Clenbuterol: Unusual Aspects of its Urinary Excretion/Detection

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CLENBUTEROL/CONTEXT

- Long established 25 pg/ml threshold/4 day withdrawal time in plasma/serum.
- RMTC proposal to go to a 140 pg/regulatory threshold in URINE with a 14 day withdrawal time.
- Goal is to control the "partitioning effect" ("anabolic like" effect) produced by high dose therapy with clenbuterol.
- Concerns with testing in urine as follows:

 SUMMARY OF CONCERNS
1998-1999, working on urinary detection of clenbuterol; an unexplained spike in urinary clenbuterol at 10 days post dosing.

 Solution: Went to plasma testing, no longer detectable after 4 days, consistent with the clenbuterol rule in place until recently.

 14 day urinary testing?? Do we have urinary spikes of clenbuterol at 10 or more days post dosing?

Clenbuterol: Background

- Member of a large (50+family) of beta-2 agonist anti-asthmatics used in humans
- Structurally related to epinephrine
- Treats "heaves" (Recurrent Airway Obstruction/Chronic Obstructive Pulmonary Disease, COPD) and Inflammatory Airway Disease (IAD) in horses.
- Administered orally; dose varies from 0.8 to 3.2 ug/kg

Clenbuterol and the Lungs

- In the lungs clenbuterol:
- Relaxes smooth muscle in the bronchi: blocks bronchospasm (also "tocolytic")
 Reduces viscosity of mucus.
- Improves mucociliary clearance.
- Small anti-inflammatory effect.
- Overall, very useful pulmonary medication.

CLENBUTEROL

- ARCI Class 3 substance.
- Clenbuterol is an important therapeutic medication in the horse.
- Pharmacological action: Prevent bronchoconstriction.
- In US, Boehringer Ingelheim, oral syrup, Ventipulmin:
- The dose varies from 0.8 to 3.2 ug/kg
- Recommended therapeutic regimen: Five to ten days.

Clenbuterol: Clinical Uses

- Allergic airway disease: "heaves"
- Viral or bacterial airway infection
- Excess airway mucus
- Inflammatory airway disease
- Assist in recovery from airway infections, including pneumonia

THE PROBLEM

CLENBUTEROL → "REPARTITIONING"

Clenbuterol: "Repartitioning"

- Repartitioning: Animals on clenbuterol and related medications gain less fat, "put on" more muscle (a"quasi-anabolic" effect).
- Dose must be high and treatment long.
- Sheep: 2 mg/kg for 42 days.
- Steers: 30 ug/kg for 98 days.
- Horses: Probability of a significant repartitioning effect after 5-10 day therapeutic regimen is small.

Clenbuterol: "Repartitioning"

- Repartitioning considered to be a problem in certain areas, especially in Quarter Horse racing.
- Reportedly being imported from Mexico and used in large repartitioning type doses for the "partitioning effect".
- Has led to pressure to more strictly regulate the use of clenbuterol in racing, especially Quarter Horse racing.

Clenbuterol: "Repartitioning"

- 2011: Standard regulatory threshold for clenbuterol, 25 pg/ml, plasma serum, 72-96 hr withdrawal time.
- 2013: Proposed RMTC regulatory threshold 140 picograms/ml in URINE.
- Withdrawal time, 14 days.
- Dosing schedule "at 0.8 mcg/kg, twice a day"

BASIS FOR CONCERNS ABOUT A URINE THRESHOLD

- In about 1998 we worked on urinary regulatory threshold for clenbuterol.
- In 1998 I did not want to put out a plasma threshold that some labs might not be able to implement, so we looked at a urinary threshold.
- Found an unexplained peak of urinary clenbuterol at 10 days after last clenbuterol dose.
- Went to a plasma test/threshold!!

