Determining of Aminoglycosides in animal tissue
by means of LC/MS-MS

Austrian Agency for Health and Food Safety (AGES)

Competence Centre for Veterinary Drugs & Hormones (CC TAHO)

<table>
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<th>QM check</th>
<th>Release</th>
</tr>
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<td>Name</td>
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<tr>
<td>Thomas Aichinger</td>
<td>05.05.2010</td>
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<td>Martin Brandtner</td>
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<td>Irina Schwaiger-Nemirova</td>
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<td>Thomas Kuhn</td>
<td>05.05.2010</td>
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</tbody>
</table>
1. **PREVIOUS VERSION**

None, this version is original

2. **PURPOSE AND SCOPE OF APPLICATION**

2.1. **Purpose**

This test procedure describes extraction of aminoglycosides from animal tissue and their qualitative and quantitative determination by means of LC/MS-MS. Aminoglycosides are antibiotics and their maximum residue limits (MRLs) are listed in Annex, table 1 of Commission Regulation (EU) 37/2010.

2.2. **Parameters tested**

Dihydrostreptomycin, Streptomycin

2.3. **Sample type**

animal tissue (muscle, liver, kidney)

3. **METHOD**

3.1. **Principle**

Aminoglycosides are extracted from animal tissue, purified by means of solid-phase extraction (SPE) and, after HPLC separation, determined by means of mass spectrometry.

3.2. **Brief description of method**

- Extraction of aminoglycosides from animal tissue with water
- Extract purification by means of solid-phase extraction (WCX)
- Measurement by means of LC-MS/MS (ESI positive)

4. **TERMS, ABBREVIATIONS AND SYMBOLS USED**

ISTD: internal standard
g (during centrifuging): symbol for normal acceleration of free fall \( (g = 9.80655) \)
rpm: rotations per minute (centrifuge)
MS: mass spectrometry
LC-MS: liquid chromatography-mass spectrometry
ESI: electrospray ionization
5. WARNINGS AND SAFETY INSTRUCTIONS
Organic solvents are potentially dangerous. All operations must be carried out such that no inhalation of the vapors or contact with the skin occurs. The corrosive potential of acids and alkalis must be kept in mind when handling them. Significant changes in pH or neutralizations must be conducted slowly to avoid a violent reaction.

Follow the safety data sheets!

6. EQUIPMENT AND APPARATUS
The company names given are indicative of the type and quality of the products. Products from other manufacturers can be used if they meet the requirements. In addition to common laboratory equipment, the following instruments and apparatus are required:

6.1. Equipment
- Balance
- pH meter
- Transfer pipette
- Overhead shaker
- Centrifuge (for 50 mL Greiner vials)
- Centrifuge (e.g. Heraeus Biofuge fresco)
- HPLC device (e.g. Agilent Technologies 1200 Series)
- MS device (e.g. Applied Biosystems 4000 Q-Trap LC/MS-MS)

6.2. Apparatus

5.2.1. Consumables
- SPE vials (Waters Sep-Pak Vac 6cc Accell Plus CM cartridges Part No. WAT054545)
- 50 mL Greiner vials
- 1.5 mL Eppendorf cups
- Plastic HPLC samples vials with insert

5.2.2. Disposable supplies
- HPLC column: Atlantis HILIC Silica 3 µm, 2,1x100 mm (Waters)
- Precolumn: Atlantis HILIC Silica 3 µm, 2,1x10 mm (Waters)

7. REAGENTS, SOLUTIONS AND TEST ORGANISMS
Determination of Aminoglycosides in animal tissue by means of LC/MS-MS

The requirements in SVA_CC_VIE_TAHO_003 (Ordering, labeling, storing, and handling of chemicals and reference substances) must be observed.

7.1. Standard and reference substances
- Streptomycin sulfate (Sigma S-6501)
- Dihydrostreptomycin sesquisulfate (Sigma D-7253)

7.2. Chemicals and reagents
The company names are given mainly as indicative of the quality of the products. Substances or products from other manufacturers may be used if the required criteria are met.
- Acetic acid p.a. ("glacial acetic acid" Merck 1.00063)
- Acetonitrile (Merck LiChrosolv 1.00030)
- Ammonium formiate (Sigma Aldrich 156264)
- Formic acid 98 %–100% (Riedel de Haen 33015)

7.3. Test organisms
None

7.4. Gases
From the central gas supply: Nitrogen 5.0 (for LC/MS)

7.5. Solutions/culture media
If not otherwise specified, an aliquot or a multiple of the specified batch can be manufactured from the solution with the required accuracy.

