

Clenbuterol in the horse: urinary concentrations determined by ELISA and GC/MS after clinical doses

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Clenbuterol is a β_2 agonist/antagonist bronchodilator marketed as Ventipulmin® and is the only member of this group of drugs approved by the US Food and Drug Administration (FDA) for use in horses. Clenbuterol is a class 3 drug in the Association of Racing Commissioners International (ARCI) classification system; therefore, its identification in postrace samples may lead to sanctions. Recently, the sensitivity of postrace testing for clenbuterol has been substantially increased. The objective of this study was to determine the 'detection times' for clenbuterol after administration of an oral clinical dose (0.8 g/kg, b.i.d.) of Ventipulmin syrup. Five horses received oral clenbuterol (0.8 g/kg, b.i.d.) for 10 days, and urine concentrations of clenbuterol were determined by an enhanced enzyme-linked immunoabsorbent assay (ELISA) test and gas chromatography/mass spectrometric (GC/MS) analysis by two different methods for 30 days after administration. Twenty-four hours after the last administration, urine concentrations of apparent clenbuterol, as measured by ELISA, averaged about 500 ng/mL, dropping to about 1 ng/mL by day 5 posttreatment. However, there was a later transient increase in the mean concentrations of apparent clenbuterol in urine, peaking at 7 ng/mL on day 10 postadministration. The urine samples were also analysed using mass spectral quantification of both the trimethylsilyl (TMS) and methane boronic acid (MBA) derivatives of clenbuterol. Analysis using the TMS method showed that, at 24 h after the last administration, the mean concentration of recovered clenbuterol was about 22 ng/mL. Thereafter, clenbuterol concentrations fell below the limit of detection of the TMS-method by day 5 after administration but became transiently detectable again at day 10, with a mean concentration of about 1 ng/mL. Derivatization with MBA offers significant advantages over TMS for the mass spectral detection of clenbuterol, primarily because MBA derivatization yields a high molecular weight base peak of 243 m/z, which is ideal for quantitative purposes. Therefore, mass spectral analyses of selected urine samples, including the transient peak on day 10, were repeated using MBA derivatization, and comparable results were obtained. The results show that clenbuterol was undetectable in horse urine by day 5 after administration. However, an unexpected secondary peak of clenbuterol was observed at day 10 after administration that averaged ~1 ng/mL. Because of this secondary peak, the detection time for clenbuterol (0.8 g/kg, b.i.d. × 10 days) is at least 11 days if the threshold for detection is set at 1 ng/mL.

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INTRODUCTION

Clenbuterol is a β_2 agonist/antagonist bronchodilator drug listed by the American Association of Equine Practitioners for use in

horses. It is also the only β_2 agonist approved by the US Food and Drug Administration (FDA) for use in horses. As a bronchodilator, clenbuterol has the potential to alter the athletic performance of horses, particularly if the animal has bronchospasm

(Sasse & Hajer, 1978). Clenbuterol is classified by the Association of Racing Commissioners International (ARCI) as a class 3 drug, and its detection in postperformance blood or urine samples may lead to significant sanctions against trainers.

Because clenbuterol is a legitimate, therapeutic medication approved for use in horses in training, there is a question regarding the length of time during which residues are likely to be detected in postadministration samples. This was brought into sharp focus in May 1998 by the development of a highly sensitive enzyme-linked immunosorbent assay (ELISA) screening test for clenbuterol at about the same time clenbuterol was approved by the FDA for use in horses¹. It seemed likely that 'detection times' for clenbuterol as Ventipulmin[®] based on the new ELISA test would be much longer than the 'detection times' based on the previously used, less-sensitive ELISA test. In addition to ELISA testing, the concentrations of clenbuterol in urine samples were also determined using two different gas chromatography/mass spectrometric (GC/MS) methods: methane boronic acid (MBA) and trimethylsilyl (TMS) derivatization of clenbuterol (Fig. 1). The objective of this study was to determine the 'detection times' for clenbuterol after administration of an oral clinical dose (0.8 g/kg, b.i.d.) of Ventipulmin syrup.

MATERIALS AND METHODS

Horses

Five mature thoroughbred mares weighing 532–579 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 just before drug administration, 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 Vet-medica, Inc, St Joseph, MO, USA) was administered orally twice daily for 10 days (20 dosings) at a dosage of 0.8 g/kg. Urine samples were collected before treatment started, each day before the morning dosing, and 30 days after dosing using a Harris flush tube (24 Fr × 60 in; Seamless, Ocala, FL, USA) to collect a maximum of 500 mL of urine.

