SULFAMETHAZINE ELISA

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A competitive enzyme immunoassay for
quantitative analysis of Sulfamethazine
in various matrices

EUROPROXIMA SULFAMETHAZINE ELISA

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BRIEF INFORMATION

The Sulfamethazine ELISA is a competitive enzyme immunoassay for the screening of urine, tissue, milk and plasma samples on the presence of this antimicrobial. The test is based on antibodies against Sulfamethazine. It has good sensitivity for Sulfamethazine and a major metabolite, N_4-acetylsulfamethazine. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analysed. The ELISA kit contains all the reagents, including standards, required to perform the test. Materials and chemicals necessary for extraction or concentration steps are not included in the test-kit.

1. INTRODUCTION

Sulfamethazine (SMZ) (syn. for Sulfadimidine or Sulfamezathine or 2-sulfanylamido-4,6-dimethylpyrimidine) is widely used for therapeutic and prophylactic purposes in animal food production. Sulfonamides which are retained in food could result in allergic or toxic reactions in for these compounds sensitive consumers. Also, there are general concerns that the widespread use of antibiotics could contribute to antibiotic resistance in pathogenic organisms. Normally, tissue residues in animals are controlled by withdrawing the drug from feed before slaughter. The concentrations of the sulfonamides are then presumed to deplete to less than the maximum residue level (MRL). However, due to contaminated food or failure to observe the withdrawal period, a number of animals are reaching slaughterhouses with a substantially excessive amount of drugs still present in their tissues.
SMZ is partially metabolised in animals. Main metabolite in horses is 5-hydroxy-SMZ, in cows, calves, goats, turtles and snails it is 6-hydroxymethyl-SMZ. In man and pigs the main metabolite is N_4-acetyl-SMZ. The amount of SMZ metabolised depends on many factors, i.e. type of animal, dose and time after administration.
In the E.C. and the U.S.A. the MRL for sulfa residues in tissue and milk is 100 µg/kg. For the determination of Sulfonamides, analytical procedures based on HPLC have been developed. However, such methods are expensive and time consuming. This ELISA detects SMZ and N_4-acetyl-SMZ in urine, tissue, milk and plasma with a fast and simple sample preparation.

2. PRINCIPLE OF THE SULFAMETHAZINE ELISA

The microtiter plate based ELISA kit consists of 12 strips, each 8 wells, precoated with sheep antibodies to rabbit IgG. Specific antibodies (rabbit anti-Sulfametazine), horseradish peroxidase labelled Sulfametazine (enzyme conjugate) as well as Sulfametazine standard solution or samples are pipetted into the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised rabbit antibodies and at the same time free Sulfamethazines (in the standard solution or in the sample) and enzyme labelled Sulfamethazine compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualised by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the Sulfamethazines concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The Sulfamethazine-ELISA utilises antibodies raised in rabbits against protein conjugated Sulfamethazine. The reactivity pattern of the antibody is:

Cross- reactions:	Sulfamethazine	100%
	N_4-acetyl-SMZ	130%
	Sulfamerazine	15%

The cross-reactivity of ten other sulfa drugs (Sulfadiazine, Sulfadimethoxine, Sulfadoxine, Sulfaguanidine, Sulfamethoxazole, Sulfamethoxydiazine, Sulfapyridine, Sulfa-quinoxaline, Sulfathiazole and Sulfatroxazole) is lower than 0.5%. The calibration curve is virtually linear in the range of 0.125-10 ng/ml.
Using HPLC, the ratio of SMZ concentrations found in urine, plasma and tissue samples of treated animals (pigs) is about 10:5:2. With this ELISA, due to the detection of N_4-acetyl-SMZ, the concentrations found in urine, plasma, kidney and other tissue samples are about 30, 3, 6 and 2 times higher as compared to HPLC values. Using urine, plasma or kidney samples to control for the presence of SMZ in tissue at half MRL (0.05 mg/kg), the decision level should be 7.5 mg/l (urine) or 0.3 mg/l (plasma) or 0.3 mg/kg (kidney). Using urine or plasma samples to control for the presence of SMZ in kidney at half the MRL, the decision level should be 1.2 mg/l (urine) or 0.3 mg/l (plasma).