The requirements in SVA_CC_VIE_TAHO_004 (Handling of solutions) must be observed.

The following list of preparations for standards is only a recommendation. The requirements in SVA_CC_VIE_TAHO_006 (Guide to the handling of standard substances) must be observed.

7.5.1. Standard stock solutions (1 mg/mL)
7.5.1.1. Streptomycin stock solution (1 mg/mL)
Dissolve 13.16 mg streptomycin sulfate (6.1.) in 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask and fill up to mark.
The solution has a shelf life of four weeks.

7.5.1.2. Dihydrostreptomycin stock solution (1 mg/mL)
Dissolve 13.75 mg dihydrostreptomycin sesquisulfate (6.1.) in 0.1 % acetic acid (6.5.6.) in a 10 mL volumetric flask and fill up to mark.
The solution has a shelf life of four weeks.

### 7.5.2. Streptomycin/Dihydrostreptomycin working solution (10 µg/mL)

Dilute Standard stock solutions (6.5.1.) of Streptomycin and Dihydrostreptomycin with 0.1 % acetic acid (6.5.5.), obtaining the following concentrations:

- Streptomycin 10 µg/mL
- Dihydrostreptomycin 10 µg/mL

The solution has a shelf life of one week.

### 7.5.3. Streptomycin/Dihydrostreptomycin spiking and calibration solution (1 µg/mL)

Dilute Streptomycin/Dihydrostreptomycin working solution (6.5.2.) with 0.1 % acetic acid (6.5.5.), obtaining the following concentrations:

- Streptomycin 1 µg/mL
- Dihydrostreptomycin 1 µg/mL

The solution has a shelf life of one week.

### 7.5.4. Streptomycin/Dihydrostreptomycin—calibration solution (0,1 µg/mL)

Dilute Streptomycin/Dihydrostreptomycin working solution (6.5.2.) with 0.1 % acetic acid (6.5.5.), obtaining the following concentrations:

- Streptomycin 0,1 µg/mL
- Dihydrostreptomycin 0,1 µg/mL

The solution has a shelf life of one week.

### 7.5.5. Acetic acid 0,1%

Fill up 1 mL glacial acetic acid with water to make up 1 L.

The solution has a shelf life of 3 months.

### 7.5.6. Acetic acid 2%

Fill up 20 mL glacial acetic acid with water to make up 1 L.

The solution has a shelf life of 3 months.

### 7.5.7. Elution solution (2 % acetic acid/acetonitrile = 80/20)

Mix 160 mL 2 % acetic acid (6.5.7.) with 40 mL acetonitrile.

### 7.5.8. Eluent A (200 mM ammonium formiate, 100 mM formic acid in H2O)

Dissolve 6.31 g ammonium formiate in distilled water, add 1.88 mL conc. formic acid and fill with water to make up 500 mL.

The solution has a shelf life of one week.

### 7.5.9. Eluent B (100 mM formic acid in acetonitrile)

Fill up 1.88 mL formic acid with acetonitrile to make up 500 mL.

The solution has a shelf life of one week.

### 7.5.10. Standard solutions for the calibration curve
7.6. Disposal
Organic solvents must be disposed of in the solvent waste canisters provided in the laboratories. The further details of the disposal system are established separately at the commercial site.

8. SAMPLING, SAMPLE PREPARATION, AND HANDLING
Sample collection is not performed by the testing laboratory.
Samples are homogenized, **1,0 ± 0,01 g** filled into 50 mL Greiner vials and stored at -20°C until sample preparation.

