intensity) is used for most AAS except testosterone and Methylboldenone of which a second ion transition from precursor to product ion 2 is also employed to increase screening specificity.

D. Sample list setup for anabolic steroid screening analysis

1. Sample solvent blank
2. Blank plasma (QC negative control)
3. Blank plasma + IS (1000 pg/mL)
4. QC plasma sample (50 pg/0.5 mL for each anabolic steroid of 52 AAS)
5. QC plasma sample (500 pg/0.5 mL for each anabolic steroid of 52 AAS)
6. Sample solvent blank
7. Calibrator 1 (25 pg/0.5 mL)
8. Calibrator 2 (50 pg/0.5 mL)
9. Calibrator 3 (100 pg/0.5 mL)
10. Calibrator 4 (250 pg/0.5 mL)
11. Calibrator 5 (500 pg/0.5 mL)
12. Calibrator 6 (1000 pg/0.5 mL)
13. Calibrator 7 (2500 pg/0.5 mL)
14. Sample solvent blank
15. Sample 1
16. Sample 2
17. Sample 3
18. Sample 4
19. Sample 5
20. Sample 6
21. Sample n
22. Sample solvent blank
23. QC plasma sample (50 pg/0.5 mL for each anabolic steroid of 52 AAS)
24. QC plasma sample (500 pg/0.5 mL for each anabolic steroid of 52 AAS)
25. Sample solvent blank

Note: use screening LC gradient (Table 7) for AAS screening analysis.

E. Detection of Methylnortestosterone, Trestolone and Mibolerone in screening analysis

1. Methylnortestosterone, Trestolone and Mibolerone are not spiked to calibrators or QCs while their detection is achieved thanks to the fact explained below.
2. Detection of Methylnortestosterone and Trestolone are covered by Testosterone in calibrators in the channel of m/z 289 \rightarrow 109 since they have the same product ion m/z 109 and are not chromatographically well resolved. Similarly, detection of Mibolerone is covered by Methyltestosterone in calibrators in the channel of m/z 303 \rightarrow 109.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

3. Optionally, a separate calibrator series with Methyltestosterone, Trestolone and Mibolerone spiked is prepared and analyzed along with the calibrator series containing the 52 AAS and plasma samples as well.
4. As for possible interferences of Testosterone, Methyltestosterone and Trestolone, Methyltestosterone and Mibolerone with one another in screening and confirmation analyses, please see the discussion under “Interfering Substances” (Section XIX).

F. Sample list setup for anabolic steroid confirmation analysis

1. Sample solvent blank
2. Blank plasma (QC negative control)
3. Blank plasma + IS (1000 pg/mL)
4. QC plasma sample (50 pg/0.5 mL for a target anabolic steroid)
5. QC plasma sample (500 pg/0.5 mL for a target anabolic steroid)
6. Sample solvent blank
7. Calibrator series 1 (25 pg/0.5 mL)
8. Calibrator series 2 (50 pg/0.5 mL)
9. Calibrator series 3 (100 pg/0.5 mL)
10. Calibrator series 4 (250 pg/0.5 mL)
11. Calibrator series 5 (500 pg/0.5 mL)
12. Calibrator series 6 (1000 pg/0.5 mL)
13. Calibrator series 7 (2500 pg/0.5 mL)
14. Sample solvent blank
15. Sample 1, replicate 1
16. Sample 1, replicate 2
17. Sample 1, replicate 3
18. Sample solvent blank
19. Sample 2, replicate 1
20. Sample 2, replicate 2
21. Sample 2, replicate 3
22. Sample solvent blank
23. Sample n, replicate 1
24. Sample n, replicate 2
25. Sample n, replicate 3
26. Sample solvent blank
27. QC plasma sample (50 pg/0.5 mL for a target anabolic steroid)
28. QC plasma sample (500 pg/0.5 mL for a target anabolic steroid)
29. Sample solvent blank

Note: use confirmation LC mobile phase gradients (Tables 8-10) and three ion transitions (Table 12) for anabolic steroid confirmation analysis.

G. Criteria for Identification of Anabolic Steroids in Equine Plasma Samples

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

- a) Confirmation of the presence of anabolic steroids is performed using three specific ion transitions from each anabolic steroid and the intensity ratios of different product ions of that anabolic steroid that are calculated using SRM chromatographic peak areas. The qualifying ions and the ratios for each analyte are listed in Table 12. These values are for reference only. In practice, the intensity ratios of product ions for a positive sample should be within $\pm 25\%$ of the average value of the ratios from the calibrators and QCs analyzed along with the positive sample in the same batch.
- b) Under the UPLC-MS/MS analytical conditions, all three qualifying product ions of an anabolic steroid in a positive sample must be recognized at retention time within ± 0.05 min of that of an authentic standard spiked to equine plasma and analyzed under identical conditions. The retention times of 55 AAS under different LC mobile phase gradients for confirmation are listed in Table 11.
- c) Full product ion spectrum may be used as a supplementary evidence for identification of an anabolic steroid in a positive sample. The major product ions in a MS/MS spectrum from a positive sample must match those from an anabolic steroid standard spiked to equine plasma and analyzed under identical conditions.

Table 12. Averaged intensity ratios of major product ions of each anabolic steroid and limit of confirmation (LOC) by ion intensity ratio and by product ion spectrum

Name	Precursor mass <i>m/z</i>	Collision energy V	Product ion 1 <i>m/z</i> (100%)	Product ion 2 <i>m/z</i>	Product ion 3 <i>m/z</i>	Ion2/Ion1	Ion3/Ion1	LOC by ion ratio pg/0.5 mL	LOC by product ion spectrum Pg/0.5 mL
15beta-hydroxytestosterone	305.2	26	97.1	109	123	97 ± 2	11 ± 0.5	100	100
Delta1-Adrenosterone	299.2	22	171	223.1	147	86 ± 2	77 ± 2	50	250
Adrenosterone	301.2	26	257.1	121.1	225.1	74 ± 3	23 ± 1	250	250
1,4-Androstadien-11beta-ol-3,17-dione	301.2	20	147	173	97.1	90 ± 4	73 ± 2	100	100
16beta-hydroxytestosterone	305.2	26	97.1	109.1	123	88 ± 3		25	50
Androstatrienedione	283.2	24	147	171	159	70 ± 1	49 ± 1	50	250
6-Dehydro-19-norandrostenedione	271.2	26	149.1	105.1	107.1	56 ± 1	51 ± 1	50	100
Boldione	285.2	20	121	147.1	151.1	25 ± 1	20 ± 1	50	50
19-norandrostendione	273.2	26	109.1	197.1	83.1	40 ± 1	31 ± 1	100	100
Trenbolone	271.2	30	199	227.1	159	35 ± 1	48 ± 3	250	500
6-dehydroandrostenedione	285.2	30	149.1	105.1	107.1	91 ± 3	85 ± 1	50	250
2α-Hydroxymethylethisterone	343.2	28	139	121	109	103 ± 4	101 ± 6	100	250
Fluoxymesterone	337.2	34	131.1	181.1	241.1	63 ± 2	66 ± 4	50	100
4,9(11)-androstadien-3,17-dione	285.2	26	252.1	227.1	147.1	44 ± 2	54 ± 2	100	250
6-dehydronandrolone	273.2	26	133.1	105.1	151.1	38 ± 1	23 ± 1	50	50
Boldenone	287.2	20	121.1	135.1	173.1	54 ± 1	27 ± 1	50	50

