Competitive Enzyme Immunoassay Kit for Quantitative Analysis of Furaltadone metabolite (AMOZ)

1. Background

Nitrofurans are synthetic broad-spectrum antibiotics, which are frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. The nitrofuran drugs furaltadone, nitrofurantoin and nitrofurazone were banned from use in food animal production in the EU in 1993, and the use of furazolidone was prohibited in 1995.

The analysis of nitrofuran drugs residue needs to be based on the detection of the tissue bound metabolites of the nitrofuran parent drugs, since the parent drugs are very rapidly metabolized, and the tissue bound nitrofuran metabolites will retain for a long time, therefore the metabolites are used as the target in the detection of the abuse of nitrofurans. Furazolidone metabolite (AOZ), Furaltadone metabolite (AMOZ), Nitrofurantoin metabolite (AHD) and Nitrofurazone metabolite (SEM).

AMOZ-residues are determined most commonly by LC-MS or LC-MS/MS. Enzyme immunoassays, compared with chromatographic methods, show considerable advantages regarding sensitivity, detection limit, technical equipment and time requirement.

2. Test Principle

This ELISA kit is designed to detect AMOZ based on the principle of indirect-competitive enzyme immunoassay. The microtiter wells are coated with capture BSA-linked antigen. AMOZ in sample competes with the antigen coated on the microtiter plate for the antibody added. After the addition of enzyme conjugate, chromogenic substrate is used and the signal is measured by a spectrophotometer. The absorption is inversely proportional to AMOZ concentration in the sample.

3. Applications

This kit can be used in quantitative and qualitative analysis of AMOZ residue in honey.

4. Cross-reactions

Furaltadone metabolite(AMOZ)………………………100%
Furazolidone metabolite(AOZ)……………………..<0.1%
Nitrofurantoin metabolite(AHD)……………………<0.1%
Nitrofurazone metabolite(SEM)……………………..<0.1%
Furaltadone……………………………………………11.1%
Furazolidone…………………………………………..<0.1%
Nitrofurantoin…………………………………………..<1%
Nitrofurazone…………………………………………..<1%

5. Materials Required

5.1 Equipments

----Microtiter plate spectrophotometer (450nm/630nm)
----Rotary evaporator or nitrogen drying instruments
----Homogenizer / stomacher
----Shaker
----Vortex mixer
----Centrifuge
----Analytical balance (inductance: 0.01g)
----Graduated pipette: 10ml
----Rubber pipette bulb
----Volumetric flask: 100ml, 1L
----Glass flask: 10ml
----Polystyrene centrifuge tube: 2ml, 50ml
----Glass centrifuge tube: 10ml
----Micropipettes: 20ul-200ul, 100ul-1000ul, 250ul-multipipette

5.2 Reagents

----Ethyl acetate (AR)
-----Methanol (AR)
----n-hexane (or n-heptane) (AR)
-----Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) (AR)
----Concentrated hydrochloric acid (HCl, AR)
----Sodium hydroxide (NaOH, AR)
----Deionized water

6. Kit Components

- Microtiter plate with 96 wells coated with antigen
- Standard solutions(6 bottles)
  0ppb, 0.1ppb,0.3ppb,0.9ppb, 2.7ppb,8.1ppb
- Spiking standard solution: (1ml/bottle) …...**100ppb**
- Enzyme conjugate 12ml……………………….red cap
- Antibody solution 7ml ………..…………….green cap
- Solution A 7ml…………………………….white cap
- Solution B 7ml…………………………..…. red cap
- Stop solution 7ml………………………….yellow cap

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- 20×concentrated wash solution 40ml ……………………………………..…..transparent cap
- 2×concentrated extraction solution 50ml ………………………………………………..blue cap
- 2-Nitrobenzaldehyde 15.1mg………………black cap

7. Reagents Preparation

Solution 1: derivative reagent:

Add methanol to the bottle with 2-Nitrobenzaldehyde and diluted to 10ml. (at the concentration of 10mM).

Solution 2: 0.1M K_2HPO_4:

Weigh 22.8g $K_2HPO_4.3H_2O$ and dissolved with deionized water to 1L.

Solution 3: 1M HCl:

Transfer 8.3ml concentrated hydrochloric acid and dilute to 100ml with deionized water.

Solution 4: 1M NaOH:

Weigh 4.0g sodium hydroxide and dissolve with deionized water and dilute to 100ml.

Solution 5: extraction solution

Dilute 2×concentrated extraction solution with deionized water in the volume ratio of 1:1. This solution can be conserved for 1month at 4℃.

Solution 6: wash solution:

Dilute the 20×concentrated wash solution with deionized water in the volume ration of 1:19, which will be used to wash the plates. This diluted solution can be conserved for 1 month at 4℃.

8. Sample Preparations

8.1 Notice and precautions before operation:

(a) Please use one-off tips in the process of experiment, and change the tips when absorbing different reagent.

(b) Make sure that all experimental instruments are clean.

(c) the derivative reagent can be conserved at 2-8℃ for half a year;

(d) K_2HPO_4 solution can be stored at 2-8℃ for 3 months

(e) The HCl solution can be stored at room temperature for 3 months;

(f) The NaOH solution can be stored for 3 months at room temperature;

(g) Keep untreated samples in freeze;

(h) Treated samples can be stored for 24h at 2-8℃ in darkness .