Urine analysis for clenbuterol

The analysis of clenbuterol by ELISA technology (concentrations of petroleum ether extracts for a Neogen terbutaline ELISA) and

¹ Scientific and Veterinary Review in Horseracing, Boehringer Ingelheim Vetmedica, Inc., p. 1, 1998.

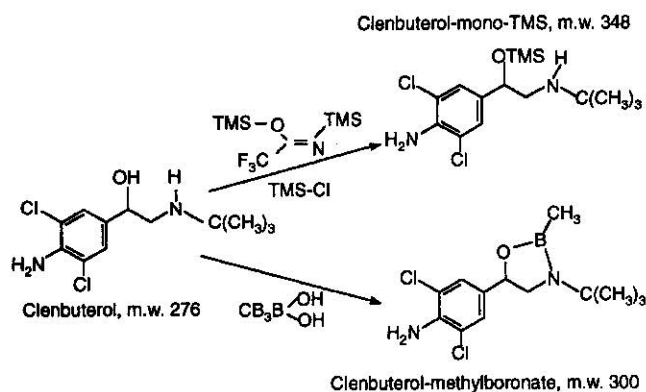


Fig. 1. Derivatization schemes for clenbuterol as verified by GD/MS to yield clenbuterol-TMS and clenbuterol-MBA. TMS = trimethylsilyl; BSTFA = N,O-bis(trimethylsilyl)-trifluoroacetamide; MBA = methanoboronic acid.

by GC/MS using TMS derivatization of hydrolyzed urine extracts (solid phase extraction with albuterol as internal standard) and method validation for TMS quantitation were described previously (Harkins *et al.*, 2000).

Clenbuterol-MBA analysis by GC/MS

This method was provided by Dr Richard Sams, Ohio State University Analytical Toxicology Laboratory and is the standard operating procedure for the confirmation of clenbuterol by GC/MS.

Enzymatic hydrolysis

Standard solutions of clenbuterol and metoprolol were prepared in methanol. Extraction standards were prepared by adding a known volume of a clenbuterol standard solution to blank urine samples at a range of 0.4–16 ng/mL. A known volume of a metoprolol standard solution (20 L of 20 g/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 were added 1 mL of glucuronidase reagent (Sigma Type L-II, 5000 units/mL) and 2 mL of 1 M sodium acetate buffer (pH 5.0). The samples were mixed briefly by vortex and incubated (water bath) at 65 °C for 3 h. After cooling (overnight, 4 °C), metoprolol (internal standard) was added to all tubes, clenbuterol was added to each standard tube at the appropriate amount, and the sample pH was adjusted to 12 ± 0.5 with 10 M NaOH (Zamecnik, 1990; Branum *et al.*, 1998).

Extraction/derivatization

The extraction and derivatization was performed as suggested by The Analytical/Toxicology Laboratory at the Ohio State University, Columbus, OH, USA (Richard Sams, personal

communication). To each tube was added 5 mL of petroleum ether. The tubes were capped and mixed by rotorack for 10 min. The tubes were centrifuged for 1 h, and the petroleum ether layer was transferred to a clean tube. The extraction was repeated, the petroleum ether layers were combined and evaporated to dryness under a stream of N_2 (<40 °C). For derivatization each sample was dissolved in 50 L of methylboronic acid solution (2 mg/mL ethyl acetate), vortexed for 15 s, and incubated at 60 °C for 60 min (Fig. 1). Each reaction mixture was diluted with 50 L ethyl acetate.

Instrumentation

The instrument used was a Hewlett-Packard Model 6890 GC equipped with a Model 5972 A mass selective detector (MS) (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. The volume injected was 1 µL in splitless mode at an injector temperature of 280 °C. Initial oven temperature was 120 °C (held 1 min), ramping at 12 °C/min to 280 °C (held 6 min). The GC to MS interface temperature was 280 °C. After diluting, 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 SIM mode with data collected from 3 to 26.33 min. Integrated areas of the two quantitative ions, ion mass 243 (clenbuterol-methylboronic acid derivative) and ion mass 276 (metoprolol-methylboronic acid derivative), were used to generate a standard curve and to quantitate clenbuterol in the samples. The qualifier ion for metoprolol-MBA was ion mass 291, and the qualifier ions for clenbuterol-MBA were ion masses 302, 300, 287, 285 and 245.

RESULTS

ELISA quantitation of clenbuterol in urine

A plot of standards giving a linear relationship for the terbitaline ELISA test (Neogen Inc, Lexington, KY, USA) indicated that addition of 1.69 ng clenbuterol/mL to the system produced 50% inhibition (I_{50} ; Fig. 2a). 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.

