Analysis for diethylstilbestrol, anabolic agents in meat and urine with GC-MS(MS)
1 Introduction

Objective
This SOP describes a quantitative method of analysis of dienestrol, hexestrol, benzestrol and diethylstilbestrol in meat.

The method consists of the following steps: destruction of meat matrix, defattening, several liquid-liquid extractions and detection by gas-chromatography - mass spectrometry (GC-MS), ionisation mode is electron impact (EI).

Scope
The method is used to perform quantitative analysis of dienestrol, hexestrol, benzestrol and diethylstilbestrol in samples of meat and urine with GC-MS(MS)

The CCß is for dienestrol 0.14 ng/g, hexestrol 0.09 ng/g, benzestrol 0.14 ng/g, diethylstilbestrol 0.10 ng/g.

The measurement of uncertainty is for dienestrol 18.08%, for hexestrol 14.99%, for benzestrol 53.63.% and for diethylstilbestrol 48.90 %.

Definitions
The definitions of CCa, CCß and measurement of uncertainty can be found in SOP ARO/501.

Background information (figure 1)
Diethylstilbestrol (DES), together with dienestrol (DE), hexestrol (HEX) and benzestrol (BE) belongs to the group of stilbenes (figure 1). Increased weight gain and improved feed conversion after administration of DES has been observed in cattle, chicken and lambs. However, in the EC the use of anabolics has been banned, and especially DES has been banned in all countries due to its carcinogenic properties.

DES has two isomers trans-DES and cis-DES (see figure 1). These isomers are in dynamic balance, this balance depend on pH, light, temperature etc. For a standard DES in ethanol this balance is approximately trans-DES: cis-DES 9:1.

Figure 1. Structure stilbenes:
In this method trans-DES-D6 as internal standard is used, during clean-up isomerizes trans-DES-D6 to cis-DES-D6. This isomerization will also occur to trans-DES. In this SOP trans-DES and trans-DES-d6 are used for quantification.

2 Apparatus

For operating instructions and maintenance status files see ARO Cardbox data-bases.

Standard laboratory glassware and equipment is used, with in addition:

**Apparatus**

2.1 Guard-column: Lichrospher 100 RP18 endcapped 5 µm (Merck, 50962).
2.2 Analytical-column: Lichrocart 125-4 RP18 endcapped (Merck, 1.16855.0001).
2.3 Vortex (Vortex-genie, Wilton & Co).
2.4 Automatic pipettes (Gilson P100, P200, P1000 and P5000).
2.5 Refrigerated centrifuge (RC-3, Sorvall).
2.6 Test tubes, 10 ml, glass (55 mm x 11.5 cm) (Renes, RB55).
2.7 Electric water bath with thermostat adjustable with nitrogen facility (Turbo Vap).
2.8 Heating module thermostat adjustable (Pierce 18790) with nitrogen facility.
2.9 Glass derivatization vials (Agilent 5182-0714) with screw caps (Agilent 5182-0717).
2.10 Ultrasonic water bath (Branson).
2.11 Rotating apparatus (Heidolph REAX2).
2.12 Glass injection vials (Agilent 5182-0714) with glass 50 µl inserts (Alltech 98024) and caps (Agilent 5182-0717).
2.13 HPLC pumps 515 (Waters).
2.14 UV detector 996 (Waters).
2.15 Autoinjector 717plus (Waters).
2.16 Fraction collector (Foxy jr.).
2.17 Gas chromatograph (Varian, type CP-3800).
2.18 Automatic injector (Varian, type CP-8400).
2.19 Mass spectrometer (Varian, type 1200L).
2.20 GC-column VF-17MS, 30 m x 0.25 mm ID, film thickness 0.15µm (Varian CP8981).
2.21 Ultrasonic finger with microtip, 50% duty cycle (Branson sonifier 250).
2.22 Moulinette S (Moulinex).
2.23 HPLC- vials (4 ml) with inserts 0.25 ml (Dispolab).
2.24 Polypropylene tubes 50 ml (Greiner).