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppb)
Urine	8.1	3
Milk	8.3	8
Serum/Plasma	8.4	1
Tissue	8.2	3

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

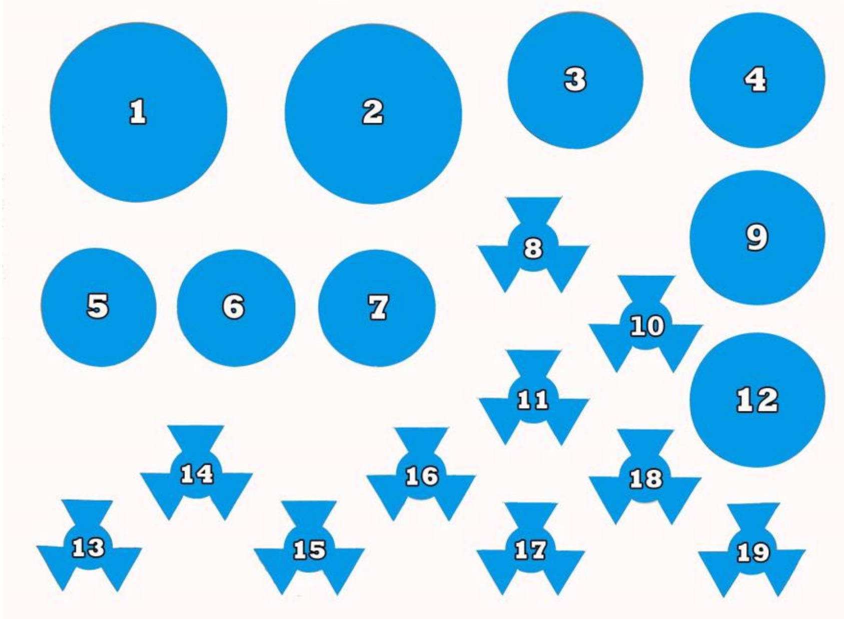
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Contents ELISA-kit:

- 1 sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready-to-use.

- Position of the reagents in the kit. For preparation of the reagents see chapter 9.



1. **Dilution buffer** (20 ml, Ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. **Conjugate** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. **Standard (**lyophilised, black cap)
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. not in use
14. not in use
15. not in use
16. not in use
17. not in use
18. not in use
19. not in use

6. EQUIPMENT AND MATERIALS REQUIRED NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (for 10 – 15 ml test tubes, with cooling, 2000 x g)
- Vortex
- Automated microplate washer or 8 channel micropipette 100-300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Siliconised glass test tubes or plastic tubes
- Micropipettes 20-200 µl, 100-1000 µl
- Multipipette with 2.5 ml combitips
- Aluminum foil or parafilm
- 1M NaOH
- 1M acetic acid
- arodisc filter 0,45 µm
- folded filter
- Tween 80
- Disodium hydrogenphosphate Na_2HPO_4
- Potassium dihydrogenphosphate KH_2PO_4
- Sodium chloride NaCl
- Distillied water

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

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8. SAMPLE PREPARATIONS

8.1 Urine samples

- Adjust the pH of urine samples (± 1 ml) to 7.5 ± 0.5 by adding 1 drop of 1 M acetic acid or 1 M NaOH (check with pH paper) and filter through an acrodisc filter 0.45 µm.
- Dilute 50 µl of the filtrate with 450 µl of sample extraction buffer, see chapter 9
- Use 50 µl in the ELISA test.

8.2 Tissue samples

- Weigh 2.5 g finely cut and subsequently homogenised tissue (meat, liver, or kidney) sample in a homogeniser (e.g. Ultra Turrax or Stomacher).
- Add 22.5 ml of sample extraction buffer (see chapter 9) and mix during 3 min.
- Centrifuge (10 min. at 2000 x g) and filter the mixture through a paper filter and collect the filtrate through an acrodisc filter 0.45 µm.
- Use 50 µl of this solution in the ELISA test.

8.3 Milk samples

- Centrifuge milk samples for 15 min. at 2000 x g at 4ºC, take samples from underneath the fat layer.
- Dilute 50 µl of the defatted milk with 5 ml of sample extraction buffer, see chapter 9.
- Use 50 µl of this solution in the ELISA test.

8.4 Serum/Plasma samples

- Dilute 50 µl of serum/plasma with 450 µl of sample extraction buffer, see chapter 9.
- Use 50 µl of this solution in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at +2°C to +8°C.

Microtiter plate

Return unused strips into the zip lock bag with desiccant and store at +2ºC to +8ºC for use in subsequent assays. Retain also the strip holder.