APPARENT URINARY CLENBUTEROL CONCENTRATIONS POST ORAL DOSING

THE SECOND FIGURE SHOWS APPARENT URINARY CONCENTRATIONS OF CLENBUTEROL AS DETERMINED BY ELISA

THE LOWER FIGURE SHOWL THE ACTUAL ELISA READINGS, BEFORE TRANSFORMATION TO URINARY CLENBUTEROL EQUIVALENTS

NOTE THAT THE CUVRE BEGINS TO GO DOWN AT DAY 8, HITS THE LOWEST POINT AT DAY 10 AND THEN, OES BACK UP.



Fig. 2. (a) Regression of ELISA standard curve for clenbuterol in equine urine using the Terbutaline ELISA test (Neogen Inc, Lexington, KY, USA): (b) individual urine concentrations of apparent clenbuterol as measured by ELISA; and (c) mean percentage of the maximum colour of the blank urine samle (which is 100% maximum colour).

ACTUAL URINARY CLENBUTEROL CONCENTRATIONS POST ORAL DOSING

INDIVIDUAL URINARY CLENBUTEROL CONCONTRATIONS AS TMS DERIVATIZED CLENBUTEROL MEASURED BY MASS SPECTROMETRY

MEAN URINARY CLENBUTEROL CONCENTRATIONS AS TMS AND BORONIC ACID DERIVATIZED CLENBUTEROL, QUANTIFIED BY MASS SPECTROMETRY.



Fig. 7. (a) Individual urine concentrations of clenbuterol as measured by the TMS-derivatization protocol for GC/MS following oral administration of clenbuterol (0.8 μ g/kg, b.i.d. for 10 days), and (b) mean comparison of methods for the analysis of clenbuterol-TMS and clenbuterol-MBA derivatives in horse urine after clenbuterol dosing (0.8 μ g/kg, b.i.d. for 10 days).



PLASMA THRESHOLD

- This 25 pg/ml plasma threshold went in to place in Kentucky and elsewhere and seemed to work very well in Thoroughbred racing.
- More recently there have been pressures to change to the very conservative 140 pg/ml RMTC urinary threshold as follows.

PRESSURE TO CHANGE CLENBUTEROL THRESHOLD

- Washington State: The lab performing the testing communicated with Commission personnel that the regulatory threshold in Washington needed change from the 25 pg/ml in plasma figure to a figure more consistent with the new proposed ARCI urinary threshold.
- This approach overlooked the defined and published regulatory threshold in place in Washington State, which was pointed out.

PRESSURES TO CHANGE CLENBUTEROL THRESHOLD

- In Florida, some months ago the laboratory appeared to change from a long in place 5 day withdrawal time and called a significant number of low concentration clenbuterol positives.
- Responding to inquiries, it was suggested that these positives would be reviewed, and things "went quiet" for a while.

PRESSURES TO CHANGE CLENBUTEROL THRESHOLD

More recently in Florida, the Laboratory has apparently gone back to more sensitive testing with a zero tolerance policy in place and a limit of detection of 5 pg/ml being used and reportedly a significant number of positives are currently on the books in Florida as we speak.

PRESSURES TO CHANGE CLENBUTEROL THRESHOLD

 More recently in Florida, the Laboratory has apparently gone back to more sensitive testing with a regulatory threshold of 5 pg/ml being put into place and a significant number of positives are currently on the books in Florida as we speak. ANALYSIS AND OPINIONS 1/ Unclear where the pressure on the laboratories to change the clenbuterol regulatory process is actually coming from.

2/ Given that we saw urinary clenbuterol concentrations at 10 days post dosing rise in an unexplained fashion, it would be appropriate to review the data on which the RMTC proposed 140 pg/ml urinary threshold is based and I would suggest that these data be made available for independent review.

SUMMARY

- 1998-1999, working on urinary detection of clenbuterol; Unexplained spike in urinary clenbuterol at 10 days post last dose.
- Solution: Went to plasma testing, reported no longer detectable after 4 days, consistent with the current clenbuterol rules in place in some jurisdictions.
- 14 day urinary testing?? Do we have urinary spikes of clenbuterol at 10 or more days post dosing?