3 Safety and environment

**Safety**

The RIVM have defined rules to work safe. These rules can be found on the intranet.

**Waste disposal**

Waste is disposed according to SOP 487. More information how to handle waste is found on the RIVM intranet.
4 Chemicals and reagents

Standards

4.1 Internal standards: Relevant internal standards are trans-diethylstilbestrol-D6, hexestrol-D4 and dienestrol-D2 (source RIVM).

Stock solutions containing 1 mg/ml are prepared by dissolving the appropriate amount of the analyte in ethanol. These solutions are stored in the dark at -20 °C for a maximum period of 2 years. Working solutions are prepared by 10-fold dilutions of the stock solutions with ethanol. These solutions are stored in the dark at 4 °C (range 1 – 10 °C) for a maximum period of 12 months.

4.2 Standards: Relevant standards are dienestrol, hexestrol, benzestrol and diethylstilbestrol (source Sigma/Steraloids)

Relevant data of the analytes are listed in table 1. Stock solutions containing 1 mg/ml are prepared by dissolving the appropriate amount of the analyte in ethanol. These solutions are stored in the dark at -20 °C for a maximum period of 2 years. Working solutions are prepared by 10-fold dilutions of the stock solutions with ethanol. These solutions are stored in the dark at 4 °C (range 1 – 10 °C) for a maximum period of 12 months.

Table 1. Information about analytes.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>CAS #</th>
<th>Formula</th>
<th>Mol. Weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dienestrol (DE)</td>
<td>84-17-3</td>
<td>C_{18}H_{18}O_{2}</td>
<td>266.3</td>
</tr>
<tr>
<td>Hexestrol (HEX)</td>
<td>84-16-2</td>
<td>C_{18}H_{22}O_{2}</td>
<td>270.4</td>
</tr>
<tr>
<td>Benzestrol (BE)</td>
<td>85-95-0</td>
<td>C_{20}H_{26}O_{2}</td>
<td>298.4</td>
</tr>
<tr>
<td>Trans-Diethylstilbestrol (DES)</td>
<td>56-53-1</td>
<td>C_{18}H_{20}O_{2}</td>
<td>268.4</td>
</tr>
</tbody>
</table>
4.3 Standards used for the calibration curve are prepared according to table 2.

Table 2. Calibration curve.

<table>
<thead>
<tr>
<th>Amount (ng)</th>
<th>μl standard</th>
<th>Corresponding Amount in μg/kg</th>
<th>μl internal standard 0.1 ng/μl (= 10 ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.625</td>
<td>12.5 μl of 0.05 ng/μl</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>1.25</td>
<td>25 μl of 0.05 ng/μl</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>25 μl of 0.1 ng/μl</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>5.0</td>
<td>50 μl of 0.1 ng/μl</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>7.5</td>
<td>75 μl of 0.1 ng/μl</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>100 μl of 0.1 ng/μl</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>12.5</td>
<td>125 μl of 0.1 ng/μl</td>
<td>2.5</td>
<td>100</td>
</tr>
</tbody>
</table>

After pipetting the required amount of standard solution into derivatization vials the standards are further processed from section 5.30.

**Chemicals**

Reference to a company and/or product is for purpose of identification and information only.

