

LC-MS/MS method for steroid esters in hair (screening)**Document:** SOP ARO/510 versie 2**Auteur:** Paul Zoontjes**Documentgebied:** ARO Methods**Autorisator:** Leen van Ginkel
(labhoofd)**Status:** Definitief

1 Introduction

This procedure describes a method for the screening of steroids esters in samples of hair.

The sample preparation consist of several of washing steps, grinding and a C18 solid phase extraction (SPE)

Detection is performed by LC connected to a triple-Q MS through an electrospray interface.

The procedure is limited validated and found to be suitable for Norclostebol acetaat, Testosteron-propionate, Testosteron-acetate, Nortestosteron-acetate, Nortestosteron-phenyl-propionate, Estradiol-benzoate, Dehydrotestosteron-un-decylenate, Clobetasol propionaat, Estradiol-acetate-17b at the level of 5 ng/g (CC β). For Testosteron-benzoate, Nortestosteron-laurate, Testosteron-iso-caproate, Estradiol-benzoate-acetaat, Estradiol-cypionate-17b, Estradiol-di-acetate, Estradiol-valerate, Estradiol-enanthate-17b, Estradiol-17-propionate at the level of 20 ng/g (CC β). The following compounds can not be analysed with this method at the level 20 ng/g, Testosteron-decanoate and Testosteron-cypionaat.

2 Apparatus

1. Automatic pipettes 1-10 ml, 10-100 μ l, 20-200 μ l, 2.0-20 μ l, 100-1000 μ l (Rainin)
2. Varifuge 3.0R, centrifuge (Heraeus).
3. Glass test tubes (10 ml) (Cardinal health, Zutphen).
4. Glass test tubes (15 ml) (Cardinal health, Zutphen).
5. Electric waterbath with thermostat adjustable $\pm 5^{\circ}\text{C}$ and nitrogen facility (TurboVap, Zymark).
6. Vortex (Vibrofix FV1).
7. Vacmaster 20 positions (Varian)
8. Shaker (Edmund Buhler)
9. Moulinex kitchen mixer (Moulinette)
10. Ultrasonic bath (Branson 521)
11. Thermos bottle
12. Freezer (Forma Scientific -80C)
13. LC-MS-MS system, Waters Ultima
14. Waters HPLC Alliance System

Reference to a company and/or product is for purpose of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute for Public Health and the Environment (RIVM) to the exclusion of others which might also be suitable.

3 Safety and environment

WARNING: The following chemicals are used in this SOP

ETHYLACETATE: Highly flammable, exposure to ethylacetate can occur when breathing contaminated air or when drinking or touching the substance or water containing it. Breathing Ethylacetate can cause dizziness, fatigue, and headaches. Ingesting ethylacetate: over long periods of time may damage your liver and kidneys. It can cause sores if large amounts touch your skin.

AMMONIA: Exposure to high levels of ammonia can cause irritation and serious burns on the skin and in the mouth, throat, lungs, and eyes. At very high levels, ammonia can even cause death.

METHANOL: Highly flammable Harmful by inhalation, in contact with skin and if swallowed irritating to eyes. Keep away from sources of ignition. Wear suitable protective clothing and gloves

ACETONITRILE: Highly flammable Harmful by inhalation, in contact with skin and if swallowed irritating to eyes. Keep away from sources of ignition. Wear suitable protective clothing and gloves

All rules regarding safety at RIVM can be found on the Intranet Insite (KAM-regels)

Waste disposal is described in ARO/SOP 487 "Procedure voor het afvoeren van afval binnen ARO".
All rules regarding waste disposal at RIVM can be found on the Intranet Insite (Regeling gevaarlijk afval)

4 Chemicals and reagents

1. Methanol (HPLC supra gradient)
2. Acetonitrile(HPLC supra gradient)
3. Ethylacetate (analytical grade)
4. Bakerbond (octadecyl C18 500mg, 40 µm, 6 ml) J.T Baker
5. HPLC column Zorbax Eclipse XDB C18 2.1*100mm 3.5 µm (Agilent)
6. Water (Milli-Q® Ultrapure Water)
7. Ammonia 25% (suprapur)
8. Thermos bottle (Dewar vessel)
9. Fluid Nitrogen

Reagents

Methanol/water 40:60 mix 300 ml water with 200 ml methanol

LC-Eluens A Methanol/water 40:60 mix 600 ml water with 400 ml methanol add 30 µl ammonia

LC-Eluens B Methanol 100 % add 30 µl ammonia

Standards and Internal Standards:

All the components (standards) are diluted from a stock solution 1 mg/ml by taking 100 µl of this solution and dilute with 9900 µl Ethanol 100 % (vortex for 30 seconds) = 10 ng/µl.

