β-AGONIST ELISA

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A competitive enzyme immunoassay for
screening and quantitative analysis of β-agonists
in various matrices

EUROPROXIMA β-AGONIST-ELISA

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BRIEF INFORMATION

This β-agonist ELISA is a competitive enzyme immunoassay for the screening of urine, faeces, feed, bile, tissue, plasma and choroid/retina samples on the presence of several β-agonists. The test is based on a mixture of antibodies raised against salbutamol and clenbuterol and has a good sensitivity for β-agonistic drugs such as clenbuterol, salbutamol, bromobuterol, cimbuterol, mapenterol, mabuterol, tulobuterol, clenpenterol, clenproperol, terbutaline, carbuterol and cimaterol. 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

β-Agonistic drugs are used in human healthcare for the treatment of for instance asthma. In recent years, it has been established that a number of β-agonistic drugs may have repartitioning effects in meat producing animals. The flow of nutrients is apparently shifted from adipose tissue towards muscle tissue. The result is an improved lean meat deposition and higher production efficiency.
The use of β-agonists as feed additives is not permitted in the European Community. Nevertheless, there have been reported several incidences of the use of clenbuterol, salbutamol, mabuterol, mapenterol and bromobuterol in a number of European countries.
Urine is still the most frequently analysed sample material, however, other sample materials are used for different reasons. In farmhouses, urine, faeces, hair and feed can be sampled. Sampling of faeces is much easier and faster than sampling of urine, and the residue levels for β-agonists are comparable. At slaughter, edible tissues (liver, kidney and muscle) can be sampled next to body fluids (plasma, urine and bile) and eye samples. Bile is one of the most suitable sample materials for the control on misuse of anabolic steroids and can be preferred for the control of both steroids and β-agonists. Plasma samples are frequently used for pharmacokinetic studies.

2. PRINCIPLE OF THE β-AGONIST-ELISA

The microtiter plate-based ELISA kit consists of 12 strips, each containing 8 wells, precoated with sheep antibodies to rabbit IgG. Specific antibodies (rabbit anti-clenbuterol and anti-salbutamol), horseradish peroxidase labelled salbutamol (enzyme conjugate) and salbutamol standards or samples are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilized rabbit antibodies and at the same time free β-agonists (in the standard solution or in the sample) and enzyme labelled salbutamol 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 substrate chromogen (tetramethylbenzidine, 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 β-agonists concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The β-agonist-ELISA utilises a mixture of antibodies raised in rabbits against clenbuterol and salbutamol.

Cross- reactions:				
	Salbutamol	100%	Tulobuterol	50%
	Clenbuterol	100%	Clenpenterol	50%
	Bromobuterol	100%	Clenproperol	50%
	Cimbuterol	75%	Carbuterol	40%
	Mapenterol	70%	Terbutaline	40%
	Mabuterol	60%	Cimaterol	10%

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.

	Procedure	Salbutamol (ppb)	Clenbuterol (ppb)	Bromobuterol (ppb)	Cimbuterol (ppb)	Mapenterol (ppb)	Mabuterol (ppb)	Tulobuterol (ppb)	Clenpenterol (ppb)	Clenproperol (ppb)	Carbuterol (ppb)	Terbutaline (ppb)	Cimaterol (ppb)
Urine	*8.1.1*	0.75	0.75	0.75	1.3	1.3	1.7	2	2	2	2.5	2.5	10
Urine	*8.1.2.2*	0.1	0.1	0.1	0.15	0.15	0.15	0.2	0.2	0.2	0.25	0.25	1.0
Faeces, liver and Kidney	*8.2*	0.25	0.25	0.25	0.35	0.35	0.4	0.5	0.5	0.5	0.6	0.6	2.5
Bile and plasma	*8.3*	0.25	0.25	0.25	0.35	0.35	0.4	0.5	0.5	0.5	0.6	0.6	2.5
Muscle (SPE)	*8.5.2*	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.4	0.4	0.5	0.5	2.0
Retina	*8.4*	0.8	0.8	0.8	1.1	1.1	1.3	1.6	1.6	1.6	2.0	2.0	8.0
Feed	*8.6*	10	10	10	13	13	17	20	20	20	25	25	100
Milk	*8.8*	-	0.03	0.03	-	-	-	-	-	-	-	-	-

