

Clenbuterol in the Horse: Confirmation and Quantitation of Serum Clenbuterol by LC–MS–MS after Oral and Intratracheal Administration*

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Abstract

Clenbuterol is a β_2 agonist/antagonist bronchodilator, and its identification in post-race samples may lead to sanctions. The objective of this study was to develop a specific and highly sensitive serum quantitation method for clenbuterol that would allow effective regulatory control of this agent in horses. Therefore, clenbuterol-*d*₉ was synthesized for use as an internal standard, an automated solid-phase extraction method was developed, and both were used in conjunction with a multiple reaction monitoring liquid chromatography–tandem mass spectrometry (LC–MS–MS) method to allow unequivocal identification and quantitation of clenbuterol in 2 mL of serum at concentrations as low as 10 pg/mL. Five horses were dosed with oral clenbuterol (0.8 μ g/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 ~7 h. Serum clenbuterol declined to 30 and 10 pg/mL at 48 and 72 h after dosing, respectively. By 96 h after dosing, the concentration was below 4 pg/mL, the limit of detection for this method. Compared with previous results obtained in parallel urinary experiments, the serum-based approach was more reliable and satisfactory for regulation of the use of clenbuterol. Clenbuterol (90 μ g) was also administered intratracheally to five 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 that intratracheal administration of clenbuterol shortly before race time can be detected with this serum test. Traditionally, equine drug testing has been dependent on urine testing because of the small volume of serum samples and the low concentrations of drugs found therein. Using LC–MS–MS testing, it is now possible to

unequivocally identify and quantitate low concentrations (10 pg/mL) of drugs in serum. Based on the utility of this approach, the speed with which new tests can be developed, and the confidence with which the findings can be applied in the forensic situation, this approach offers considerable scientific and regulatory advantages over more traditional urine testing approaches.

Introduction

Clenbuterol is a bronchodilator listed by the American Association of Equine Practitioners for use in horses (1). Because it has the potential to alter the athletic performance of racing horses, particularly if the horse has bronchospasm, clenbuterol is classified by the Association of Racing Commissioners International (ARCI) as a class 3 medication, and its detection in post-performance samples may lead to significant sanctions against trainers.

Previous work has shown that clenbuterol can be detected in urine samples at concentrations of 1000–20,000 pg/mL using trimethylsilyl (TMS) derivatization and at concentrations of 1000 pg/mL or less using methanboronic acid (MBA) derivatization (2). However, the latter method is problematic in that it is very corrosive to GC columns and mass spectrometric detector components.

The use of urinary measurements for the control of clenbuterol has created concerns among regulators, horsemen, and veterinarians for two important reasons. First, clenbuterol can persist in urine for at least four weeks after a prolonged course of therapy (3). Second, urine samples can oscillate between positive and negative. In recent work from our group, urine samples became essentially “negative” (< 250 pg/mL) about 5 days after dosing but then became clearly “positive” again with mean concentrations > 1000 pg/mL at day 10 (2). These findings, as well as the unsatisfactory characteristics of urine as a forensic sample, led to the pursuit of a highly sensitive and specific serum quantitation method for clenbuterol. The objective of this study was to develop a specific and highly

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sensitive serum quantitation method for clenbuterol that would allow for effective regulatory control of this agent in horses.

Materials and Methods

Horses

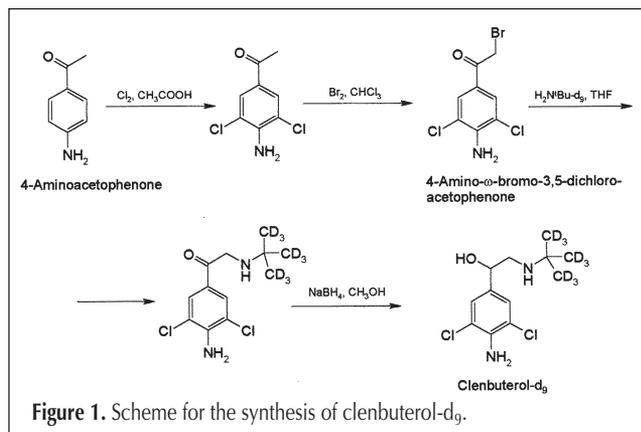
Five mature Thoroughbred mares weighing 489–535 kg were used for this experiment. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to assure 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 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.

Clenbuterol (Ventipulmin syrup, Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) was administered orally at a dosage of 0.8 µg/kg at 8 a.m. and 5 p.m. for 10.5 days (21 dosings). Using Vacutainer® tubes (SST Gel and Clot Activator, Becton Dickinson, Franklin Lakes, NJ), serum samples were collected before treatment started and then each day before the morning dosing. Furthermore, after the 21st dosing (the morning of the 11th day), samples were collected at 1, 2, 4, 6, and 8 h after treatment. After that, samples were collected each morning for the next 14 days.

