

CARAZOLOL ELISA

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

EUROPROXIMA CARAZOLOL ELISA

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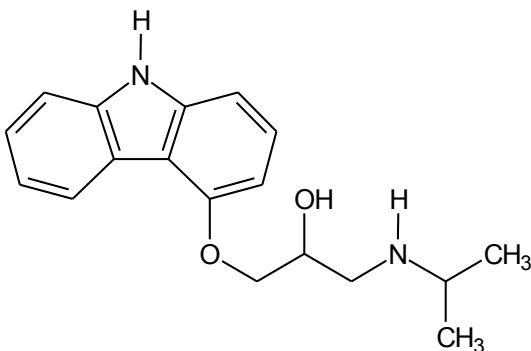
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BRIEF INFORMATION

The carazolol ELISA is a competitive enzyme immunoassay for measurement of the concentration of carazolol in various matrices. 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 structure of carazolol

Carazolol [1-(9H-Carbazol-4-yloxy)-3-(isopropylamino)-2-propanol] is a nonspecific β -adrenergic blocking agent that is used primarily in pigs to prevent sudden death due to stress during transport 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 a tranquilliser or β -blocker such as carazolol to prevent stress during transportation of pigs. Carazolol is also used in female pigs to relieve the stress of parturition and in male pigs to placate and prevent frenzy during mating. A less widespread use is in the prevention of reproductive stress in dairy cows.

In the EU, maximum residue limits (MRLs) have been established for both pigs and cattle. The compound is listed in Annex I of Council Regulation (EEC) No. 2377/90. The marker residue is the parent substance.

2. PRINCIPLE OF THE CARAZOLOL ELISA

The microtiter plate based carazolol ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled carazolol and standard solution or sample are added to the wells. Free carazolol from the samples or standards and carazolol-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 carazolol-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound carazolol-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 carazolol concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

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

Cross-reactivity:	Carazolol	100%
	Clenbuterol	< 0.02%
	Salbutamol	< 0.02%
	Adrenaline	< 0.02%
	Epinephrine	< 0.02%
	Alprenolone	< 0.02%

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 ng/ml (ppb)
Tissue	8.2	0.3
Urine	8.1	2.2
Liver, Kidney	8.3	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 (2°C to 8°C) 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:

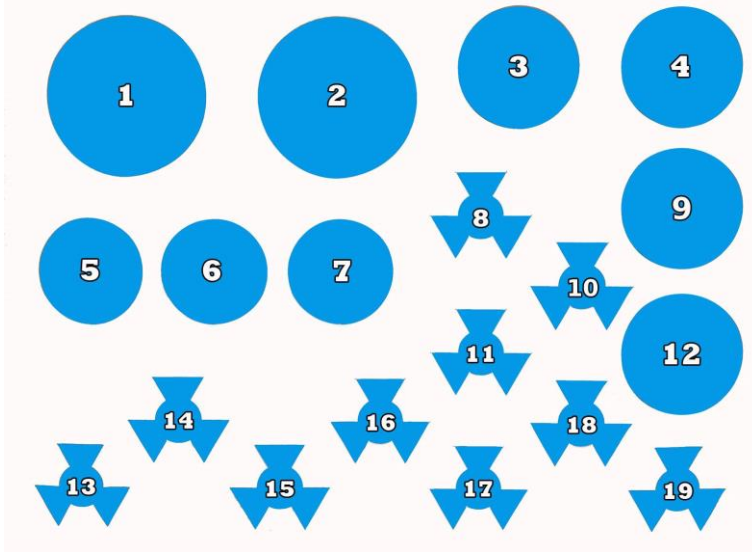
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells ($E_{450nm} < 0.8$).

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 μ l; 100x concentrated)
9. not in use
10. **Antibody solution** (100 μ l; 100x concentrated)
11. not in use
12. not in use
13. **Zero Standard solution** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.156 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.313 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.625 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.25 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **2.50 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **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
- 50 ml tubes with screw cap (polypropylene)
- Acetonitril
- Petroleum ether
- 5N NaOH
- 1N HCl
- 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 +4°C.
- 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 PREPARATION

8.1 Urine

- Centrifuged at 2000 x g for 5 minutes.
- Pipette 50 μ l of the supernatant into a glass tube.
- Add 350 μ l of dilution buffer and mix thoroughly
- Use 50 μ l of the sample in the ELISA.