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:

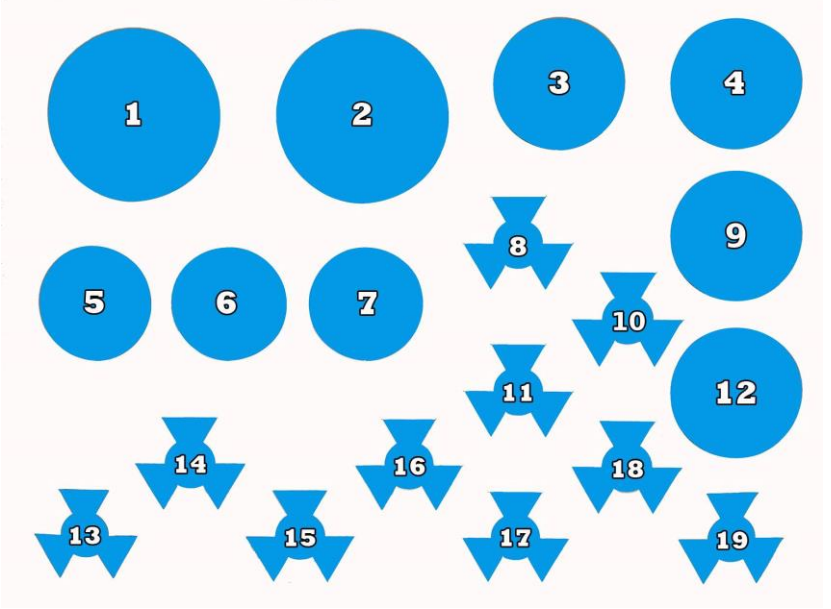
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed 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. not in use
8. **Standard 100 ng/ml** (1.0 ml, Ready to use)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero Standard solution** (2 ml, Ready to use)
14. **Standard solution 1** (1ml, Ready to use) **0.062 ng/ml**
15. **Standard solution 2** (1ml, Ready to use) **0.125 ng/ml**
16. **Standard solution 3** (1ml, Ready to use) **0.25 ng/ml**
17. **Standard solution 4** (1ml, Ready to use) **0.5 ng/ml**
18. **Standard solution 5** (1ml, Ready to use) **1.0 ng/ml**
19. **Standard solution 6** (1ml, Ready to use) **2.0 ng/ml**

6. EQUIPMENT REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Glass tubes
- Gloves
- Fume hood
- Homogeniser
- Vortex, mixer
- Centrifuge (4000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 0.5 – 10 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- 0.1 M HCl
- 0.2 M Sodium Acetate
- Helix Pomatia juice
- 0.25M Sodium Carbonate
- 1 M NaOH
- 10 M NaOH
- Isobutanol
- 0.1 M Phosphate buffer (pH 6)
- SPE columns 3 ml
- Methanol
- 1 M Acetic acid
- Ammonium hydroxide
- Ethylacetate
- Tris
- Calcium Chloride
- Pronase E from Streptomyces griseus
- 0.5 µm Filter
- SPEC MP1 Columns
- Tertiair Butyl Methyl Ether
- 0.1 M Disodium Tetraborate
- Di- Sodium Hydrogen Phosphate
- Potassium Di-Hydrogen Phosphate
- Potassium Chloride
- Sodium Chloride
- Tween 20
- Filter
- Phosphoric acid 0.2M
- 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 PREPARATION

8.1 Urine samples

Urine samples can be applied directly after a 5 times dilution in sample dilution buffer. Alternatively, to lower background of the urine samples, extraction procedures such as a simple Liquid-Liquid Extraction (LLE) or a Solid Phase Extraction (SPE) can be used.