Rinsing buffer

The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

<u>Substrate solution</u>
The substrate solution (ready-to-use) precipitates at 4ºC.
Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

<u>Standard solutions</u>
Prepare a dilution range of the Sulfamethazine standard.
Add 2.0 ml of dilution buffer to the vial of Sulfamethazine standard and mix. This Sulfamethazine solution contains 10 ng/ml.
Pipette 0.5 ml of this Sulfamethazine solution into a siliconised glass or plastic tube and add 0.5 ml of dilution buffer. Concentration of this diluted Sulfamethazine solution is 5 ng/ml.
Continue to make a dilution range of 1, 0.5, 0.25 and 0.125 ng/ml.

<u>Conjugate solution</u>
Reconstitute the vial of lyophilised conjugate (Sulfamethazine-HRP) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.

<u>Antibody solution</u>
Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.

<u>Sample extraction buffer</u>
Prepare this buffer as follows: [Quantities indicated are for 1 liter buffer.]

$Na_2HPO_4\ 2H_2O$	0.96 g
KH_2PO_4	0.17 g
NaCl	9 g
Tween 80	0.5 ml

Add to 1000 ml with distilled water.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, un-bound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results. Basically, manual rinsing or rinsing with automatic plate wash equipment can be done as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl of dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

4. Add 25 µl of conjugate (Sulfamethazine-HRP) to all wells, except wells H1 and H2.

5. Add 25 µl of antibody solution to all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate for 1 min.

7. Incubate for 1 hour in the dark at 4ºC (2ºC - 8ºC).

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well. Incubate 30 min. at room temperature (20ºC - 25ºC).

10. Add 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) value of the blank wells A1 and A2 from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (percentage maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the Sulfamethazine equivalent concentration (ng/ml) on a logarithmic X-axis.

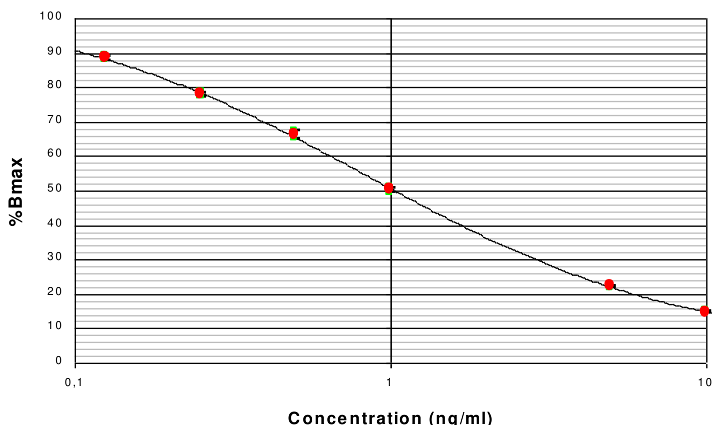

Figure 1 : Example of a calibration curve

The amount of Sulfamethazine in the samples is expressed as Sulfamethazine equivalents. The Sulfamethazine equivalents in the extracts (ng/ml) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

Urine samples
Applying the ELISA on urine samples, the calculated Sulfamethazine equivalents have to be multiplied by a factor 10.

Tissue samples
Applying the ELISA on tissue samples, the calculated Sulfamethazine equivalents have to be multiplied by a factor 10.

Milk samples
Applying the ELISA on milk samples, the calculated Sulfamethazine equivalents have to be multiplied by a factor 100.

Serum/Plasma samples

Applying the ELISA on serum/plasma samples, the calculated Sulfamethazine equivalents have to be multiplied by a factor 10.

12. LITERATURE

Nouws, J.F.M., T.B. Vree, H.J. Breukink, M. Braakman, F. Driessens and A. Smulders. Dose dependent disposition of sulphadimidine and of its N_4-acetyl and hydroxy metabolites in plasma and milk of dairy cows. The Veterinary Quarterly (1985) 7, 177-186.

Nouws, J.F.M., T.B. Vree, H.J. Breukink, A.S.J.P.A.M. van Miert and Jan Grondel. Pharmacokinetics, hydroxylation and acetylation of sulphadimidine in mammals, birds, fish, reptiles and molluscs. Proceedings of the third Congress of the European Association for Veterinary Pharmacology and Toxicology, August 25-29 1985, Ghent, Belgium. Eds. A.S.J.P.A.M. van Miert, M.G. Bogeart and M. Debackere, MTP Press Limited.

13. ORDERING INFORMATION

For ordering the Sulfamethazine ELISA kit, please use cat. code 5101SUL.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

R-Biopharm Nederland B.V.
Beijerinckweg 18
6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl