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Determination of Ivermectin and Zilpaterol in Cattle Plasma by Immunoassay

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Abstract

Competitive enzyme-linked immunosorbent assays (ELISA) for the detection of the veterinary drugs, ivermectin and zilpaterol were developed. Rabbit polyclonal antibodies were produced and used for development of the assays. The lower detection limit for both ivermectin and zilpaterol assays was 0.1 ppb. The ivermectin assay range is from 0.1 ppb to 3.6 ppb and the zilpaterol assay has a calibration range of 1 to 40 ppb. Samples of EDTA-plasma were collected from cows, calves and sheep. Acetonitrile was used to extract the drugs from the plasma. After dilution of the liquid extracts, each assay was run for total of 60 minutes (ivermectin) or 90 minutes (zilpaterol). The percent recovery from ivermectin spiked cow and calf plasma was determined to be within the range of 87-103% whereas zilpaterol recovery studies gave a range of 97-137% in the assay. Stability studies showed that both ivermectin and zilpaterol kits are stable for over 1 year at 4°C based upon accelerated stability prediction models. The results indicate that the immunoassay kits developed in this study can be used as rapid and accurate tools for determination of ivermectin and zilpaterol in blood samples.

Introduction

Ivermectin is a semi-synthetic derivative of avermectin B_1 and consists of an 80:20 mixture of the equipotent homologous 22,23 dehydro B_{1a} and B_{1b}. It is active at extremely low dosage against a wide variety of nematode and arthropod parasites, and widely used for the treatment and control of parasites in cattle, horses, sheep, swine and dogs. Due to the high potency of ivermectin, effective doses given to cattle and sheep are 0.1 mg kg^{-1} and lower. Therefore, very sensitive assays would be needed. Current US federal regulations for ivermectin tolerances are 15 ppb through 1600 ppb from liver as a target tissue for America bison, reindeer, sheep, swine and cattle. For muscle samples, ivermectin tolerance levels are 20 ppb and 650 ppb for swine and cattle, respectively. Studies indicate that plasma levels are a better monitor of tissue levels than urine since excretion in urine is less than 2% of the circulating ivermectin. Instrumental analysis of ivermectin for biological samples would need time-consuming extraction and complicated clean-up procedures. Therefore, routine screening of large numbers of biological samples should be simple and easy using collected blood samples.

Zilpaterol is a β-adrenergic repartition agent demonstrated to enhance carcass leanness, improve growth rates, and decrease feed consumption in cattle and sheep. It was approved by US FDA in 2006 and its tolerance is 12 ppb for cattle liver. However, Canada (2 ppb from muscle) and Brazil (1 ppb from urine) have lower maximum residue limits (MRL) for cattle. Instrumental analyses such as GC-MS and LC-MS/MS have been developed for analyzing zilpaterol. However, these methods require stringent clean-up procedures and skilled operators making them impractical for the high throughput needed for routine residue monitoring. For screening purposes, ELISA offers the advantages of simplicity, high throughput, rapid turnaround time and portability. In this study, competitive ELISAs were developed and evaluated allowing for simple and accurate determination of ivermectin and zilpaterol levels in the blood of cattle and sheep.

Methodology

Polyclonal antibody production:

In the development of these assays, ivermectin or zilpaterol was coupled to a protein carrier, which served as an immunogen, and horse radish peroxidase (HRP) which served as the enzyme conjugate. The antibody was produced through the immunization of New Zealand white rabbits. The antiserum was collected monthly.

Sample preparation:

❖ For Ivermectin assay

1. Transfer 0.5 mL of plasma into a glass vial.
2. Add 0.1 mL of Extraction Buffer.
3. Vortex for 10 seconds.
4. Add 0.9 mL of pure Acetonitrile.
5. Vortex at least 1 minute.
6. Let it stand until clean top layer appears (≈ 30 seconds).
7. Dilute the clean top layer 1:5 with Dilution Buffer.
8. Vortex 30 seconds.
9. Ready for assay.
10. The sample results must be multiplied by 15.

❖ For Zilpaterol assay

1. Transfer 2 mL of plasma into a glass vial.
2. Add 2 mL of pure Acetonitrile.
3. Vortex for 30 seconds.
4. Let it stand for 10 minutes.
5. Vortex at least 1 minute.
6. Allow few minutes until clean top layer appears.
7. Centrifuge for 1 minute at 6,000 rpm.
8. Dilute the supernatant 1:5 with deionized water.
9. Vortex 30 seconds.
10. Ready for assay.