8.1.1 **Direct method for urine**

Urine samples can be applied directly after a five times dilution in sample dilution buffer.

- Centrifuge for 5 minutes at 2000 x g.
- Pipette 50 µl of the supernatant into a glass tube, add 200 µl of sample dilution buffer and mix thoroughly (5 times diluted samples).
- Use 50 µl of diluted sample in the ELISA.

8.1.2 **Extraction procedures for urine**

REMARK I: In urine, most of the hydroxyl group(s) containing β-agonists (such as salbutamol, terbutaline and carbuterol) are present as glucuronide and/or as sulphate. For the direct method, enzymatic hydrolysis (using Helix pomatia juice) is not necessary. However, applying extraction methods, enzymatic hydrolysis prior to the extraction of urine samples is recommended for an effective extraction of the hydroxyl group(s) containing β-agonists.

8.1.2.1 Hydrolysis of urine samples

- Add 2 ml of 0.2 M acetate buffer (see chapter 9) to 2 ml of the urine sample
- Check the pH and if necessary, adjust the pH to 4.8 with a few drops of 1 M acetic acid
- Add 20 µl of Helix pomatia Juice
- Incubate overnight at 37ºC or during 2 hours at 55ºC
- Cool to room temperature before applying samples to one of the extraction procedures

8.1.2.2 Liquid-liquid Extraction (LLE) procedure

- After hydrolysis (8.1.2.1), add 2 ml of 0.25 M Sodium carbonate buffer pH 9.8
- Check and/or adjust pH (9.8 ± 0.2) with 1 M NaOH
- Add 2 ml of isobutanol to 1.5 ml of the diluted urine sample, mix (vortex) for 1 min and centrifuge (2-5 min at 2000 x g)
- Evaporate 1 ml of the upper layer (isobutanol) at 50ºC under a mild stream of nitrogen
- Dissolve the residue in 250 µl of sample dilution buffer (see chapter 9)
- Pipette 50 µl in the microtiter plate.

8.1.2.3 Solid Phase Extraction (SPE) procedure

- After hydrolysis (8.1.2.1), add 2 ml of 0.1 M phosphate buffer (pH 6.0) and adjust the pH to 6.0 ± 1.0 by adding drops of 1 M NaOH.
- The urine samples are purified by means of SPE columns as follows:
- Activate the columns by washing successively with 2 ml of 100% methanol and with 2 ml of 0.1 M phosphate buffer (pH 6.0).
- Transfer the hydrolysed and pH 6.0 adjusted urine samples onto the activated columns and draw them slowly through the columns (it should at least take 2 min; do not dry the columns.)
- Wash the columns with 1 ml of 1.0 M acetic acid. Dry the column under vacuum for 5 min.
- Wash the column with 6 ml of 100% methanol. Dry the column under vacuum for 2 min.
- Elute the β-agonists with 5 ml of 2% ammonium hydroxide in ethyl acetate.
- Evaporate the eluent to dryness at 50ºC under a mild stream of nitrogen.
- Dissolve the residue in 500 µl of sample dilution buffer (see chapter 9) and pipette 50 µl in the microtiter plate.

8.2. Faeces, liver, kidney

- To 1 g of homogenised faeces or tissue (liver or kidney) sample, 5 ml of 0.1 M HCl are added.
- Mix for 1 min. on a vortex and centrifuge for 10 min. at 2000 x g and at 4ºC.
- Transfer 2 ml of the supernatant (approximately 0.4 g of sample) into another tube and add 2 ml of 0.2 M acetate buffer (pH 4.8), check the pH and if necessary adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.
- Add 20 µl of Helix pomatia juice and incubate overnight at 37ºC or during 2 hours at 55ºC.
- After this incubation, the pH has to be adjusted to 9.8 ± 0.2 by adding 0.25 ml of 1 M Sodium carbonate pH 9.8 and, if necessary, a few drops of 1 M NaOH.
- Add 4 ml of isobutanol , mix (vortex) during 30 seconds and centrifuge for 5 min. (2000 x g).
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube and evaporate to dryness under a mild stream of nitrogen at 50ºC. Add 500 µl of sample dilution (see chapter 9) buffer to the residue and mix (vortex) during 30 seconds. Pipette 50 µl of this solution in the microtiter plate.