ACKNOWLEDGEMENTS

The research presented here has been in large part supported by supported by gifts rants from the National, and State divisions of the Horsemen's Benevolent and Protective Association which support is deeply appreciated. The continuing support of the University of Kentucky, the Director and faculty of the Gluck Equine Research Center and the University of Kentucky Research Foundation are also gratefully acknowledged.

Acknowledgements

 The research presented here has been supported by grants from the Kentucky Science and Engineering Foundation [KSEF] the Kentucky Racing Commission and the Kentucky Equine Drug Research Council, the National, Florida and other divisions of the Horsemen's Benevolent and Protective Association, The Grayson-Jockey Club Research Foundation, the National Institutes of Environmental Health Sciences, and a gift from Mrs. John Hay Whitney. The continuing support of the University of Kentucky, the College of Agriculture, the Director and Faculty of the Gluck Equine Research Center and the University of Kentucky Equine Research Foundation are also gratefully acknowledged.

Clenbuterol: Detection time events

- Tested for years in US by standard ELISA.
- About a 72 hour detection time.
- No significant concerns.
- Enhanced ELISA introduced, Derby, 1988.
- Up to 30 day detection time in urine.
- Multiple identifications across US.
- Concentrations in urine variable.
- New detection strategy required.

Clenbuterol Analysis

- Recovery: Zymark Automated Recovery System.
- Previous recoveries: vacuum system.
- Horse urine is notoriously variable in viscosity.
- This is a computer controlled positivepressure apparatus that drives the urine through the extraction column.

Clenbuterol Analysis

- No derivatization step: Another source of error eliminated
- Liquid Chromatography Mass Spec-Mass Spec (LC-MS-MS)
- Derivatization: Methane boronic acid ("moronic acid") for Gas Chromatography



4-Aminoacetophenone

4-Amino-ω-bromo-3,5-dichloroacetophenone





Clenbuterol-d₉

Figure 1 Scheme for the synthesis of clenbuterol-d₉

Clenbuterol LC-MS-MS Method

- D₉ clenbuterol internal standard
- Zymark positive pressure extraction system
- Liquid Chromatography: no derivatization
- MS-MS MRM detection
- Highly sensitive: LOD about 4 pg/ml



SERUM WASHOUT OF CLENBUTEROL AFTER 10 DAY DOSING



Serum Testing for Clenbuterol

- LC-MS-MS based testing
- Highly sensitive parts per trillion (PPT)
- Serum-based testing: Better correlation to pharmacological effect than urine testing
- Detects intra-tracheal administration in minutes
- Fairer to horse and horseman
- Allows rapid test development

Clenbuterol Approach

- Highly sensitive detection of clenbuterol (10 picograms/ml)
- Measured plasma clenbuterol and followed elimination from plasma
- Smooth, reproducible elimination curve
- Plasma concentrations fall below that needed for pharmacological activity by 72 hours

Clenbuterol Approach

- This approach allows one to confidently set a "cut-off" for clenbuterol in plasma.
- It seems probable that an intra-tracheal administration would also be detectable by this approach.

Clenbuterol Regulation

- US Thresholds, urine.
- 1ng/ml, urine, Ohio
- 5ng/ml urine, California
- CANADA: 72 hour detection time
- AUSTRALIA: 72-96 hours after last dose.
- EUROPE: no data



TAKE HOME MESSAGE

Probably more research on bronchodilators than any other single group of equine therapeutic agents.

Relatively well established "detection time" data for clenbuterol rable amount of "detection time" data.



Synthesis of Clenbuterol-d9

TAKE HOME MESSAGE

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Clenbuterol and its two major metabolites

Serum concentrations of clenbuterol following intratracheal injection of 90 µg.

