A HIGHLY SENSITIVE LC/MS/MS DETECTION METHOD FOR CLENBUTEROL IN EQUINE SERUM

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ABSTRACT

A highly sensitive LC/MS/MS-based method for detection of the broncholdilator clenbuterol (4-amino-α-[(tert-butyl-d9-amino) methyl]-3,5-dichlorobenzyl alcohol) in equine serum samples has been developed. The method evolved from application of several recent developments in the authors' laboratory. First, deuterated clenbuterol (clenbuterol-d9) was synthesised as an internal standard. Secondly, solid phase extraction was run on a Zymark RapidTrace automated system, which afforded improved precision during extraction of low concentration samples. And thirdly, quantitative analyses were performed on a Hewlett-Packard 1050 HPLC equipped with a Micromass Quattro-II MS/MS set in ESI positive mode. The mobile phase was initially 0.05% formic acid + 5% acetonitrile in water, programmed along a linear gradient to 0.05% formic acid in acetonitrile. Quantitation of clenbuterol was based on the 277 m/z to 203 m/z transition corrected by the internal standard (clenbuterol-d9) 286 m/z to 204 m/z transition. The LC/MS/MS detector was optimised for detection of clenbuterol by adjusting argon levels in the intermediate gas cell for maximum production of the clenbuterol 203 m/z product ion. The standard curves in blank serum (0.01–1 ng/ml) were highly reproducible (r=0.9999) when fit to a secondary quadratic function, with an estimated limit of detection <10 pg/ml, and assay recovery was about 70%.

Five horses were administered oral clenbuterol (0.8 µg/kg, bid) for 10 days, and serum was collected for 14 days thereafter. Serum clenbuterol showed mean trough concentrations of ~150 pg/ml. After the last dose on Day 10, serum clenbuterol reached a peak of ~500 pg/ml and then declined with a half-life of roughly 7 h. Compared with previous results obtained from urine measurement of clenbuterol (Harkins *et al.* 2001), the serum-based approach proved to be more reliable and therefore more satisfactory for regulation of the use of clenbuterol.

Clenbuterol (90 µg) was also administered intra-tracheally to 5 horses. Peak serum concentrations of ~230 pg/ml were detected 10 min after administration, dropping to ~50 pg/ml within 30 min and declining much more slowly thereafter. These observations suggest the LC/MS/MS-based serum method can detect intra-tracheal clenbuterol when administered shortly before race time.

INTRODUCTION

Clenbuterol, a bronchodilator approved by the American Association of Equine Practitioners for use in horses, has the potential to alter athletic performance of racehorses, particularly if the horse has bronchospasm. This drug is classified by the Association of Racing Commissioners International (ARCI) as a Class 3 agent, meaning that its detection in post race samples may lead to significant penalties against trainers.

Previous work from the authors' laboratory has shown that clenbuterol can be detected readily in post administration urine samples at concentrations of 1–20 ng/ml using trimethylsilyl (TMS) derivatisation, and at concentrations of 1 ng/ml or less using methaneboronic acid (MBA) derivatisation methods with GC/MS (Harkins *et al.* 2001). The latter method for derivatisation is not preferred, however, because MBA is highly damaging to the GC column and the MS detector.

The application of urinary measurements for clenbuterol regulation raises concerns among regulators, horsemen and veterinarians for important reasons. First, clenbuterol persists in urine for at least 4 weeks after a prolonged therapeutic regimen. Secondly, recent work has shown that urine samples became negative (defined as <250 pg/ml) 5 days after dosing, but

of such hydroxylated metabolites of cocaine in the horse, although they are known to appear in human urine (Zhang and Foltz 1990; Steele *et al.* 1993; ElSohly *et al.* 1998) and have in fact been found to arise in equine liver microsomal preparations incubated with cocaine (C. Uboh, personal communication). We specifically note that para-OH-BZE was the hydroxylated metabolite present to the highest extent, and it is therefore our expectation that its measurement in equine urine will prove useful in the differentiation between actual equine cocaine metabolism and post collection contamination of samples. This is especially true with regard to hydrolytic cleavages such as to BZE which, as seen in Figure 4, can arise from cocaine spontaneously when spiked into equine urine.