All reagents must be of analytical grade, unless otherwise specified

4.4.1 Methanol (HPLC solvent B).
4.4.2 Chloroform.
4.4.3 Tertiair-ButylMethylEther (TBME).
4.4.4 Sodium hydroxide.
4.4.5 NaOH, 1 mol/l, dissolve 40 g sodium hydroxide in water and add water to a final volume of 1000 ml.
4.4.6 Hydrochloric acid, 37% solution.
4.4.7 HCl solution, 5 mol/l, add 490 g hydrochloric acid 37% to 510 g of water.
4.4.8 Ethanol.
4.4.9 n-Heptane.
4.4.10 Isooctane.
4.4.11 Acetonitrile.
4.4.12 Water; Milli-Q, Waters
4.4.13 HPLC solvent A: acetonitrile/water 50:50 v/v-%, solvent B: Methanol
4.4.14 N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey-Nagel).
4.4.15 Ammonium iodide (Fluka).
4.4.16 Dithioerythreitol (Sigma).
4.4.17 MSTFA++: consists of a mixture of N-methyl-N trimethylsilyltrifluoroacetamide (MSTFA): ammonium iodide: dithioerythreitol (1000:2:4, v/w/w-%).
4.4.18 Acetic acid (Merck, art. 63)
4.4.19 Sodium acetate (Merck, art. 6268).
4.4.20 Acetate buffer 2 mol/l, pH=5.2. Dissolve 25.2 g acetic acid and 129.5 g sodium acetate in 800 ml of water. Adjust the pH to 5.2 ± 0.1 and add water to a final volume of 1000 ml.
4.4.21 Beta-glucuronidase/sulfatase (suc d’Helix Pomatia containing 100.000 units β-glucuronidase/sulfatase per ml. (Industr.Biol. France, 213473).

5 Procedure
Samples of urine and meat are stored in the dark at approximately -20°C, but not higher than -10°C, until analysis, or at approximately 40°C (range 1 - 10°C) if analysis is foreseen to be within 2 days. As a rule quality control samples are included. Details on these samples are always included within the study plan.

If a laboratory sample is considered suitable for analysis (adequate sample size, proper storage history and representative for the analysis) the first step in the analytical procedure is the preparation of a primary extract, including procedures for deconjugation and if appropriate (e.g. tissue) for defatting.

**Preparation of a primary meat-extract**

5.1 Weigh 5 g of well homogenized meat in a tube, add 10 ng of internal standard DES-D6, HEX-D4, DE-D2 (100 µl from standard solution of 0.1 ng/µl).

5.2 Add 4 ml of water and vortex meat/water mixture for 30 seconds.

5.3 Ultrasonificate the meat/water mixture for 60 seconds.

5.4 Add 10 ml methanol and rotate for 15 minutes.

5.5 Centrifugate 10 minutes at 4500 rpm, transfer the water/methanol layer to a 10 ml tube and evaporate the water/methanol at 55°C under a gentle stream of nitrogen.

5.6 Add 6 ml methanol to the dried extract, vortex for 1 minute and rotate for 15 minutes.

5.7 Centrifugate 10 minutes at 3000 rpm, combine the water/methanol layer with the corresponding 10 ml tube used in 5.5 and evaporate the water/methanol at 55°C under a gentle stream of nitrogen till a volume of 1 ml.

5.8 Add 4 ml methanol and 4 ml n-heptane to the extract, vortex for 20 seconds.

5.9 Centrifugate 10 minutes at 3000 rpm.

5.10 Remove and discard the n-heptane layer.

5.11 Add 4 ml n-hexane and repeat 5.9 and 5.10.

5.12 Evaporate at 55°C under a gentle stream of nitrogen till < 1 ml and add 3 ml of water.

5.13 Continue at point 5.17.

**Preparation of a primary urine-extract**

5.14 Pipette 5 ml of urine in a tube, add 10 ng of internal standard DES-D6, HEX-D4, DE-D2 (100 µl from standard solution of 0.1 ng/µl).

5.15 Add 1 ml of 2.0 mol/L acetate buffer, check pH (5.2 ± 0.2) and add acetic acid if necessary.

5.16 Add 0.05 ml β-glucuronidase/sulfatase and incubate during the night at 37°C or 2 hours at 50°C. Cool down to room temperature.