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

Oxandrolone	307.2	20	229.1	121.1	253.1	73 ± 3	56 ± 4	100	500
Androstenedione	287.2	26	97	109	123	82 ± 2	8.6 ± 0.3	50	100
Nandrolone	275.2	26	109	145.1	135.1	38 ± 1	25 ± 1	50	250
Norlutin	299.2	26	109.1	231.1	145.1	26 ± 1	37 ± 1	100	250
9-Dehydrotestosterone	287.2	28	147	145.1	121	62 ± 2	42 ± 1	50	100
Methyldienolone	287.2	30	159	135	161	68 ± 2	67 ± 3	25	250
6-Dehydrotestosterone	287.2	28	133.1	105.1	151.1	46 ± 1	26 ± 1	50	100
Methylboldenone	301.2	20	121.1	149.1	173.1	89 ± 3	19 ± 1	25	100
1-androstendione	287.2	26	185.1	143.1	203.1				1000
Gestodene	311.2	24	109.1	201	159	34 ± 1	37 ± 2	50	500
Epiboldenone	287.2	20	121.1	135.1	173.1	43 ± 3	15 ± 0.5	50	100
Gestrinone	309.2	28	241.1	199.1	262.1	92 ± 3	34 ± 1	50	100
Epinandrolone	275.2	26	109	145.1	199.1	51 ± 2	17 ± 1	250	250
Trestolone	289.2	30	107.1	119.1	159.1	61 ± 1	50 ± 2	100	100
Testosterone	289.2	26	97.1	109	123	101 ± 2	9.5 ± 0.2	50	100
Ethinyltestosterone	313.2	26	97.1	109.1	123.1	99 ± 3	9.7 ± 0.5	25	50
Methylnortestosterone	289.2	26	109.1	213.1	231.1	54 ± 2	35 ± 1	25	100
Hydroxyprogesterone	331.2	26	109	97.1	253.1	91 ± 2	16 ± 0.7	50	100
9-dehydromethyltestosterone	301.2	28	159.1	147	253.1	84 ± 2	56 ± 2	50	100
Norclostebol	309.2	26	143	213.1	237.1	42 ± 1	29 ± 2	100	100
Melengestrol	355.2	26	279.2	236.1	237.1	66 ± 2	56 ± 3	50	250
Norgestrel	313.2	28	109.1	135.1	245.1	56 ± 1	26 ± 1	100	500
1-testosterone	289.2	26	187.1	145.1	131.1	30 ± 2	30 ± 1	50	250
Mibolerone	303.2	28	107.1	121.1	245.1	83 ± 1	21 ± 1	50	250
Methyltestosterone	303.2	26	109	97.1	227.1	92 ± 2	14 ± 0.7	25	100
Turinabol	335.2	20	155	149.1	161.1	68 ± 2	26 ± 1	50	100
Epitestosterone	289.2	26	109.1	97.1	123	93 ± 3	11 ± 1	50	100
Metenolone	303.2	26	187.1	83.1	145	93 ± 1	33 ± 1	100	100
Clostebol	323.2	24	143	131	251.1	42 ± 2	9.3 ± 0.3	50	250
Bolasterone	317.2	26	97.1	123	203.1	24 ± 0.3	12 ± 0.2	50	100
altrenogest	311.2	26	227.1	251	225	24 ± 1	30 ± 0.4	50	50
1-dehydromethandrostenolone	303.2	26	201.1	145.1	159.1	68 ± 5	29 ± 3	50	500
Methylclostebol	337.2	26	143	131	189.1	53 ± 1	12 ± 0.5	50	250
Norethandrolone	303.2	28	109.1	135.1	231.1	44 ± 1	17 ± 0.4	50	250
Calusterone	317.2	26	97.1	123	203.1	29 ± 1	5.5 ± 0.2	50	25
Tetrahydrogestrinone	313.2	28	241	159	266	83 ± 2	29 ± 1	100	250
Danazol	338.2	32	148	120	105.1	91 ± 3	65 ± 2	100	500
Norbolethone	317.2	26	109	227.1	245.1	52 ± 2	49 ± 2	25	1000
Stanozolol	329.3	42	329.2	107.1	121.1	69 ± 3	65 ± 1	100	100

H. Criteria for Anabolic Steroid Quantitation

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

1. Determination of Anabolic Steroids

- a) The ion transition used for quantitation of each AAS is “precursor ion → product ion 1 listed in Table 11”. Examples are listed below.
- b) The ion transition used for quantification of boldenone is “ m/z 287 → 121”.
- c) The ion transition used for quantification of nandrolone is “ m/z 275 → 109”.
- d) The ion transition used for quantification of testosterone is “ m/z 289 → 97”.
- e) The ion transition used for quantification of stanozolol is “ m/z 329 → 329”.
- f) The ion transition used for testosterone- d_3 (internal standard) is “ m/z 292 → 97”.
- g) The ion transition used for stanozolol- d_3 (internal standard) is “ m/z 342 → 342”.
- h) Testosterone- d_3 is used as an internal standard for quantification of all the anabolic steroids except danazol and stanozolol.
- i) Stanozolol- d_3 is used for quantification of danazol and stanozolol.
- j) Plot the peak area ratios of each quantifying ion for each calibrator versus the concentration of each anabolic steroid in the calibrator (internal calibration). Use the Xcaliber software’s Quantification function to perform calibration and quantification. Print the compound summary quantification report and calibration curve. The correlation should be greater than 0.98.
- k) Examine the reported concentrations for all samples. The accuracy of concentrations for QC samples should be between 80% and 120 %.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

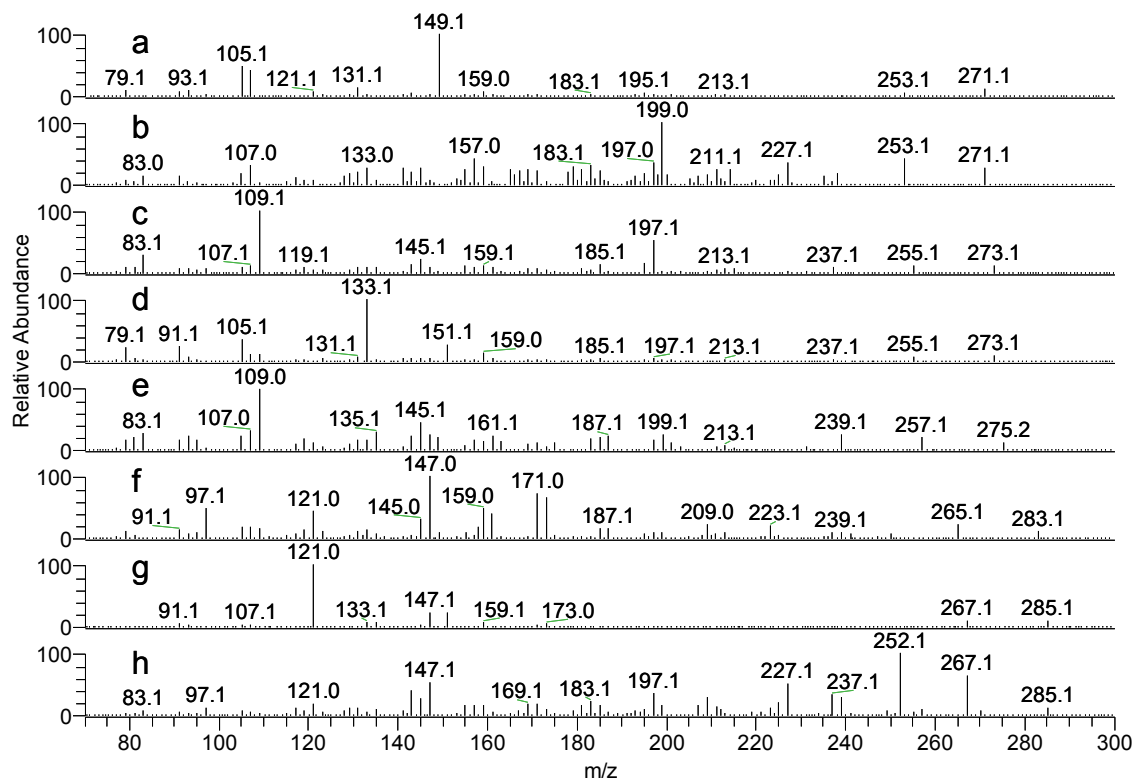


Figure 2. Product ion spectra of 6-dehydro-19-norandrostedione (m/z 271) at collision energy (CE) of 26 V (a), trenbolone (m/z 271) at CE of 30 V (b), Norandrostedione (m/z 273) at CE of 26 V (c), 6-Dehydronandrolone (m/z 273) at CE of 26 V (d), Nandrolone (m/z 275) at CE of 26 V (e), Androstatriendione (m/z 283) at CE of 24 V (f), Boldione (m/z 285) at CE of 20 V (g), and 9-Dehydroandrostedione (m/z 285) at CE of 26 V (h). Each analyte at concentration of 1.0 $\mu\text{g/mL}$ in methanol/formate buffer (2 mM, pH 3.4) (50/50, v/v) was infused at a flow rate of 5 $\mu\text{L/min}$ into ESI source.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