9. IMPLEMENTATION

9.1. Preparing the control samples
The control samples are used to calculate the recovery rate and the current retention times and to determine the ion ratios (see 9.).
Spike suitable negative pool samples as follows:

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration of analytes (in ng/mL standard solution or in µg/kg tissue)</th>
<th>Solution (0,1 µg/mL) (6.5.4)</th>
<th>Solution (1 µg/mL) (6.5.3.)</th>
<th>Elutionslösung (6.5.7.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 ng/mL (= 100 µg/kg)</td>
<td>100 µL</td>
<td>-</td>
<td>+ 900 µL</td>
</tr>
<tr>
<td>2</td>
<td>20 ng/mL (= 200 µg/kg)</td>
<td>200 µL</td>
<td>-</td>
<td>+ 800 µL</td>
</tr>
<tr>
<td>3</td>
<td>50 ng/mL (= 500 µg/kg)</td>
<td>-</td>
<td>50 µL</td>
<td>+ 950 µL</td>
</tr>
<tr>
<td>4</td>
<td>100 ng/mL (= 1000 µg/kg)</td>
<td>-</td>
<td>100 µL</td>
<td>+ 900 µL</td>
</tr>
<tr>
<td>5</td>
<td>150 ng/mL (= 1500 µg/kg)</td>
<td>-</td>
<td>150 µL</td>
<td>+ 850 µL</td>
</tr>
<tr>
<td>6</td>
<td>200 ng/mL (= 2000 µg/kg)</td>
<td>-</td>
<td>200 µL</td>
<td>+ 800 µL</td>
</tr>
<tr>
<td>7</td>
<td>250 ng/mL (= 2500 µg/kg)</td>
<td>-</td>
<td>250 µL</td>
<td>+ 750 µL</td>
</tr>
</tbody>
</table>

This calibration curve is an example and can be adapted.

81x145 DOT 500 µg/kg 500 µL Streptomycin/Dihydrostreptomycin spiking and calibration solution 1 µg/mL (6.5.3.)

9.2. Sample preparation

9.2.1. Extraction
- Bring samples and control samples to room temperature
- Spike one control sample with 500 µL and a second with 1000 µL Streptomycin/
Dihydrostreptomycin spiking and calibration solution 1 µg/mL (6.5.3.)
- add 25 mL water and shake 30 min with the overhead shaker
- Centrifuge (10 min at 3000 rpm) [Note: Do not centrifuge at > 3000 rpm because this can damage (deform, break) the Greiner vials!]

**9.2.2. Solid phase extraction WCX**
- Condition column with 2 x 5 mL 2 % acetic acid (6.5.6.)
- Wash with 2 x 5 mL water (do not allow to run dry)
- Put some wadding into the columns (prevents clogging of the columns)
- transfer the entire centrifuged extract on the column
- Remove wadding
- Wash the column with 2 x 5 mL water
- Elute into a 10 mL volumetric flask with 2 x 5 mL elution solution (6.5.7)
- Fill up volumetric flask with acetonitrile to 10 mL
- Centrifuge 1 mL of the eluent 10 min at 13000 rpm
- Transfer centrifuged solution into a plastic HPLC vial

**9.3. Measurement/testing**
Analyze the sample extracts in the HPLC sample vials e. g. under the following conditions:

**HPLC:**

- **HPLC column:** Atlantis HILIC Silica 3µm, 2,1x100mm (Waters)
- **Precolumn:** Atlantis HILIC Silica 3µm, 2,1x10mm (Waters)
- **Eluent:**
  - Eluent A (200 mM ammonium formiate, 100 mM formic acid in H₂O) (6.5.8.)
  - Eluent B (100 mM formic acid in acetonitrile) (6.5.9.)
- **Gradient:**
  - 0 min: 10% A, 90% B
  - 6 min: 60% A, 40% B
  - 10 min: 60% A, 40% B
  - 10,1 min: 10% A, 90% B
  - 20 min: 10% A, 90% B

- **Injection volume:** 20 µL
- **HPLC flow:** 0,3 mL/min
- **column temperature::** 30°C

**Mass spectrometry detection:**

- **Ionization mode:** ESI positive
**Test Procedure**  CC_VIE_TAHO_233  Version: 01

**Competence Centre for Veterinary Drugs & Hormones (CC TAHO)**

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10. EVALUATION

10.1. Evaluation/calculation

Use the corresponding software (e.g. Analyst) to perform qualitative and quantitative evaluation on the obtained measurement results:

- Identify the signals (peaks) using the reaction times and ion traces.
- Integrate the peaks to calculate the area values.
- Create the calibration curve related to the internal standard (dihydrostreptomycin) from the results of the calibration standard (6.5.12) and calculate the associated concentrations. Save the results.
- Print the results of the analysis ("report").
- Transfer the data into a Microsoft Excel template for further calculations.

Using Microsoft Excel, carry out the additional calculations below:

- Relative retention times and retention time deviations for the corresponding fragment ions;
- Ion ratios of the corresponding fragment ions for identification according to EU criteria (9.1.2);
- Determine recovery rates.

\[
WFR \text{ (dot.Probe)} = \frac{c_{\text{dot kalk}}}{c_{\text{dot erw}}} \times 100 \% 
\]

- \( WFR \) ...... recovery rate
- \( c_{\text{dot kalk}} \) .... calculated concentration of the spiked control sample (measured) [µg/kg]
- \( c_{\text{dot erw}} \) .... expected concentration of the spiked control sample (theoretical) [µg/kg]

- Calculate analyte content in the sample.

\[
c (\text{analyte sample}) = \frac{c_{\text{pur kalk}}}{WFR} \times [\mu g/kg \text{ tissue}]
\]

- \( c_{\text{pur kalk}} \) .... calculated concentration sample (measured) [µg/kg]

**Remark:** Take the weighed amount of the sample and any potential dilution factors into account during calculation of the analyte content!

9.1.1. Evaluation screening

a) Check the recovery rates of standard solutions, control samples and samples:
aa) The recovery time of the analyte in question must correspond to the average retention time of the spiked control samples, at a tolerance of ± 5 %.

ab) The retention times of the corresponding fragment ions must correspond, at a tolerance of 0,2 min.

ac) The ratio of the retention time of the analyte in question to that of the suitable internal standard (i.e.: relative retention time) must correspond to that of the standard solutions or of the spiked control samples, at a tolerance of ± 5 %

Analytes that meet these criteria are considered as suspected positives. Otherwise, the analyte is "not detectable".

b) Check the relative ion intensities:
The relative ion intensities of the corresponding fragment ions are calculated for all samples (calibration standards, control samples, samples) based on the ion trace with the highest intensity (greatest peak area). An analyte is then considered to be a "suspected positive" if the relative ion intensity of the sample lies within the following tolerance ranges, which are based on the mean ion ratios of the calibration standards and control samples:

<table>
<thead>
<tr>
<th>Relative ion intensity</th>
<th>Maximum permissible relative deviations (tolerance range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50 %</td>
<td>± 40 %</td>
</tr>
<tr>
<td>&gt; 20 %-50 %</td>
<td>± 50 %</td>
</tr>
<tr>
<td>&gt; 10 %-20 %</td>
<td>± 60 %</td>
</tr>
<tr>
<td>≤ 10 %</td>
<td>± 80 %</td>
</tr>
</tbody>
</table>

If the relative ion intensities are outside these computed ranges, the analyte is considered "not detectable".

c) Check the internal standards (if applicable): if a possible internal standard of the sample is found to be less than 20% of the mean concentration of the accompanying control samples, or the ISTD is not detectable at all, the sample is classified as "not evaluable" and must be repeated. If in doubt, consult the responsible supervisor.

d) Compare the analyte content (corrected for recovery rate) in the sample with the detection limit: If the analyte content is above the detection limit, the substance is considered as a "suspected positive"; otherwise, the analyte is considered "not detectable".

e) Check the analyte content with respect to the calibration range:
If the analyte content is higher than the highest calibration standard, the measurement must be repeated with a suitable dilution. If necessary, a new partial sample must be extracted and spiked with a correspondingly higher amount of a possible internal standard, so that the concentration is consistent with the required dilution.
If in doubt, consult the responsible supervisor.

If Dihydrostreptomycin and/or streptomycin is detected in a sample during screening ("suspected positive"), the further procedure must be clarified with the responsible superior (possibly repetition of the extraction to ensure reliability). In any case, the measurement results must be evaluated according to 9.1.2.

If a sample is evaluated as “not evaluable,” weigh a new aliquot of the sample and repeat the analysis.

If in doubt, the specific procedure should be clarified with the responsible supervisor.

