GENERIC PROMAZINE ELISA

(5201PROM[3]04.20)

A competitive enzyme immunoassay for screening and quantitative analysis of several promazines in various matrices

EUROPROXIMA GENERIC PROMAZINE ELISA

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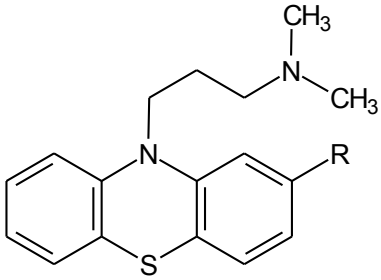
BRIEF INFORMATION

The generic promazine ELISA is a competitive enzyme immunoassay for measurement of the concentration of several promazines (chlorpromazine, acepromazine, propionylpromazine, promazine). 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 analysed.
The ELISA kit contains all the reagents to perform the assay. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of the promazines: chlorpromazine with R = Cl, acepromazine with R = $COCH_3$, propionylpromazine with R = COC_2H_5 and promazine with R =H.

Promazines belong to the phenothiazine class of antipsychotics. These agents are tranquillisers that act on the central nervous system causing calmness, drowsiness and an indifference to the surroundings. Tranquillisers are commonly used to reduce stress during transportation of food producing animals from farm to slaughterhouse. 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 (PSE meat) caused by stress-induced accelerated glycogen metabolism in muscle. Such meat is less marketable and pig farmers may be financially penalized. Therefore, it is common practice for farmers to use tranquillisers such as promazines to prevent stress during transportation of pigs. Furthermore, promazines are used in treating restlessness and agitation of horses and domestic cattle. In the EU the use of phenothiazine derivatives (chlorpromazine, acepromazine, propionylpromazine) to treat animals intended for human consumption is totally prohibited. There exists no Maximum Residue Limits (MRLs) for these agents.

2. PRINCIPLE OF THE GENERIC PROMAZINE ELISA

The microtiter plate based promazine ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labelled chlorpromazine and chlorpromazine standard solution or sample are added to the wells. Free chlorpromazine from the samples or standards and chlorpromazine-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 60 minutes the non-bound reagents are removed in a washing step. The amount of bound chlorpromazine-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound chlorpromazine-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 chlorpromazine concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The generic promazine ELISA utilizes antibodies raised in rabbit against protein conjugated chlorpromazine. The reactivity pattern of the antibody is:

Cross-reactivity:	propionyl promazine	130%
	acepromazine	110%
	chlorpromazine	100%
	promazine	50%
	chlorprothixene	20%
	fluphenazine	<0.01%
	xylazine	<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	8.2	4.3
Urine	8.1	1.1
Liver	8.3	0.2
Kidney	8.3	0.3

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:

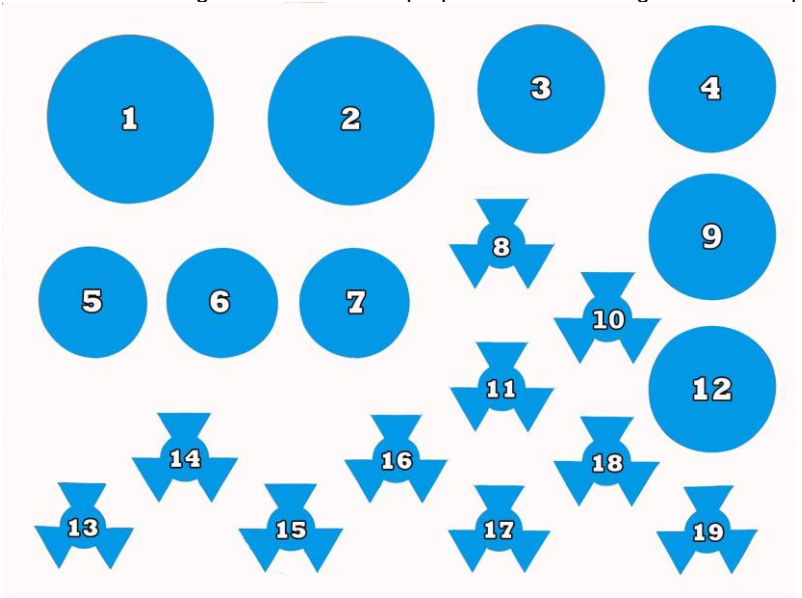
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

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. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. **Standard** (lyophilized, 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 BUT NOT PROVIDED

- 50 ml tubes with screw cap (polypropylene)
- Scales and weighing vessels
- Evaporation equipment
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Vortex
- Centrifuge (2000 x g)
- Automated microplate washer or 8 channel micropipette 100 - 300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Micropipettes 20-200 µl, 100-1000 µl
- Multipipette with 2.5 ml combitips
- Petroleum ether
- Sodium hydroxide 5M
- Hydrogen chloride 1M
- Dichloromethane
- N-Hexane
- Ethanol 100%
- Methanol 100%
- Distilled 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.