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

Synthesis and characterization of deuterated clenbuterol-d₉

Figure 1 shows the synthesis of clenbuterol-d₉ (4-amino-α-[(tert-butyl-d₉-amino)methyl]-3,5-dichlorobenzyl alcohol). The tert-butylamine-d₉ (98 atom % D, minimum quality) was from Isotec, Inc. (Miamisburg, OH). All other reagents and solvents



were from Aldrich (Milwaukee, WI) and were analytical grade or better. The substrate for this synthesis was 4-amino-acetophenone. 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 (4). The reaction of the bromoketone with deuterated tert-butylamine (H₂NtBu-d₉) in tetrahydrofuran gave the ketoamine in moderate yield, which, after reduction to alcohol with sodium borohydride in methanol, yielded clenbuterol-d₉. The final product was purified by column chromatography on silica gel and crystallized from ethyl ether.

Characterization data for 4-amino- α -[(tert-butyl-d₉-amino)methyl]-3,5-dichlorobenzyl alcohol: colorless crystals from ethyl ether, melting point 182–185°C; ¹H-NMR (300 MHz, CDCl₃): d (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).

Validation of quantitative liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for serum clenbuterol

The LC–MS–MS method for the quantitation of clenbuterol was validated by examining the measurement of consistency of results (within run and between run), correlation (coefficient of determination of the standard curve), and recovery of the assay. The within-run precision was calculated from similar responses from six repeats of these three standards in one run. The between-run precision was determined by comparing the calculated response (in picograms per milliliter backfit of the standard curve) of the low (10 pg/mL), middle (100 pg/mL), and high (1000 pg/mL) standards over six consecutive daily runs. Standard curve correlation was measured by the mean coefficient of determination (*r*²) for six consecutive daily runs (5). The recovery was determined by comparing the response (in area) of low (50 pg/mL), high (500 pg/mL), and the deuterated clenbuterol (25,000 pg/mL) methanol standards to the equivalent extracted standards. The limit of detection (LOD) was calculated as the mean of the assay background plus two times the standard deviation of the mean for 122 runs. The limit of quantitation (LOQ) was likewise calculated as five times the standard deviation of the mean (6).

Development of a LC–MS–MS method for clenbuterol

Clenbuterol and clenbuterol-d₉ were dissolved at 10 µg/mL in 1:1 acetonitrile/0.05% formic acid and examined by direct infusion ESI⁺ MS. Mass spectra derived following optimization of tuning revealed intense [M+H]⁺ molecular ions with little post-source fragmentation (Figure 2), indicating the appropriateness of this approach for development of a sensitive quantitative procedure. Negative ion ESI was not investigated because of the lack of negatively ionizable functionalities. High-performance liquid chromatography (HPLC) of clenbuterol in acidified organic/aqueous mobile phases revealed the propensity for excellent chromatography with little tailing and no need for post-column flow splitting or acidification by infusion of acid. Other candidate compounds of related structure (salbutamol, isoproterenol, terbutaline, ephedrine) were investigated as potential internal standards before the development of the d₉

compound and generally found to provide relatively broad peaks, with the exception of ephedrine, which was considered suitable both during HPLC and during extraction, but which did not enable as low an LOQ or the high correlation coefficients achieved with clenbuterol- d_9 . The extraction method was based on existing SPE methods developed for other basic drugs and found to provide adequate recovery.

Serum analysis for clenbuterol by LC-MS-MS

Standard solutions of clenbuterol and clenbuterol- d_9 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 10–1000 pg/mL. A known amount of a clenbuterol- d_9 standard (50 μ g of 1 μ g/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 of 0.1M sodium phosphate buffer (pH 6) was added, and the sample pH was adjusted as needed to 6.0 ± 0.5 with 1M NaOH or 1M HCl. Standards were prepared at the following concentrations: 0.0, 10, 50, 100, 500, and 1000 pg/mL from appropriate concentrated stocks in mobile phase.

Extraction procedure. Solid-phase extraction (SPE) was performed on the Rapid Trace[®] SPE Workstation (Zymark Corp., Hopkinton, MA). Clean Screen SPE columns (Worldwide Monitoring #ZSDAU020) were conditioned by sequential addition of 3 mL of methanol, 3 mL of water, and 1 mL of 0.1M sodium phosphate buffer (pH 6). The samples were loaded at 1.5 mL/min, and the column was washed sequentially with 2 mL of water, 2 mL of 1M acetic acid, and 4 mL of methanol. The SPE column was dried with N_2 gas for 5 min then eluted with 3 mL of dichloromethane/isopropanol/ NH_4OH , conc. (78:20:2) at 1.5 mL/min. The eluent was evaporated to dryness under a stream of N_2 (< 40°C). Each sample was dissolved in 50 μ L of Solvent A (see Instrumentation).

Instrumentation. Extracts were analyzed with a Hewlett-Packard model 1050 LC equipped with a Micromass VG Quattro-II MS-MS (Micromass, Beverly, MA) set in electrospray ionization-positive mode (ESI⁺). The column was a Luna phenyl/hexyl 2 mm \times 250 mm \times 5 μ m (Phenomenex, Torrance, CA). The mobile phase was a linear gradient of Solvent A (0.05% v/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- μ L aliquot was injected onto the LC-MS-MS. Transitions monitored for clenbuterol and its deuterated internal standard are summarized in Table I. Quantitation of clenbuterol was based on the m/z 277 \rightarrow 203 transition corrected by the internal standard (clenbuterol- d_9) m/z 286 \rightarrow 204 transition.