For mix 1 = take 500 µl from each 10 ng/µl solution (Table 1 mix 1), evaporate to dryness, dissolve in 5000 µl Ethanol (vortex for 30 seconds) = 1.0 ng/µl mix 1.

For mix 2 = take 500 µl from each 10 ng/µl solution (Table 1 mix 2), evaporate to dryness, dissolve in 5000 µl Ethanol (vortex for 30 seconds) = 1.0 ng/µl mix 1.

For internal standard: take 500 µl from the 10 ng/µl solution (Table 1 mix 3), evaporate to dryness, dissolve in 5000 µl Ethanol (vortex for 30 seconds) = 1.0 ng/µl internal standard.

Make sub sequential 1 to 10 dilutions of this standards and internal standard as needed

Table 1 Compound list

	Name Mix 1	Mass (g/mol)	MRM ESI +
1	Norclostebol acetaat	350.0	351.2 > 291.20
2	Testosteron-propionate	344.5	345.2 > 109.17
3	Testosteron-acetate	330.2	331.5 > 289.40
4	Nortestosteron-acetate	316.4	317.2 > 257.50
5	Nortestosteron-phenyl-propionate	406.2	407.3 > 105.2
6	Testosteron-benzoate	392.5	393.4 > 253.20
7	Nortestosteron-laurate	456.4	457.2 > 257.20
8	Testosteron-decanoate	442.0	443.4 > 97.39

9	Testosteron-iso-caproate	386.0	387.4 > 109.19
10	Estradiol-benzoate	376.0	377.5 > 105.05
11	Dehydro-testosteron-un-decylenate	452.2	453.3 > 135.20
12	Estradiol-benzoate-acetaat	418.0	419.0 > 105.20
13	Testosteron Cypionaat	412.6	413.2 > 97.20
14	Estradiol-di-acetate	378.6	374.3 > 255.26

	Name Mix 2	Mass (g/mol)	MRM ESI -
1	Clobetasol propionaat	467.0	465.2 > 429.30
2	Estradiol-cyprionate-17b	396.6	395.2 > 253.20
3	Estradiol-valerate	356.5	355.2 > 253.30
4	Estradiol-enanthate-17b	384.6	383.2 > 253.20
5	Estradiol-17-propionate	328.5	327.5 > 253.20
6	Estradiol-acetate-17b	314.4	313.3 > 253.20

	Name	Mass (g/mol)	MRM ESI +
	Internal standard (mix 3)		
1	testosteron-phenyl-propionate	420.2	421.2 > 105.25

5 Procedure

Washing

Before the analysis the hair samples have to be washed, cut, and homogenized, put the complete sample in a 500 ml plastic jar.

1. Fill the jar with water, ($\pm 37^\circ\text{C}$) close and shake for 15 minutes with a shaker
2. Lift the top a bit and pour the water out the jar, repeat this step till the water stays clear.
3. In most cases 7 to 10 times, in all cases wash at least 7 times.
4. Dry the hair with a oven at $\pm 55^\circ\text{C}$.

Grinding

1. Pour liquid nitrogen in a thermos bottle.
2. Dip the hair in the liquid nitrogen and put the hair in a Moulinex kitchen mixer.
3. Pour some liquid nitrogen in the mixer and mix. Take the mixer and shake it during the run for about 15 seconds. If the sample isn't mixing, pour a bit more fluid nitrogen in the mixer.
4. Pour the sample in a clean jar, and leave it for a hour with room temperature. The liquid nitrogen will evaporate.

(Alternative for grinding is cutting the hair with a scissor in pieces of ± 1.0 centimeter)

Sample preparation

1. Weigh 0.5 gram of the grinded hair in a glass 10 ml tube.
2. Add 5 ng Testosteron-phenyl-propionate to each sample
3. Add 6 ml Methanol 100%

4. Put the samples for 2 hours in a ultrasonic bad
5. Centrifuge 10 min at 4000 rpm
6. Place the samples in freezer (-80°C) for 1 hour
7. Transfer supernatant in new 15 ml tube
8. Add 12 ml water
9. Mix with a pipette

Solid Phase Extraction

with Baker Bond Octadecyl 40 µm, 500mg, 6ml columns (7020-26)

Use suitable SPE-rack

Conditioning

10. Activate SPE columns with 5 mL Acetonitrile. Do not allow column to run dry
11. Activate SPE columns with 5 mL methanol. Do not allow column to run dry
12. Activate SPE columns with 5 mL Water. Do not allow column to run dry