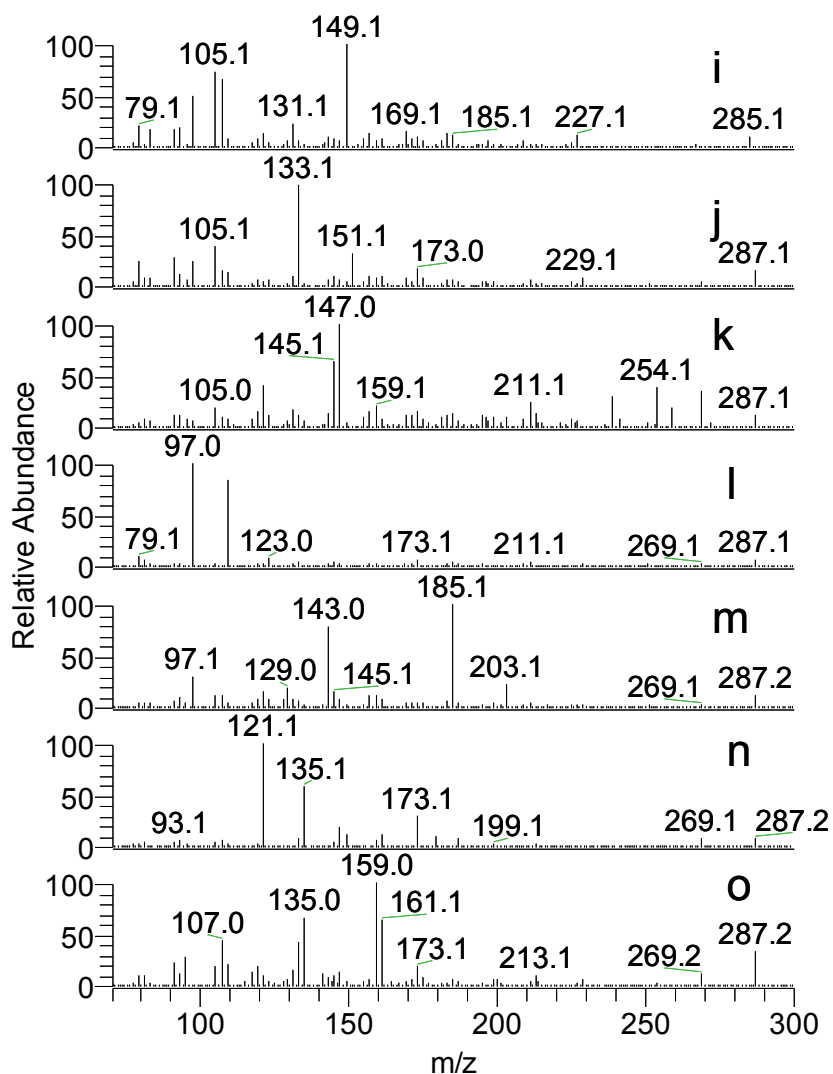


Figure 2 (continued). Product ion spectra of 6-Dehydroandrostenedione (m/z 285) at CE of 30 V(i), 6-Dehydrotestosterone (m/z 287) at CE of 28 V (j), 9-Dehydrotestosterone (m/z 287) at CE of 28 V (k), androstenedione (m/z 287) at CE of 26 V (l), 1-Androstenedione (m/z 287) at CE of 26 V (m), Boldenone (m/z 287) at CE of 20 V (n), and Methyldienolone (m/z 287) at CE of 30 V (o).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

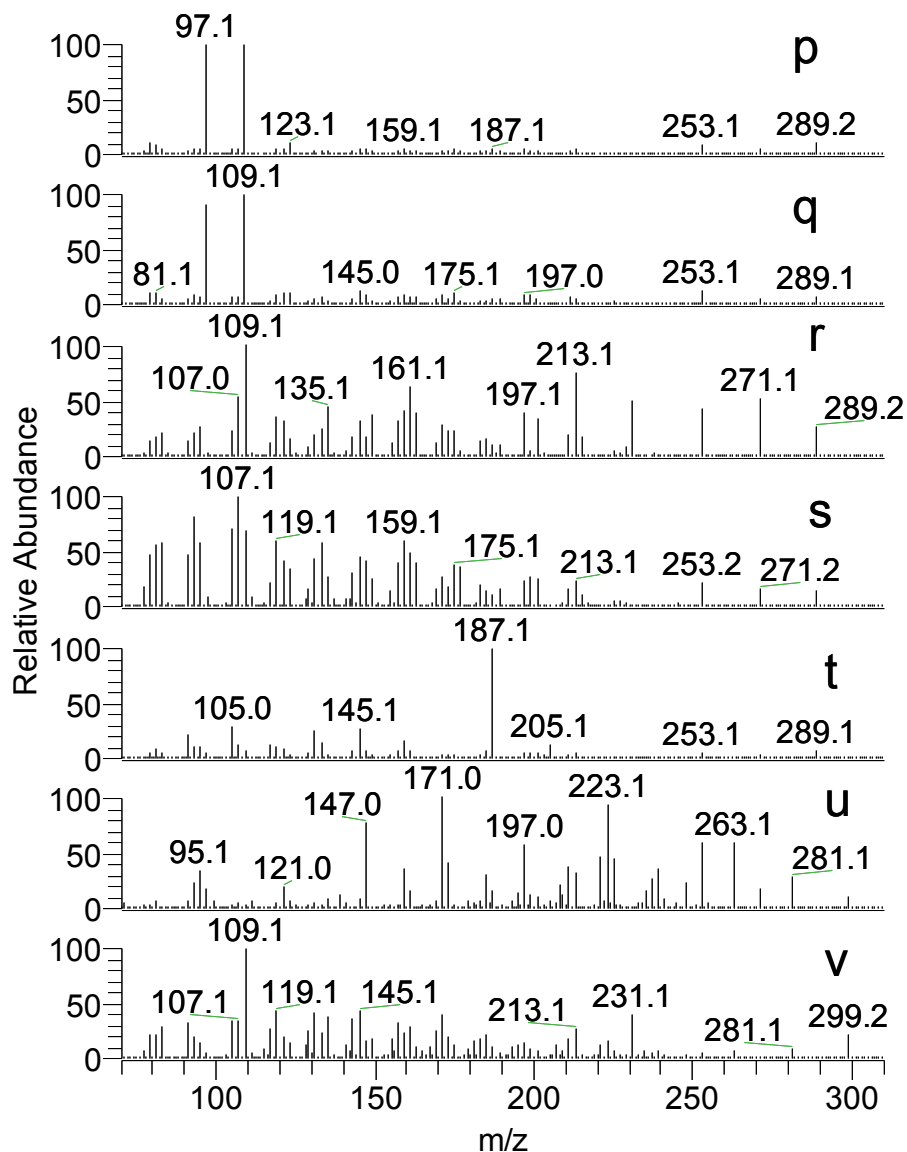


Figure 2 (continued). Product ion spectra of Testosterone (m/z 289) at CE of 26 V (p), Epitestosterone (m/z 289) at CE of 26 V (q), Methyltestosterone (m/z 289) at CE of 26 V (r), Trestolone (m/z 289) at CE of 30 V (s), 1-Testosterone (m/z 289) at CE of 26 V (t), Δ^1 -Adrenosterone (m/z 299) at CE of 22 V (u), and Norlutone (m/z 299) at CE of 26 V (v). Un-annotated major peaks are m/z 109 (p), m/z 97 (q), m/z 231 and 253 (r), m/z 93 (s), and m/z 253 (u).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

