

SULFAMETHAZINE ELISA

A competitive enzyme immunoassay for
quantitative analysis of Sulfamethazine
in urine, tissue, milk and plasma

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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, N₄-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 [1]. 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 N₄-acetyl-SMZ. The amount of SMZ metabolised depends on many factors, i.e. type of animal, dose and time after administration [2].

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 N₄-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-Sulfamethazine), horseradish peroxidase labelled Sulfamethazine (enzyme conjugate) as well as Sulfamethazine 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 N₄-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).

12. LITERATURE

1. 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 N₄-acetyl and hydroxy metabolites in plasma and milk of dairy cows. *The Veterinary Quarterly* (1985) 7, 177-186.
2. 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. Bogear and M. Debackere, MTP Press Limited.

13. ORDERING INFORMATION

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

14. LAST MUTATIONS

Pipette schedule of the standard curve is adapted

EuroProxima B.V.
Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

TEL: + 31 26 3630364
FAX: + 31 26 3645111
Web-site: <http://www.europroxima.com>
E-mail: info@europroxima.com

The amount of Sulfamethazine in the samples is expressed as Sulfamethazine equivalents. The Sulfamethazine equivalents in the extracts (ng/ml) corresponding to the % 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 (urine is 10 x diluted in sample extraction buffer).

Tissue samples

Applying the ELISA on tissue samples, the calculated Sulfamethazine equivalents have to be multiplied by a factor 10 (0.1 g sample/ml buffer).

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.

The limit of detection (LOD) is calculated as $X_n + 3SD$ ($X \geq 20$). For extraction procedures the LOD is calculated reflecting the respective concentration steps. The LOD is determined under optimal conditions. Cut-off values need critical consideration.

<u>Matrix</u>	<u>Procedure</u>	<u>LOD</u>
Urine	Dilution with buffer	3 ppb
Milk	De-fatting and dilution with buffer	8 ppb
Serum/Plasma	Dilution with buffer	1 ppb
Tissue	Extraction with buffer and filtration	3 ppb

4. HANDLING AND STORAGE

- Store the kit at +2°C to +8°C in a dark place.
- Be aware: sulfamethazine, adsorb to glass. Always use siliconised glass tubes or plastic ones.
- After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
- It is advised to unpack the sealed microtiter plate, reconstitute or dilute the kit components, immediately before use.
- In order to avoid condensation in the ELISA plates allow the sealed plate to equilibrate to room temperature before opening it.
- After the lyophilised kit components have been reconstituted, these components are only guaranteed for 1 week (stored at +2°C to +8°C in the dark). For prolonged storage, aliquot reconstituted components and store at -20°C.
- Any direct action of light on the chromogen solution should be avoided.

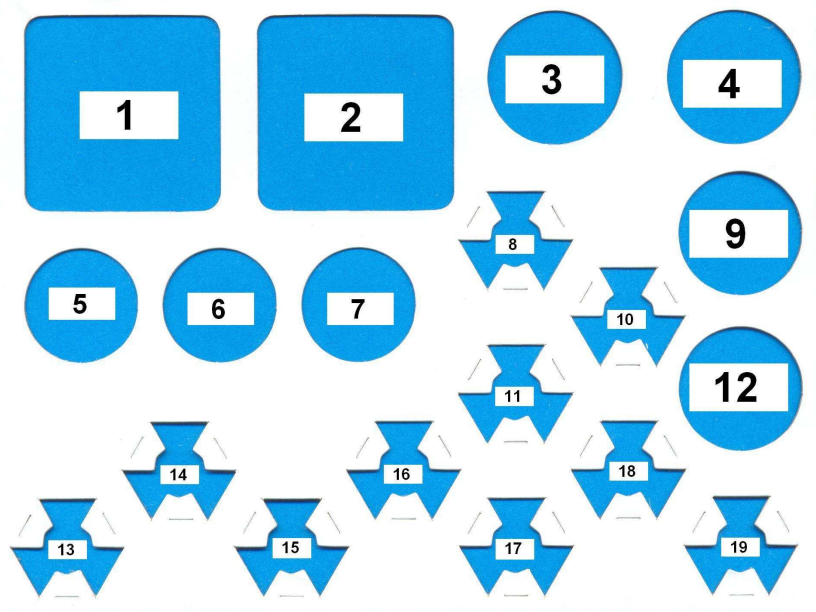
If the following phenomena are observed, this may indicate a degeneration of the reagents:

- A blue colouring of the chromogen solution before putting it into the wells,
- A weak or absent colour reaction of the first standard (zero standard) ($E_{450nm} < 0.8$).

