AZAPERONE - AZAPEROL ELISA

5201AZA[5]05.20

A competitive enzyme immunoassay for screening and quantitative analysis of azaperone-azaperol in various matrices

EUROPROXIMA AZAPERONE - AZAPEROL ELISA

A competitive enzyme immunoassay for screening and quantitative analysis of azaperone-azaperol in various matrices

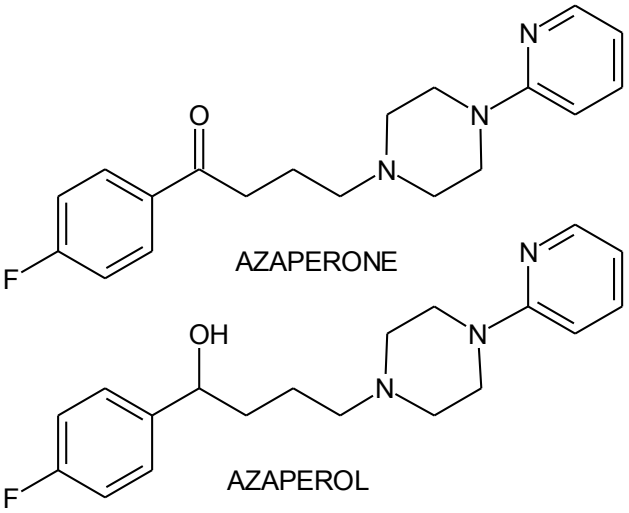
TABLE OF CONTENTS

The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

BRIEF INFORMATION

The azaperone-azaperol ELISA is a competitive enzyme immunoassay for measurement of the concentration of azaperone-azaperol. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analyzed.
The ELISA kit contains all reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structures of azaperone and azaperol

Azaperone [1-(4-fluorophenyl)-4-[4(2-pyridinyl)-1-piperazinyl]-1-butanone] is a neuroleptic tranquillizer belonging to the class of butyrophenones. Tranquillizers cause calmness, drowsiness and an indifference to the surroundings. In veterinary medicine, azaperone is used in pigs for a wide variety of indications, e.g. anti-aggressiveness, obstetrics, stress, sedation and anaesthesia. Pigs are particularly sensitive to stress and this can cause high mortality rates. In addition, stressed pigs produce poor quality meat, which is pale, soft and exudative and consequently, pig farmers may be financially penalized. Therefore, it is common practice for farmers to use a tranquillizer such as azaperone to prevent stress during transportation of pigs.
Azaperone has a number of effects on the central nervous system. As other neuroleptics, it is able to antagonize apomorphine and amphetamine-induced behavioural effects, mediated by brain catecholamines, especially dopamine. Therefore, it is thought to act by blocking dopamine receptors in the brain.

The major metabolite of azaperone is azaperol. For control purposes the marker residue is therefore the sum of azaperone and azaperol, as both compounds show pharmacological activity.
In the EU azaperone is listed in Annex I of Council Regulation (EEC) No. 2377/90. Maximum residue limits (MRLs) of the marker residue have been set at 100 µg/kg in porcine kidney and muscle tissue.

2. PRINCIPLE OF THE AZAPERONE-AZAPEROL ELISA

The microtiter plate based azaperone-azaperol ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled azaperol and standard solution or sample are added to wells. Free azaperol from the samples or standards and azaperol-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 1 hour the non-bound reagents are removed in a washing step. The amount of bound azaperol-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound azaperol-HRP conjugate transforms the colourless 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. The optical density is inversely proportional to the azaperol concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The azaperone-azaperol ELISA utilizes antibodies raised in rabbit against protein conjugated azaperone-azaperol. The reactivity pattern of the antibody is:

Cross-reactivity:

Azaperol	100%
Azaperone	100%
Haloperidol	< 0.01%

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)
Tissue	Dilution	3
Liver	Dilution	5
Urine	Dilution	0.2
Kidney	Extraction	10

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:

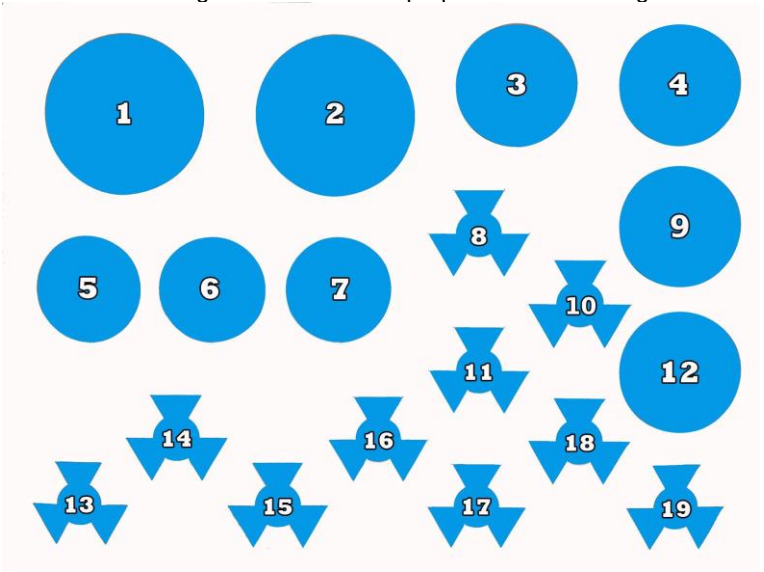
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (30 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate solution** (100 µl; 100x concentrated)
9. not in use
10. **Antibody solution** (100 µl; 100x concentrated)
11. not in use
12. not in use
13. **Zero Standard solution** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use) **0.0157 ng/ml**
15. **Standard solution 2** (1 ml, Ready-to-use) **0.0313 ng/ml**
16. **Standard solution 3** (1 ml, Ready-to-use) **0.0625 ng/ml**
17. **Standard solution 4** (1 ml, Ready-to-use) **0.125 ng/ml**
18. **Standard solution 5** (1 ml, Ready-to-use) **0.25 ng/ml**
19. **Standard solution 6** (1 ml, Ready-to-use) **0.5 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Acetonitril
- Petroleum ether
- 5N NaOH
- Dichloromethane
- 1 M HCl
- Methanol 100%
- Ethanol 100%
- Distilled water
- 50 ml tubes with screw cap (polypropylene)

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.