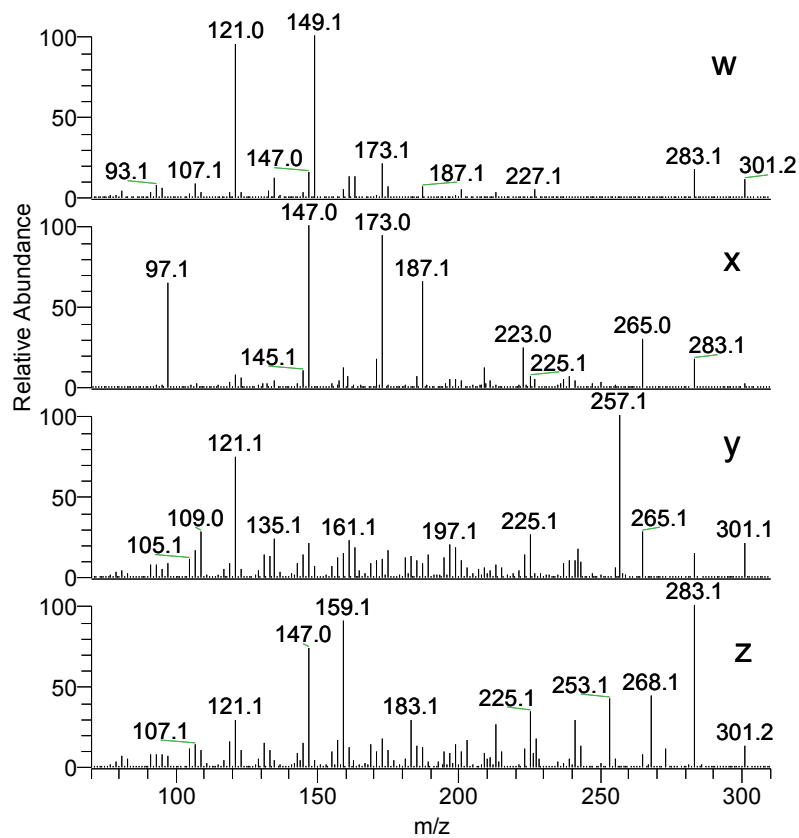


Figure 2 (continued). Product ion spectra of Methylboldenone (m/z 301) at CE of 20 V (w), 11 β -Hydroxy-boldione (m/z 301) at CE of 20 V (x), Adrenosterone (m/z 301) at CE of 26 V (y), and 9-Dehydromethyltestosterone (m/z 301) at CE of 28 V (z).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

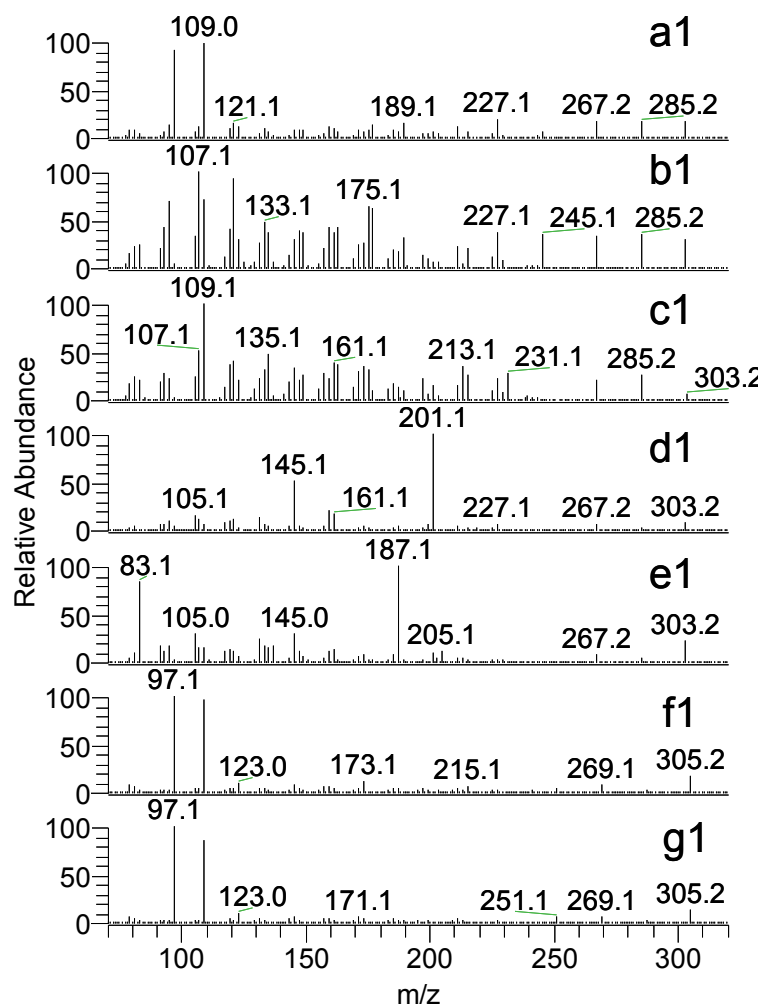


Figure 2 (continued). Product ion spectra of Methyltestosterone (m/z 303) at CE of 26 V (a1), Mibolerone (m/z 303) at CE of 28 V (b1), Norethandrolone (m/z 303) at CE of 28 V (c1), Methyl-1-testosterone (m/z 303) at CE of 26 V (d1), Methenolone (m/z 303) at CE of 26 V (e1), 15 β -Hydroxytestosterone (m/z 305) at CE of 26 V (f1), and 16 β -Hydroxytestosterone (m/z 305) at CE of 26 V (g1). Un-annotated major peaks are m/z 97 (a1), m/z 95, 109, and 121 (b1), m/z 109 (f1), and m/z 109 (g1).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

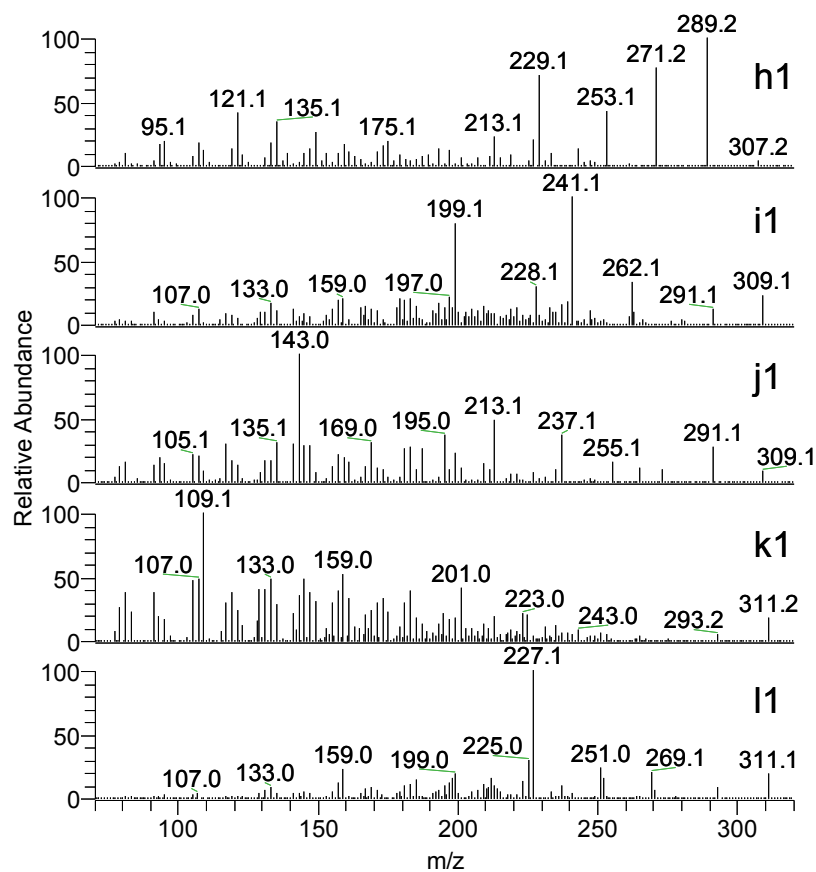


Figure 2 (continued). Product ion spectra of Oxandrolone (m/z 307) at CE of 20 V (h1), Gestrinone (m/z 309) at CE of 28 V (i1), Norclostebol (m/z 309) at CE of 26 V (j1), Gestodene (m/z 311) at CE of 24 V (k1), and Altrenogest (m/z 311) at CE of 26 V (l1).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