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then became positive again with mean concentrations >1 ng/ml at Day 10 (Harkins *et al.* 2001). These concerns led to the consideration of serum as an alternative matrix with potentially superior properties for quantitative forensic purposes, and to the pursuit of a highly sensitive and specific serum quantitation method for clenbuterol.

Factors that, when applied in concert, enabled development of a sensitive method included: 1) synthesis of deuterated clenbuterol-d9 as an internal standard; 2) application of highly precise automated solid-phase extraction based on a Zymark RapidTrace positive-displacement; and 3) application of LC/MS/MS-based multiple reaction monitoring (MRM). Concentrations as low as 10 pg/ml with a limit of detection (LOD) of 4 pg/ml could be measured, roughly 10,000-fold more sensitive than current testing methods, enabling the detection and quantification of clenbuterol in only 2 ml of serum.

Application of this technology to serum samples from horses dosed with clenbuterol (0.8 µg/ml, bid x 10 days) allows for the measurement of steady-state trough and washout concentrations. This technology also allowed for the detection of clenbuterol in serum samples within minutes of intra-tracheal injection and for hours thereafter. This new testing methodology should therefore provide racing chemists and regulators with an extremely sensitive and highly reliable approach to regulating the use of clenbuterol in racehorses.

MATERIALS AND METHODS

Horses

Five mature Thoroughbred mares weighing 489–535 kg were maintained on grass hay and feed (12% protein), ie a 50:50 mixture of oats and an alfalfa-based protein pellet. They were fed twice daily. The animals were vaccinated annually for tetanus and were de-wormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). A routine clinical examination was performed before each experiment to confirm that the animals were healthy and sound. Horses were kept in a 20-acre field until they were placed in box stalls where they were provided with water and hay *ad libitum*. Animals used in these experiments were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care Use Committee, which also approved the experimental protocol.

Oral clenbuterol (0.8 µg/kg; Ventipulmin Syrup, Boehringer Ingelheim Vetmedica, Inc., Missouri, USA) was administered at 8.00 am and 4.00 pm for 10.5 days (21 dosings). Serum samples were collected by means of Vacutainer tubes (SST Gel and Clot Activator, Becton Dickinson, New Jersey, USA), before treatment started and then each day before the morning dosing. After the 21st dosing, samples were collected each morning for the next 14 days.

In a second experiment, injectable clenbuterol (Ventipulmin, Boehringer Ingelheim Ltd, Ontario, Canada) was administered intra-tracheally (IT, 90 µg) to 5 horses. Serum samples were collected before treatment and at 10, 20 and 30 min and at 1, 2, 4, 8 and 24 h after treatment.

Synthesis and characterisation of deuterated clenbuterol-d9

Clenbuterol-d9 (4-amino-α-[(tert-butyl-d9-amino) methyl]-3,5-dichlorobenzyl alcohol) was synthesised with 4-amino-acetophenone as substrate. Chlorination of 4-amino-acetophenone in acetic acid gave the 3,5-dichloro derivative which, after bromination in chloroform, provided 4-amino-ω-bromo-3,5-dichloro-acetophenone (Keck *et al.* 1972). The reaction of this bromoketone with deuterated *tert*-butylamine (H_2N-tBu-d9) in tetrahydrofuran gave the ketoamine analogue of clenbuterol-d9 in moderate yield. This was reduced to the alcohol with sodium borohydride in methanol, which yielded clenbuterol-d9. The final product was purified by column chromatography on silica gel and crystallised from ethyl ether.

Characterisation data for *4-amino-α-[(tert-butyl-d9-amino)methyl]-3,5-dichlorobenzyl alcohol):* colourless crystals from ethyl ether, m.p. 182-185°C; ^{1}H-nmr (300 MHz, $CDCl_3$): δ (ppm) 2.91 (dd, 1 H, J 10.5 Hz, J 12.0 Hz), 3.18 (dd, 1H, J 2.4 Hz, J 12.0 Hz), 4.48 (s, 2H), 5.30 (dd, 1H, J 2.4 Hz, J 10.5 Hz), 7.29 (s, 2H).