8.2 Honey:

----Weigh 1.0±0.05g of honey sample into a 50ml polystyrene centrifuge tube, add 4ml deionized water, 0.5ml 1M HCl(**solution** 3) and 100µl derivative reagent (**solution** 1), shake completely for 2min;

----Incubate at 37 ℃ over night (about 16h);

----Add 5ml 0.1M K_2HPO_4 (**solution** 2), 0.4ml 1M NaOH (**solution** 4) and 5ml ethyl acetate, shake fiercely for 30s;

----Centrifuge at room temperature (20-25℃) for 10min, at least 3000g;

----Take 2.5ml of the supernatant organic phase into a 10ml clean glass tube, dry with 50°C nitrogen gas or rotary evaporator;

----Dissolve the dry leftover with 1ml n-hexane (or n-heptane), vortex for 30s, then add 1ml extraction solution (**solution** 5), and vortex for 1min to mix it completely.

----Centrifuge at room temperature (20-25°C) for 5min, at least 3000g;

----Remove the supernatant organic phase, and take 50µl of the substrate water phase for assay.

9. Assay process

9.1 Notice before assay:

9.1.1 Make sure all reagents and microwells are all at room temperature (20-25℃).

9.1.2 Return all the rest reagents to 2-8℃ immediately after used.

9.1.3 Washing the microwells correctly is an important step in the process of assay; it is the vital factor to the reproducibility of the ELISA analysis.

9.1.4 Avoid the light and cover the microwells during incubation.

9.2 Assay Steps:

9.2.1 Take all reagents out at room temperature (20-25℃) for more than 30min, shake gently before use.

9.2.2 Get the microwells needed out and return the rest into the zip-lock bag at 2-8℃ immediately.

9.2.3 The diluted wash solution should be rewarmed to be at room temperature before use.

9.2.4 **Number:** Numbered every microwell positions and all standards and samples should be run in duplicate. Record the standards and samples positions.

9.2.5 **Add standard solution / sample and antibody solution**: add 50µl of standard solution or prepared sample to corresponding wells, add 50µl antibody solution. Mix gently by shaking the plate manually and incubate for 30min at 25℃ with cover.

9.2.6 **Wash**: Remove the cover gently and pour the liquid out of the wells and rinse the microwells with 250µl diluted wash solution (**solution** 6) at interval of 10s for 4-5 times. Absorb the residual water with absorbent paper (the rest air bubble can be eliminated with unused tip).

9.2.7 **Add enzyme conjugate:** add enzyme conjugate 100µl to each well, mix gently, incubate for 30min at 25℃ in darkness with cover, take out and repeat the Wash Step;

9.2.8 **Coloration**: Add 50µl solution A and 50µl solution B to each well. Mix gently by rocking the plate manually and incubate for 15min at 25℃ with cover(see 12.8).

9.2.9 **Measure**: Add 50µl the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450nm (It's suggested measure with the dual-wavelength of 450/630nm. Read the result within 5min after addition of stop solution.)

10. Results

10.1 Percentage absorbance

The mean values of the absorbance values obtained for the standards and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100%. The zero standard is thus made equal to 100% and the absorbance values are quoted in percentages.

$$\text{Absorbance}\,(\%) = \frac{B}{B_0} \times 100\%$$

B ——absorbance standard (or sample)

B_0 ——absorbance zero standard

10.2 Standard Curve

To draw a standard curve: Take the absorbance value of standards as y-axis, semi logarithmic of the concentration of the AMOZ standard solution (ppb) as x-axis.

----The AMOZ concentration of each sample (ppb), which can be read from the calibration curve, is multiplied by the corresponding dilution factor of each sample followed, and the actual concentration of sample is obtained.

Please notice:

Special software has been developed for all data analysis, which can be provided on request.

Dilution factor of samples: 2.

11. Sensitivity, accuracy and precision

Sensitivity: **0.1ppb**

Detection limit………………………………..………..0.2ppb.

Accuracy……………………………………………90±20%

Precision: CV of the ELISA kit is less than 10%.

12. Notice

12.1 The mean values of the absorbance values obtained for the standards and the samples will be reduced if the reagents and samples have not been regulated to room temperature (20-25℃).

12.2 Do not allow microwells to dry between steps to avoid unsuccessful reproducibility and operate the next step immediately after tap the microwells holder.

12.3. Shake each reagent gently before using.

12.4. Keep your skin away from the stop solution for it is the 2M H_2SO_4 solution.

12.5 Don't use the kits out of date. Don't exchange the reagents of different batches, or else it will drop the sensitivity.

12.6 Keep the ELISA kits at 2-8℃,do not freeze. Seal rest microwell plates, Avoid straight sunlight during all incubations. Covering the microtiter plates is recommended.

12.7 Substrate solution should be abandoned if it turns colors. The reagents may be deteriorated if the absorbance value (450/630nm) of the zero standard is less than 0.5 (A450nm<0.5).

12.8 The coloration reaction needs 10-15min after the addition of solution A and solution B; But you can prolong the incubation time to 20min or more if the color is too light to be determined, never exceed 25min, on the contrary, shorten the incubation time properly.

12.9 The optimal reaction temperature is 25℃. Higher or lower temperature will lead to the changes of sensitivity and absorbance values.

13. Storage condition and storage period

Storage condition: 2-8℃.

Storage period: 12 months**.**

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