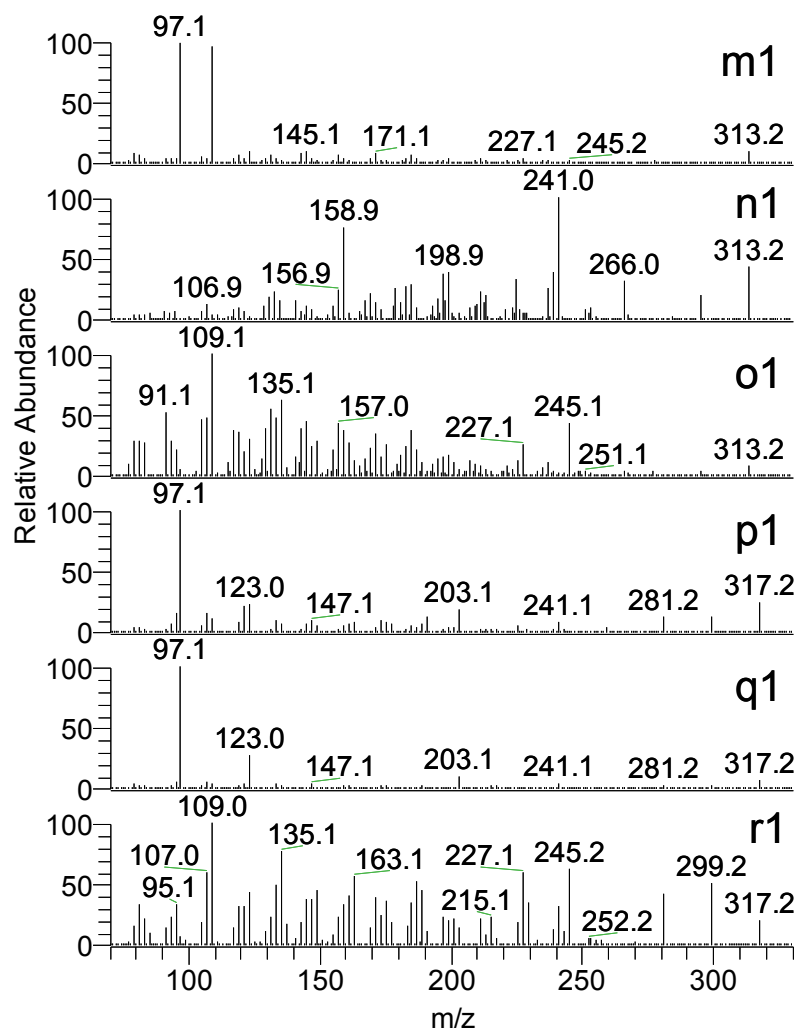


Figure 2 (continued). Product ion spectra of Ethinyltestosterone (m/z 313) at CE of 26 V (m1), THG (m/z 313) at CE of 28 V (n1), Norgestrel (m/z 313) at CE of 28 V (o1), Bolasterone (m/z 317) at CE of 26 V (p1), Calusterone (m/z 317) at CE of 26 V (q1), and Norbolethone (m/z 317) at CE of 26 V (r1). Un-annotated major peaks are m/z 109 (m1), and m/z 281 (r1).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

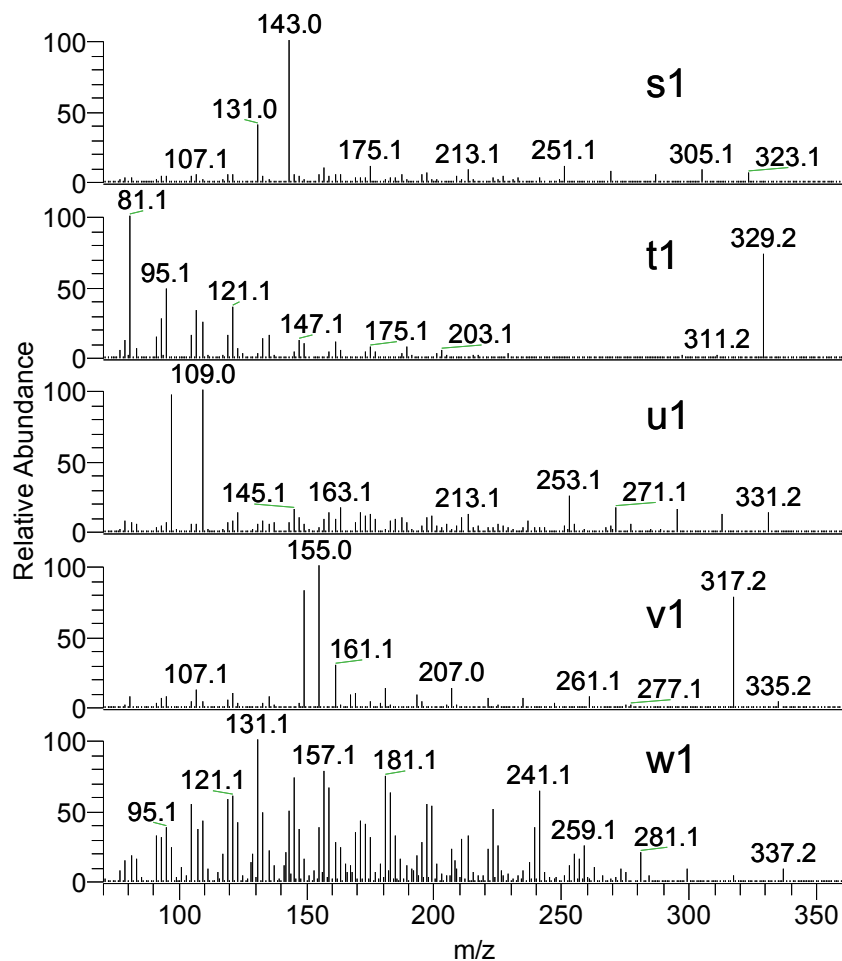


Figure 2 (continued). Product ion spectra of Clostebol (m/z 323) at CE of 24 V (s1), Stanozolol (m/z 329) at CE of 42 V (t1), Hydroxyprogesterone (m/z 331) at CE of 26 V (u1), Turinabol (m/z 335) at CE of 20 V (v1), and Fluoxymesterone (m/z 337) at CE of 34 V (w1). Un-annotated major peaks are m/z 97 (u1), m/z 149 (v1), m/z 145, 199 and 223 (w1).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