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

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 (20°C - 25°C).
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.

O.D. standard (or sample)

-----x 100 = % maximal absorbance

O.D. zero standard

Calibration curve:

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

Calibrationcurve Sulphamethazine

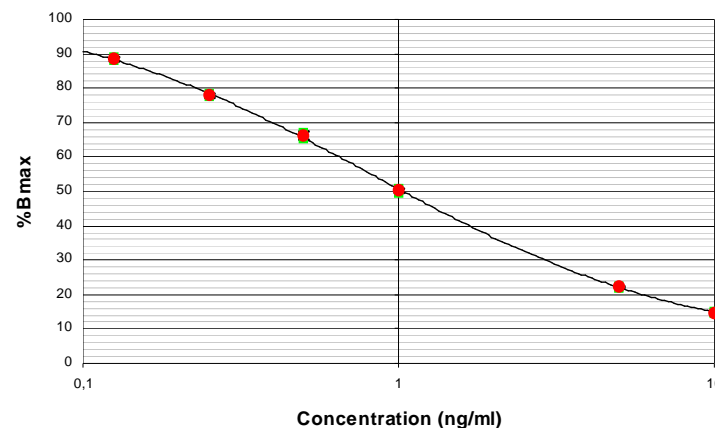


Figure 1 : Example of a calibration curve

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 (Sample treatment) and prepare reagents according to Chapter 9 (Preparation of samples).
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 I1,2).
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 4°C (2°C - 8°C).

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

7. PRECAUTIONS

- Good laboratory practise should be employed when using this kit. Safety clothing should be worn and contact with skin and/or mucous membranes avoided.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area. Do not pipette by mouth.
- The stop solution contains 0.5 M sulfuric acid (H₂SO₄). Do not allow the reagent to get into contact with skin and/or eyes.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past their expiration date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which tends to crystallize at temperatures below +4°C.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure is necessary to maintain good precision and accuracy.
- In case of contact of any reagent with eyes or skin, rinse immediately with plenty of water.

8. SAMPLE TREATMENT

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 μ m.
- Dilute 50 μ l of the filtrate with 450 μ l of sample extraction buffer.
- Use 50 μ l in the ELISA test.

Tissue samples

- Weigh 2.5 g finely cut and subsequently homogenised tissue (meat, liver, or kidney) sample in a homogeniser (for instance Ultra Turrax or Stomacher).
- Add 22.5 ml of sample extraction buffer* 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 μ m.
- Use 50 μ l of this solution in the ELISA test.

Milk samples

- Centrifuge milk samples for 15 min. at 2000 x g at 4°C, take samples from underneath the fat layer.
- Dilute 50 μ l of the defatted milk with 5 ml of sample extraction buffer*
- Use 50 μ l of this solution in the ELISA test.

Serum/Plasma samples

- Dilute 50 μ l of serum/plasma with 450 μ l of sample extraction buffer*
- Use 50 μ l of this solution in the ELISA test.

*For sample extraction buffer: see Chapter 9 "Preparation of reagents".

9. PREPARATION OF REAGENTS

The reagents included in the test-kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

Before beginning the test, the reagents should be brought up to ambient temperature.

Microtiter plate

Return unused strips into the zip lock bag with desiccant and store at +2°C to +8°C 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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate solution

The substrate solution (ready-to-use) precipitates at 4°C.

Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

Standard solutions

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.

Keep the dilution of the dilution range in the dark at room temperature until use. Store at -20°C.

Conjugate solution

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

Store the vial immediately after use in the dark at +2°C to +8°C.

Antibody solution

Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.

Store the vial immediately after use in the dark at +2°C to +8°C.

Sample extraction buffer

Sample extraction buffer is not provided in the kit. Prepare this buffer as follows: [Quantities indicated are for 1 liter buffer.]

Na ₂ HPO ₄ 2H ₂ O	0.96 g
KH ₂ PO ₄	0.17 g
NaCl	9 g
Tween 80	0.5 ml
Distilled water add	1000 ml