Residue analysis of alfa- and beta-trenbolone in bovine urine, meat, liver and fish

Document:	SOP ARO/519 versie 1	**Auteur:**	Frederike Braam - van Tricht (Onderzoeksmedewerker)
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1 Introduction

Summary

This method describes the analysis of 17α- and 17β-trenbolone (17-hydroxy-19-norandrosta-4, 9, 11-trien-3-one) in samples of urine, meat, liver and fish.

Meat and fish samples are digested with protease. Urine and liver samples are hydrolysed with suc d'Helix Pomatia.

Clean-up is based on liquid-liquid extraction and immunoaffinity chromatography (IAC).

The analytes are separated by liquid chromatography and detected with electrospray triple quad mass spectrometry (LC-MSMS).

2 Apparatus

Besides standard laboratory glassware and equipment is used:

- Automatic pipettes 1-10 ml, 10-100 µl, 20-200 µl, 2.0-20 µl, 100-1000 µl (Rainin).
- Moulinette S (Moulinex)
- Polypropylene test tubes 50 ml, 30/115 mm (Greiner Bio-One, art. 210296)
- pH indicator paper: pH 1-10 (Merck, 9526).
- pH-meter, Schott pH-meter CG 837.
- Centrifuge Heraeus, Varifuge 3.0 R with swing out rotor 5315 (Heraeus).
- Disposable pasteur pipettes (Elkay)
- Glass tubes 10 ml (Renes, custom made, 31.00.50).
- Vortex-Genie 2 (Scientific Industries)
- Electric waterbath with thermostat adjustable ± 5°C and nitrogen facility (TurboVap, Zymark).
- Thermostatic heating block (Thermolyne 17600 Dri-Bath) with nitrogen facility 50°C.
- Ultrasonic waterbath Bransonic 521
- LC-vials and inserts, 100 µl
- HPLC column Zorbax Eclipse XDB C_{18} 2.1*100mm 3.5 µm (Agilent)
- Waters HPLC Alliance 2695 System.
- LC-MS-MS system, Waters Ultima.
  - Temperature column thermostat: 60ºC
  - Autosampler temperature: 4ºC
  - Eluens A: 10:90 v/v% Methanol : Ammoniumacetate 5mM (see Chemicals)
  - Eluens B: 90:10 v/v% Methanol : Ammoniumacetate 5mM (see Chemicals)
  - Injection volume: 10 µl
  - Flow: 0.3 ml/min.
  - Solvent delay: 0-4 min., 10-15 min.

Table 1. Gradient LC-system, total runtime: 15 minutes

	Time (min.)	Eluens A	Eluens B

1	0.0	60	40
2	10.0	20	80
3	10.1	0	100
4	12.0	0	100
5	12.5	60	40
6	15.0	60	40

Table 2. Tune parameters product scan measurements

Ionization mode	ES+
Capillary (kV)	3.50
Cone (V)	50
RF lens 1	30
Aperture (V)	0.5
RF lens 2	0.5
Source temperature (°C)	110
Desolvation temperature (°C)	350
Cone gas flow (L/Hr)	109
Desolvation gas flow (L/Hr)	825
LM 1 resolution	12.5
HM 1 resolution	12.5
Ion energy 1	0
Entrance	20
Collision energy	20
Exit	10
LM 2 Resolution	14.5
HM 2 Resolution	14.5
Ion Energy 2	7.1
Multiplier	650
Collision cell pressure (mbar)	2.70e-3

Table 3. *MRM transitions measured.*

Component	**$[M+H]^+$**	**Transition 1**	**Transition 2**	**Retention time (min.)**
17α-Trenbolone	271.3	253.3	199.11	6.1

17β-Trenbolone	271.3	253.3	199.11	5.2
17β-Trenbolone-D3	274.4	256.4	-	5.2

3 Safety and environment

Health and safety

Standard RIVM health and safety procedures have to be followed. These procedures can be found on the RIVM intranet.

Waste disposal

Standard RIVM procedures have to be followed Use the appropriate waste containers for disposal of organic solvents and samples of animal origin.

4 Chemicals and reagents

4.1 Chemicals

All listed chemicals are of p.a. quality or better, unless stated otherwise.