8.3 Bile and plasma

- To 1 ml of bile or plasma sample 4 ml of 0.1 M HCl is added.
- Mix for 1 min. using a vortex and centrifuge for 10 min. at 2000 x g, at 4ºC.
- Transfer 2 ml of the supernatant into another tube and add 2 ml of 0.2 M acetate buffer (pH 4.8), check the pH and if necessary, adjust the pH to 4.8 ± 0.1 with a few drops of 1 M NaOH.
- Add 20 µl of Helix pomatia juice and incubate overnight at 37ºC or during 2 hours at 55ºC.
- After this incubation, the pH has to be adjusted to 9.8 ± 0.2 by adding 0.25 ml of 1 M Sodium carbonate and a few drops of 1 M NaOH.
- Add 4 ml of isobutanol, mix (vortex) during 30 seconds and centrifuge for 5 min. at 2000 x g.
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube and evaporate to dryness under a mild stream of nitrogen at 50ºC. Add 500 µl of sample dilution buffer (see chapter 9) to the residue and mix (vortex) during 30 seconds. Pipette 50 µl of this solution in the microtiter plate,

8.4 Choroid/retina

- Remove the 'tissue' from top of the eyeball.
- Cut open the eyeball at the side of the lens.
- Remove the vitreous humour and fold the eye inside out.
- The choroid and retina are collected in a glass tube.
- The previously weighed glass tube is weighed again.
- The difference in weight is the weight of the choroid/retina tissue.
- Add 4 ml of 0.1 M Tris buffer containing 1.25 mg pronase E (see chapter 9).
- Incubate overnight at 55ºC and centrifuge (10 min at 2000 x g).
- Pipette 2 ml of the supernatant into a test tube and adjust the pH to 9.4 ± 0.2 by adding a few drops of NaOH (10 M).
- Add 4 ml of isobutanol.
- After vortexing for 30 sec, wait for 5 min (phase separation).
- Subsequently, pipette 2 ml of the upper layer (isobutanol) into a glass tube.
- Evaporate the isobutanol to dryness at 60ºC under a mild stream of nitrogen.
- Dissolve the residue in 1 ml of sample dilution buffer (see chapter 9).
- If the weight of the choroid/retina sample is 1 g than the dissolved residue contains 0.25 g sample/ml.
- Pipette 50 µl of this solution in the microtiter plate.

8.5 Muscle samples

8.5.1 Liquid extraction method

- Homogenise approximately 10 g of tissue
- Weight 1 g of the homogenised sample and transfer into a glass tube
- Add 4 ml of 0.1 M Tris buffer containing 1.25 mg pronase E (see chapter 9)
- Incubate overnight at 55ºC and centrifuge (10 min at 2000 x g)
- Pipette 2 ml of the supernatant into a test tube and adjust the pH to 9.4 ± 0.2 by adding a few drops of NaOH (10 M)
- Add 4 ml of isobutanol

- Vortex for 30 sec and wait for 5 min (phase separation)
- Pipette 2 ml of the upper layer (isobutanol) into a glass tube
- Evaporate the isobutanol to dryness at 60ºC under a mild stream of nitrogen
- Dissolve the residue in 250 µl of sample dilution buffer (see chapter 9).
- Pipette 50 µl of this solution in the microtiter plate.

