Intratracheal clenbuterol in the horse: its pharmacological efficacy and analytical detection

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Clenbuterol, a β_2 agonist/antagonist, is the only bronchodilator approved by the US Food and Drug Administration for use in horses. The Association of Racing Commissioners International classifies clenbuterol as a class 3 agent, and, as such, its identification in post-race samples may lead to sanctions. Anecdotal reports suggest that clenbuterol may have been administered by intratracheal (IT) injection to obtain beneficial effects and avoid post-race detection. The objectives of this study were (1) to measure the pharmacological efficacy of IT dose of clenbuterol and (2) to determine the analytical findings in urine in the presence and absence of furosemide. When administered intratracheally (90 μg/horse) to horses suffering from chronic obstructive pulmonary disease (COPD), clenbuterol had effects that were not significantly different from those of saline. In parallel experiments using a behavior chamber, no significant effects of IT clenbuterol on heart rate or spontaneous locomotor activity were observed. Clenbuterol concentrations in the urine were also measured after IT dose in the presence and absence of furosemide. Four horses were administered i.v. furosemide (5 mg/kg), and four horses were administered saline (5 mL). Two hours later, all horses were administrated clenbuterol (IT, 90 μg), and the furosemide-treated horses received a second dose of furosemide (2.5 mg/kg, i.v.). Three hours after clenbuterol dose (1 h after hypothetical 'post-time'), the mean specific gravity of urine samples from furosemide-treated horses was 1.024, well above the 1.010 concentration at which furosemide is considered to interfere with drug detection. There was no interference by furosemide with 'enhanced' ELISA screening of clenbuterol equivalents in extracted and concentrated samples. Similarly, furosemide had no effect on mass spectral identification or quantification of clenbuterol in these samples. These results suggest that the IT dose of clenbuterol (90 μg) is, in pharmacological terms, indistinguishable from the dose of saline, and that, using extracted samples, clenbuterol dose is readily detectable at 3 h after dosing. Furthermore, concomitant dose of furosemide does not interfere with detection or confirmation of clenbuterol.

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INTRODUCTION

Clenbuterol is a β_2 agonist/antagonist bronchodilator approved by the American Association of Equine Practitioners and is the only β_2 agonist approved by the United States Food and Drug Administration for use in horses. As a bronchodilator, it may have the potential to alter the athletic performance of animals, particularly if the horse has bronchospasm. Furthermore, clenbuterol may stimulate the cardiac and central nervous systems, which could translate to positive effects on the performance of racing horses. Clenbuterol is also classified by the Association of Racing Commissioners International as a class 3 agent, and its detection in post-race samples may lead to significant sanctions against trainers. In 1998, highly sensitive screening procedures

for clenbuterol were introduced, leading to the identification and reporting of concentrations of clenbuterol and/or its metabolites in the range of 100–1000 pg/mL.

The basic pharmacological actions of clenbuterol and its therapeutic efficacy in the horse are well characterized. At an oral dose range of 0.8–3.2 μg/kg BID for 5–10 days, clenbuterol relieves airway obstruction in heaves-affected horses. It is, therefore, effective in the treatment of chronic obstructive pulmonary disease (COPD), which is one of its primary indications in the horse (Sasse & Hajer, 1978). Other effects in the respiratory tract include modest anti-inflammatory and mucokinetic actions. Therefore, clenbuterol is often used as either a primary or a supportive medication in the treatment of non-infectious and infectious respiratory diseases.

This study was based on anecdotal reports that low doses of clenbuterol were being administered IT to horses about 2 h before the race time to enhance performance. Furthermore, to interfere with detection of clenbuterol, these reports suggest that, in addition to the approved dose of furosemide administered 4 h before race time, the horses also received a second dose of furosemide at the same time as the IT clenbuterol dose (2 h before post-race). The rationale behind this approach is that a small amount of clenbuterol IT before a race should produce bronchodilation and improve racing performance and the second dose of furosemide may render the small amount of clenbuterol undetectable.

This communication seeks to answer questions about the efficacy of IT clenbuterol in racehorses. With regard to the pharmacological actions, bronchodilator effects were studied at Michigan State University using horses with COPD, and effects on heart rate and behavior were investigated using a behavior chamber method developed at the Maxwell H. Gluck Equine Research Center. Quantitative analytical methods for clenbuterol and its metabolites were developed and validated and then used to investigate the relationship between clenbuterol dose and analytical findings in urine with and without furosemide dose.

The second question addressed was the relationship between clenbuterol dose and analytical findings in urine. To this end, quantitative analytical methods for clenbuterol and its metabolites have been developed and validated. Subsequently, clenbuterol was administered to horses after the anecdotally reported schedules.

METHODS AND MATERIALS

Clinically normal horses

Eight mature Thoroughbred mares and geldings weighing 492–612 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, USA). 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*. At least 7 days elapsed between experiments. 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.

Chronic obstructive pulmonary disease (COPD) horses

To study bronchodilator effects of IT clenbuterol, seven COPD-affected horses (four geldings, three mares) aged 17–28 years were used because they developed bronchospasm when stabled and are the best models for testing the effect of bronchodilator agents in horses (Robinson *et al.*, 1993; Derksen *et al.*, 1996). Horses were maintained at pasture and fed a complete pelleted feed until they had no clinical signs of airway obstruction. Horses were then housed in stalls, bedded on straw, and fed dusty hay until they exhibited characteristic clinical signs of heaves, at which time lung function was measured. Atropine (0.02 mg/kg, i.v.) was administered, and after 15 min measurement of lung function was repeated to verify the reversibility of the airway obstruction. Horses were then returned to pasture for at least 30 days.