Any reference of a product and/or manufacturer is only for information and identification and is not a recommendation of the mentioned product and/or manufacturer or an exclusion by the National Institute Of Public Health And The Environment (RIVM) of other products and/or manufacturers which might also suitable.

- Acetic acid (Baker, 6152)
- Acetic acid 1M. Dilute 6.0 g acetic acid with water to a final volume of 100 ml
- Sodium acetate (Merck, 6268)
- Acetate buffer 2M, pH 5.2. Dissolve 25.2 g acetic acid and 129.5 g sodium acetate in 800 ml of water. Adjust the pH with acetic acid or with sodium hydroxide at 5.2 ± 0.1 and add water to a final volume of 1000 ml
- Sodium hydroxide (NaOH) (Baker, 0402)
- Sodium hydroxide 1M. Dissolve 4.0 g sodium hydroxide in 100 ml water
- Ammonium acetate (Baker, 0390)
- Hydrochloric acid (HCl), 37% solution (Merck, 317)
- TRIS (Tris(hydroxymethyl)-amino-methane) (Merck, 8382)
- TRIS buffer 0.1M, pH 9.5. Dissolve 12.1 g TRIS in 800 ml of water. Adjust the pH to 9.5 ± 0.1 and add water to a final volume of 1000 ml
- Heptane (Biosolve, 08050502)
- n-Butanol (Merck, 1990)
- Methanol (Biosolve, 13686)
- Ethanol absolute (Baker, 8006)
- TBME (tert-Butyl methyl ether) (Biosolve, 13890602)

- *Suc d'Helix Pomatia juice (S.H.P.)*, Enzymatic activities, β-glucuronidase: 100.000 FU/ml, Sulfatase: 1.000.000 FU/ml, Industrie Biologique, France, code IBR 2134
- Subtilisin (Protease), (Sigma, P-5380)
- Extract solution: heptane/butanol (80:20 v/v%). Add 200 ml butanol to 800 ml heptane
- IAC-eluting solution 70:30 v/v% ethanol:water. Add 300 ml water to a final volume of 1000 ml
- IAC-columns for trenbolone (Randox®, TB 2186, containing 5 columns, 20 x concentrated wash-buffer and 5 x concentrated storage-buffer)
- Column Wash buffer concentrate (Randox® 0EA06, only available with columns)
- IAC wash buffer: Dilute 50 ml Column Wash buffer concentrate with water to a final volume of 1000 ml
- Column Storage buffer concentrate (Randox® 0EA05, only available with columns)
- IAC storage buffer: Dilute 200 ml Column Storage buffer concentrate with water to a final volume of 1000 ml
- Ammoniumacetate 5mM. Dissolve 0.39 g ammonium acetate in 1000 ml water
- Eluens A: 10:90 v/v% Methanol : Ammoniumacetate 5mM. Add 100 ml methanol to 900 ml ammoniumacetate 5mM
- Eluens B: 90:10 v/v% Methanol : Ammoniumacetate 5mM. Add 100 ml ammoniumacetate 5mM to 900 ml methanol
- Milli-Q-water from Milli-Q-water apparatus
- 17α-Trenbolone (0.1 mg) ampoule 91M3359, RIVM, Bilthoven, The Netherlands
- 17β-Trenbolone (0.1 mg) ampoule 91M4365, RIVM, Bilthoven, The Netherlands
- 17β-Trenbolone-D3 (0.1 mg) ampoule 2005C0003, RIVM, Bilthoven, The Netherland

4.2 Preparation and storage of standard and internal standard solutions

- Stock solutions of each component, containing 1 mg/ml of the component

  Stock solutions of the components containing 1 mg/ml are prepared in ethanol and stored in an amber vial at –20°C. These solutions are stored for a maximum period of 5 years.

- Working solutions of each component (including internal standards), containing 10 ng/µl

  Working solutions containing 10 ng/µl are prepared in ethanol (in 2 steps of 1:10) by diluting 1 ml of the stock solution in a volumetric flask of 10 ml. These solutions are stored for a maximum period of 12 months.

- Working solutions of each component (including internal standards), containing 1 ng/µl

  Working solutions containing 1 ng/µl are prepared in ethanol by diluting 1 ml of the working solution in a volumetric flask of 10 ml. These solutions are stored for a maximum period of 12 months.