Sampling and washing:

13. Apply sample
14. Wash with 5 ml water
15. Wash with 5 ml methanol/water 40:60 v/v

Elution:

16. Elute with 4 mL of acetonitrile in glass tube
17. Elute with 4 mL of ethyl acetate in the same glass tube

Reconstitution:

18. Evaporate the eluate to dryness at 50-55 °C in a TurboVap
19. Add 200 µL MeOH/water 40:60 v/v-% + 0.003% Ammonia, mix on Vortex and put in ultrasonic bath for 5 min.
20. Transfer the MeOH/water 40:60 v/v-% + 0.003% Ammonia in a eppendorf vial
21. Centrifuge 10 min at 14000 rpm
22. Transfer the solution into an injection vial. The sample is ready for injection.

Calibration curve

23. Construct a calibration curve by pipet resp. 0, 0.5, 1.0, 2, 4, 6, 8, and 10 ng from Mix 1 +2 and 5 ng Testosteron-phenyl-propionate in a glass tube.
24. Evaporate the eluate to dryness at 50-55 °C in a TurboVap
25. Reconstitute in 200 µl MEOH/water 40:60 v/v-% + 0.003% Ammonia

LC-MSMS

LC: Waters Waters HPLC Alliance System, MSMS: Micromass QUATRO Ultima

LC Column: Zorbax Eclipse XDB-C18 Narrow Bore RR 2.1 * 100 MM 3.5 Micron (Agilent)

Temperature column thermostat: 60°C

Autosampler temperature: 20 °C

Solvent A: MEOH/water 40:60 + 0.003% Ammonia

Solvent B: MEOH 100% + 0.003% Ammonia

Injection volume: 40 µL

Flowrate: 0.25 mL min⁻¹

Table 2. Gradient LC-system, total runtime: 20 minutes

	Time (min.)	Flow	Eluens A	Eluens B
1	0.0	0.25	70	30
2	8.0	0.30	0	100
3	13.0	0.30	0	100
4	13.5	0.30	70	30
5	20.0	0.25	70	30

Table 3. Tune parameters product scan measurements

Ionization mode	ES+	ES-
Capillary (kV)	3.5	3.5
Cone voltage (V)	50	50
RF lens 1	0.3	0.3
Aperture (V)	0.0	0.0
RF lens 2	0.3	0.3
Source temperature (°C)	30	30
Desolvation temperature (°C)	350	350
Cone gas flow (L/Hr)	100	600
Desolvation gas flow (L/Hr)	100	600
LM 1 resolution	14.0	14.0
HM 1 resolution	14.0	14.0
Ion energy 1	1.0	1.0
Entrance	-1	-1
Collision	0	0
Exit	2	2
LM 2 Resolution	15.0	15.0
HM 2 Resolution	15.0	15.0
Ion Energy 2	1.0	1.0
Multiplier	650	650
Collision cell pressure (mbar)	Off	Off
Solvent Delay	Mix 1	Mix2
Solvent Delay Start :	0-4 min	0-4 min
Solvent Delay End :	16-20 min	12-20 min

Table 4 MS-conditions

Compound Mix 1	Precursor (M/Z)	Product (M/Z)	Dwell (s)	Coll Energy (eV)	Delay (s)
Function 1 MRM, Time 5.0 to 10.0 min					
Norclostebol acetaat	351.20	291.20	0.050	20.00	0.050

testosteron-propionate	345.50	109.17	0.050	20.00	0.050
testosteron-acetate	331.20	289.40	0.050	23.00	0.050
nortestosteron-acetate	317.20	257.50	0.050	18.00	0.050
Function 2 MRM, Time 6.0 to 12.0 min					
nortestosteron-phenyl-propionate	407.30	105.20	0.050	20.00	0.050
testosteron-benzoate	393.50	253.20	0.050	20.00	0.050
testosteron-iso-caproate	387.40	109.19	0.050	28.00	0.050
estradiol-benzoate	377.50	105.05	0.050	20.00	0.050
estradiol-di-acetate	374.30	255.26	0.050	15.00	0.050
Function 3 MRM, Time 9.0 to 16.0 min					
nortestosteron-laurate	457.20	257.20	0.050	20.00	0.050
dehydro-testosteron-un-decylenate	453.30	135.20	0.050	20.00	0.050
testosteron-decanoate	443.40	271.32	0.050	20.00	0.050
estradiol-benzoate-acetaat	419.20	105.20	0.050	20.00	0.050
Testosteron Cypionaat	413.30	107.20	0.050	28.00	0.050
Function 2 MRM, Time 6.0 to 12.0 min					
Internal standard					
testosteron-phenyl-propionate	421.20	105.25	0.050	20.00	0.050

Compound Mix 2	Precursor (M/Z)	Product (M/Z)	Dwell (s)	Coll Energy (eV)	Delay (s)
Function 2 MRM, Time 4.5 to 12.0 min					
clobetasol propionaat	465.20	429.30	0.050	16.00	0.050
estradiol-cyprionate-17b	395.20	253.30	0.050	30.00	0.050
estradiol-valerate	355.20	253.20	0.050	40.00	0.050
estradiol-enanthate-17b	383.20	253.20	0.050	28.00	0.050
estradiol-17-propionate	327.50	253.13	0.050	33.00	0.050
estradiol-acetate-17b	313.30	253.30	0.050	30.00	0.050

7 Validation and Measurement uncertainty

The method described in this SOP was validated conform Commission Decision 2002/657. See Annex 1 for an overview of the validation results.

8 Quality control

Take 4 blank samples of hair and a chemical blank for the quality control samples.

Add 5 ng Testosteron-phenyl-propionate to each sample.

For the blank control samples :

1 = blank

2 = spike with 2.5 ng mix 1 and mix 2

3 = spike with 5.0 ng mix 1 and mix 2

4 = spike with 10.0 ng mix 1 and mix 2

5 = 0.5 mL of water instead of 0.5 g hair and treat as sample.

10 Relating documents

SOP ARO/501, Method validation using ResVal, revision 2, 21april 2004, RIVM.

Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

Annex

Overview of validation results

Since there were no relevant isotope labeled internal standards were available the method can not be categorized as a quantitative method, and is therefore a qualitative screening method. According to the 2002/657/EC the following validation parameters have to be determined: Selectivity/specificity and the Detection capability (CC_B).

Validation was performed by analysing 20 hair samples. The samples were also spiked at 5 and 20 ng/g. The selectivity/specificity is demonstrated by the analysis of the blank samples. There should be no signal present at the expected retention time of the analyt. All samples were from different animals. By doing so the matrix effect and indirect the ruggedness of the method is determined. The validation was performed on different days.

To determine the CC_B in at least 95% of the spiked samples the analyte of interest should be detected (≤ 5% false compliant results).

In the table below an overview of the validation result is given

Table 5. Overview of the validation results (n=20 blank, and the same 20 samples spiked at 5 and 20 ng/g)

Compound	Blank samples	Spiked samples 5 ng/g	Blank samples	Spiked samples 20 ng/g	(
Norclostebol acetaat	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Testosteron-propionate	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Testosteron-acetate	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Nortestosteron-acetate	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Nortestosteron-phenyl-propionate	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Testosteron-benzoate	20 compliant	13 non-compliant (65%)	20 compliant	20 non-compliant (100%)	(
Nortestosteron-laurate	1 compliant	18 non-compliant (90%)	20 compliant	20 non-compliant (100%)	(
Testosteron-decanoate	5 compliant	18 non-compliant (90%)	13 compliant	20 non-compliant (100%)	(
Testosteron-iso-caproate	20 compliant	7 non-compliant (35%)	20 compliant	20 non-compliant (100%)	(
Estradiol-benzoate	1 compliant	19 non-compliant (95%)	1 compliant	20 non-compliant (100%)	(
Dehydrotestosteron-un-decylenate	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	(
Estradiol-benzoate-acetaat	9 compliant	19 non-compliant (95%)	20 compliant	20 non-compliant (100%)	(
Testosteron-cypionaat	13 compliant	16 non-compliant (80%)	17 compliant	17 non-compliant (85%)	(

Estradiol-di-acetate	10 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	
Clobetasol propionaat	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	
Estradiol-cyprionate-17b	19 compliant	0 non-compliant (0%)	20 compliant	20 non-compliant (100%)	
Estradiol-valerate	18 compliant	17 non-compliant (85%)	20 compliant	20 non-compliant (100%)	
Estradiol-enanthate-17b	8 compliant	17 non-compliant (85%)	20 compliant	20 non-compliant (100%)	
Estradiol-17-propionate	16 compliant	12 non-compliant (60%)	20 compliant	20 non-compliant (100%)	
Estradiol-acetate-17b	20 compliant	20 non-compliant (100%)	20 compliant	20 non-compliant (100%)	

n.d. not detectable

Depending on the number of non-compliant detected (>95%) the CC_B is set on 5 or 20 ng/g.

Documentbeheer

Algemeen

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