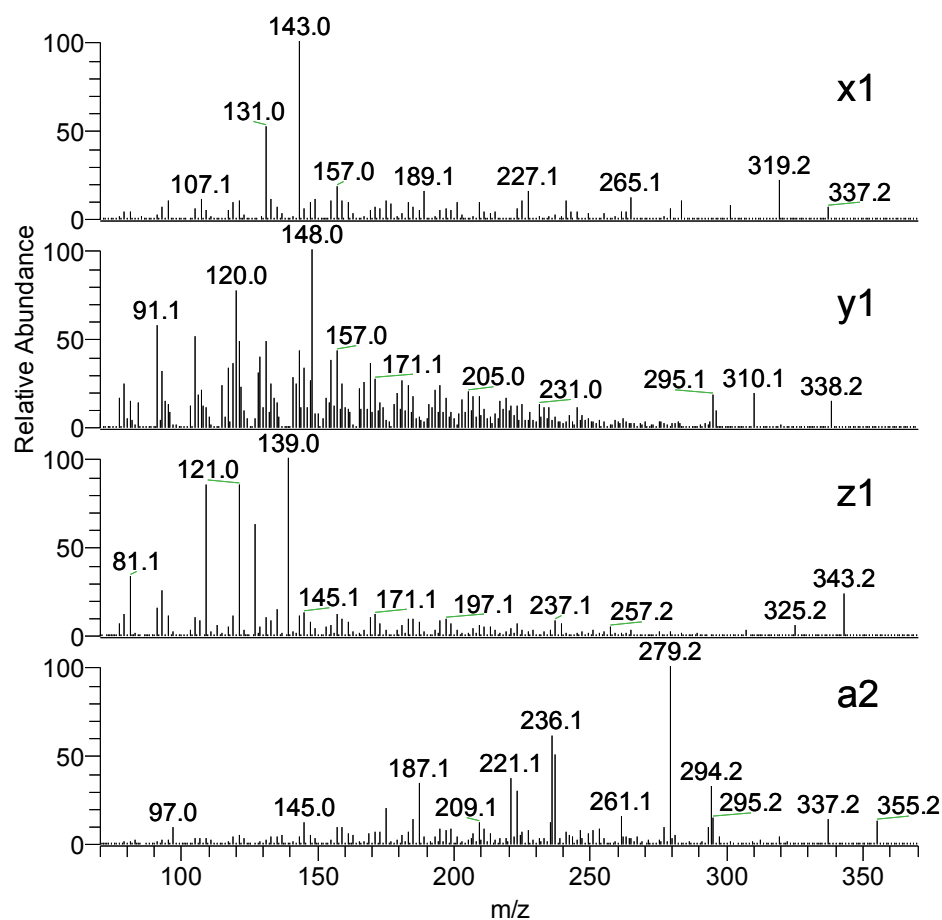


Figure 2 (continued). Product ion spectra of Methylclostebol (m/z 337) at CE of 26 V (x1), Danazol (m/z 338) at CE of 32 V (y1), 2α -Hydroxymethylethisterone (m/z 343) at CE of 28 V (z1), and Melengestrol (m/z 355) at CE of 26 V (a2). Un-annotated major peaks are m/z 105 (y1), m/z 109 and 127 (z1), and m/z 237 (a2).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

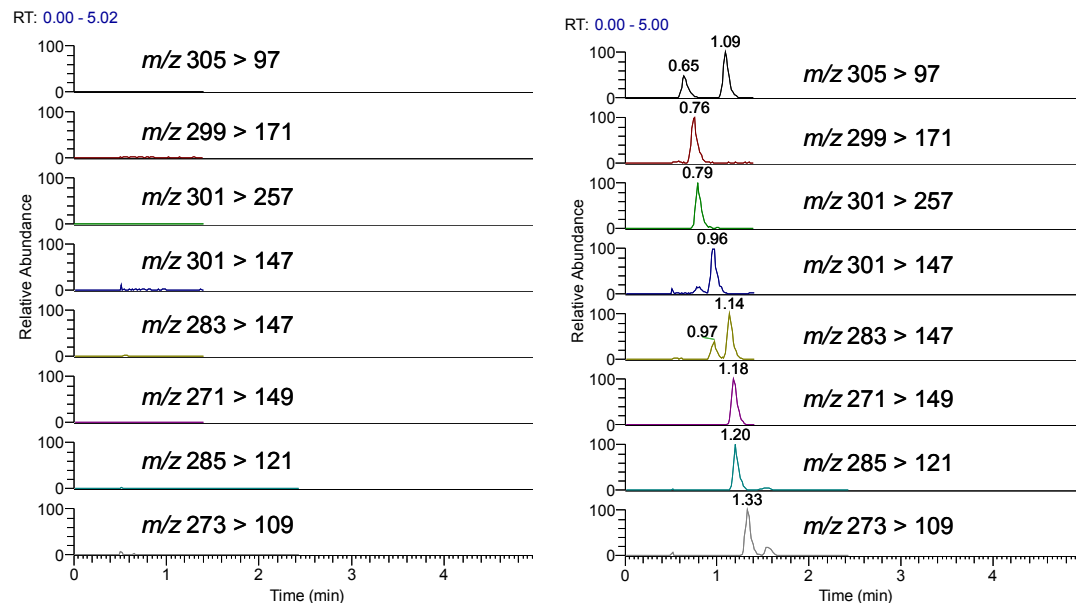


Figure 3. LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of 15 β -Hydroxytestosterone at retention time (t_R) of 0.65 min (m/z 305 > 97), 16 β -Hydroxytestosterone at t_R of 1.09 min (m/z 305 > 97), Δ 1-Adrenosterone at t_R of 0.76 min (m/z 299 > 171), Adrenosterone at t_R of 0.79 min (m/z 301 > 257), 11 β -Hydroxyboldione at t_R of 0.96 min (m/z 301 > 147), Androstatriendione at t_R of 1.14 min (m/z 283 > 147), 6-Dehydro-19-norandrostenedione at t_R of 1.18 min (m/z 271 > 149), Boldione at t_R of 1.20 min (m/z 287 > 121), and 19-Norandrostenedione at t_R of 1.33 min (m/z 273 > 109).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

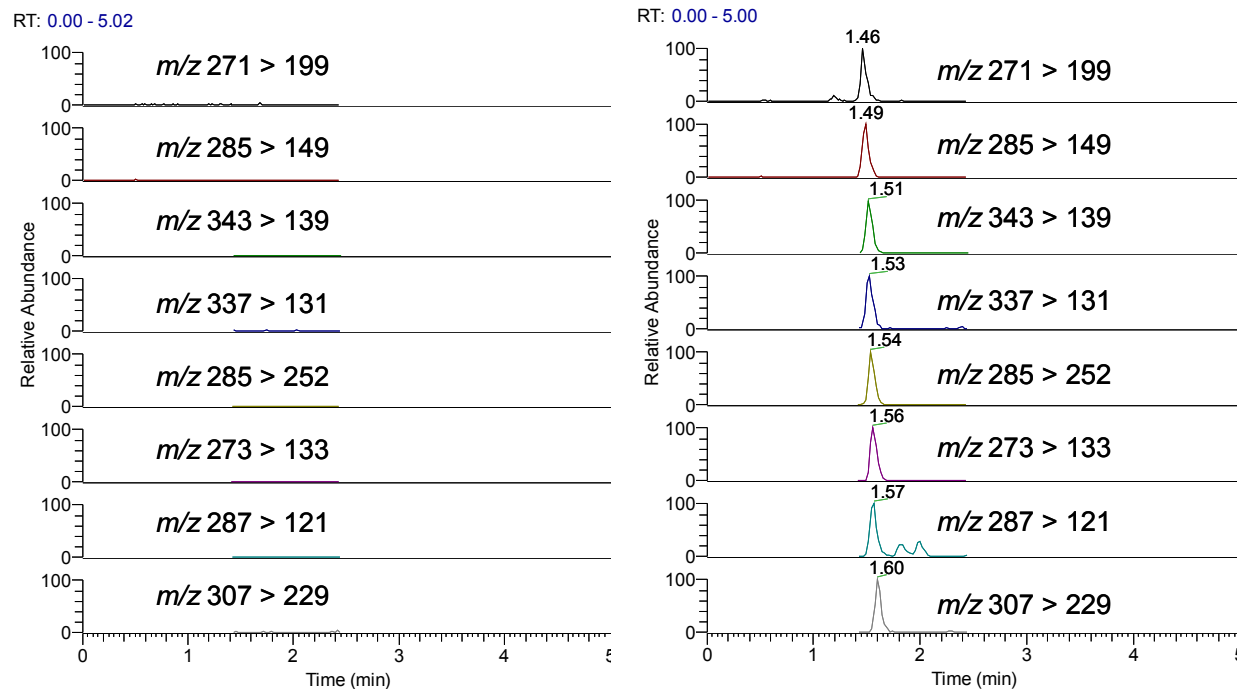


Figure 3 (continued). LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of Trenbolone at t_R of 1.46 min (m/z 271 > 199), 6-Dehydroandrostenedione at t_R of 1.49 min (m/z 285 > 149), 2 α -Hydroxymethylethisterone at t_R of 1.51 min (m/z 343 > 139), Fluoxymesterone at t_R of 1.53 min (m/z 337 > 131), 9-Dehydroandrostenedione at t_R of 1.54 min (m/z 285 > 252), 6-Dehydronandrolone at t_R of 1.56 min (m/z 273 > 133), Boldenone at t_R of 1.57 min (m/z 287 > 121), and Oxandrolone at t_R of 1.60 min (m/z 307 > 229).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