- Mixture of 17α-Trenbolone and 17β-Trenbolone, containing 0.1 ng/µl of each component

  This mixture is prepared in ethanol by diluting 1 ml of the working solutions (containing 1 ng/µl) in a volumetric flask of 10 ml.

  This solution is stored for a maximum period of 12 months.

- Mixture of 17α-Trenbolone and 17β-Trenbolone, containing 0.01 ng/µl of each component

  This mixture is prepared in ethanol by diluting 1 ml of the working solutions (containing 0.1 ng/µl) in a volumetric flask of 10 ml.

  This solution is stored for a maximum period of 12 months.

4.3 Controls and blanks

Calibration standards and a set of spiked samples are prepared by adding the specified volumes of the calibration standard mixture.

Table 4. *Preparation of a calibration curve and spiked control samples*

According to concentration	**Calibration curve** Pipette directly in 2 ml vial	**Spiked samples** Add to 5 ml/g sample material (urine, meat, liver, fish)
6 ppb	30 µl 1.0 ng/µl	-
5 ppb	25 µl 1.0 ng/µl	25 µl 1.0 ng/µl
3 ppb	150 µl 0.1 ng/µl	-
2 ppb	100 µl 0.1 ng/µl	100 µl 0.1 ng/µl
1.5 ppb	-	75 µl 0.1 ng/µl
1 ppb	50 µl 0.1 ng/µl	50 µl 0.1 ng/µl
0.5 ppb	25 µl 0.1 ng/µl	25 µl 0.1 ng/µl
0.2 ppb	100 µl 0.01 ng/µl	-
0 ppb	-	-

Each standard and sample is also spiked with 1 ng/µl(g) (= 50 µl) internal standard

5 Procedure

5.1 Preparation of a primary extract

If a laboratory sample is considered suitable for analysis (adequate sample size, proper storage history and representative for the study) the first step in the analytical procedure is the preparation of a primary extract, including procedures for deconjugation or digestion.

- **Sample pretreatment of urine.**

  From the laboratory sample a test portion of 5.0 ml urine is pipetted into a 50 ml plastic centrifuge tube. 5 ng Internal standard, 17β-Trenbolone-D3 (corresponding to 1 ng/ml) is added.

  For deconjugation of glucuronide- and sulfate-conjugates, adjust the pH to 5.2 with 2 ml acetate-buffer. The pH is checked with pH paper. When necessary the pH is adjusted to 5.2 with diluted

acetic acid or NaOH. Add 75 µl *suc d'Helix Pomatia* and vortex. The sample is incubated and shaken during 3 hours at 37°C.

- **Sample pretreatment of meat, fish and liver**

Meat, fish and liver samples are crushed and homogenized in a Moulinette S. A homogeneous test portion of 5.0 g is weighed into a 50 ml centrifuge tube. 5 ng Internal standard, 17β-Trenbolone-D3 (corresponding to 1 ng/g) is added.

Meat and fish

Digestion: add 10 ml 0.1M Tris-buffer pH 9.5 and 5 mg subtilisin. Vortex for 30 seconds and incubate the sample for two hours at 50°C.

Liver

Hydrolysis: add 10 ml 2 Mol/l acetate buffer pH 5.2 and 50 µl of *suc d'Helix Pomatia* are added. The pH is checked with pH paper. When necessary the pH is adjusted to 5.2 with diluted acetic acid or NaOH.

Overnight incubation at 37°C.

5.2 Liquid/liquid extraction

- **Urine samples**

To the deconjugated urine sample (5.1) 10 ml heptane:butanol (80:20 v/v%) is added.

Vortex for 1 minute. Centrifuge during 10 minutes at 3600 rpm.

The organic layer is transferred to a clean glass tube with a disposable pasteur pipette and evaporated to dryness under a stream of nitrogen in a heating block or electric water bath at 55°C (Turbovap).

The dry residue is dissolved in 250 µl of ethanol and 5 ml of water. The samples are further processed following 5.3.

- **Meat, fish and liver samples**

To the digested samples of meat and fish (5.1) and the hydrolysed samples of liver (5.1), 2 ml HCl 37% and 10 ml TBME are added.

