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

**Urine samples**

Applying the EIA on urine samples, the calculated Sulfadiazine equivalents have to be multiplied by the applied dilution factor (10, 100 or 1000 times).

**Tissue samples**

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

**Milk samples**

Applying the EIA on milk samples, the calculated Sulfadiazine equivalents have to be multiplied by the applied dilution factor (10 or 100 times).

**Plasma samples**

Applying the EIA on plasma samples, the calculated Sulfadiazine equivalents have to be multiplied by the applied dilution factor (10 or 100 times).

11. **LITERATURE**


12. **ORDERING INFORMATION**

For ordering the Sulfadiazine EIA kit, please use cat. code 5101SUDA1p.
BRIEF INFORMATION

The Sulfadiazine EIA is a competitive enzyme immunoassay for the screening of urine, tissue, plasma and milk samples on the presence of this anti-infective agent. The test is based on antibodies against Sulfadiazine. It has good sensitivity for Sulfadiazine, its major metabolite N₂-acetyl sulfadiazine and Sulfamerazine. With this EIA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 samples can be analyzed. The EIA 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

Sulfadiazine (SDZ) and Sulfamerazine (SMR) are widely used for therapeutic and prophylactic purposes in animal diseases. Sulfonamides which are retained in food could result in allergic or toxic reactions in 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 faillure to observe the withdrawal period, a number of animals are reaching slaughterhouses with a substantially excessive amount of drugs still present in their tissues. Various factors (e.g. dosage, age, gender, species differences, molecule structure) affect the metabolism and pharmacokinetic behaviour of Sulfonamides and their metabolites. In pigs, the N₂-acetylated derivatives of SDZ and SMR are the main metabolites (1). In plasma of pigs, calves and cows, the N₂-acetylated SDZ: SDZ is about 1:6 (also for SMR) (1,2). In urine of pigs, calves and cows, SMR is excreted mainly as N₂-SMR (10-20 times higher than the parent drug). The concentration of N₂-SDZ in urine is 2-4 times higher than the parent drug (1,2).

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 EIA detects SDZ, N₂-acetyl-SDZ and SMR in urine, tissue, plasma and milk with a fast and simple sample preparation.

2. PRINCIPLE OF THE SULFADIAZINE EIA

The microtiter plate based EIA kit consists of 12 strips, each 8 wells, precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibodies (rabbit anti-SDZ), enzyme labelled SDZ (enzyme conjugate) and SDZ standard or sample are added to the precoated wells. The specific antibodies are bound by the immobilised anti-rabbit antibodies and at the same time free SDZ (in the standard solution or in the sample) and enzyme labelled SDZ 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 visualized by the addition of chromogen substrate (tetrathylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product.

The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the SDZ concentration in the sample.

7. Incubate for 1 hour in the dark at 4°C.
8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
9. Pipet 100 μl substrate solution into each well. Incubate 30 min. at room temperature (25°C).
10. Add 100 μl stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

10. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the 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 Sulfadiazine equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.1 - 10 ng/ml range.

Figure 1 : Example of a calibration curve
9. ASSAY PROCEDURE

- **Rinsing protocol**

  In EIA'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 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 7 (Sample treatment) and prepare reagents according to chapter 8.

  Microtiter plate is ready to use, do not wash.

  2. Pipet 100 μl dilution buffer in duplicate (well A1, A2).

  Pipet 50 μl dilution buffer in duplicate (well B1, B2).

  Pipet 50 μl of each standard dilution in duplicate (well C1,2 to F1,2).

  3. Pipet 50 μl of each sample solution in duplicate into the remaining wells of the microtiter plate (42 samples; 84 wells).