5.17 Add 5 ml of TBME, vortex for 30 seconds.

5.18 Centrifugate 10 minutes at 3000 rpm.

5.19 Pipet the TBME in a 10 ml tube and evaporate the TBME at 50°C under a gentle stream of nitrogen.

5.20 Repeat 5.17 to 5.19.

5.21 Dissolve the dry residue in 1 ml chloroform by placing the tube for 1 minute in an ultrasonic waterbath.

5.22 Extract the chloroform with 3 ml NaOH 1 mol/l (vortex for 30 seconds).

5.23 Centrifugate 10 minutes at 3200 rpm.

5.24 Pipet the NaOH layer into a centrifugation tube in which is 0.6 ml HCl 5 mol/l.

5.25 Extract twice with 5 ml of TBME, evaporate the TBME at 50°C under a gentle stream of nitrogen.

5.26 Dissolve the dry extract in 120 µl HPLC-eluens.

5.27 Transfer the extract into a HPLC-vial.

5.28 100 µl is injected onto the LC-system and fractions are collected and evaporated at 55°C under a stream of nitrogen.

5.29 Dissolve the dried LC fraction in 0.4 ml ethanol.

5.30 Transfer the residue into a derivatization vial.
5.31 The ethanol of the LC fraction and from the calibration curve (table 2) is evaporated and 20 µl MSTFA++ is added.

5.32 The vials are vortexed and the reaction mixture is incubated during 1 hour at 60°C.

5.33 After incubation the reaction mixture is evaporated to dryness under a stream of nitrogen at 55°C and the derivatized residue is dissolved in 50 µl isooctane.

5.34 The residue is transferred into a glass injection vial and transferred into the automatic injector of the GC-MS.

**Analytical Method**

**Table 3. HPLC gradient.**

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>% solvent A</th>
<th>% solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>15.1</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**LC fraction collection:**

- Flow-rate: 0.7 ml/min.
- Injection volume: 100 µl.
- Detection: 230 nm.
- LC retention time of the compounds is determined by injection of a standard mixture of 25 µl of 1 ng/µl, from this injection is the correct time window determined which should be collected, usually starting 0.75 minutes before the retention time of the first eluting compound and ending 0.75 minute after the last eluting compound.
- The eluens is removed under a stream of nitrogen in a water bath at 55°C.

**GC-MS**

- Injection 2 µl pulsed splitless 260°C
- Initial oven temperature 110°C (1 minute)
- Temperature is increased by 20°C/min to 340°C, remains for 5 minutes at 340°C
- Temperature transfer line 280°C
- Constant flow mode, 1.1 ml/min
Table 4. Measured ions for screening selected ion monitoring (SIM).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>m/z</th>
<th>Dwell (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dienestrol</td>
<td>410*</td>
<td>20</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>207*</td>
<td>20</td>
</tr>
<tr>
<td>Hexestrol-D4</td>
<td>209**</td>
<td>20</td>
</tr>
<tr>
<td>t-Diethylstilbestrol</td>
<td>412*</td>
<td>20</td>
</tr>
<tr>
<td>t-Diethylstilbestrol-D6</td>
<td>418**</td>
<td>20</td>
</tr>
<tr>
<td>Benzestrol</td>
<td>193/207*</td>
<td>20</td>
</tr>
</tbody>
</table>

* Ion used for quantification, ** Internal standard

Note: for benzestrol is HEX-D4 used as internal standard.

The suitability of the GC-MS system is checked by injection of the lowest standard of the calibration curve. If the S/N for all compounds is higher then 6 the system is considered as suitable for analysis of all the samples.

For confirmation conform EC/2002/657 the measurement has to be performed conform the conditions as described in table 5.

Table 5. GC-MS-MS Measured transitions.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Dwell time (secs)</th>
<th>MRM I</th>
<th>Col. Energy (Volt)</th>
<th>MRM II</th>
<th>Col. Energy (Volt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dienestrol</td>
<td>0.01</td>
<td>410 &gt; 381</td>
<td>-5</td>
<td>410 &gt; 395*</td>
<td>-5</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>0.01</td>
<td>207 &gt; 179</td>
<td>-15</td>
<td>207 &gt; 191*</td>
<td>-15 V</td>
</tr>
<tr>
<td>Hexestrol-D4</td>
<td>0.01</td>
<td>209 &gt; 193**</td>
<td>-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Diethylstilbestrol</td>
<td>0.01</td>
<td>412 &gt; 217*</td>
<td>-20</td>
<td>412 &gt; 383</td>
<td>-15</td>
</tr>
<tr>
<td>t-Diethylstilbestrol-D6</td>
<td>0.01</td>
<td>418 &gt; 220**</td>
<td>-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzestrol</td>
<td>0.03</td>
<td>207 &gt; 179</td>
<td>-5</td>
<td>207 &gt; 191*</td>
<td>-5</td>
</tr>
</tbody>
</table>

