Residue analysis of alfa- and beta-trenbolone in bovine urine, meat, liver and fish
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 C18 2.1*100mm 3.5 µm (Agilent)
- Waters HPLC Alliance 2695 System.
- LC-MS-MS system, Waters Ultima.
  - Temperature column thermostat: 60°C
  - Autosampler temperature: 4°C
  - 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>
<thead>
<tr>
<th>Time (min.)</th>
<th>Eluens A</th>
<th>Eluens B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>10.1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>12.0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>15.0</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2. Tune parameters product scan measurements

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

Table 3. MRM transitions measured.

<table>
<thead>
<tr>
<th>Component</th>
<th>[M+H]^+</th>
<th>Transition 1</th>
<th>Transition 2</th>
<th>Retention time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-Trenbolone</td>
<td>271.3</td>
<td>253.3</td>
<td>199.11</td>
<td>6.1</td>
</tr>
</tbody>
</table>
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)
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 $17\alpha$-Trenbolone and $17\beta$-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

<table>
<thead>
<tr>
<th>According to concentration</th>
<th>Calibration curve</th>
<th>Spiked samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ppb</td>
<td>30 µl 1.0 ng/µl</td>
<td>-</td>
</tr>
<tr>
<td>5 ppb</td>
<td>25 µl 1.0 ng/µl</td>
<td>25 µl 1.0 ng/µl</td>
</tr>
<tr>
<td>3 ppb</td>
<td>150 µl 0.1 ng/µl</td>
<td>-</td>
</tr>
<tr>
<td>2 ppb</td>
<td>100 µl 0.1 ng/µl</td>
<td>100 µl 0.1 ng/µl</td>
</tr>
<tr>
<td>1.5 ppb</td>
<td>-</td>
<td>75 µl 0.1 ng/µl</td>
</tr>
<tr>
<td>1 ppb</td>
<td>50 µl 0.1 ng/µl</td>
<td>50 µl 0.1 ng/µl</td>
</tr>
<tr>
<td>0.5 ppb</td>
<td>25 µl 0.1 ng/µl</td>
<td>25 µl 0.1 ng/µl</td>
</tr>
<tr>
<td>0.2 ppb</td>
<td>100 µl 0.01 ng/µl</td>
<td>-</td>
</tr>
<tr>
<td>0 ppb</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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, $17\beta$-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

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

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

<table>
<thead>
<tr>
<th>matrix</th>
<th>urine</th>
<th>Liver</th>
<th>meat</th>
<th>fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Average (ppb)</td>
<td>1.028</td>
<td>0.489</td>
<td>2.190</td>
<td>1.438</td>
</tr>
<tr>
<td>SD (ppb)</td>
<td>0.038</td>
<td>0.096</td>
<td>0.838</td>
<td>0.068</td>
</tr>
<tr>
<td>$t_{13(0.05)}$ Critical</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
</tr>
<tr>
<td>$t_{calculated}$</td>
<td>0.38</td>
<td>9.82</td>
<td>4.41</td>
<td>7.36</td>
</tr>
<tr>
<td>conclusion t-test</td>
<td>no significant difference</td>
<td>significant difference *)</td>
<td>significant difference *)</td>
<td>significant difference *)</td>
</tr>
<tr>
<td>F critical</td>
<td>$F_{9,4} = 8.905$</td>
<td>$F_{9,4} = 8.905$</td>
<td>$F_{4,9} = 4.718$</td>
<td>$F_{9,4} = 8.905$</td>
</tr>
<tr>
<td>F calculated (F≥1)</td>
<td>8.035</td>
<td>1.257</td>
<td>60.950</td>
<td>2.481</td>
</tr>
<tr>
<td>conclusion F-test</td>
<td>no significant difference</td>
<td>no significant difference</td>
<td>significant difference **)</td>
<td>no significant difference</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>matrix</th>
<th>urine</th>
<th>liver</th>
<th>meat</th>
<th>fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Average (ppb)</td>
<td>1.150</td>
<td>0.960</td>
<td>1.176</td>
<td>1.086</td>
</tr>
<tr>
<td>SD (ppb)</td>
<td>0.048</td>
<td>0.057</td>
<td>0.080</td>
<td>0.010</td>
</tr>
</tbody>
</table>
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 blank 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

References

• H.J. van Rossum et al, Multi residue analysis anabolic agents, SOP ARO/113.Revision 4, 21 January 1997, RIVM.
• A.A.M. Stolker, Procedure for the validation of analytical methods, SOP ARO/425. revision 1, 28 April 1997, RIVM.

• Report: European Commission Decision laying down requirements for analytical methods to be used for detecting certain substances and residue thereof in live animals and animal products according to Council Directive 96/256/EC. (Revision of Commission Decision 93/256/EC)


• N. Haagsma, G. Ellen, W.G. De Ruig, and R.W. Stephany (1991), Begrippen bij de bepaling van residuen in voedingsmiddelen van dierlijke oorsprong. Overleggroep Residu-Analyse (ORA), Werkgroep Kwaliteit Analyse, De Ware(n) Chemicus, 21, 82-95.

• G. Maghuin-Rogister, G. van Vyncht, P. Gaspar (1996), Université de Liège,
  S.O.P. 1, Extraction and purification method for Trenbolone 17α et 17β determination in liver by GC-MS,
  S.O.P. 2 Anabolic steroid purification on immunoaffinity column (I.A.C.) Analytical procedure,
  S.O.P. 3 GC-MS-Trenbolone 17α and 17β Analytical procedure.


• Heitzman, Chemical and physical data for residue substances (1994), page 9/1 and 9/15.

• Livestock Control BV, Wijk bij Duurstede, Holland (1992), Ridascreen Trenbolone assay rs 2601.


1. Spaan, Preparation and validation of reference standards, S.O.P. ARO/374, revision 0
15 June 1994, RIVM.


Annex

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

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

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

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

Algemeen

Invoerdatum: 13 april 2007
Wijzigingsdatum: 1 juni 2007
Controledatum: 9 juli 2012
Publicatiedatum: 11 juli 2007

Wijzigingen ten opzichte van vorige versie:
Vervangt SOP ARO/443 in verband met omzetting naar HTML
Toegevoegd liver en fish

Beoordelaars

Marco Blokland (Onderzoeker)
Saskia Sterk (afdelingshoofd)

Hyperlinks