Assay protocol:

1. Add 50 µL of HRP conjugate to each well.
2. Add 50 µL of calibrator or sample extract.
3. Add 50 µL of antibody solution to the wells and mix gently for 30 seconds.
4. Incubate the wells for 30 minutes in the Ivermectin assay.
   Incubate the wells for 60 minutes in the Zilpaterol assay.
1. Dump the well contents and wash 5 times with deionized water.
2. Add 100 µL of substrate solution into each well and incubate for 30 minutes.
3. Add 100 µL of stop solution and measure absorbance at 450 nm – 650 nm.
4. The concentration is calculated from the calibration curve multiplied by the total dilution factor used during the sample extract preparation.

Results

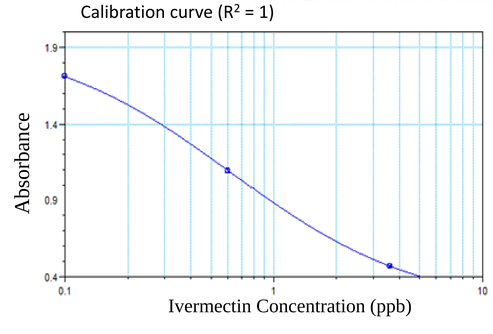
Table 1. Recovery of Ivermectin spiked in blood of calf and cow.

Calf	Ivermectin (ppb)	Recovery (%)
Blank	ND	---
5 ppb	4.59	92
10 ppb	10.3	103
20 ppb	20.3	102

Cow	Ivermectin (ppb)	Recovery (%)
Blank	1.21	---
5 ppb	5.55	87
10 ppb	11.2	100
20 ppb	20.1	94

Table 2. Curve and OD data of Ivermectin calibrators.

Calibration curve (R^2 = 1)



Conc. (ppb)	OD	Average OD ± SD	%RSD	%B0
0	1.921 2.011	1.966 ± 0.064	3.3	100
0.1	1.709 1.708	1.708 ± 0.001	0.0	87
0.6	1.107 1.087	1.097 ± 0.014	1.3	56
3.6	0.476 0.463	0.470 ± 0.009	1.9	24

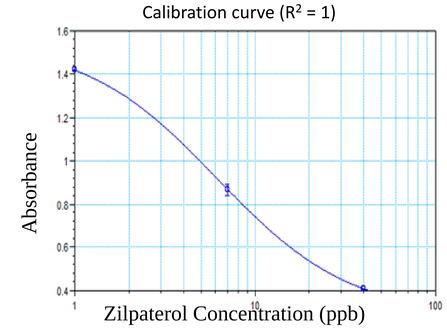
Results

Table 3. Recovery of Zilpaterol spiked in blood of calf, cow and sheep.

	Spike	Zilpaterol (ppb)	Recovery (%)
Calf	Blank	<1 ppb	---
	1 ppb	1.20	120
Cow	Blank	<1 ppb	---
	1 ppb	1.37	137
Sheep	Blank	<1 ppb	---
	1 ppb	0.97	97

Table 4. Curve and OD data of Zilpaterol calibrators.

Calibration curve (R^2 = 1)



Conc. (ppb)	OD	Average OD ± SD	%RSD	%B0
0	1.510 1.594	1.552 ± 0.059	3.8	100
1	1.417 1.421	1.419 ± 0.003	0.2	91
7	0.888 0.852	0.870 ± 0.025	2.9	56
40	0.408 0.410	0.409 ± 0.002	0.4	26

Discussion

Competitive immunoassays were developed for the determination of ivermectin and zilpaterol in animal blood (cattle and sheep). Sample cleanup and preparation is easy without the use of filters, solid phase extraction cartridges or immunoaffinity columns. Once collected, the animal's plasma can be used in the preparatory steps for both ivermectin and zilpaterol assays. Sample preparation takes little time and requires only 5 minutes for ivermectin and about 15 minutes for zilpaterol. Recoveries of ivermectin spiked in calf and cow blood ranged from 87% to 103%. Ivermectin determinations from untreated calf blood samples were below the detection level (ND) however cow blood levels were slightly elevated. A low residual level could be a result of Ivermectin treatment that had been done on the herd 6 months previous or it may be normal base line variability. Due to this reason, the recovery calculation from spiked cow blood samples included subtraction of the blank value from the spiked blood levels. A low level of zilpaterol (1 ppb) was chosen for spike and recovery testing since this is the target MRL in cattle urine in Brazil. Although the spiked level is close to the detection limit of the zilpaterol ELISA the range of recovery was found to be 97-137% in plasma samples (Table 3). The Ivermectin Plate Kit is stable longer than 1 year when stored at 4 °C. The Zipaterol Plate Kit is stable for over 60 days at 37 °C which conservatively predicts a shelf life of over 12 months at 4 °C . These results indicate that the immunoassays developed in this study can be used as rapid and accurate tools for the determination of ivermectin and zilpaterol residue levels in animal blood samples.

References

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