8.2 Tissue

- Weigh 1 g of homogenized tissue in a glass tube.
- Add 3 ml 100% methanol, vortex and mix head-over-head for 10 minutes.
- Centrifuge at 2000 x g for 5 minutes.
- Transfer 1 ml of the supernatant to a glass tube (volume tube 4 ml).
- Evaporate under a mild stream of nitrogen at +50°C.
- Reconstitute the residue with 1 ml sample dilution buffer (see Chapter 9) and add 1 ml of n-hexane.
- Vortex for 1 minute.
- Centrifuge at 2000 x g for 5 minutes.
- Remove the upper (n-hexane) layer.
- Use 50 μ l of the clear layer underneath 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 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 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 5N 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 μ l 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 μ l of sample in another tube, add 960 μ l of dilution buffer and mix (vortex) for 30 sec.
- For kidney samples, pipette 25 μ l of sample in another tube, add 975 μ l of dilution buffer and mix (vortex) for 30 sec.
- Use 50 μ l of the sample 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 +2°C to +8°C. Prepare reagents fresh before use.

Microtiter plate

Return unused strips into the resealable bag with desiccant and store at +2°C to +8°C 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 (20°C to 25°C) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution.

Sample dilution buffer

Prepare this buffer as follows:

Take 18 ml dilution buffer, add 2 ml 100% methanol, mix.

Conjugate solution (100 µl)

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 (100 µl)

The antibody is delivered 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (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 +4°C. 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 μ l) 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 μ l of zero standard in duplicate (wells H1, H2, blank).
Pipette 50 μ l of zero standard (Bmax) in duplicate (wells A1, A2).
Pipette 50 μ l of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.156, 0.313, 0.625, 1.25, 2.5 and 5.0 ng/ml).
3. Pipette 50 μ l of each sample solution in duplicate into the remaining wells of the microtiter plate.
4. Pipette 25 μ l of conjugate (carazolol-HRP) to all wells, except H1 and H2.
5. Pipette 25 μ l 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 (20°C to 25°C).
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 (20°C to 25°C).
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.

O.D. standard (or sample)

-----x 100 = percentage maximal absorbance

O.D. zero standard/ Bmax

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

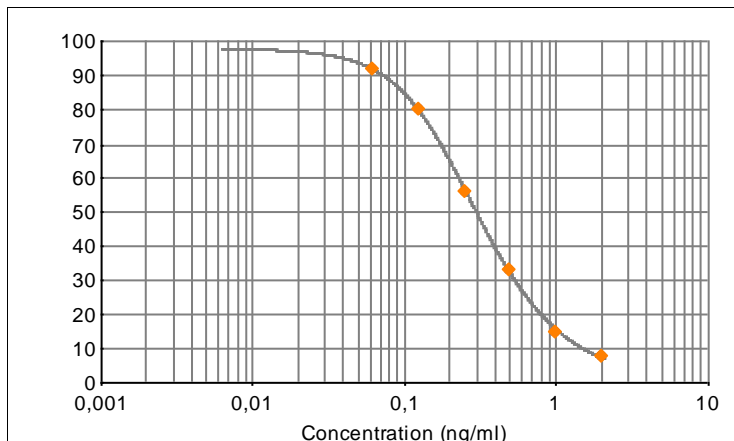


Figure 1 : Example of a calibration curve

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

Urine

To obtain the carazolol content in urine samples, the calculated carazolol concentration has to be multiplied by a factor 8.

Tissue

To obtain the carazolol content in tissue samples, the calculated carazolol concentration has to be multiplied by a factor 4.

Liver

To obtain the carazolol content in liver samples, the calculated carazolol concentration has to be multiplied by a factor 25.

Kidney

To obtain the carazolol content in kidney samples, the calculated carazolol 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, Veterinary 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 carazolol ELISA kit please use cat. code 5201CARA.

14. REVISION HISTORY

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