  4. Add 25 μl conjugate (Sulfadiazine-HRPO) to all wells, except wells A1 and A2.

  5. Add 25 μl antibody solution to all wells, except wells A1 and A2.

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

3. SPECIFICITY AND SENSITIVITY

The SDZ-EIA utilizes antibodies raised in rabbits against protein conjugated SDZ. The reactivity pattern of the antibody is:

<table>
<thead>
<tr>
<th>Cross-reactions</th>
<th>SDZ</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-acetyl-DZ</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>Sulfaquinoxaline</td>
<td>3%</td>
</tr>
</tbody>
</table>

The cross-reactivity of eight other sulfa drugs (Sulfadimidine, Sulfadimethoxine, Sulfadoxine, Sulfaguanidine, Sulfamethoxazole, Sulfadoxine, Sulfamethoxydiazine, Sulfaquinoxaline and Sulfamethsuxidine) is smaller than 0.5%. The calibration curve is virtually linear in the range of 0.1-10 ng/ml.

4. HANDLING AND STORAGE

- Store the kit at +2°C to +8°C in a dark place.
- 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;
- After the lyophilized 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) (E450nm < 0.6).

5. KIT CONTENTS

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

Contents EIA-kit:

- 1 sealed dry (96-wells) Microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready to use, do not wash.
- 1 vial containing lyophilized standard solution of Sulfadiazine (lilac cap),
- 1 vial containing lyophilized conjugate (peroxidase conjugated Sulfadiazine, blue cap),
- 1 vial containing lyophilized anti-Sulfadiazine antibodies (gold cap)
- 1 vial containing the substrate solution, ready to use (12 ml),
- 1 vial containing dilution buffer pH 7, ready to use (20 ml, white cap),
- 1 vial containing stop solution, ready to use (15 ml, red cap),
- 1 vial containing rinsing buffer, 10 times concentrated (60 ml).
6. PRECAUTIONS
- The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- 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 intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the undersurface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which cristalizes at 4°C.
- Optimal results will be obtained by strict adherence to this protocol.

7. SAMPLE TREATMENT

Urine samples
Dilute urine samples 10, 100 or 1000 times in sample extraction buffer*. Check or adjust the pH (7.5 ± 0.5). If necessary adjust the pH by adding a few drops of 0.1 M HAC or 0.1 M NaOH. Centrifugate (5 min. at 2000 rpm) the diluted and pH-adjusted sample and pipet 50 μl aliquots in the microtiter plate.

Tissue samples
Weigh 2.5 g finely cut and subsequently homogenized tissue (meat, liver, or kidney) sample in a homogenizer (for instance Ultra Turrax or Stomacher). Add 22.5 ml of sample extraction buffer* and mix during 3 min. Centrifugate (10 min. at 10,000 rpm) and filtrate a portion, through an acrodic filter 0.45 μm and use filtrated solution for the test.

Milk samples
Centrifuge milk samples for 15 min. at 2,000 g at 4°C, take samples from underneath the fatlayer, dilute the defatted milk 10 or 100 times in sample extraction buffer* and use this solution for the test.

Plasma samples
Dilute 100 μl plasma samples 10 or 100 times in sample extraction buffer* and use this solution for the test.

* for sample extraction buffer: see chapter 8 "Preparation of reagents".

8. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at 2 - 8 °C.

Prepare reagents freshly before use

Microtiter plate
Return unused strips into plastic ziplock bag with desiccant and store at 2°C-8°C for use in subsequent assays. Retain also the stripholder.

Rinsing buffer
The rinsing buffer is delivered 10 times concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (4 ml concentrated rinsing buffer + 36 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 Sulfadiazine standard. Add 2.0 ml of dilution buffer to the vial of Sulfadiazine standard and mix. This Sulfadiazine solution contains 10 ng/ml. Pipet 100 μl of this Sulfadiazine solution into a glass tube and add 900 μl of dilution buffer. Concentration of this diluted Sulfadiazine solution is 1 ng/ml. Continue to make a dilution range of 0.5 and 0.1 ng/ml. Keep the standard dilution range in the dark at room temperature until use.

Store at -20°C.

Conjugate solution
Reconstitute the vial of lyophilized conjugate (Sulphadiazine-HRPO) with 3 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 lyophilized antibodies with 3 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.]

Na₂HPO₄·2H₂O 0.96 g
KH₂PO₄ 0.17 g
NaCl 9 g
Tween 20 0.5 ml
Distilled water add to 1000 ml