**9.1.2. Evaluation confirmation**

a) Check the retention times of standard solutions, control samples and samples:

   aa) The retention time of the analyte in question must agree with the mean retention time of the spiked control sample within a tolerance of ±5%.
   ab) The retention time of the corresponding fragment ions must agree within a tolerance of 0.2 min.
   ac) The relationship of the retention time for the analyte in question and that of an appropriate internal standard (i.e., relative retention time) must correspond to that of the standard solutions or the spiked control samples within a tolerance of ±2.5%.

b) Check the relative ion intensities:
For the correct identification of an analyte, the ion ratios of the corresponding fragment ions must be compared with those of the standards or control samples and lie within an established tolerance limit. According to the Commission Decision 2002/657/EC the following tolerances are permitted:

<table>
<thead>
<tr>
<th>Relative ion intensity</th>
<th>Maximum permissible relative deviations (tolerance range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50 %</td>
<td>± 20 %</td>
</tr>
<tr>
<td>&gt; 20 %–50 %</td>
<td>± 25 %</td>
</tr>
<tr>
<td>&gt; 10 %–20 %</td>
<td>± 30 %</td>
</tr>
<tr>
<td>≤ 10 %</td>
<td>± 50 %</td>
</tr>
</tbody>
</table>

c) Check the internal standards (if applicable):
The concentration of a possible internal standard of the sample in question must be higher than 20 % of the average concentration of control samples.

d) The analyte content in the sample must be above its detection limit (see Section 10).

e) The measured value must be within the calibration range.
A substance is considered to be confirmed if all of the listed criteria are met. The mean value for analyte content in parallel samples is used in the presentation of quantitative results.

If one of the three criteria specified under a), b), and d) is not met, then the substance is considered “not detectable” providing that criteria c) and e) are met. Conversely, if criterion c) is not met, further procedures must be clarified with the responsible supervisor. If criterion e) is not met, a suitable dilution of the measurement solution must be carried out. If necessary, a new partial sample must be extracted and spiked with a correspondingly higher amount of an internal standard (if applicable), so that the concentration is consistent with the required dilution. If in doubt, consult the responsible supervisor.

10.2. Documentation

At a minimum, the Test Report must contain:

- Name(s) or initial(s) of the analyst(s) (preparing samples and measurement)
- Date of the sample preparation and measurement
- List of tested samples (with clearly attributable sample names or numbers)
- List of standards used and control samples
- Raw data of the samples and control samples
- Results of the samples
- Any deviations from the test procedure.

10.3. Presentation of results

- Analyte not detectable: “ND (LOD: ... µg/kg)”
- Analyte detectable, however, less than detection limit: “detectable < LOQ (LOD: ... µg/kg; LOQ: ... µg/kg)”
- Content of the detected analyte is quantifiable: “xx.y µg/kg (LOD: ... µg/kg; LOQ: ... µg/kg)”

11. VALIDATION

The results of the validation are available in electronic form at:
L:\taho\QM\Validierung\Streptomycin LC-MS\Streptomycin-Niere

12. REFERENCES

12.1. Standards, laws and guidelines

Test Procedure  CC_VIE_TAHO_233  Version: 01

Competence Centre for Veterinary Drugs & Hormones (CC TAHO)

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Location: in electronic form at: L:\taho\QM\Gesetzliche Grundlagen

12.2. QM documents and document templates
- SVA_CC_VIE_TAHO_003 (Ordering, labeling, storing, and handling of chemicals and reference substances)
- SVA_CC_VIE_TAHO_004 (Handling of solutions)
- SVA_CC_VIE_TAHO_006 (Guide to handling of standard substances)
- SVA_CC_VIE_TAHO_005 (Preparation of control samples)
- PV Vorlage TAHO dot_QMT_PV_01

13. APPENDICES
None

14. DISTRIBUTION

<table>
<thead>
<tr>
<th>Minimum distribution list for information regarding this test procedure:</th>
<th>Information format</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institute Director for the respective institute</td>
<td>Paper  IT  Training</td>
</tr>
<tr>
<td>Department Head(s) of the respective department(s)</td>
<td>X</td>
</tr>
<tr>
<td>Head of the implementing center or competence center</td>
<td>X</td>
</tr>
<tr>
<td>Staff performing the test procedure or parts of it</td>
<td>X  X</td>
</tr>
</tbody>
</table>

QM-coding: PV_CC_VIE_TAHO_233_01  Status: Valid as of: 06.05.2010  Page 12 of 12