The mean concentration of apparent clenbuterol on day 1 measured by ELISA technology was 485 ng/mL (urine sample was collected 15 h after the last administration). This concentration dropped rapidly to ~15 ng/mL on day 2 and reached 1 ng/mL on day 5. However, the concentrations of apparent clenbuterol began to increase again on day 9, peaking at ~7 ng/mL 10 days after dosing. Figure 2b shows the consistency of the secondary peak on day 10 among the five horses. Figure 2c shows the mean optical densities of the urine samples.

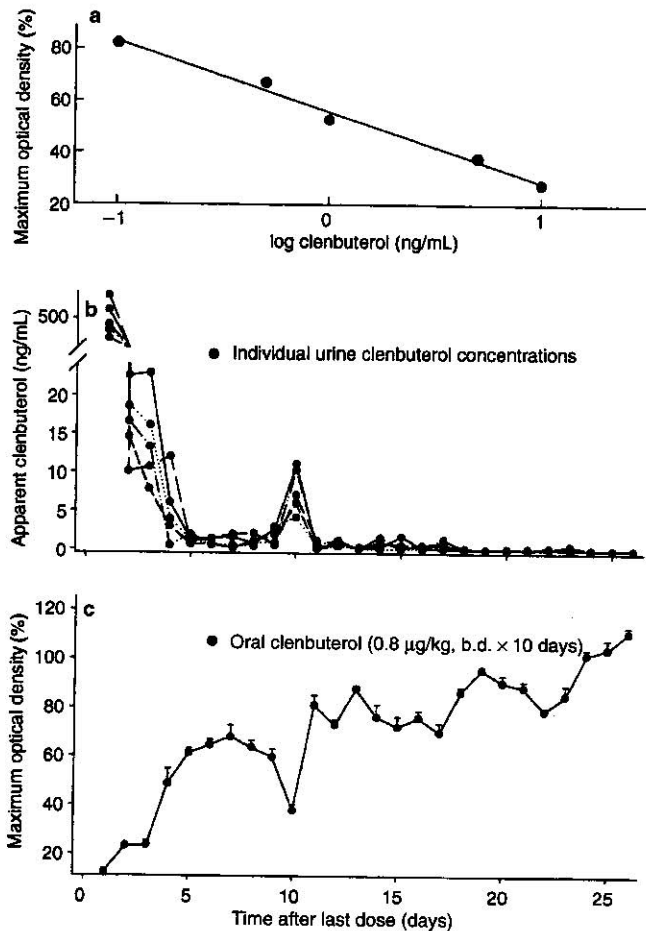


Fig. 2. (a) Regression of ELISA standard curve for clenbuterol in equine urine using the Terbitaline ELISA test (Neogen Inc, Lexington, KY, USA); (b) individual urine concentrations of apparent clenbuterol as measured by ELISA; and (c) mean percentage of the maximum colour of the blank urine sample (which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

Mass spectral identifications of clenbuterol-MBA and clenbuterol-TMS

The total ion chromatograms of clenbuterol-TMS and clenbuterol-MBA are shown in Fig. 3(a,b), respectively. Note the single peak in both chromatograms, which indicates there were no side products and no multiple derivatives. Figure 4a shows the full scan mass spectrum of the peak at 10.5 min seen in Fig. 3a. Figure 4b shows the mass spectrum of the higher range (270–355 m/z) to better illustrate the ions in that range. The hypothetical origin of the base peak (86 m/z) by intramolecular α -cleavage with loss of the aminodichlorobenzy-OTMS fragment (loss of 262 amu as a neutral radical) is shown in Fig. 4c.

Figure 5a shows the full scan mass spectrum of the peak at 12.1 min seen in Fig. 3b. The hypothetical origin of the base peak (243 m/z) by α -cleavage of the *tert*-butyl group (loss of 57 amu as a neutral radical) is shown in Fig. 5b. Figure 6(a,b) was generated by the integration of ions 86 and 243 m/z, respectively, and the areas were calculated relative to the internal standards to provide standard curves suitable for interpolation of unknowns.