8. SAMPLE PREPARATION

8.1 Urine

Urine samples can be applied to the wells after a 1:10 dilution in dilution buffer.

- All samples are centrifuged at 2000 x g for 5 minutes.
- Pipette 50 µl of the supernatant into a glass tube.
- Add 450 µl of dilution buffer and mix thoroughly.
- Use 50 µl of this solution in the ELISA.

8.2 Tissue/liver

- Weigh 1 gram of homogenized sample in a glass tube.
- Add 3 ml 90% methanol (9 ml methanol 100%/1 ml distilled water), vortex, mix head over head for 30 minutes.
- Centrifuge at 2000 x g for 10 minutes.
- Dilute 10 µl supernatant with 490 µl dilution buffer, vortex.
- Use 50 µl of this solution in the ELISA.

8.3 Kidney

- Weigh 5 g of homogenized sample in a glass tube.
- Add 15 ml of acetonitrile, 5 ml of 1 N HCl and 10 ml of petroleum ether.
- Mix (head over head) for 15 minutes.
- Centrifuge at 2000 x g for 10 minutes.
- Remove the petroleum ether and decant the liquid phase in another glass tube.
- Reduce the liquid phase at approximately 5 ml by evaporation under a mild stream of nitrogen at +50°C.
- Add 3 ml of 5N NaOH and 20 ml of dichloromethane.
- Mix (head over head) for 15 minutes.
- Centrifuge the samples tube at 2000 x g for 10 minutes.
- Remove the upper layer and evaporate the layer underneath till dryness under a mild stream of nitrogen at +50°C.
- Add 500 µl of ethanol and 4.5 ml of dilution buffer to the residue (1 g of sample/ml).
- Vortex for 1 minute.
- Store overnight at +4°C in the dark.
- Samples must be diluted in dilution buffer before applying.
- Pipette 25 µl of sample in another glass tube, add 975 µl of dilution buffer and vortex for 30 sec.
- Use 50 µl of this solution in the ELISA.

9. 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 +2ºC to +8ºC. Prepare reagents fresh before use.

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

Dilution buffer
The dilution buffer is 10x concentrated. Before dilution (10 ml buffer + 90 ml distilled water) the concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution.

Conjugate solution
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at +2°C to +8°C.

Antibody
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation (1 min. 1000 x g). Add 5 µl of the concentrated antibody to 495 µl dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at +2°C to +8°C

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

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound 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 performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
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 from 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 zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.0157, 0.0313, 0.0625, 0.125, 0.25 and 0.5 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

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

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

6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

7. Incubate for 1 hour in the dark at room temperature (20ºC to 25ºC).

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.

10. Incubate 30 minutes in the dark at room temperature (20ºC to 25ºC).

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

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) 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/ Bmax (wells A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

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

Alternative for calibration curve:
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

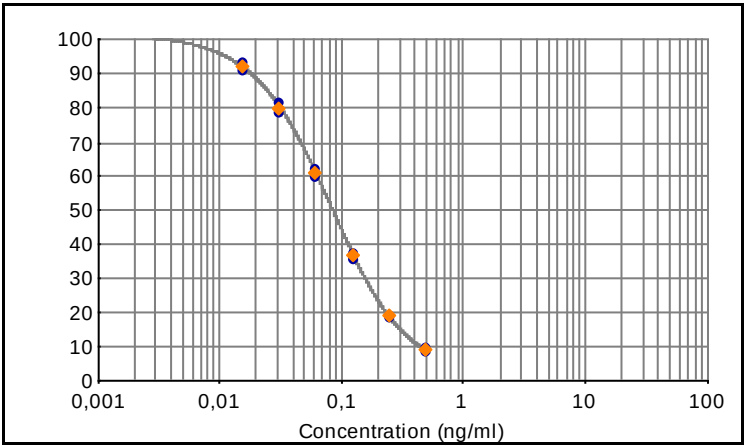


Figure 1 : Example of a calibration curve

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

Urine
To obtain the azaperone-azaperol content in urine samples, the calculated azaperone-azaperol concentration has to be multiplied by a factor 10.

Tissue/liver
To obtain the azaperone-azaperol content in tissue/liver samples, the calculated azaperone-azaperol concentration has to be multiplied by a factor 200.

Kidney
To obtain the azaperone-azaperol content in kidney samples, the calculated azaperone-azaperol concentration has to be multiplied by a factor 40.

12. LITERATURE

Cooper, J., Delahaut, P., Fodey, T.L. and Elliott, C.T. Analyst, 2004, **129**, 169.

Health and Consumer Protection, Veterinairy Inspections, Belgium, 1516-1998, http://ec.europa.eu/food/fs/inspections/vi/reports/belgium/vi_rep_belg_1516-1998_fr.pdf.

Council Regulation (EEC) No 2377/90 of 26 June 1990. Off. J. Eur. Commun. 1990, **L224**, 1.

13. ORDERING INFORMATION

For ordering the azaperone-azaperol ELISA kit please use cat. code 5201AZA.

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