8.5.2. Solid phase extraction method

- Homogenise approximately 10 g of tissue
- Weigh 1 g of the homogenised sample and transfer into a glass tube
- Add 4 ml of 0.1 M Tris buffer containing 1.25 mg pronase E (see chapter 9)
- Incubate overnight at 55ºC and centrifuge (10 min at 2000 x g)
- Pipette 2 ml of the supernatant into a test tube and adjust the pH to 9.4 ± 0.2 by adding a few drops of NaOH (10 M)
- Filter the solution through a 0.5µm filter
- For further sample clean-up, use SPEC MP1 columns
- Activate the columns by washing successively with 250 µl of 100% methanol and 250 µl of 0.1 M acetic acid
- Transfer 1 ml of the filtrate onto the columns and draw it slowly through the columns, flow 1 ml/min (do not dry the columns!!)
- Wash the columns with 500 µl of a solution of 100% methanol/0.1 M Acetic acid (1:1)
- Elute the β-agonists with 3 ml of 2% ammonium hydroxide in ethyl acetate
- Evaporate the eluent to dryness at 50ºC under a mild stream of nitrogen
- Dissolve the residue in 500 µl of sample dilution buffer
- Pipette 50 µl of this solution in the microtiter plate

8.6 Feed samples

- Approximately 50 – 100 g of sample is ground and pulverised into a fine powder.
- To 5 g of the ground feed sample, 25 ml of 100 % methanol and 25 ml of 0.2 M phosphoric acid are added.
- Shake for 30 minutes at room temperature and centrifuge for 15 min. at 2000 x g and at 4ºC.
- Pipette 0.25 ml of the supernatant and add 1 ml of sample dilution buffer (see chapter 9), check the pH (7.4 ± 0.2) and adjust if necessary, by a few drops of 1 M NaOH.
- Mix (vortex) for 30 seconds and centrifuge at 2000 x g for 10 min.
- Pipette 50 µl of the supernatant in the microtiter plate.

8.7 Hair samples

- An amount of 0.1 g of pre-washed hair is weighed into a test-tube and 2.5 ml of 5 M NaOH is added.
- The mixture is heated for 10 min. at 95ºC in a water-bath.
- Cool to room temperature and add 5 ml of tertiair butyl methyl ether.
- Mix the content in the tubes for 30 min. (head over head).
- Centrifuge the mixtures for 5 min. at 2000 x g.
- Transfer 2.5 ml of upper layer into another tube and evaporate at 50ºC under a mild stream of nitrogen.

- Dissolve the residue in 500 µl of sample dilution buffer (see chapter 9) and pipette 50 µl into the microtiter plate.

8.8 Skimmed milk powder and Milk samples

- To 100 g of skimmed milk powder an amount of 600 ml distilled water is added.
- The solubilised skimmed milk samples and milk samples are treated as follows:
- Defat approximately 5 ml of milk sample.
- Store the milk samples at 4ºC followed by centrifugation for 10 min at 4ºC and at 2000 x g.
- Remove the upper fat layer.
- Transfer 1 ml of defatted milk into a glass tube.
- Add 1 ml of 0.1 M di-Sodium-tetraborate, pH 8.
- Check the pH and add a few drops of 1 M NaOH to obtain a pH ≥ 8.
- Add 4 ml of tertiar-Butyl-methyl-ether and vortex for 30 seconds.
- Keep the tubes on a table to obtain phase separation.
- Pipette 2 ml of (upper layer) into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at a temperature of 50ºC.
- Dissolve the residue in 250 µl sample dilution buffer (see chapter 9)
- Pipette 50 µl of this solution in the microtiter plate.

9. PREPARATION OF REAGENTS

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

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready to use, 12 ml) precipitates at 4ºC. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

Standard solution (100 ng/ml)
To prepare standards in the appropriate matrix or to prepare spikes use the standard solution containing 100 ng salbutamol per ml. Dilute the standard in the sample matrix to make a dilution range of 2.0, 1.0, 0.5, 0.25, 0.125 and 0.062 ng/ml. Also the zero standard should be from the same matrix.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (SB-HRPO) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use.
Store the vial immediately after use in the dark at +2ºC to +8ºC.

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 +2ºC to +8ºC.