Serum analysis for clenbuterol by LC/MS/MS

Standard solutions of clenbuterol and clenbuterol-d9 were prepared in methanol. Extraction standards were prepared by the addition of a known amount of a clenbuterol solution to blank serum samples at a range of 0.01–1.0 ng/ml. A known amount of a clenbuterol-d9 standard (50 µl of 1 µg/ml in 10% mobile phase in H_2O) was added to each sample, standard and blank as an internal standard.

The serum samples, standards and blanks (2 ml/sample) were placed in culture tubes. Then 2 ml 0.1 M $NaHPO_4$ buffer, pH 6 was added, and the sample pH was adjusted as needed to 6.0 ± 0.5 with 1 M NaOH or 1 M HCl. Standards were prepared at the following concentrations: 0.0, 0.01,

TABLE 1: Settings used for clenbuterol and clenbuterol-d_9 data acquisition by LC-MS/MS

Precursor ion, mz	Product ion, mz	Dwell time, s	Collision energy, eV	Cone voltage, V
277	259	0.01	16	19
277	203	0.05	16	19
277	57	0.01	16	19
279	205	0.01	16	19
279	261	0.01	16	19
279	57	0.01	16	19
286	204	0.05	16	19
288	206	0.01	16	19

0.05, 0.1, 0.5 and 1 ng/ml from appropriate concentrated stocks in mobile phase.

Solid phase extraction was performed on the RapidTrace SPE Workstation (Zymark Corporation, Massachusetts, USA). Clean Screen SPE columns (Worldwide Monitoring #ZSDAU020, United Chemical Technologies, Pennsylvania, USA) were conditioned by sequential addition of 3 ml methanol, 3 ml water and 1 ml 0.1 M sodium phosphate buffer, pH 6. The samples were then loaded and the column was washed sequentially with 2 ml water, 2 ml 1 M acetic acid and 4 ml methanol. The column was eluted with 3 ml dichloromethane/isopropanol/NH_4OH, conc. (78/20/2). The eluent was evaporated to dryness under a stream of N_2 (<40°C). Each sample was dissolved in 50 µl mobile phase.

Extracts were analysed with a Hewlett-Packard Model 1050 LC equipped with a Micromass VG Quattro-II MS/MS (Micromass, Massachusetts, USA) set in ESI-positive mode. The column was a Luna phenyl/hexyl 2 mm x 250 mm x 5 µ reverse phase column (Phenomenex, California, USA). The mobile phase was a gradient of solvent A (0.05% w/v formic acid in water + 5% v/v acetonitrile) and Solvent B (0.05% w/v formic acid in acetonitrile), flowing at 0.45 ml/min throughout and which varied as follows: 0 min, 100% A; 1 min, 100% A; 5 min, 10.5% A and 89.5% B; 10 min, 10.5% A and 89.5% B; 10.5 min, 100% A; 15 min, 100% A. Each sample was transferred to an autosampler vial from which a 25 µl aliquot was injected onto the LC/MS/MS. Transitions monitored for clenbuterol and its deuterated internal standard are summarised in Table 1. Quantitation of clenbuterol was based on the 277 m/z to 203 m/z transition corrected by the internal standard (clenbuterol-d9) 286 m/z to 204 m/z transition.

MS/MS

Full scan electrospray ionisation (ESI) mass spectra were obtained on analytical standards at 10 µg/ml in 1:1 acetonitrile:0.05% formic acid (aq), pH 3, by infusion at 0.6 ml/h with a Harvard syringe pump (Massachusetts, USA) into the electrospray probe of a Quattro II MS/MS set in positive ion mode. The Quattro II MS was tuned for positive mode by direct infusion of 10 µg/ml of clenbuterol in 1:1 acetonitrile:0.05% formic acid (aq). The peak shape and intensity of the monoprotonated clenbuterol 277 m/z ion were optimised by adjustment of capillary, HV lens, cone voltage, skimmer lens and RF lens settings. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimising settings for maximum production of the 203 m/z product ion. Generally, the collision gas was set to 1 x 10^{-3} mbar, the source cone voltage was set at +19 v, the collision energy was set at -16v, the capillary of the ESI probe was set at +2.6 kvolts, skimmer was set at 1.0 v and the HV lens was set at +0.5 kvolts. Source temperature was set at 120°C.