Vortex for 1 minute. Centrifuge during 10 minutes at 3600 rpm.

The organic layer is transferred to a clean glass tube with a disposable pasteur pipette and evaporated to dryness under a stream of nitrogen in a heating block or electric water bath at 55°C (Turbovap).

For defatting: dissolve the dry residue in 5 ml of 30% of methanol:water v/v% and wash twice with 3 ml heptane (each time centrifuged for 3 minutes at 3600 rpm). The heptane layer is discarded. The methanol-water layer in the tube is further processed following 5.3.

5.3 Immuno affinity Chromatography (IAC)

To allow detection and identification of low concentrations of analysis in extracts of biological samples adequate extract clean-up is necessary. One of the most powerful-techniques, immuno affinity chromatography (IAC), is used for these samples.

During analyses the IAC-columns should not run dry.

- **Urine, meat, fish and liver**

  The IAC-column is conditioned by washing it twice with 5 ml of wash-buffer. The total sample mixture (5.2) is applied to the IAC-column.

  After sample application the column is washed twice with 5 ml of wash-buffer and with 5 ml of water and eluted with 5 ml of IAC-eluting solution. The eluate is evaporated to dryness under a stream of nitrogen in a heating block or electric water bath at 55°C. The residue is dissolved in 500 µl of ethanol and further processed in 5.4.

- **Storage of the IAC-column**

  The IAC columns are washed with 5 ml of IAC-eluting solution and three times with 5 ml of storage-buffer, leaving the last 5 ml in the column. The columns are stored at 4°C.

5.4 Detection by LC-MSMS.

- **Preparation of urine, meat, fish and liver samples for LC-MSMS**

  The residue obtained at 5.3 is transferred to a 2 ml vial. The ethanol is evaporated to dryness under a stream of nitrogen in a heating block at 50°C. After evaporation 25 µl methanol:water (40/60 v/v%) is added. The vial is vortexed for 30 seconds. The mixture is transferred into an insert and placed in the autosampler for LC-MSMS analysis.

6 Calculation

The selected area of the standard is divided by the selected area of the internal standard for the combination of compound and internal standard. The ratio is the response variable.

A calibration curve is constructed by linear curve fitting using least squares linear regression calculation.

Unknown concentrations are calculated by interpolation.

7 Validation and Measurement uncertainty

The method described in this SOP was validated for urine conform SOP ARO/475. The validation level used for the validation was 1 ng/ml.

A summary of the validation results is given in the table below.

The summaries of validation results for different compounds are stored on a central location, to be found using CB\AMAP keyword *validatie.*

Table 5. *Performance characteristics of the method*

Compound	transition	internal standard	CCα	CCβ	Accuracy at 1 ng/ml (%)	U (%)
17α-trenbolone	271.3 > 253.3	17β-trenbolone-D3	0.04	0.07	108.0	24
	271.3 > 199.1		0.05	0.08	109.7	22
17β-trenbolone	271.3 > 253.3	17β-trenbolone-D3	0.03	0.05	104.8	8
	271.3 > 199.1		0.03	0.06	106.3	14

To determine if the validation results of urine samples are representative for liver, meat and fish samples, spiked samples (1 ng/ml or 1 ng/g) of each matrix were analysed and calculated (n=5).

To control this process, also 5 different samples of urine were analysed.

To compare the results of the different matrices, the following statistic tests were used:

- t-test for the comparison of means (accuracy)
- F-test for the comparison of standard deviations (repeatability)

Table 6. *t-test and F-test results for 17α-trenbolone, 1 ng/ml or 1 ng/g*

matrix	urine	Liver	meat	fish
n	5	5	5	5
Average (ppb)	1.028	0.489	2.190	1.438
SD (ppb)	0.038	0.096	0.838	0.068
$t_{13\ (0,05)}$ critical	2.16	2.16	2.16	2.16
t calculated	0.38	9.82	4.41	7.36
conclusion t-test	no significant difference	significant difference *)	significant difference *)	significant difference *)
F critical	$F_{9,4}$ = 8.905	$F_{9,4}$ = 8.905	$F_{4,9}$ = 4.718	$F_{9,4}$ = 8.905
F calculated (F≥1)	8.035	1.257	60.950	2.481
conclusion F-test	no significant difference	no significant difference	significant difference **)	no significant difference