Measurement of lung function

Pleural pressure was estimated using a latex condom sealed over the distal end of a polypropylene catheter (3 mm inside diameter, 4.4 mm outside diameter, 240 cm long). The balloon was passed to the distal third of the esophagus and connected to a pressure transducer (Validyne Model DP/45-35, Northridge, CA, USA). The pressure transducer was calibrated before each study with a water manometer. The position of the esophageal balloon was adjusted to obtain the maximal change in pleural pressure (ΔPpl_{max}) during a tidal breath. Flow rate was collected using a pneumotachograph (No. 5 Fleisch; Rusch International, Deluth, GA, USA) fitted in a facemask placed over the horse's muzzle and sealed with a rubber shroud and tape. The pneumotachograph was connected to a pressure transducer (Validyne DP/45-22) that provided a signal proportional to flow. A lung function computer (Buxco model LS-14, Sharon, CT, USA) integrated the flow signal to provide tidal volume. The computer also calculated pulmonary resistance (R_L) and dynamic elastance (E_{dyn}). The pneumotachograph/transducer/computer system was calibrated with a 2-L syringe. Thirty consecutive breaths were used to calculate R_L, E_{dyn}, and ΔPpl_{max} during each measurement period.

A cross-over design with two treatments was used. Horses were randomly assigned to the initial treatment, and a 7-day washout period was allowed between treatments. Horses were housed in stalls, bedded on straw, and fed dusty hay. Horses qualified for the study when induced airway obstruction resulted in a ΔPpl_{max} during tidal breathing of at least 25 cm H_2O. Horses were treated with an IT injection of either saline (3 mL)

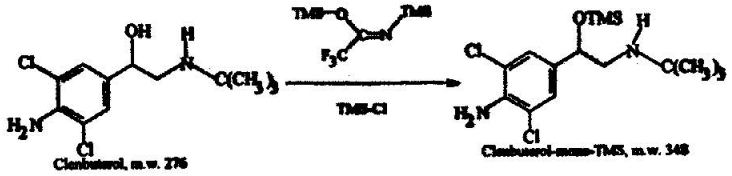

Fig. 1. Derivatization scheme for clenbuterol as verified by GC/MS to yield clenbuterol-TMS. TMS = trimethylsilyl

or injectable clenbuterol solution (90 μg; Ventipulmin, Boehringer Ingelheim, Ltd, Burlington, Ont., Canada). Injections were prepared through a 20-gauge 1.5-in needle inserted between the tracheal rings midway between the larynx and the thoracic inlet.

Lung function was measured before IT injections and 0.25, 0.5, 1, 2, and 4 h later. Horses remained in the laboratory up to the 0.5-h measurement and then were returned to their stalls between all subsequent measurement periods. Horses returned to pasture for at least 7 days between each treatment. The study protocol was approved by the All University Animal Use and Care Committee at Michigan State University.

Locomotor chamber studies

The locomotor chambers have been described previously (Harkins *et al.*, 1997). Briefly, two 3.4 × 3.4 m box stalls were equipped with Minibeam sensors (SM31E and SM2A31R, Banner Engineering, Minneapolis, MN) spaced equally around the stall 45 cm above the floor. Each time the horse disrupted the beam of light, an interruption was scored, and this output was summed and recorded on a data logger (CR10, Campbell Scientific, Inc., Logan, UT, USA).

Heart rates (HR) were recorded at 1 min intervals during each experiment by an on-board HR computer (Polar CIC Inc. Port Washington, NY, USA). An elastic strap with a receiver and transmitter attached was placed around the chest of the horse. The transmitter was connected to two electrodes placed on shaved areas of the sternum and left side of the anterior chest. Electrode gel was used to insure proper conduction of the HR signal.

Behavioral experiments followed a rigorous standard protocol to reduce variability from extraneous effects. A horse was placed in each behavior stall at 07.00 hours, and the HR strap was attached. The horse was allowed to acclimate to the stall for 7 h. Recording of locomotor and HR activities was begun at 14.00 hours. Baseline activity was recorded for 30 min, then the control and experimental treatments were administered. Locomotor and HR data were recorded for 14 h until 05.00 hours the following morning. The total number of interruptions was summed every 15 min.

To validate the behavior chamber, terbutaline (1.5 μg/kg, i.v.), a β_2 agonist known to increase locomotor activity and HR, was administered as a positive control. Injectable clenbuterol (Ventipulmin, Boehringer Ingelheim, Ltd, Burlington, Ont., Canada) was administered IT (90 μg) to eight horses. Control horses received an equal volume of saline, IT. In a separate behavior chamber experiment, i.v. furosemide (Lasix®, Hoechst-Roussel, Somerville, NJ, USA) was administered at a dose of 250 mg. Both control and experimental trials were run simultaneously, and each horse was used as its own control.

Detection of intratracheal clenbuterol dose

For urine analysis, four horses received 250 mg furosemide i.v., and four other horses received 5 mL of saline. Two hours after, all horses were administered 90 μg of clenbuterol IT and a second dose of i.v. furosemide (125 mg) or saline (2.5 mL). Urine for clenbuterol analysis was collected before the first dose of furosemide/saline (−2 h), and at −1.75, −1.5, −1.25, −1, and −0.5 h. Urine was also collected immediately before dose of clenbuterol and the second furosemide/saline dose (0 h) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, and 6 h after clenbuterol dose. This series of analytical experiments was designed and executed as part of a Testing Integrity Program (TIP) cooperative effort. Results generated by the University of Kentucky group are presented here.

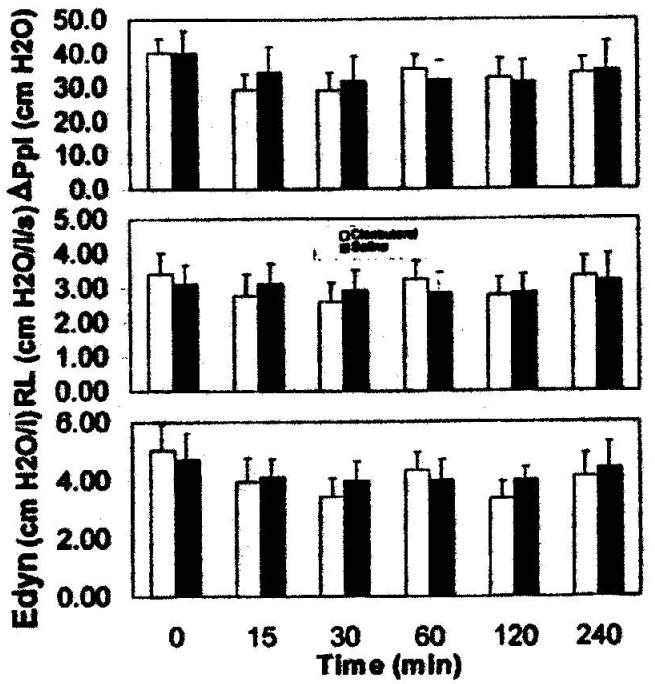

Fig. 2. Comparison of the effects of IT dose of clenbuterol (90 μg, white bars) and saline (dark bars) on maximal change in pleural pressure during tidal breathing (ΔPpl_{max}, top), pulmonary resistance (R_L, middle), and dynamic elastance (E_{dyn}, bottom). See text for statistical information.

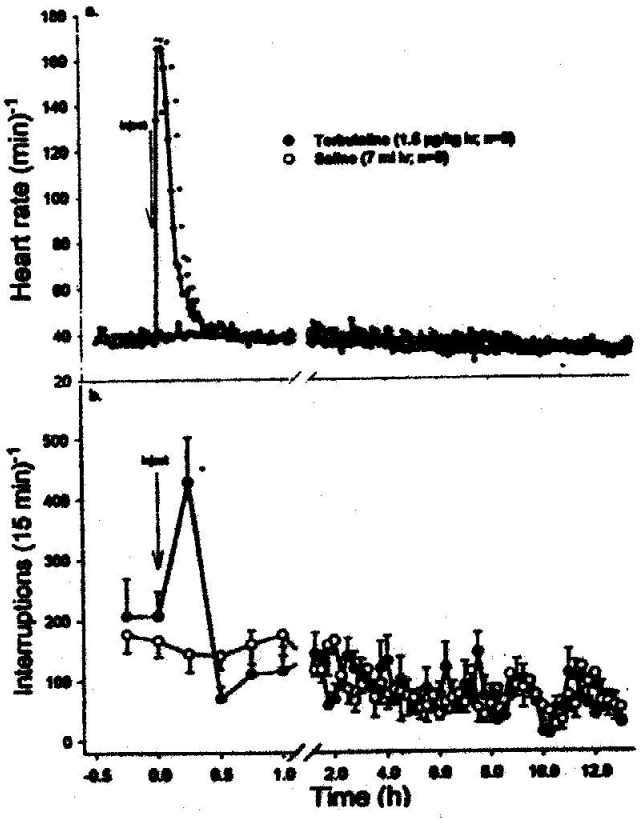

Fig. 3. Effect of terbutaline or saline on (a) HR and (b) spontaneous locomotor activity after i.v. injection. *, Significantly different from control values ($P < 0.05$).

Clenbuterol analysis by ELISA

Extraction. A standard solution (1 mg/mL) of clenbuterol was prepared in methanol. Extraction standards were prepared by the addition of a known amount of a clenbuterol solution (diluted in water) to blank urine samples at a range of 0.005–0.5 ng/mL.

The urine samples, standards, and blanks (2 mL/sample) were placed in culture tubes. Saturated sodium bicarbonate (1 mL) and 1 M sodium hydroxide (700 μL) were added to adjust the sample pH to about 11. Petroleum ether (4 mL) was added, and the tubes were mixed on a rotorack for 20 min followed by centrifugation (750 × *g*, 60 min at 4 °C). The petroleum ether layer was pipetted into a clean tube, and another 4 mL petroleum ether was added to the extraction tubes. The tubes were centrifuged again, and the petroleum ether fractions were combined. The extract was evaporated to dryness under a stream of nitrogen (< 40 °C), and the samples were reconstituted in 100 μL ELISA assay buffer and vortexed for 15 sec.

ELISA. The ELISA test was based on previously established methodology. Neogen, Inc. (Lexington, KY, USA) terbutaline (Bronchodilator Group) ELISA kits were used throughout. Appropriate clenbuterol standards were prepared in Neogen assay buffer, and a standard curve of these standards was generated on each plate along with the extracted samples. Each well received 20 μL of extracted urine sample or extracted standard solution and 180 μL of terbutaline-horseradish peroxidase complex solution. The wells were gently mixed for 1 min and incubated at room temperature (24 °C) for 45 min. Each well was washed three times with 300 μL of Neogen wash buffer, and Neogen Kentucky Blue substrate (150 μL) was then added. The plate was agitated for 30 min at 24 °C. The absorption at 650 nm of each well was measured on a universal microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Clenbuterol-trimethylsilyl analysis by GC/MS

Enzymatic hydrolysis. Standard solutions of clenbuterol and albuterol were prepared in methanol. Extraction standards were prepared by the addition of a known amount of a clenbuterol solution to blank urine samples at a range of 0.4–16 ng/mL. A known amount of an albuterol standard (20 μL of 20 μL/mL methanol solution) was added to each sample, standard, and blank as an internal standard.

The urine samples, standards, and blanks (5 mL/sample) were placed in culture tubes. To each sample was added 1 mL β-glucuronidase solution (Type L-II, from *Patella vulgata* 5000 U/mL, Sigma Chemical Co., St Louis, MO, USA) and 2 mL of 1 M sodium acetate buffer (pH 5.0). The samples were mixed briefly by vortex and incubated in a water bath at 65 °C for 3 h. After cooling (overnight, 4 °C), the clenbuterol standard and albuterol internal standard were added appropriately, and the samples were sonicated for 90 sec, 2 mL of 0.1 M sodium phosphate buffer (pH 6) were added, and the sample pH was adjusted to 6.0 ± 0.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

Extraction/derivatization. Clean Screen solid phase extraction (SPE) columns (United Chemicals Technologies-Worldwide Monitoring, Bristol, PA, USA) were conditioned by sequentially adding methanol (3 mL), water (3 mL), and 1 mL of 0.1 M sodium phosphate buffer (pH 6.0). The samples were then loaded, and the column was sequentially washed with water (2 mL), 1 M acetic acid (2 mL), and methanol (4 mL). The column was eluted with 3 mL of dichloromethane/isopropanol/ammonium hydroxide (78/20/2). The eluent was evaporated to dryness under a stream of nitrogen (< 40 °C). For derivatization, each sample was dissolved in 40 μL of *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Pierce, Rockford, IL, USA), vortexed for 15 sec, and incubated at 75 °C for 45 min (Fig. 1).

Instrumentation. The instrument used was a Hewlett-Packard Model 6890 GC equipped with a Model 5972A mass selective (MS) detector (Hewlett-Packard, Palo Alto, CA, USA). The column was an MDN-5S, 30 m × 250 μm × 0.25 μm (Supelco, Bellefonte, PA, USA). The carrier gas was helium with a flow of 1 mL/min. A volume of 1 μL was injected in splitless mode at an injector temperature of 250 °C. Initial oven temperature was 150 °C (held 2 min), ramping at 7.5 °C/min to 280 °C (held 4 min). The GC to MS interface temperature was 280 °C. After

cooling, each derivatized sample was transferred to an autosampler vial. An aliquot (1 μL) was injected onto the GC/MS, and the MS was run in selected ion monitoring (SIM) mode with data collected from 3 to 21.33 min. Integrated areas of the two quantitative ions, ion mass 86 (clenbuterol-trimethylsilyl derivative) and ion mass 369 (albuterol-trimethylsilyl derivative), were used to generate a standard curve and to quantitate clenbuterol in the samples. The qualifier ions for albuterol-trimethylsilyl (TMS) were ion masses 440, 147, 86, and 73. The qualifier ions for clenbuterol-TMS were ion masses 262, 243, 212, and 73.

GC/MS method validation

The GC/MS method for the quantitation of clenbuterol was validated by examining the measurement of consistency of results (within-run and between-run), linearity (coefficient of regression 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 ng/mL backfit of the standard curve) of the low (0.4 ng/mL), middle (4 ng/mL), and high (16 ng/mL) standards over six consecutive daily runs. The linearity was the mean coefficient of regression (r) for six consecutive daily runs. The recovery was determined by comparing the response (in area) of low and high standards and the response from derivatized equivalent methanol standards.

Statistical analysis

Data are presented as means ± SEM. Analysis of variance with repeated measures (SAS Institute Inc., 1985) was used to compare control and treatment values for each dosage. Effects of treatment on pulmonary function were evaluated by repeated-measures ANOVA with time and treatment (clenbuterol or saline) as the main effects. Significance was set at $P < 0.05$.

RESULTS

Effect of IT clenbuterol on lung function

Dose of atropine demonstrated that all horses used in the study developed reversible bronchospasm when stabled. Fifteen minutes after atropine dose, ΔPpl_{max} decreased from 44.3 ± 6.2 to 12.3 ± 2.8 cm H_2O, R_L decreased from 3.2 ± 0.4 to 1.4 ± 0.5 cm H_2O/L/sec, and E_{dyn} decreased from 10.2 ± 6.6 to 1.9 ± 0.7 cm H_2O/L.

The effect of IT clenbuterol and saline on ΔPpl_{max}, R_L, and E_{dyn} are shown in Fig. 2. Before clenbuterol or saline dose, lung function values indicated considerable airway obstruction and did not differ between the treatment groups. Repeated measures ANOVA of ΔPpl_{max}, R_L, and E_{dyn} indicated that there was a highly significant effect of time. The ΔPpl_{max}, R_L, and E_{dyn} were all decreased significantly below baseline at 15 min and the decrease continued through 4 h, 30 min, and 2 h, respectively (Fig. 2). However, there were no significant differences between

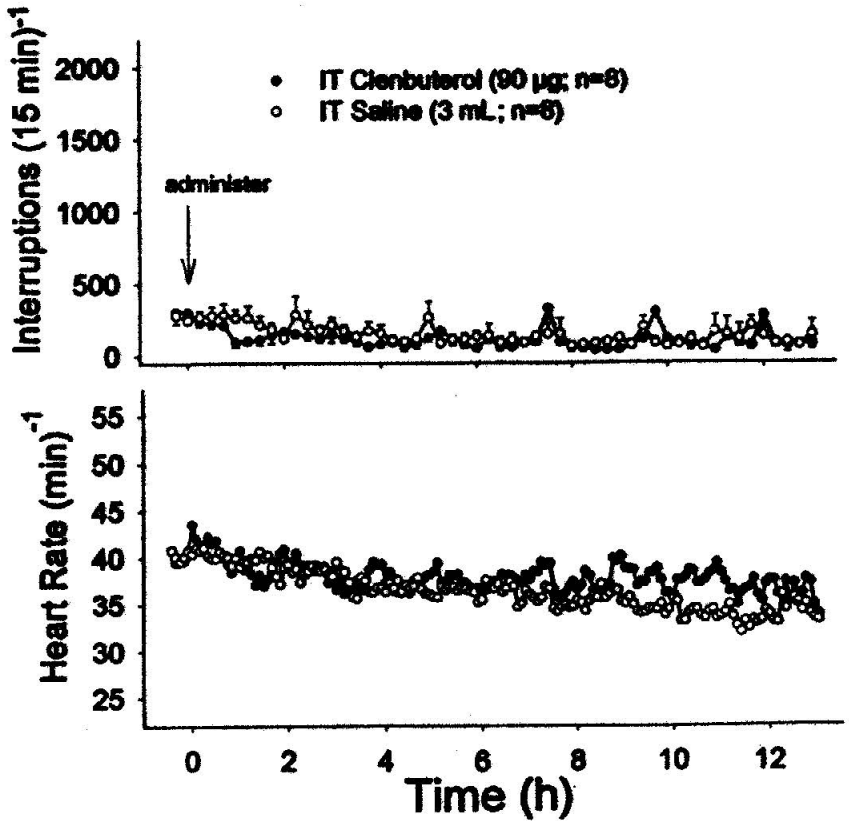

Fig. 4. Spontaneous locomotor activity and HR after IT dose of clenbuterol (90 μg)

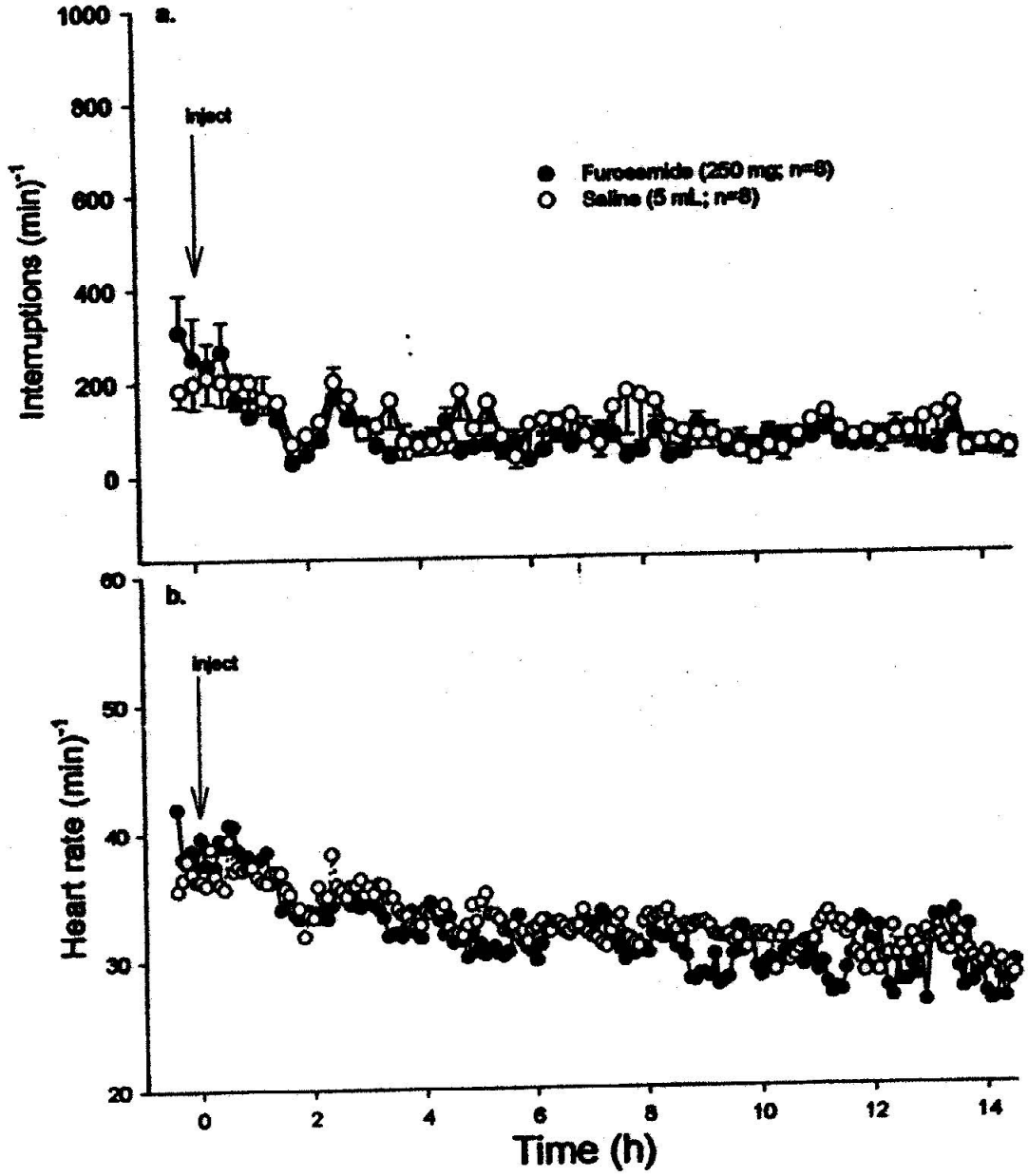

Fig. 5. Locomotor activity and HR of control and furosemide-treated horses.

clenbuterol and saline, i.e. there was no significant main effect of drug and no significant drug/time interaction.

HR and behavior chamber

The stimulating effect of terbutaline on HR and locomotor activity as a 'positive control' is presented in Fig. 3. Both effects of terbutaline occurred immediately and were short-lived, with HR and locomotor activity returning to pretreatment concentrations by 15 min after injection.

In contrast, there were no differences in locomotor activity and HR between IT doses of clenbuterol (90 μg) and saline (3 mL; Fig. 4). Figure 5 shows the locomotor activities and HR of control and furosemide-treated horses. There was no significant difference between treatment groups for either variable.

Mass spectral identifications of clenbuterol-TMS

The chromatography and full scan mass spectrometry are demonstrated for clenbuterol-TMS (Fig. 6) and matches the methodology where SIM of key ions in the spectrum were used for quantitative and qualifier ions. Figure 7b shows the type of linear standard curve that the method generates using this combination of derivatization and chromatography. Note the single peak in the chromatogram (Fig. 6a), which indicates there were no side products and no multiple derivatives.

Standard curves for the ELISA test and GC/MS analyses

A standard curve for the terbutaline ELISA test (Neogen Inc, Lexington, KY, USA) indicated that addition of 1.69 ng clen-

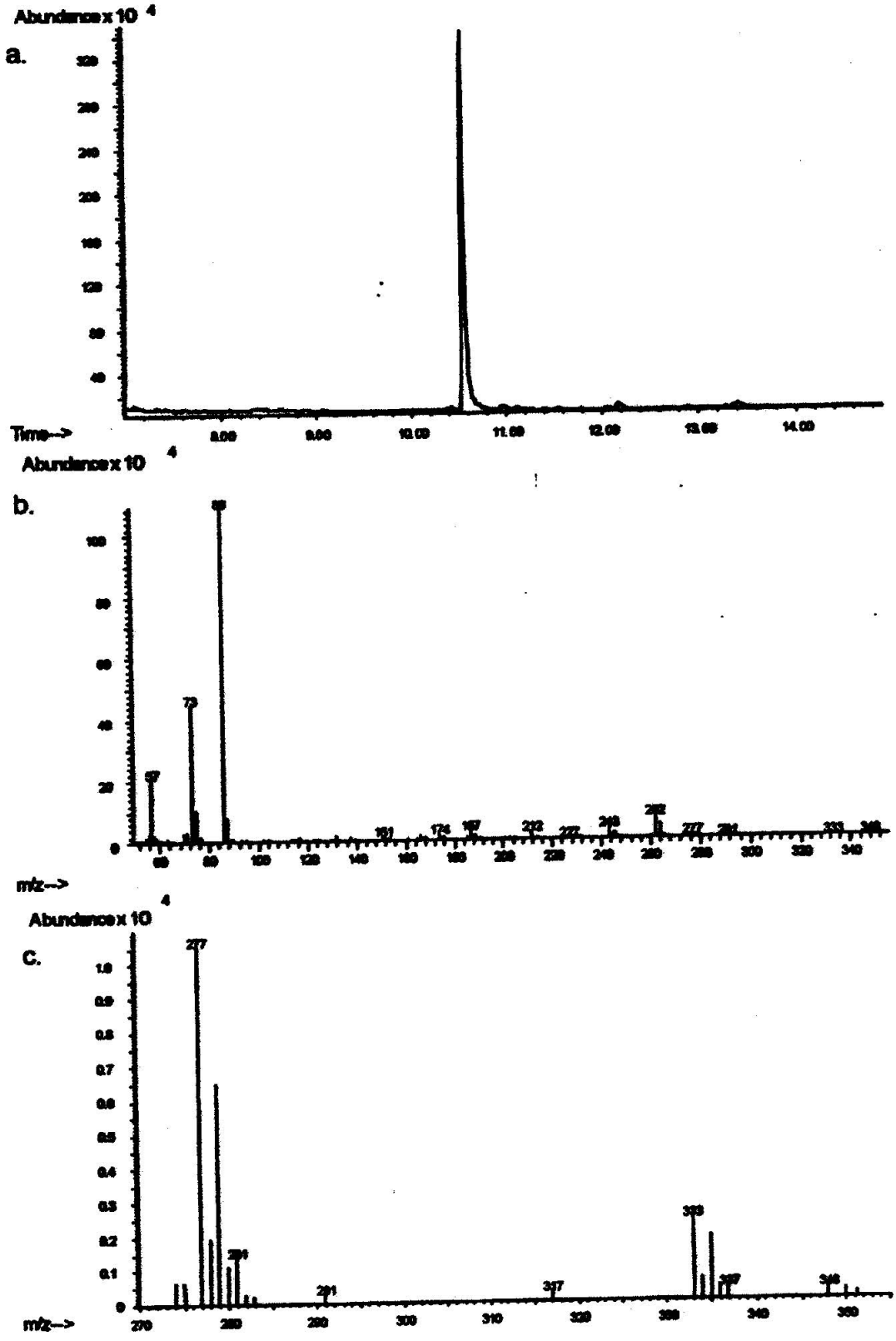

Fig. 6. (a) Total ion chromatogram of clenbuterol-TMS. (b) Full-scan mass spectrum of peak at 10.5 min seen in Fig. 6(a). (c) The mass spectrum of the higher range (270–355 m/z) to better illustrate the ions in that range.

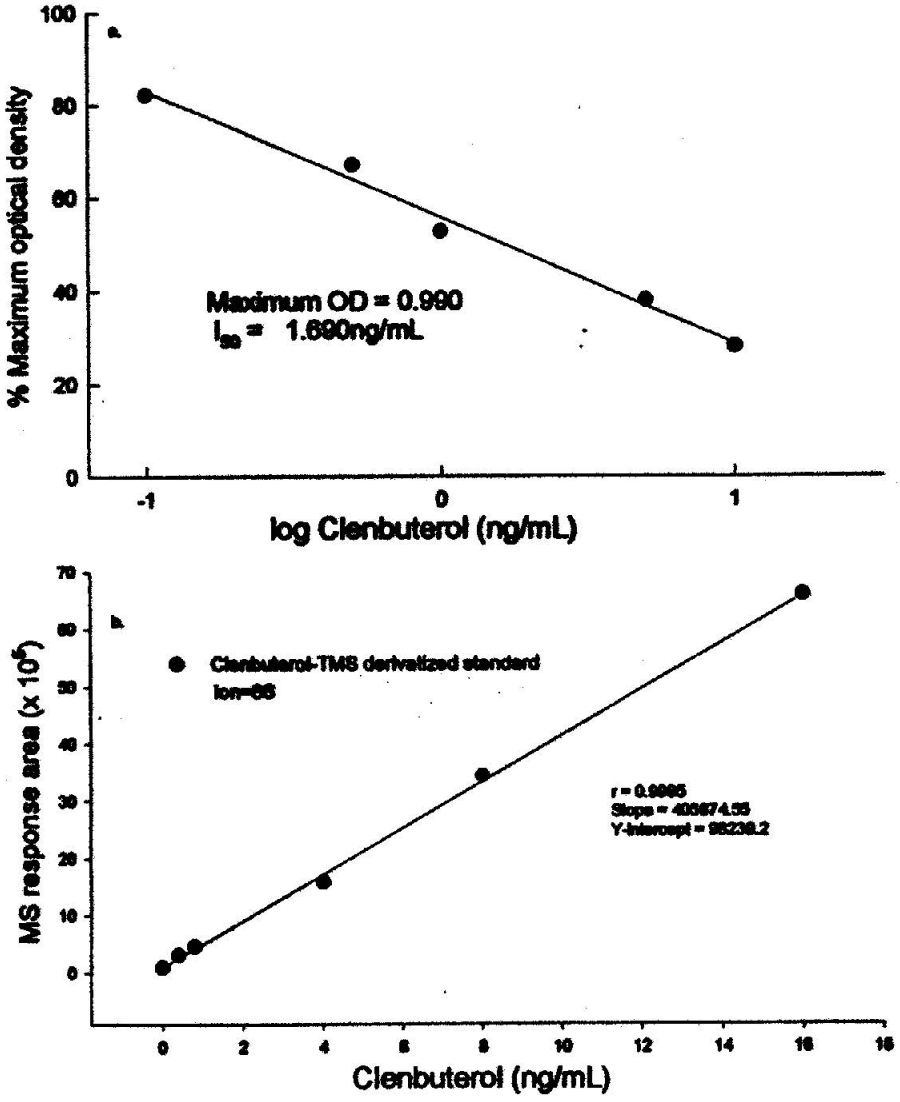

Fig. 7. (a) Regression of typical ELISA standard curve for clenbuterol in equine urine using the Terbutaline ELISA test (Neogen Inc. Lexington, KY, USA). (b) An example of a standard curve for derivatized clenbuterol-TMS extracted from urine and generated by least squares fit of integrated GC/MS areas for Ion 86.

buterol/mL to the system produced 50% inhibition (I_{50}; Fig. 7a). Higher concentrations of clenbuterol increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 10 ng/mL of clenbuterol.

As shown in Fig. 6b, chromatographic peaks of ion 86 were integrated, and the areas were calculated relative to the internal standard (ion 243) to provide a standard curve suitable for interpolation of unknowns. This approach to quantitations of clenbuterol yielded linear standard curves with r^2 values > 0.99.

Validation of quantitative GC/MS method for urine clenbuterol

The within-run precision was determined for the low (0.4 ng/mL: CV = 16%), middle (4 ng/mL: CV = 5.9%), and high (16 ng/mL: CV = 8.8%) concentrations of the clenbuterol standard curve, with a mean CV of 10.2%. The between-run precision was determined for the low (0.4 ng/mL: CV = 21%), middle (4 ng/mL: CV = 5.0%), and high (16 ng/mL: CV = 1.3%) concentrations of the clenbuterol standard curve, with a mean CV of 9.1%. The mean r for the assay was 0.9996 (0.0001 SEM). The recovery was determined at two concentrations; the mean recovery was 65.7%.

ELISA and GC/MS quantitation of clenbuterol in urine

After dosing with clenbuterol (90 μg IT) and furosemide (250 and 125 mg at −2 and 0 h, respectively), the specific gravity of urine from the furosemide-treated horses decreased sharply after the first dose, increased gradually for 2 h, then decreased again after the second dose (Fig. 8a). Note the consistent specific gravity of the saline-treated horses. The concentrations of apparent clenbuterol in urine after IT injection (90 μg) measured by ELISA technology reached 2.8 and 5.2 ng/mL at 6 h after dosing in clenbuterol-treated horses receiving saline and furosemide, respectively (Fig. 8b). Figure 8c shows the concentration of clenbuterol recovered from urine and measured by the TMS-derivatization protocol for GC/MS. The concentration of recovered clenbuterol rose rapidly after about 1 h and reached peaks of 9.0 and 11.7 ng/mL 4 h after dosing in horses receiving saline and furosemide, respectively.

DISCUSSION

Before 1998, the sensitivity of screening for clenbuterol by ELISA was not less than 1 ng/mL. With the introduction of enhanced sensitivity screening for clenbuterol, as described in this paper, and the advent of improved confirmation techniques, proceedings ('positive calls') before State Racing Commissioners have been initiated following clenbuterol identifications at concentrations as low as 100 pg/mL in urine. This represents more than a 10-fold increase in the sensitivity of testing.

To address the anecdotal reports of the IT dose of clenbuterol, it was important to determine if this mode of dose produced pharmacological effects in the respiratory tract or other critical systems of performance horses. Secondly, it was important to determine the duration of any effects after the IT dose.

To evaluate the effect of IT dose, horses with COPD were used because they have airway obstruction that is reversible with bronchodilator drugs (Robinson *et al.*, 1993; Derksen *et al.*, 1996), which was confirmed by treating the horses with atropine. This muscarinic antagonist is a reliable bronchodilator in COPD-affected animals and, in the present population of animals, caused a significant reduction in R_L, E_{dyn} and ΔPpl_{max}.

Figure 2 shows that during the 4 h after dose, the IT dose of clenbuterol produced no changes in R_L, E_{dyn} and ΔPpl_{max} that were different from those after the saline dose. Although there was an effect of time, the time effects of IT injection were trivial when compared with the major decreases in response to atropine dose. A placebo effect on lung function of similar magnitude has been observed in a few bronchodilator trials involving horses with COPD (Derksen *et al.*, 1996, 1999). Most likely, this effect is the result of moving horses from a dusty stall to a clean laboratory for measurements of lung function. In summary, IT clenbuterol produced no detectable pharmacological effects in COPD horses, which are very sensitive to the bronchodilating effects of β_2 agonists drugs.

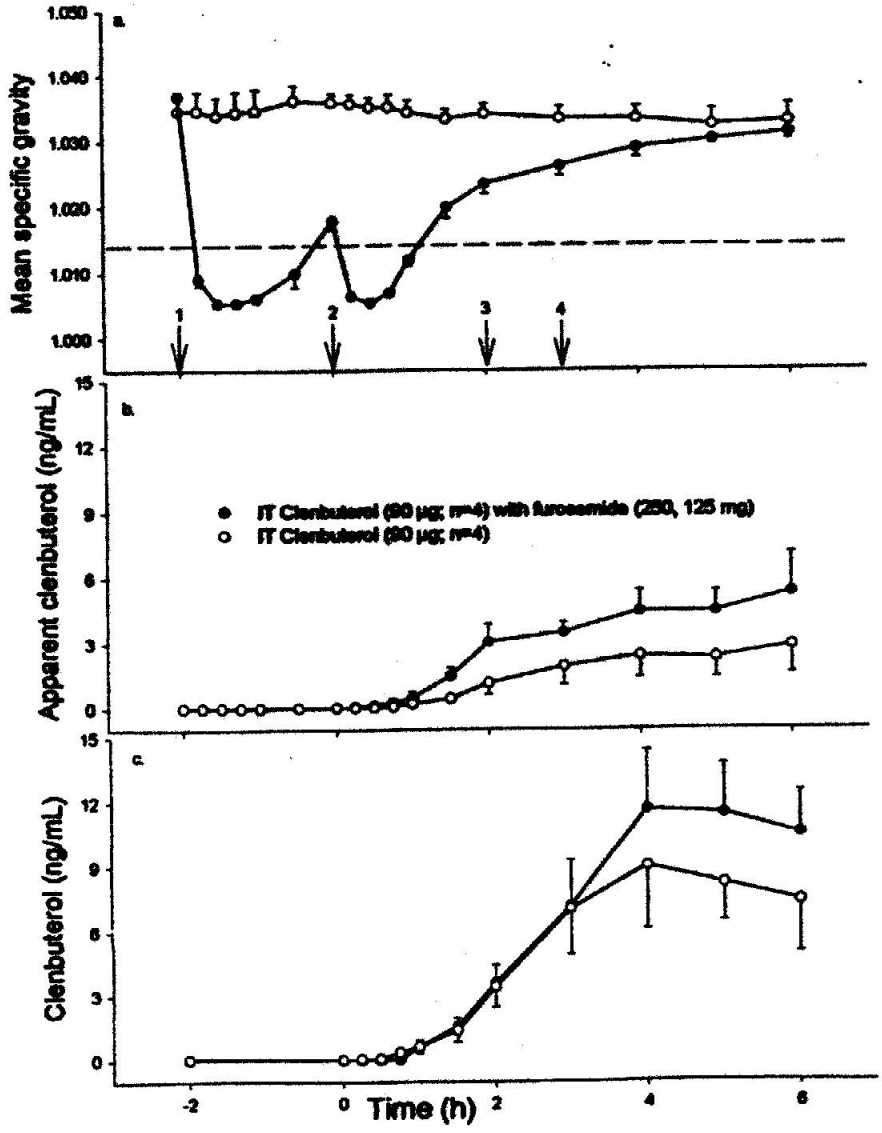

Fig. 8. (a) Specific gravity of urine samples; urine concentrations of (b) apparent clenbuterol as measured by ELISA and (c) clenbuterol as measured by the TMS-derivatization protocol for GC/MS after IT dose of clenbuterol (90 µg) with i.v. saline or furosemide (250 and 125 mg at −2 and 0 h, respectively). Numbers show: (1) time of approved injection of furosemide (4 h before a race), (2) time of unapproved injection of furosemide and clenbuterol (0 h), (3) hypothetical post-time for race 2 h later, and (4) hypothetical time of urine collection after race.

If IT clenbuterol has no effect in horses with COPD, it is unlikely to have any effect in horses with healthy airways for the following reasons. First, the airways of healthy horses are no more sensitive to β_2-agonists than are airways of COPD-affected horses (LeBlanc *et al.*, 1991), and secondly, bronchodilator drugs have little effect in normal horses (Shapland *et al.*, 1981). This is especially true because the dose of clenbuterol used in the IT experiments is only 25% of the standard clinical i.v. or oral dose.

Supposedly, the smaller IT dose was thought to be effective without being detected in post-race samples. However, clenbuterol was clearly detectable by both ELISA screening and the TMS-derivatization protocol for GC/MS (Fig. 8b and c, respectively). Furthermore, in the furosemide experiment, the mean specific gravity of urine 3 h after clenbuterol dose (1 h after hypothetical 'post time') was 1.024, well above the 1.010 concentration at which furosemide is considered to interfere with drug detection (Fig. 8a).

The lack of any locomotor or HR effects suggests that IT dose of clenbuterol is unlikely to be associated with positive effects on the performance of racing horses, whether affected by COPD or clinically normal. Therefore, the locomotor chamber data support the conclusions of the work in COPD horses and together suggest that the potential of clenbuterol (90 μg, IT) to produce significant pharmacological effects in the respiratory, cardiovascular, or central nervous systems is small. Furthermore, the lack of any effect after furosemide treatment suggests that horses gain no calming effect from the diuretic.

These findings are consistent with previous studies that suggest clenbuterol gives no definitive advantage on cardio-respiratory variables in healthy horses. Rose *et al.* (1983) concluded that i.v. clenbuterol had no untoward effects on the circulatory system of exercising horses. Kallings *et al.* (1991) found that oral clenbuterol did not cause any major effects on cardio-respiratory and blood lactate variables in healthy horses performing submaximal exercise. Slocombe *et al.* (1992) found that i.v. clenbuterol had no effects on the mechanics of breathing during exercise.

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