Sample Dilution buffer
To prepare additional dilution buffer dissolve in 1 L of distilled water

Na_2HPO_4	1.15g
KH_2PO_4	0.20g
KCl	0.20g
NaCl	30.0g
Tween 20	0.5ml

0.2 M Sodium Acetate buffer
Dissolve 16.4 g sodium acetate in bi-distilled water. Adjust pH to 4.8 with acetic acid.

Tris buffer pH 8.0
dissolve 24.2 g Tris and 14.7 g $CaCl_2$ in 1 L H_2O, adjust pH.

2% ammonium hydroxide in ethyl acetate
After preparation this solution appears turbid. Prepare the solution at least 12 hours before use and leave at room temperature.

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 for at least 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 at least 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).
   Pipette 50 µl of zero standard in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2, i.e. 0.062, 0.125, 0.25, 0.5, 1.0 and 2.0 ng/ml).
   Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

3. Add 25 µl of conjugate (SB-HRPO) to all wells, except wells H1 and H2.

4. Add 25 µl of antibody solution to all wells, except wells H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds.

6. Incubate for 1 hour in the dark at 4ºC. (2ºC - 8ºC)

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

8. Pipette 100 µl substrate solution into each well. Incubate 30 min. at room temperature (20ºC - 25ºC).

9. Add 100 µl stop solution to each well.

10. 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 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 A1 and A2) 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 = percentage maximal absorbance
O.D. zero standard

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the salbutamol equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.06 - 2 ng/ml range.

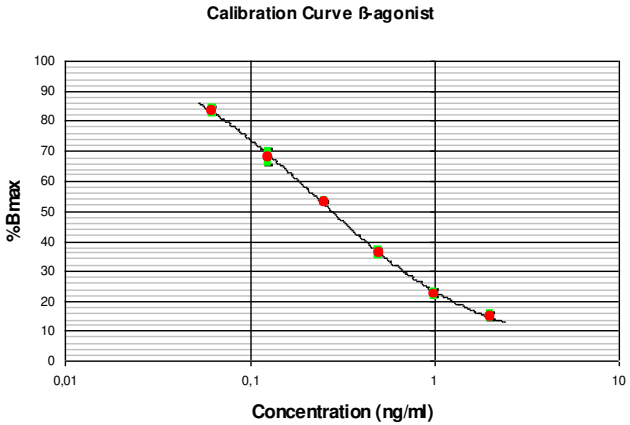


Figure 1 : Example of a calibration curve

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

Urine samples (direct)
Applying the ELISA on urine samples, the calculated salbutamol equivalents have to be multiplied by a factor 5.

Urine samples (liquid-liquid extraction)
The salbutamol equivalents are equal to the concentration in urine (dilution factor 1).

Urine samples (solid phase extraction)
The salbutamol equivalents have to be divided by a factor 4.

Faeces, liver, kidney
The salbutamol equivalents measured in the extracts have to be multiplied by a factor 2.5.

Bile and plasma
The salbutamol equivalents in the extracts have to be multiplied by a factor 2.5.

Choroid/retina
For choroid/retina, the calculated salbutamol equivalents have to be multiplied by a factor of 4.

Muscle samples (liquid extraction method)
The calculated salbutamol equivalents in the extracts are equal to the salbutamol equivalents in muscle .

Muscle samples (SPE extraction method)
The calculated salbutamol equivalents read from the standard curve have to be multiplied by a factor 2,5.

Feed samples
Applying the ELISA on feed samples, the calculated salbutamol equivalents have to be multiplied by a factor 50.

Hair samples

The salbutamol equivalents found in the hair extracts have to be multiplied by a factor 10.

Milk samples
The salbutamol equivalents found in the milk samples have to be divided by a factor 2.

Skimmed milk powder
The salbutamol equivalents found in the solubilised milk powder samples have to be multiplied by a factor 3.5.

12. LITERATURE

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13. ORDERING INFORMATION

For ordering the β-Agonist ELISA kit please use cat. code 5061BAG.

14. REVISION HISTORY

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

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