8. SAMPLE PREPARATIONS

8.1 Urine
- All samples are centrifuged at 2000 x g for 5 minutes.
- Pipette 20 µl of the supernatant into a glass tube.
- Add 480 µl of sample dilution buffer (see chapter 9) and mix thoroughly.
- Use 50 µl of this solution in the ELISA.

8.2 Tissue
- Weigh 1 g of homogenized sample in a glass tube.
- Add 4 ml 45% methanol (4.5 ml 100% methanol and 5.5 ml distilled water), vortex, mix head-over-head for 30 minutes.
- Centrifuge at 2000 x g for 10 minutes.
- Dilute 50 µl supernatant with 450 µl sample dilution buffer (see chapter 9), vortex.
- Use 50 µl of this solution in the ELISA.

8.3 Liver and kidney
- Weigh 5 g of homogenised sample in a glass tube,
- Add 15 ml of acetonitrile, 5 ml of 1 M 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 transfer the liquid phase in a glass tube.
- Reduce the liquid phase to approximately 5 ml by evaporation under a mild stream of nitrogen at +50°C.
- Add 3 ml of 5M NaOH and 20 ml of dichloromethane.
- Mix (head-over-head) for 15 minutes.
- Centrifuge the samples 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.
- Vortex for 1 minute.
- Store overnight at +4°C in the dark.
- For liver samples, pipette 40 µl of sample in another tube, add 960 µl of dilution buffer and mix (vortex) for 30 sec.
- For kidney samples, pipette 25 µl of sample in another tube, add 975 µl of dilution buffer and mix (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
This buffer is used for the dilution of conjugate, antibody, samples and to prepare the sample 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.

Sample dilution buffer
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows: Take 18 ml dilution buffer, add 2 ml 100% methanol, mix and store this buffer at +4°C until use.

Standards
Prepare a dilution range of chlorpromazine standards. Add 2 ml of sample dilution buffer to the lyophilized chlorpromazine standard and mix. This solution contains 9 ng chlorpromazine per ml.
Pipette 0.25 ml of this solution into a glass tube and add 0.5 ml of sample dilution buffer. Continue to make a dilution range of 3, 1, 0.33, 0.11 and 0.04 ng/ml.

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

Antibody
Reconstitute the vial of lyophilized promazine antibody with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Rinsing buffer (30 ml)
The rinsing buffer is delivered 20x 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 solution (12 ml)
The substrate solution (ready-to-use) tends to precipitate 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.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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 rim (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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing equipment

When using automatic plate washing equipment, make sure that all wells can be aspirated completely and that the rinsing solution is correctly 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 (well H1, H2, blank).

3. Pipette 50 µl of zero standard in duplicate (Bmax; well A1, A2).

4. Pipette 50 µl of each standard dilution in duplicate (B1,2 to G1,2)

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

6. Add 25 µl diluted conjugate (chlorpromazine-HRP) to all wells except wells H1 and H2.

7. Add 25 µl diluted antibody solution to all wells, except wells H1 and H2.

8. Seal the microtiter plate and shake the plate a few seconds.

9. Incubate for 1 hour in the dark at room temperature (20°C to 25°C).

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

11. Pipette 100 µl substrate solution into each well.

12. Incubate 30 minutes at room temperature (20°C to 25°C).

13. Add 100 µl stop solution to each well.

14. Read the optical density (OD) 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 (% 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 absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is logit and the X-axis is logarithmic.

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

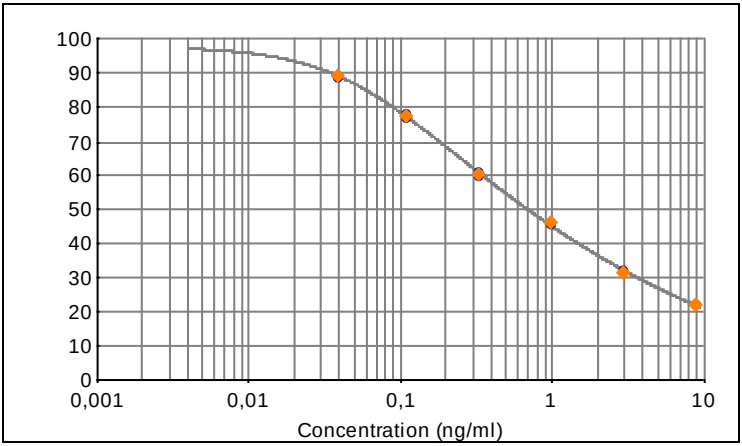


Figure 1: Example of a calibration curve

8.1 Urine

To obtain the content of promazines in urine samples, the calculated chlorpromazine concentration has to be multiplied by a factor 25.

8.2 Tissue

To obtain the content of promazines in tissue samples, the calculated chlorpromazine concentration has to be multiplied by a factor 50.

8.3 Liver and kidney

Using the extracton method, the promazine equivalents can be read directly from the calibration curve.

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,

13. ORDERING INFORMATION

For ordering the generic promazine ELISA kit please use cat. code 5201PROM.

14. REVISION HISTORY

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

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