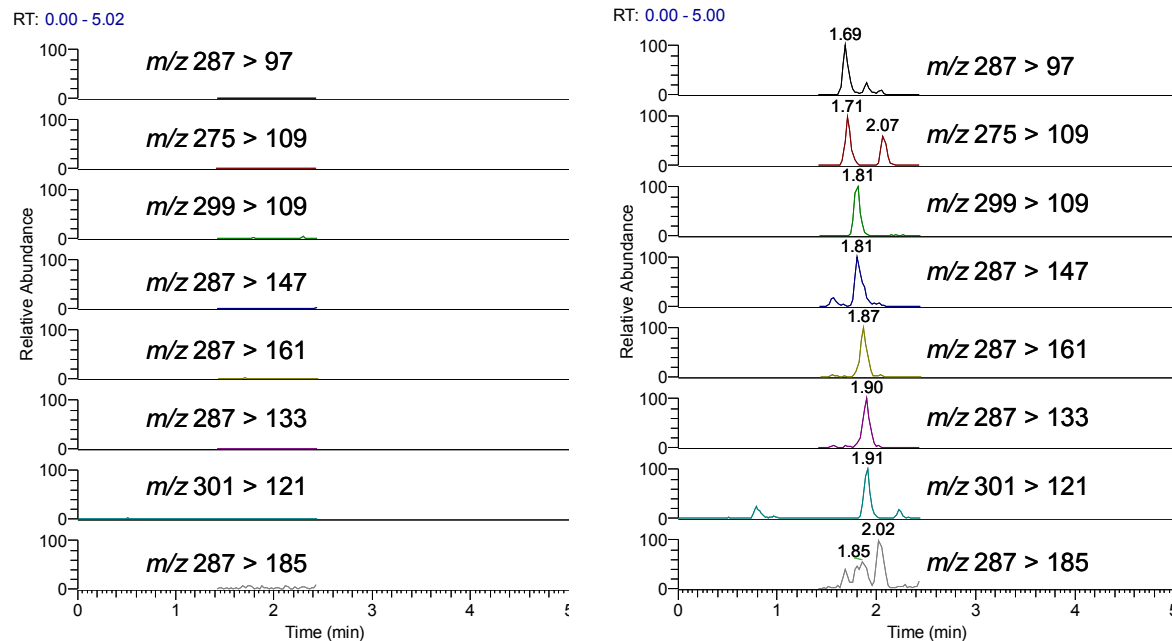


Figure 3 (continued). LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of Androstenedione at t_R of 1.69 min (m/z 287 > 97), Nandrolone at t_R of 1.71 min (m/z 275 > 109), Epinandrolone at t_R of 2.07 min (m/z 275 > 109), Norlutin at t_R of 1.81 min (m/z 299 > 109), 9-Dehydrotestosterone at t_R of 1.81 min (m/z 287 > 147), Methyldienolone at t_R of 1.87 min (m/z 287 > 161), 6-Dehydrotestosterone at t_R of 1.90 min (m/z 287 > 133), Methylboldenone at t_R of 1.91 min (m/z 301 > 121), and 1-Androstenedione at t_R of 2.02 min (m/z 287 > 185).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

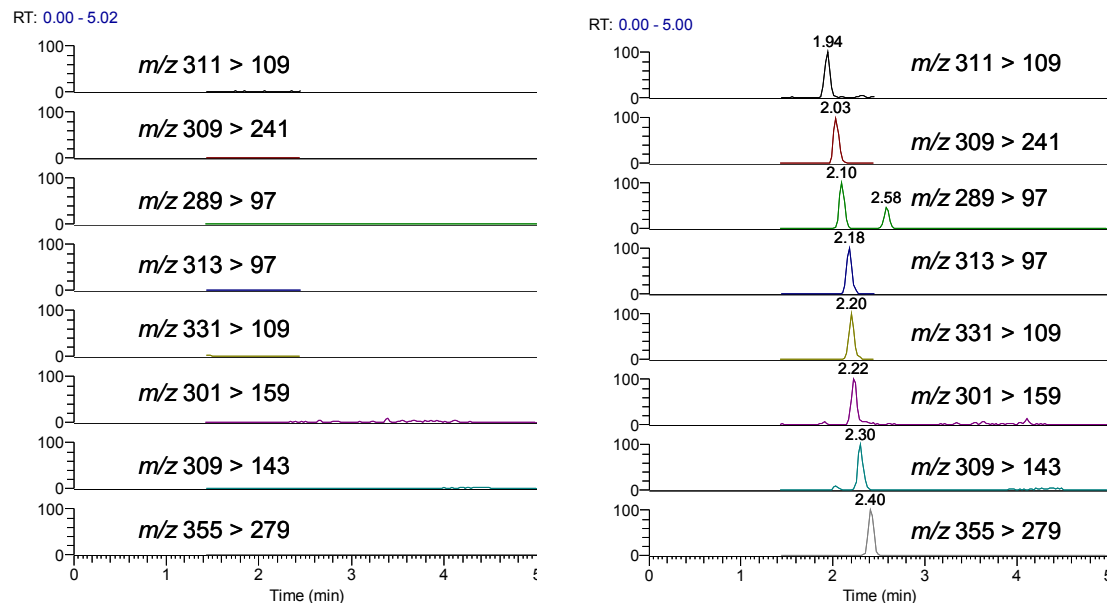


Figure 3 (continued). LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of Gestodene at t_R of 1.94 min (m/z 311 > 109), Gestrinone at t_R of 2.03 min (m/z 309 > 241), Testosterone at t_R of 2.10 min (m/z 289 > 97), Epitestosterone at t_R of 2.58 min (m/z 289 > 97), Ethinyltestosterone at t_R of 2.18 min (m/z 313 > 97), Hydroxyprogesterone at t_R of 2.20 min (m/z 331 > 109), 9-Dehydromethyltestosterone at t_R of 2.22 min (m/z 301 > 159), Norclostebol at t_R of 2.30 min (m/z 309 > 143), and Melengestrol at t_R of 2.40 min (m/z 355 > 279).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

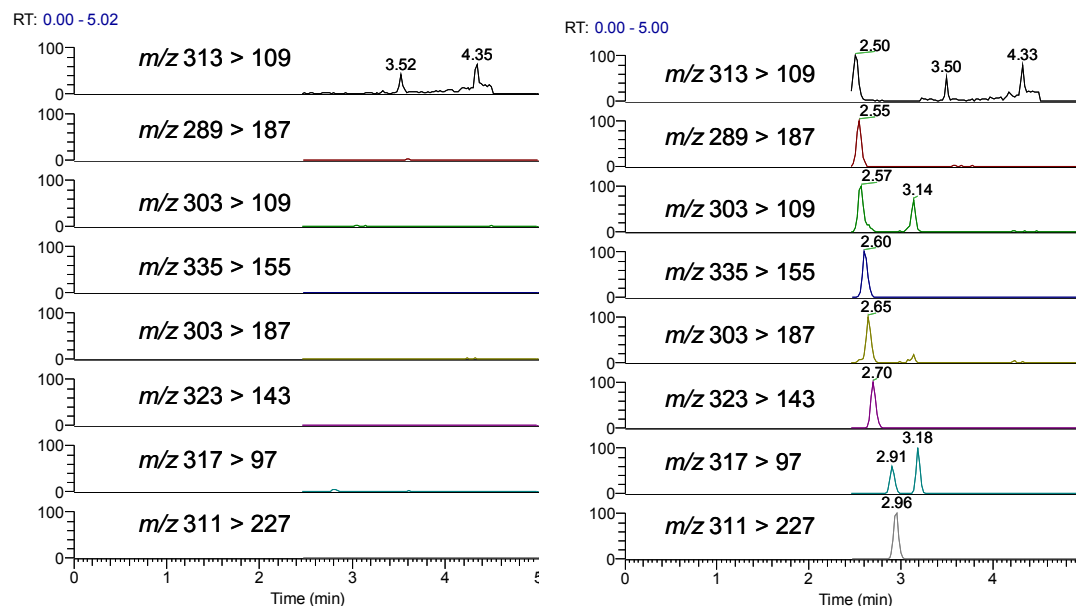


Figure 3 (continued). LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of Norgestrel at t_R of 2.50 min (m/z 313 > 109), 1-Testosterone at t_R of 2.55 min (m/z 289 > 187), Methyltestosterone at t_R of 2.57 min (m/z 303 > 109), Norethandrolone at t_R of 3.14 min (m/z 303 > 109), Turinabol at t_R of 2.60 min (m/z 335 > 155), Methenolone at t_R of 2.65 min (m/z 303 > 187), Clostebol at t_R of 2.70 min (m/z 323 > 143), Bolasterone at t_R of 2.91 min (m/z 317 > 97), Calusterone at t_R of 3.18 min (m/z 317 > 97), and Altrenogest at t_R of 2.96 min (m/z 311 > 227).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

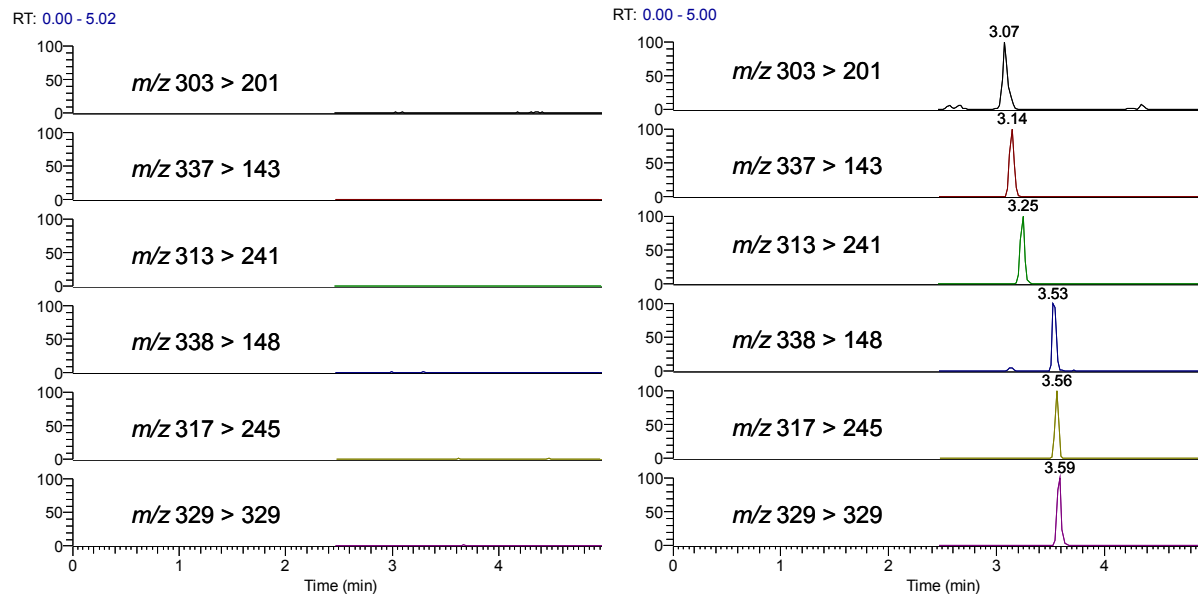


Figure 3 (continued). LC-MS/SRM chromatograms of blank-plasma extract (left panel) and AAS spiked to blank plasma (each at 500 pg/0.5 mL) (right panel) showing the peaks of Methyl-1-testosterone at t_R of 3.07 min (m/z 303 > 201), Methylclostebol at t_R of 3.14 min (m/z 337 > 143), THG at t_R of 3.25 min (m/z 313 > 241), Danazol at t_R of 3.53 min (m/z 338 > 148), Norbolethone at t_R of 3.56 min (m/z 317 > 245), and Stanozolol at t_R of 3.59 min (m/z 329 > 329).

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

XVII. CRITERIA FOR REPORTING A SAMPLE AS A POSITIVE FINDING FOR THE PRESENCE OF AN ANABOLIC STEROID

Report a test sample as a positive per this standard operating procedure for an anabolic steroid if the sample contains one of the 55 AAS at a concentration greater than a set value (see PA Harness Racing Commission's rule and PA Horse Racing Commission's rule for details) with 95 % Confidence Interval, and all of the following criteria are met:

- The test sample contains an anabolic steroid according to the chromatographic and product ion ratio criteria described in XVI (F).
- The concentration of an anabolic steroid quantified in a test sample is above the limit of quantification of the UPLC-MS/MS method.
- The signal-to-noise ratio of the least abundant qualifying ion for an anabolic steroid in each replicate of the test sample is greater than 10.
- The concentration of an anabolic steroid in the test sample is greater than the limit of confirmation of the UPLC-MS/MS method.
- The concentration of an endogenous AAS (Testosterone, Epitestosterone, Androstenedione) exceeds a well established threshold value.

XVIII. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Confidence Determination Report
4. Sample List
5. Tune Page Settings
6. LC Method
7. MS Method
8. Screening Report
9. Quantification Report
10. Quantification Calibration Curve
11. SRM Chromatogram Comparison for confirmation including blank plasma, blank solvent immediately before the positive sample, the positive sample, a positive QC sample.
12. SRM Chromatograms for product ion intensity ratio comparison including the 3 ion transition traces for the positive sample and a positive QC sample.
13. Product ion spectrum comparison between the positive sample and a positive QC sample.

Other Required Documentation

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

In addition to the positive data packet, the following documentation is required:

Sample list print-out that is maintained in the three-ring binder
Routine instrument usage checklist completion (and maintenance log if needed)
Sample Analysis logbook, indicating date, project, operator initials, and listing of official samples and sample identification.

Data packets for samples determined to be negative must contain the following elements:

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Confidence Determination Report
4. Screening Report
5. Quantification Report

XIX. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of the anabolic steroids by this procedure.

Occasionally, an unknown substance from racehorse plasma exhibits signals in the channels of ion transitions m/z 301 \rightarrow 121 and 149, and may cause false positive for Methylboldenone. However, intensity ratio of the SRM m/z 301 \rightarrow 149 to m/z 301 \rightarrow 121 from the unknown substance is remarkably different from that from authentic Methylboldenone. It is easily distinguishable from Methylboldenone by product ion intensity ratio comparison. Additionally, full product ion spectrum of the unknown substance is significantly different from that of authentic Methylboldenone spiked to blank plasma.

In screening analysis, either methylnortestosterone or trestolone is not chromatographically well resolved from testosterone, and thus, either one contributes to testosterone's signal in the channel of m/z 289 \rightarrow 109, but not in the channel of m/z 289 \rightarrow 97. Similarly, mibolerone is not chromatographically well separated from methyltestosterone, and the former adds to the latter's intensity in the channel of m/z 303 \rightarrow 109, but not in the channel of m/z 303 \rightarrow 97. These results can be taken advantage of in both screening analysis and confirmation analysis. In screening analysis, a calibrator series with testosterone spiked covers methylnortestosterone and trestolone, as a methyltestosterone-containing calibrator series does mibolerone. In confirmation analysis, however, testosterone, methylnortestosterone and trestolone do not interfere with each other, nor do methyltestosterone and mibolerone, since their product ion profiles are different.

Other AAS with isobaric precursor ions are either chromatographically or mass spectrometrically (by specific precursor-to-product transition) resolved and do not interfere with each other in both screening and confirmation analyses.

This SOP has been Approved By the Laboratory Director and Acknowledged By the Quality Assurance Officer and By the Lab Supervisor.

XX. REFERENCES:

1. Youwen You, Cornelius E. Uboh, Lawrence R. Soma, Fuyu Guan, Xiaoqing Li, Jeffrey A. Rudy, Ying Liu and Jinwen Chen. A Selective and High –Throughput UPLC-MS/MS Method for Detection, Quantification and Confirmation of Anabolic Steroids in Equine Plasma. *Rapid Communication in Mass Spectrometry*, submitted 2/2009.
2. Fuyu Guan, Cornelius E. Uboh, Lawrence R. Soma, Yi Luo, Jeffery Rudy, and Thomas Tobin. Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography and tandem mass spectrometry. *Journal of Chromatography B*, 829: 56-68 (2005).
3. Fuyu Guan, Cornelius E. Uboh, Lawrence R. Soma, Yi Luo and Scott Peterman. Collision-induced dissociation Pathways of anabolic steroids by electrospray ionization tandem mass spectrometry. *Journal of American Society for Mass Spectrometry*, 17: 477-489 (2006).