Quantitation of serum clenbuterol using LC/MS/MS

Clenbuterol standard curves were generated by automated data collection and fitting of resultant data to a second order quadratic curve with either MassLynx (Micromass, Massachusetts, USA) or Excel (Microsoft Corporation) software. The 2

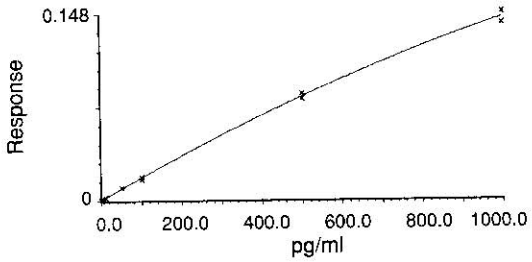

Fig 1: Typical calibration curve for clenbuterol (m/z 277--> 203) with clenbuterol-d9 (m/z 286-->204) as internal standard (IS) and acquisition by LC-ESI-MS/MS with MRM data acquisition. The curve in this case was fitted to a second order equation, calculated as -3.95401 x $10^{-8}x^2$ + 0.000182846x + 0.000626380. The origin was excluded, point weighting was 1/x, and response was calculated based on the measured area x (IS concentration/IS area). The coefficient of determination was 0.998457.

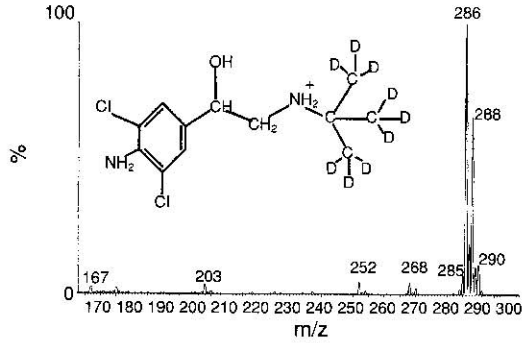

Fig 2: Clenbuterol-d9 full scan ESI-MS obtained by direct infusion at 10 µg/ml in acetonitrile:0.05% formic acid, 1:1. Inset reveals structure including locations of deuteriums.

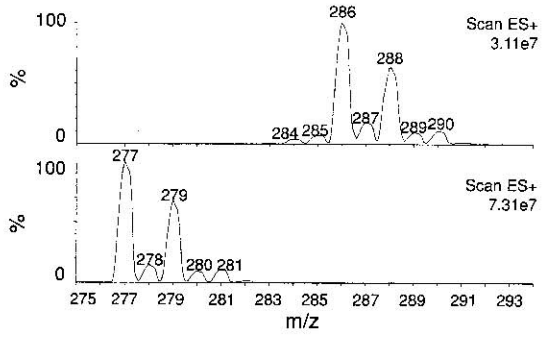

Fig 3: Full scan ESI(+)-MS of clenbuterol-d9 [top] in comparison to clenbuterol-d0, with emphasis placed on the vicinity immediate to each compound's molecular ion group.

methods gave generally equivalent results. Typical results showed a range of MS response areas from approximately 400 (background) for 0 ng/ml to 20,000 for 1 ng/ml with excellent point fit to the curve. A typical calibration curve as generated by MassLynx software for clenbuterol is shown in Figure 1, with response in this example based on the measured area x (I.S. concentration/I.S. area).

Validation of quantitative LC/MS/MS method for serum clenbuterol

The LC/MS/MS method for the quantitation of clenbuterol was validated by measurement of the consistency of results (within-run and between-run), correlation of standard values (coefficient of regression of the standard curve) and recovery of the assay. The within-run precision was calculated from similar responses from 6 repeats of low (0.01 ng/ml), middle (0.1 ng/ml), and high (1 ng/ml) standards in one run. The between-run precision was determined by comparing the calculated response (in ng/ml backfit of the standard curve) of these 3 standards over 6 consecutive daily runs. The recovery was determined by comparing the response (in area) of low and high standards and the response from equivalent methanol standards. The limit of detection was calculated as the mean of the assay background plus 2 times the standard deviation of the mean for 12 runs. Limit of quantitation was determined as the mean of the assay background plus 6 times the standard deviation of the mean for 12 runs.

RESULTS

Purity and mass spectral characteristics of clenbuterol-d9

The ESI(+)-MS full scan of clenbuterol-d9 is shown in Figure 2. Peak intensities neighbouring 286 m/z suggested that there were small amounts of d8 and d7 in this preparation, roughly 7% and 2%, respectively. This is emphasised by comparison to undeuterated clenbuterol, shown in Figure 3, in which no peaks were present immediately below the major molecular ion at 277 m/z. Of more immediate concern was whether this preparation contained undeuterated clenbuterol-d0, which would arise from undeuterated contaminants in *tert*-butylamine-d9 during the original reaction. Direct infusion and HPLC measurements estimated the amount of clenbuterol-d0 at no more than 0.01%, lending assurance that even relatively large amounts of this internal standard could be added without serious contamination of clenbuterol.

Quantitation of serum clenbuterol using LC/MS/MS

Clenbuterol quantitative measurements were enhanced by optimisation of the yield of product ions from the compound's most prevalent transition, specifically m/z 277–>203. In addition to regular tuning parameters, special emphasis was placed on optimising collision gas pressure for production of the 203 m/z ion, as shown in Figure 4. Optimum yield occurs in the vicinity of 1×10^{-3} mbar.

Clenbuterol elicits several ions with electrospray MS, and these in turn offer product ions of significant intensity, as listed in Table 2. Qualitative identification of clenbuterol was assured by locating qualifier transitions during gradient HPLC analysis. The area ratios for these various transitions are summarised in Table 3.

Typical standard curves for clenbuterol in serum are best described by second order quadratic regressions, which yield a small positive Y-intercept, and have correlation coefficients r=1.0000.

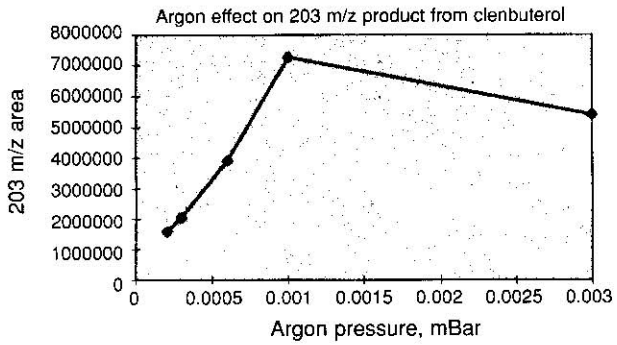

Fig 4: Optimisation of argon gas pressure for collision-induced dissociation of clenbuterol 277 m/z ion to produce the 203 m/z product ion.

TABLE 2: Principal product ions arising from selected ions observed in the full scan ESI(+)-MS of clenbuterol

Precursor ion, m/z	Principal product ion(s), m/z
277	259, 203, 57
279	261, 205, 57
281	263, 207, 57
259	203, 57
261	205, 57
203	168, 132

TABLE 3: Relative areas of clenbuterol transitions as revealed on chromatography via gradient HPLC-MS/MS

Transition	Relative area
m/z 277-> 203	100%
m/z 279 -> 205	64%
m/z 277 -> 259	14%
m/z 279 -> 261	7.3%
m/z 277 -> 57	1.4%
m/z 279 -> 57	0.7%

Validation of quantitative LC/MS/MS method for serum clenbuterol

The within-run precision was determined for the low (0.01 ng/ml: CV = 18%), middle (0.1 ng/ml: CV=3.7%) and high (1 ng/ml: CV=0.7%) concentrations of the clenbuterol standard curve, with a mean CV of 7.4%. The between-run precision was determined for the low (0.01 ng/ml: CV=29%), middle (0.1 ng/ml: CV=7.2%) and high (1 ng/ml: CV=0.1%) concentrations of the clenbuterol standard curve, with a mean CV of 12%. Correlation coefficients for standard curves, r, were routinely 0.999 or greater. Assay recovery was determined at 2 concentrations; the mean recovery was 76%. The limit of detection for the LC/MS/MS method for the quantitation of clenbuterol was 0.004 ng/ml (n=12) and the calculated limit of quantitation is approximately equal to the lowest standard used (10 pg/ml) at 0.014 ng/ml.

Data acquired with the ESI(+)-LC-MS/MS clenbuterol method

The oral administration regimen was followed by serum collection and analysis, providing the data listed in Table 4 for 5 horses, showing the clenbuterol concentration in serum each day before the morning dosing. Note that serum was not collected on Days 4 and 5. A gradual increase could be seen in serum clenbuterol concentrations over the course of the 10-day dosing, suggesting that clenbuterol had not yet reached steady state by Day 10. The maximum trough concentration of clenbuterol in serum was about 200 pg/ml on Day 10. Serum clenbuterol declined rapidly at the end of dosing and, by Day 14, was no longer detectable. Serum clenbuterol declined rapidly with a half-life of approximately 7 h.

Table 5 shows the serum concentrations of clenbuterol in serum following intra-tracheal injection of the agent. The mean peak serum concentration of 223 pg/ml was measured at the first sampling after treatment (10 min), suggesting a rapid absorption from the respiratory tract. Serum clenbuterol decreased rapidly during the first hour, then fell more slowly from 1 h (40 pg/ml) to 8 h (29 pg/ml). At 24 h, the serum concentration was estimated at 6 pg/ml, which was above the calculated limit of detection (4 pg/ml).

Discussion

To develop a quantitative analytical method for serum clenbuterol, deuterated clenbuterol was first synthesised as an internal standard. The data reported here are based on use of clenbuterol-d9 as internal standard, which, in terms of deuterated product, was >99.9% pure. Purity of the internal standard is critical due to the low LOD. The <10

TABLE 4: Trough serum clenbuterol (pg/ml) concentrations during and after treatment (0.8 µg/kg bid x 10 days)

Day	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
1	98	137	67	148	89
2	122	139	95	232	112
3	124	128	96	150	118
6	112	115	111	159	102
7	137	162	131	184	103
8	138	116	125	202	108
9	186	175	142	214	126
10	214	181	173	214	272
11	100	64	67	111	123
12	13	6	15	114	28
13	nd	nd	8	40	17
14	nd	nd	nd	nd	nd

nd = not detected

pg/ml LOD for this method is at least 10,000 times more sensitive than the regulatory threshold for plasma furosemide (100 ng/ml). Because the clenbuterol-d9 contained a very small amount (<0.01%) of non-deuterated clenbuterol, the assay yielded a small positive Y-intercept. A major source of variability in drug recovery is the sample-to-sample variability in viscosity of equine serum and especially urine samples. These viscosity differences affect the rates at which samples can be extracted in vacuum-driven (negative-pressure), solid-phase extraction devices. The Zymark programmable positive-displacement solid-phase extraction system allowed for consistent recovery and quantitation of clenbuterol as low as 10 pg/ml from only 2 ml of serum. Furthermore, the LC-MS/MS system was optimised for detection of very low concentrations of clenbuterol in serum. Use of gradient HPLC for chromatography enabled elimination of the derivatisation steps necessary for GC/MS, and therefore removed a significant source of variability. Finally, use of MRM detection significantly reduced background noise, thereby enabling high levels of accuracy and reproducibility.

Application of our serum assay to horses receiving a 10-day course of clenbuterol is in good agreement with the earlier report of Kallings *et al.* (1991) concerning the uptake and elimination of clenbuterol in the horse. At the initiation of treatment, serum levels of drug reached a plateau within 2 days. The trough concentrations of clenbuterol during this plateau averaged 125–150 pg/ml between Days 1 and 8 of treatment but, by Day 10, exceeded 200 pg/ml. The trough concentrations that we report are less than those reported by Kallings *et al.* (250–400 pg/ml), but this may simply be a function of the dose schedules and sampling times used in the 2 studies.

TABLE 5: Serum concentration (pg/ml) of clenbuterol following intra-tracheal injection of 90 µg

Hours	Horse 1	Horse 2	Horse 3	Horse 4
0.17	104	273	365	153
0.33	96	154	191	80
0.5	42	45	128	35
1	38	33	48	39
2	31	20	50	40
4	33	24	59	31
8	21	20	35	37
24	7	2	3	13

A major concern raised during the Association of Racing Commissioners International workshop on clenbuterol was the inability of urine testing to detect intra-tracheal injection or nebulisation of clenbuterol shortly prior to racing. The high sensitivity of our method allowed us to detect such intra-tracheal administration up to 24 h after administration. Ten minutes after intra-tracheal injection of 90 mg clenbuterol, it was present in high concentrations (200 pg/ml) in serum. It declined rapidly to 50 pg/ml at 30 min but then decreased more slowly to reach the limit of detection of the method by 24 h. The fear that detection of intra-tracheal clenbuterol will be hampered by co-administration of furosemide is not a concern when serum concentrations are measured. Furosemide would decrease serum volume and therefore raise the concentration of clenbuterol.

Comparison of the data presented here with the lung function data of Robinson *et al.* (2001) support Robinson's estimated pharmacological threshold concentration of clenbuterol (30 pg/ml). By 2 h after intra-tracheal injection there were no obvious effects on lung function; serum levels were close to and below the suggested pharmacological threshold.

The findings support the established position of Agriculture Canada that a 72 h 'detection-time' is appropriate for clenbuterol. By 72 h after dosing, the serum concentrations of clenbuterol were ~10 pg/ml, much below the estimated 'no pharmacological effect' threshold for this agent. The manufacturer's recommended dosing schedule (bid) also suggests that the pharmacological effect of a single dose of clenbuterol is unlikely to last longer than 24 h. Consistent with this, the apparent plasma half-life of clenbuterol of about 7 h also suggests that any pharmacological effects of this agent will be gone by 72 h after the last administration.

If the threshold for the pharmacological effect of clenbuterol is between 30 and 100 pg/ml and

the LOD of this method is <10 pg/ml, then this method is capable of detecting a pharmacologically effective dose of clenbuterol administered by any route or mechanism, including intra-tracheal administration and nebulisation. A further advantage of a serum test is that serum is unaffected by the administration of diuretics or other agents that can dilute urine samples. Quality advice can now be given about when to withdraw clenbuterol from a horse in training. Because of the log-linear nature of the decline in the serum concentrations of clenbuterol, a 'washout' curve can be used to estimate the probability of clenbuterol serum concentrations exceeding a threshold level at certain times after the last administration.

In summary, review of current regulatory approaches to clenbuterol strongly recommended development of a sensitive serum test for this agent. A serum test was developed that unequivocally identifies and quantifies clenbuterol in serum down to 10 pg/ml. This threshold is about 10,000 times more sensitive than the only other widely accepted plasma regulatory threshold, that for furosemide. Review of recent research suggests that the 10 pg/ml serum concentration of clenbuterol is at least one third less than the minimum serum concentration of clenbuterol required for a pharmacological effect. This serum threshold is therefore a 'no pharmacological effect' threshold for this agent. According to this cut-off, there is a 95% probability that any given serum sample will be negative for clenbuterol 96 h after the last dose. Our technology is a highly effective regulatory approach because it readily detects concentrations of clenbuterol of 30 pg/ml or greater that are likely to be associated with a pharmacological effect, and can detect intra-tracheal administration of this drug within minutes of administration. Furthermore, this technique is inherently more reliable than urine testing because serum concentrations of drugs are not known to be affected by the administration of furosemide or other diuretics or 'milk shakes'. Finally, there is at the time of writing no reason that highly sensitive serum testing cannot be extended to regulatory control of other bronchodilating agents and perhaps many, if not most, other therapeutic agents used in horses in training.

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