* Trace used for quantification, ** internal standard

6 Calculation

Calculation

Areas of the selected ion of the standard and of the internal standard are calculated. The ratio is the response variable. Quantitative results are obtained by constructing linear curve fitting using least squares linear regression calculation of the response variable versus the concentration. Unknown concentrations are calculated by interpolation.

Quantification is only valid if:

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

Process control
Each series contains a blanc and a spiked sample (1 µg/kg or 1 µg/l) and all samples are spiked with deuterated internal standards, both should fulfill the criteria as under calculation.

To judge if a sample is compliant or non-compliant commission decision 2002/657 is used.

7 Validation and Measurement uncertainty

The method described in this SOP was validated conform SOP ARO/475

The validation level used is 1.0 ng/ml or 1.0 ng/kg. See Annex 1 for an overview of the validation results.

To determine if the validation results of meat samples are representative for urine, spiked samples of urine (1 ng/ml) and meat (1 ng/g) were analyzed and calculated (n=10).

To control this process, also 10 different samples of meat were analyzed.

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>
<thead>
<tr>
<th>matrix</th>
<th>Dienestrol</th>
<th>Hexestrol</th>
<th>t-Diethylstilbestrol</th>
<th>Benzestrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Average meat (ng/g)</td>
<td>0.96</td>
<td>0.94</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td>CV meat (%)</td>
<td>4.5</td>
<td>14.9</td>
<td>4.7</td>
<td>5.2</td>
</tr>
<tr>
<td>t 18 (0.05) critical</td>
<td>1.7</td>
<td>1.7</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>t calculated</td>
<td>1.5</td>
<td>2.6</td>
<td>0.7</td>
<td>2.1</td>
</tr>
<tr>
<td>conclusion t-test</td>
<td>No difference</td>
<td>Difference</td>
<td>No difference</td>
<td>Difference</td>
</tr>
<tr>
<td>F 9,9 critical</td>
<td>3.2</td>
<td>3.2</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>F calculated (F ≥1)</td>
<td>2.1</td>
<td>3201</td>
<td>183</td>
<td>5.2</td>
</tr>
<tr>
<td>conclusion F-test</td>
<td>No difference</td>
<td>Difference</td>
<td>Difference</td>
<td>Difference</td>
</tr>
</tbody>
</table>

The F-test and t-test show that in general there is a significant difference between the meat and urine matrix. Evaluation of the results show that the standard deviation for urine is very small compared to the meat method causing the significant difference between the matrices. Overall, it can be concluded that the method performance is better for urine then for meat.

9 Responsibilities

See ARO-MIS FUNC for the organization of the responsibilities within ARO.

10 Relating documents


ARODOC 4913, Vergelijkend onderzoek in Nederland naar de aantoonbaarheid van diethylstilbestrol (des) in kalver- en runderurine door middel van verschillende chemische methoden, Stephany (rapporteur), 1982
Documentbeheer

Algemeen
Invoerdatum: 20 april 2007
Wijzigingsdatum: 25 juli 2008
Controledatum: 24 juli 2013
Publicatiedatum: 25 juli 2008
Wijzigingen ten opzichte van vorige versie:
Toevoegen van matrix en techniek aan titel, techniek aan scope. Inhoudelijk geen wijzigingen.

Beoordelaars
Marco Blokland (Onderzoeker)
Saskia Sterk (afdelingshoofd)

Hyperlinks