Table 7. *t-test and F-test results for 17β-trenbolone, 1 ng/ml or 1 ng/g*

matrix	urine	liver	meat	fish
n	5	5	5	5
Average (ppb)	1.150	0.960	1.176	1.086
SD (ppb)	0.048	0.057	0.080	0.010

t13 (0,05) critical	2.16	2.16	2.16	2.16
t calculated	5.58	2.81	5.08	3.54
conclusion t-test	significant difference ***)	significant difference ***)	significant difference ***)	significant difference ***)
F critical	F9,4 = 8.905	F9,4 = 8.905	F4,9 = 4.718	F9,4 = 8.905
F calculated (F≥1)	1.816	2.555	4.929	13.381
conclusion F-test	no significant difference	no significant difference	significant difference ****)	significant difference *****)

*) tested method less accurate for this component then validated method

**) tested method less precise for this component then validated method, due to the lack of deuterated α-trenbolone-D3

***) tested method gives a significant difference compared to the validated method. However, due to the small standard deviation the results are acceptable at this level

****) although significant different, the standard deviation is still very small and acceptable for this level

*****) the tested method is *more* precise for this component then validated method

8 Quality control

- **Calculation**

Quantification is only valid if:

- The maximum of the signal originating from the analyte has an S/N ratio >3.
- In the blank control samples all the internal standards are visible.
- In the spiked control samples all components are visible (S/N ratio >10 for internal standards, >6 for the non-deuterated compounds).

- **Process control**

The process is controlled by spiking blanc samples with a known amount of standard.

9 Responsibilities

Technician: responsible for the analysis and registration of abnormalities

Study director: authorize in case the procedure differs from the normal situation

10 Relating documents

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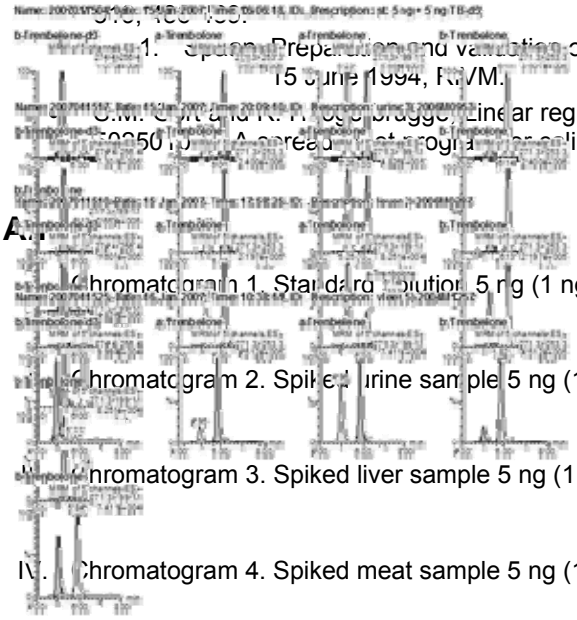
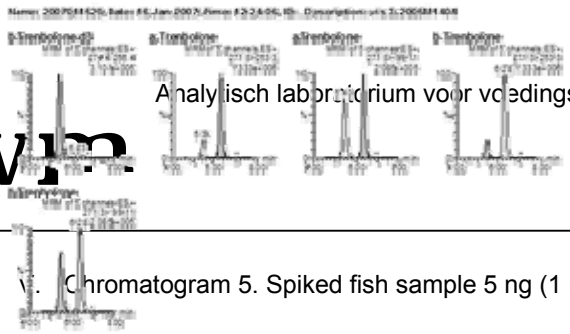


A. Chromatogram 1. Standard solution 5 ng (1 ng/ml)

Chromatogram 2. Spiked urine sample 5 ng (1 ng/ml)

Chromatogram 3. Spiked liver sample 5 ng (1 ng/g)

Chromatogram 4. Spiked meat sample 5 ng (1 ng/g)



vi. Chromatogram 5. Spiked fish sample 5 ng (1 ng/g)

Documentbeheer

Algemeen

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Hyperlinks