MS-MS

Full scan ESI⁺ mass spectra were obtained on clenbuterol (Sigma, St. Louis, MO) and its internal standard at 10 μ g/mL in 1:1 acetonitrile/0.05% formic acid (aq), pH 3, by infusion at 0.6 mL/h with a Harvard syringe pump (Holliston, MA). The Quattro II MS was tuned for positive mode by direct infusion of 10,000 pg/ μ L of clenbuterol in 1:1 acetonitrile/0.05% formic acid (aq). The peak shape and intensity of the monoprotonated clenbuterol m/z 277 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Collision gas (argon) and collision energy were adjusted for collision-induced dissociation (CID) in the central hexapole by optimizing settings for maximum production of the m/z 203 product ion. Generally, the collision gas was set to 1×10^{-3} mbar, the source cone voltage was set at +19 V, the collision energy was set at -16 V, 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 (Micromass MassLynx software, Beverly, MA) and fitting of resultant data to a second-order quadratic curve generated with Microsoft Excel software (www.microsoft.com). Sigma Plot (SPSS, Inc) enabled convenient plotting of standard curves where necessary. Typical results showed a range of MS response areas from approximately 400 (background) for 0 pg/mL to 20,000 for 1000 pg/mL with excellent point fit to the curve.

Results

Purity and mass spectral characteristics of clenbuterol- d_9

The ESI⁺ MS full scan of clenbuterol- d_9 is shown in Figure 2. Intensities of peaks immediately preceding m/z 286 suggested that there were small amounts of d_8 and d_7 in this preparation, roughly 7% and 2%, respectively. This is emphasized by comparison to undeuterated clenbuterol (Figure 3), in which no peaks were present immediately preceding the major molecular ion at m/z 277. Of more immediate concern was whether this preparation contained undeuterated clenbuterol- d_0 , which would arise from undeuterated contaminants in the tert-butylamine- d_9 during the original reaction. Direct infusion and HPLC measurements estimated the amount of clenbuterol- d_0 at no more than 0.01%, lending assurance that even relatively

Table I. Settings Used for Clenbuterol and Clenbuterol- d_9 Data Acquisition by ESI⁺ LC-MS-MS

Precursor ion (m/z)	Product ion (m/z)	Dwell (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

large amounts of this internal standard could be added without creating problems for the accurate quantitation of clenbuterol.

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

The within-run precision was determined for the low (10 pg/mL: CV = 17.9%), middle (100 pg/mL: CV = 3.7%), and high (1000 pg/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 (10 pg/mL: CV = 29.3%), middle (100 pg/mL: CV = 7.2%), and high (1000 pg/mL: CV = 0.1%) concentrations of the clenbuterol standard curve, with a mean CV of 12.2%. The mean r^2 for the assays were 0.99989 ± 0.00006 SD. The recovery was determined at two concentrations: low (50 pg/mL) = 86.5%, high (500 pg/mL) = 76.8; the recovery for the deuterated clenbuterol was 65.1%. The LOD for the LC-MS-MS method for the detection of clenbuterol was 4 pg/mL ($n = 12$), and the LOQ was 13 pg/mL ($n = 12$).

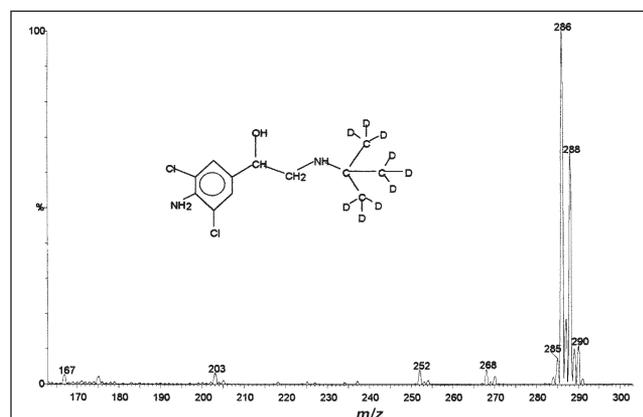


Figure 2. Full scan ESI+ MS of clenbuterol- d_9 obtained by direct infusion of a 10- μ g/mL solution in acetonitrile/0.05% formic acid (aq) (1:1). Inset displays structure including location of deuteriums.

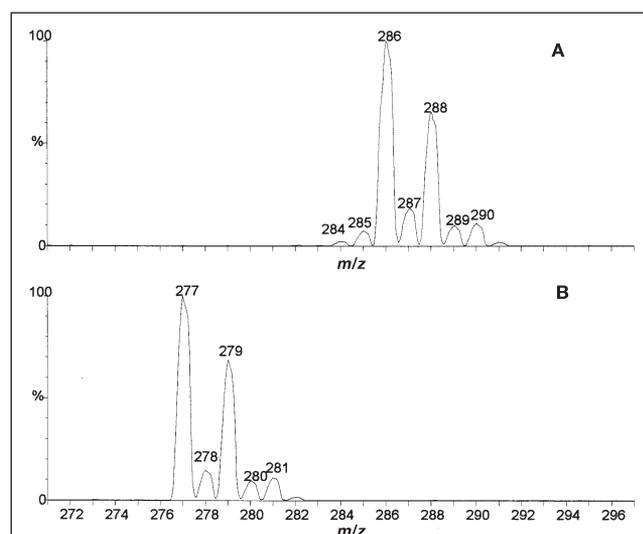


Figure 3. Full scan ESI+ MS of clenbuterol- d_9 (A) and clenbuterol- d_0 (B) with emphasis placed on the spectral window immediately adjacent to the respective compounds' molecular ion group. Spectra were obtained by direct infusion of a 10- μ g/mL solution in acetonitrile/0.05% formic acid (aq) (1:1).

Multiple reaction monitoring of clenbuterol

Figure 4 shows product ions for the two major $[M+H]^+$ molecular ions of clenbuterol, at m/z 277 and 279. Clenbuterol quantitative measurements were enhanced by maximization of the yield of product ions, specifically by optimization of collision gas pressure and tuning parameters which resulted in significant yield of the m/z 203 ion from 277. Optimum yield for the m/z 203 ion occurred with argon gas pressure at roughly 1×10^{-3} mbar (data not shown).

Principal product ions arising from clenbuterol and the internal standard clenbuterol- d_9 $[M+H]^+$ molecular ions were simultaneously optimized by focusing on the m/z 277 \rightarrow 203 transition of clenbuterol. Table II lists the principal product ions observed during direct infusion-MS-MS experiments both for the $[M+H]^+$ molecular ions and the major water loss peaks arising from the largest molecular ion of each compound.

Figure 5 depicts the fragmentation mechanism proposed for the m/z 277 \rightarrow 203 transition of clenbuterol. Protonation is suggested to occur at the much more basic t-butylamino nitrogen: compare model compounds t-butylamine [$pK_a = 10.8$] and 2,4-dichloroaniline [$pK_a = 2.05$] (7). Dehydration accounts for the m/z 259 product ion, which then undergoes McLafferty rearrangement (8) involving shift of a tert-butyl proton and dis-

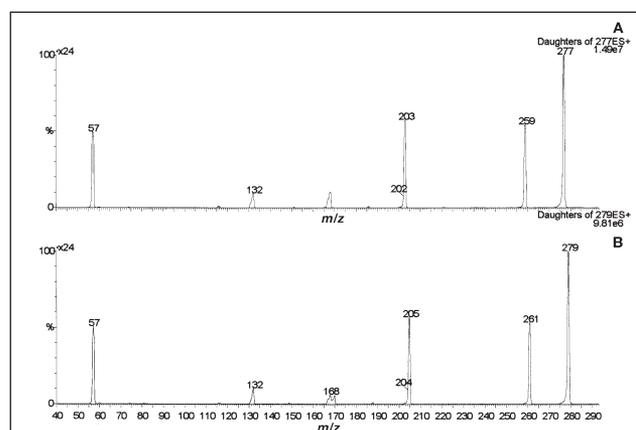


Figure 4. ESI+ MS-MS daughter ion spectra of clenbuterol molecular ions m/z 277 (A) and m/z 279 (B). Spectra were obtained by direct infusion of a 10- μ g/mL solution in acetonitrile/0.05% formic acid (aq) (1:1). Note that the mass-to-charge ratio region from roughly 40 to 185 has been expanded for clarity of detail.

Table II. Principal Product Ions Observed from Clenbuterol Molecular Ions (m/z 277, 279, and 281) and Principal Water Loss Ion (m/z 259) during ESI+ MS-MS*

Clenbuterol		Clenbuterol- d_9	
Precursor ion (m/z)	Principal product ion(s) (m/z)	Precursor ion (m/z)	Principal product ion(s) (m/z)
277	259, 203, 57	286	268, 204, 66
279	261, 205, 57	288	270, 206, 66
281	263, 207, 57	290	272, 208, 66
259	203, 57	268	204, 66

* Right side of table enables comparison with principal product ions observed from the equivalent ions of the internal standard clenbuterol- d_9 .

charge of a neutral 2-methylpropylene molecule. This reaction mechanism is supported by observations with clenbuterol- d_9 , whose m/z 286 $[M+H]^+$ molecular ion elicits an m/z 268 dehydration fragment with collapse to an m/z 204 ion, the analogous fragment to the clenbuterol- d_0 m/z 203 ion (Table I). The additional 1 amu of this fragment must arise specifically from transfer of a deuterium atom. Other $[M+H]^+$ molecular ions in Table II (m/z 279 and 281 for clenbuterol and m/z 288 and 290 for clenbuterol- d_9) follow suit, respectively adding two or four amu units to product ions depending upon the isotopic contribution of chlorine atoms. The m/z 57 (clenbuterol) and 66 (clenbuterol- d_9) ions are uniform throughout as they represent charged tert-butyl groups unaffected by chlorine isotopic contributions.

Figure 6 demonstrates simultaneity of product ion transitions acquired for clenbuterol, including the 277 \rightarrow 203 quantitative transition and five qualifier transitions observed as nested sets during gradient HPLC. It is our recommendation that clenbuterol confirmation include at least several of such qualifier transitions as diagnostic for accuracy in assignment of the presence of clenbuterol, as is typically done according to labo-

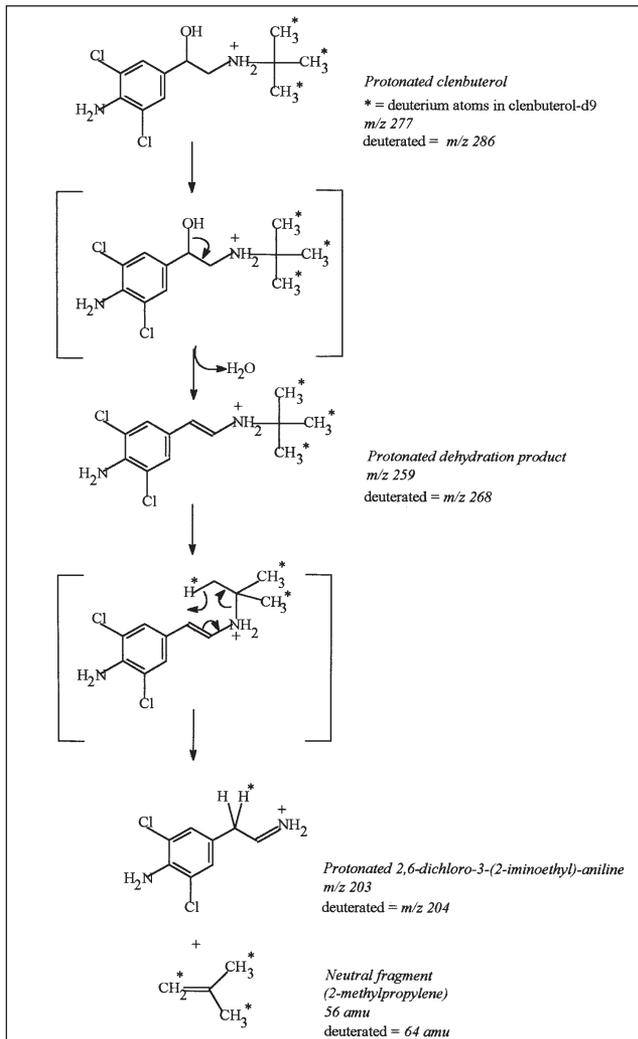


Figure 5. Scheme showing the fragmentation mechanism for the transition from m/z 277 to 203 during ESI⁺ MS-MS of clenbuterol. Note that the asterisks denote the location of deuterium atoms in clenbuterol- d_9 .

ratory standard operating procedures (SOP) during selected ion monitoring (SIM) mass spectrometry. The areas for the specific product ions measured as in Figure 6 relative to the largest transition were as follows: m/z 277 \rightarrow 203, 100%; m/z 279 \rightarrow 205, 64%; m/z 277 \rightarrow 259, 14%; m/z 279 \rightarrow 261, 7.3%; m/z 277 \rightarrow 57, 1.4%; and m/z 279 \rightarrow 57, 0.7%.

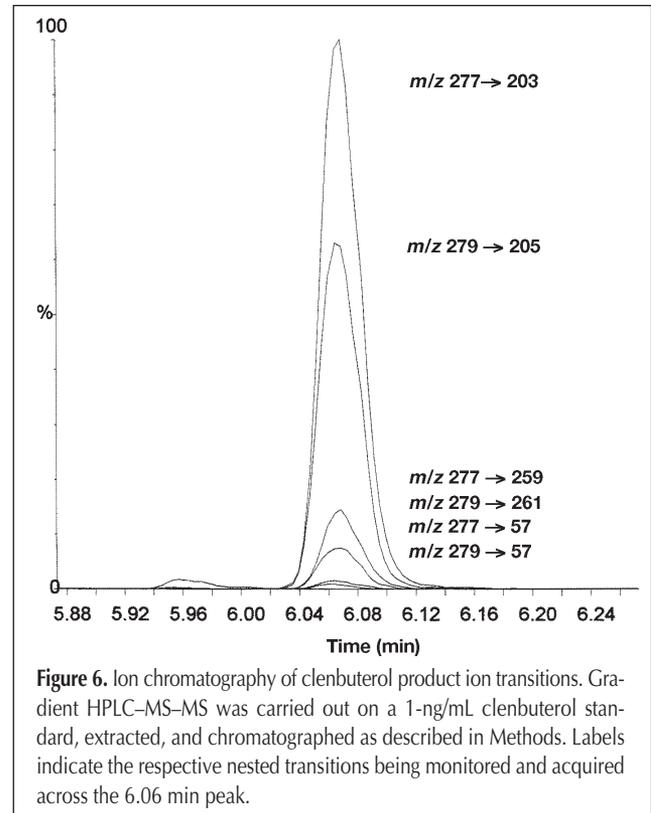


Figure 6. Ion chromatography of clenbuterol product ion transitions. Gradient HPLC-MS-MS was carried out on a 1-ng/mL clenbuterol standard, extracted, and chromatographed as described in Methods. Labels indicate the respective nested transitions being monitored and acquired across the 6.06 min peak.

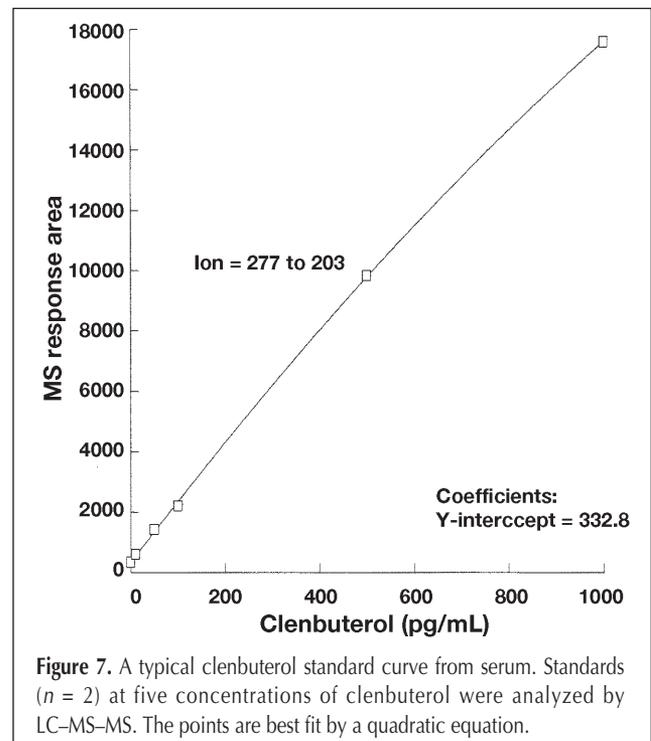


Figure 7. A typical clenbuterol standard curve from serum. Standards ($n = 2$) at five concentrations of clenbuterol were analyzed by LC-MS-MS. The points are best fit by a quadratic equation.

Quantitation of serum clenbuterol using LC-MS-MS

Figure 7 shows a typical standard curve for clenbuterol in serum using the LC-MS-MS methodology described. Duplicate standards at five concentrations (0.01, 0.05, 0.1, 0.5, 1.0 ng/mL) of clenbuterol supplemented into horse serum (plus blank, 0 ng/mL) were analyzed by LC-MS-MS. The standard curve is best described by a second-order quadratic regression,

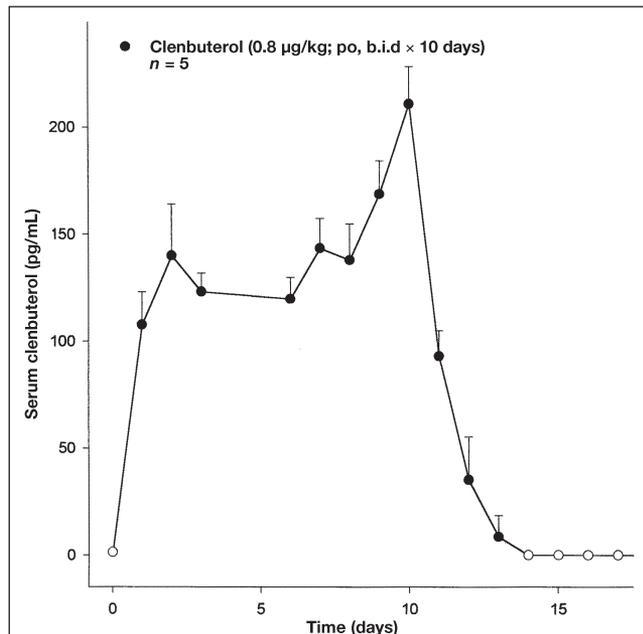


Figure 8. Trough serum concentrations during and after clenbuterol treatment (0.8 µg/kg BID × 10 days). Open circles indicate values that were below the limit of detection.

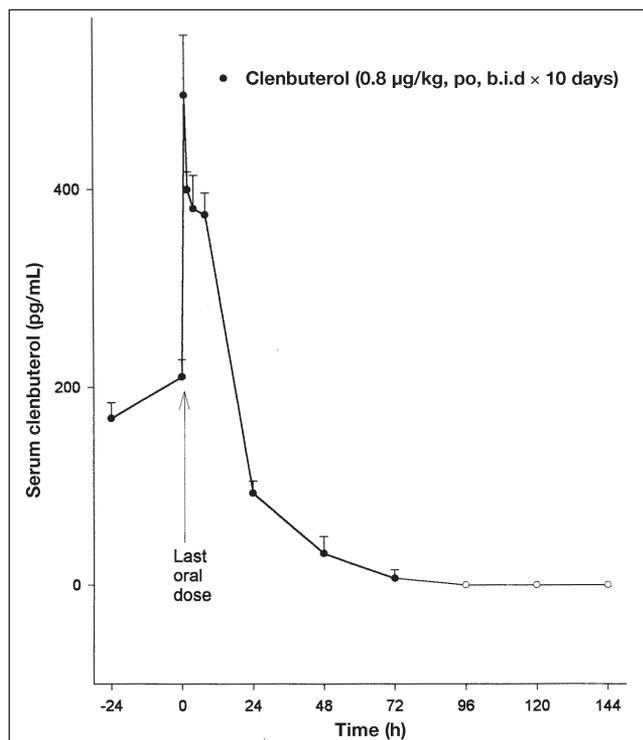


Figure 9. Serum concentrations after last dose of clenbuterol. Open circles indicate values that were below the limit of detection. (The 96 h point is the same as Day 14 in Figure 8.)

which yields a small positive *Y*-intercept and has a correlation coefficient $r^2 = 0.99986$.

Figure 8 shows the clenbuterol concentration in serum each day before the morning dosing. (No serum was collected on days 4 and 5). There was a gradual increase in serum clenbuterol concentration during the 10-day dosing. The maximal trough concentration of clenbuterol in serum was about 200 pg/mL on day 10. Serum clenbuterol rapidly declined at the end of dosing, and by day 14 (4 days after last dosing), clenbuterol was no longer detectable in serum. Figure 9 shows the detailed time course of serum clenbuterol concentrations through 72 h after the last dose. The maximal serum concentration at 1 h after dosing was almost 500 pg/mL, considerably higher than the daily trough concentrations seen in Figure 8. Thereafter, serum clenbuterol declined rapidly with a half-life of ~7 h. By 24 h after dosing, the concentration was less than 100 pg/mL and was ~30 pg/mL 48 h after dosing. The concentration fell below the limit of detection by 96 h, which was the same loss of detection seen at day 14 in Figure 8.

In Figure 10, the serum clenbuterol is plotted on a log scale with a dotted line drawn at 30 pg/mL, the estimated threshold serum concentration for pharmacologic effect (9). Using the 30-pg/mL cutoff and the data collected, there is a < 75%, < 5%, and

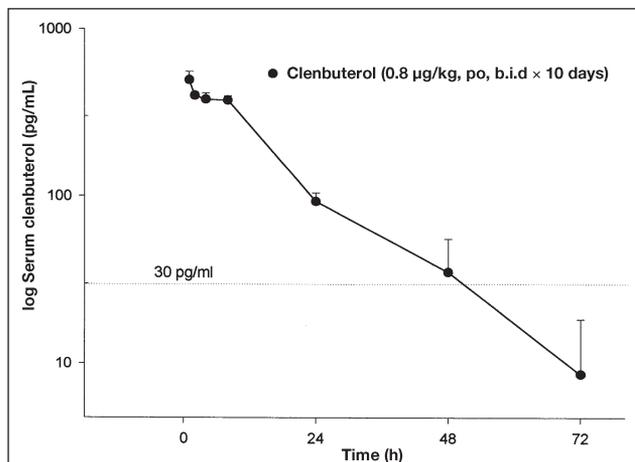


Figure 10. Log of serum clenbuterol through 72 h after last dose. Dotted line at 30 pg/mL represents the estimated threshold for pharmacologic effect.

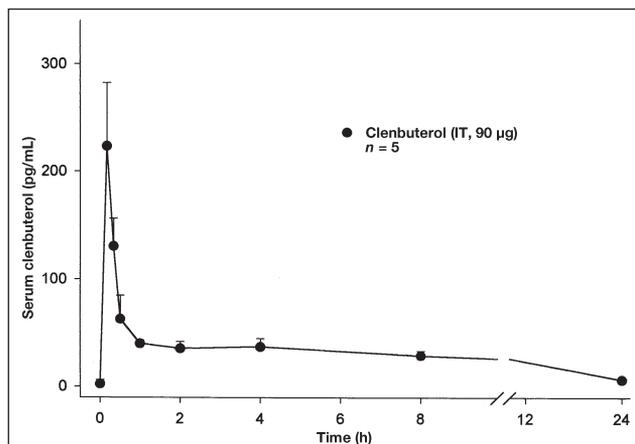


Figure 11. Serum concentration of clenbuterol following 90 µg intratracheal injection.

< 0.05% chance that a horse will be positive for clenbuterol at 48, 72, and 96 h after administration, respectively, using a rate of administration similar to this study. These values were obtained by applying the central limit theorem of statistics to a sampling distribution of the sample mean in a *t*-test distribution with the single assumption that the low sample number ($n = 5$) is representative of the population in a binomial distribution (5).

Figure 11 shows the serum concentration of clenbuterol in serum following IT injection of the agent. Mean peak serum concentration (223 pg/mL) was measured at the first sampling after treatment (10 min), suggesting very rapid absorption of clenbuterol from the respiratory tract. Serum clenbuterol decreased rapidly during the first hour, then fell more slowly from 1 h (39.8 pg/mL) to 8 h (28.7 pg/mL). At 24 h, the serum concentration was still 6.4 pg/mL, which was above the calculated limit of detection (4 pg/mL).

Discussion

The serum testing approach to the regulation of clenbuterol described in this paper offers a practical method for regulatory control of clenbuterol in racing horses. Unlike urine testing, there is good correlation between serum concentrations and pharmacological effects. Furthermore, serum testing avoids certain problems inherent with urine testing; for example, the prolonged (up to 28 days) urinary elimination of clenbuterol or its metabolites effectively precludes the use of clenbuterol as a therapeutic agent. Finally, serum testing allows immediate detection and control of the use of clenbuterol by the intratracheal route or by nebulization, which is not afforded by urinary testing.

In the United States, the regulation of clenbuterol use has been made even more difficult by the development of very sensitive methods for its detection in urine. These methods were developed in response to the reported administration of clenbuterol by nebulization or IT injection. However, detection in urine does not allow regulators to distinguish these improper administrations shortly before race time from long-term washout days or weeks after a legitimate course of oral treatment. The data in this paper show that serum concentrations can lead to more precise and appropriate regulatory decisions.

Application of the serum assay to horses receiving a 10-day course of clenbuterol has generally confirmed an earlier study (10) concerning its uptake and elimination in the horse. Serum trough levels reached an apparent plateau within 2 days (125–150 pg/mL) but then slowly increased (> 200 pg/mL) for unknown reasons, although they are likely related to the factors that lead to the prolonged, and somewhat cyclical, washout of clenbuterol in urine after higher oral doses for 28 days (1,3). The concentrations are less than those reported earlier (10) (250–400 pg/mL), but this may be due to differences in dose schedules and sampling times. In that study, clenbuterol was administered every 12 h, whereas we administered it at a schedule likely to be used in practice at the racetrack (i.e., twice in a working day at 8 a.m. and 5 p.m.). Our trough samples were taken just before the morning dose, 15 h after the last dose com-

pared to 12 h in the other study. Examination of the washout curve after cessation of administration (Figure 9) shows that the extra 3 h is sufficient to account for the apparent differences.

The effective concentration (EC_{50}) of clenbuterol ranges from 0.9 to 6.8 nM (280 to 2180 pg/mL) (11). Serum clenbuterol only reached this concentration immediately after administration and trough values were well below the EC_{50} . However, the 12 h trough values from an earlier study (10) were mainly within the EC_{50} . These results show that it is important to administer clenbuterol at 12-h intervals to maintain a therapeutic blood level.

The data on the elimination of clenbuterol from blood are in agreement with previously published reports (10,12). In this study, the apparent half-life of clenbuterol in serum was 7 h compared to 10.4 h in an earlier study (10).

Based on the *in vitro* pharmacology of clenbuterol (11,13), the threshold concentration for a pharmacological effect on β_2 -adrenoceptors was calculated to be at least 31 pg/mL (9). From published elimination data (10,12), it would be highly unlikely for this concentration to be present 72 h after the last dose of a 10-day treatment regime (0.8 mg/kg q 12 h). This study supports that conclusion because serum concentrations of clenbuterol (10 pg/mL) were well below the calculated pharmacological threshold (31 pg/mL) at 72 h after the last dose (9).

One of the major concerns raised during the ARCI workshop on clenbuterol was the inability of urine testing to detect IT injection or nebulization of clenbuterol just before a race. The high sensitivity of our method allows for immediate detection of clenbuterol after IT administration. Ten minutes after IT injection (90 μ g), it was present in high concentrations (> 200 pg/mL) in serum. The fear that detection of IT clenbuterol will be hampered by coadministration of furosemide is not a concern when serum concentrations are measured because the action of furosemide would be to decrease serum volume, thereby raising the concentration of clenbuterol.

Serum concentrations after IT injection (90 μ g) agreed with a recent study from this group on the effects of that dosing on lung function. Working with horses affected with chronic obstructive pulmonary disease (COPD), there was a transient improvement in lung function immediately after IT injection, although the degree of improvement did not attain statistical significance based on the current sample number (9). This response was consistent with transient serum concentrations of clenbuterol that approach the EC_{50} following such an injection.

The data obtained after IT injection of clenbuterol also support the estimated pharmacological threshold concentration (31 pg/mL) calculated in an earlier study (9). At 1 and 2 h after IT injection, when there was no effect on lung function, serum levels were close to or below the suggested pharmacological threshold (9).

The findings in this paper and the published information about the pharmacological activity of clenbuterol are in good agreement with the longtime position of Agriculture Canada that a 72-h "detection time"/"withdrawal time" is appropriate for this agent. By 72 h after the last administration, serum concentration is only one third of the minimal concentration calculated to have a pharmacological effect. The apparent half-life

of clenbuterol in serum (7–10 h) also suggests there is little likelihood of a pharmacological effect at 72 h after the last administration.

Finally, the results reported in this paper have major implications for the future art and science of equine drug testing. To date, the regulatory control of high potency drugs in racing horses has depended virtually entirely on the detection of residues of these drugs and/or their metabolites in post-race urine samples.

However, regulatory strategies based on detection of urinary metabolites have technical limitations. Analytical standards for equine urinary metabolites are not readily available, so quantitation of metabolites in urine may not be possible. Secondly, the metabolites available may be chemically unstable or difficult to crystallize and, therefore, of limited utility for quantitative forensic work. Additionally, recovery of metabolites for urine samples requires carefully defined hydrolytic and recovery procedures, which must be optimized for each individual metabolite. Finally, no matter how carefully each of these steps are defined and performed, urinary drug concentrations are still subject to the standard renal influences in urine volume, specific gravity, and pH. These factors make it difficult to quantitate metabolite concentrations in equine urine, and such information is of limited pharmacological significance.

On the other hand, the data presented here show it is now possible to unequivocally identify and accurately quantitate very low concentrations of drugs or metabolites in small volumes of serum. This approach allows for the immediate targeting of parent drug during new test development, rather than searching for and identifying unknown metabolites. Secondly, standards for parent drugs are almost always available, and the stability of the parent drug is generally sufficient for accurate quantitative work. Finally, the concentrations of parent drug identified in serum can be confidently applied in the forensic arena. Based on the utility of this approach, the speed with which new, more effective, and more interpretable drug tests can be developed, and the confidence with which the findings can be applied in the forensic situation, the expanded application of this technology to equine drug testing merits consideration.

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