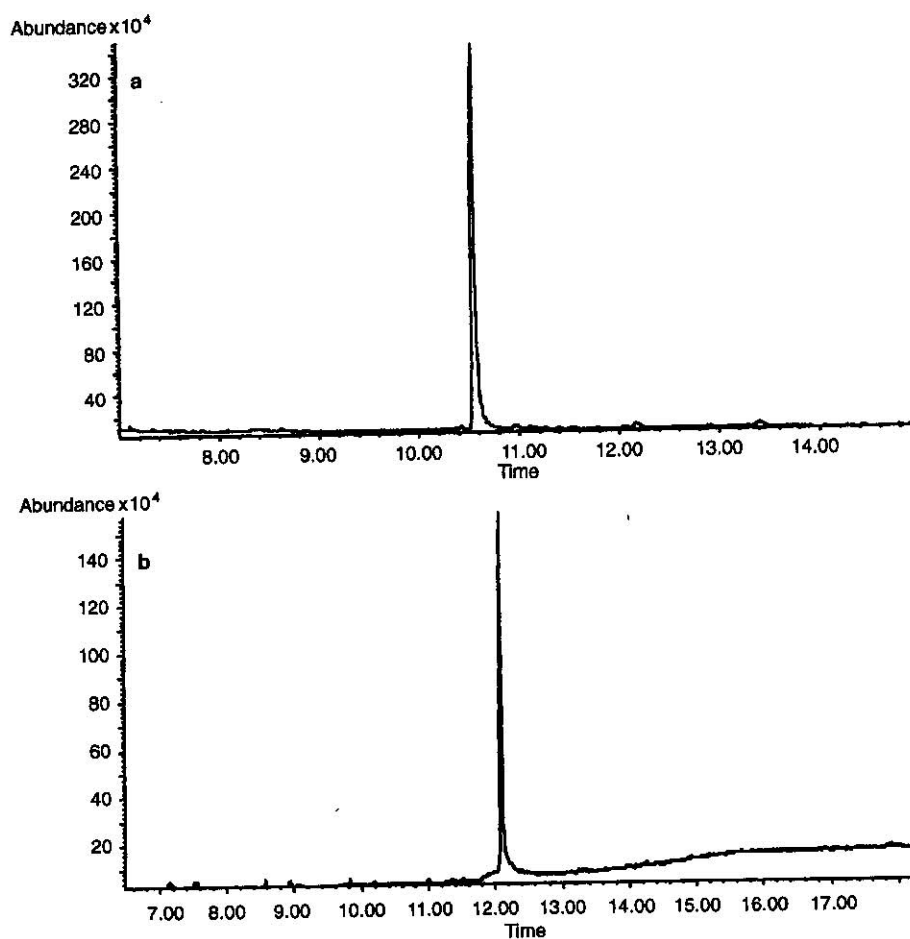


Fig. 3. Total ion chromatograms of (a) clenbuterol-TMS and (b) clenbuterol-MBA.

Figure 7a shows the urine concentrations of clenbuterol as measured by TMS-derivatization protocol for GC/MS of the five individual horses. Note the consistent, cyclic patterns of the urine concentrations: all horses were negative on day 5, two became positive on day 6, all were negative on days 7 and 8, all became positive on days 9 and 10, all but one became negative on day 11, and then there was another increase on day 12. (The apparent clenbuterol increase in individual urine samples at day 10 was also measured by ELISA; Fig. 2b).

The highest mean concentration of clenbuterol in urine occurred on the first day after the end of administration (22.0 ng/mL) and decreased to 2.0 ng/mL by day 3 (Fig. 7b). This figure also shows the comparison of the methods for the analysis of clenbuterol-MBA (days 3 and 8–11) and clenbuterol-TMS derivatives in horse urine.

DISCUSSION

The goal of this study was to generate a data base to provide guidelines for veterinarians and regulators regarding the period after administration in which residues of clenbuterol would likely be detected in urine. The dosage used was the manufacturer's lowest recommended dose of 0.8 g/kg, b.d. for 10 days, twice the manufacturer's suggested minimum therapeutic period of 5 days.

Regulatory control of clenbuterol generally involves ELISA screening of posttrace urine samples, followed by mass spectral confirmation of its presence. Until recently, the sensitivity of routine screening for clenbuterol was about 5 ng/mL. At this sensitivity, a 3–5 day withdrawal time for clenbuterol generally would yield a 'negative' posttrace screening test, and the sample would not be evaluated further.

As clenbuterol is recommended for twice-a-day administration and its plasma half-life is relatively short (~7 h), a 3 days withdrawal time for this medication would seem appropriate. Indeed, for 20 years the 3 days withdrawal time has been the standard regulatory approach to clenbuterol in Canada (Canadian Pari-Mutuel Agency, 1994).

However, with the recent introduction of enhanced ELISA testing, the lower limit of detection of screening methods for clenbuterol decreased to about 0.1 ng/mL, and there was a large concomitant increase in the number of clenbuterol identifications. It is likely that at least some of these identifications were associated with clenbuterol administrations that had terminated 5 days or more prior to collection of the urine samples in question, especially if the dose was large and/or the duration of administration was prolonged.

In this regard, review of the ELISA data (Fig. 2b) shows that apparent clenbuterol was virtually undetectable by day 5 but began to increase consistently in all horses on days 9–10

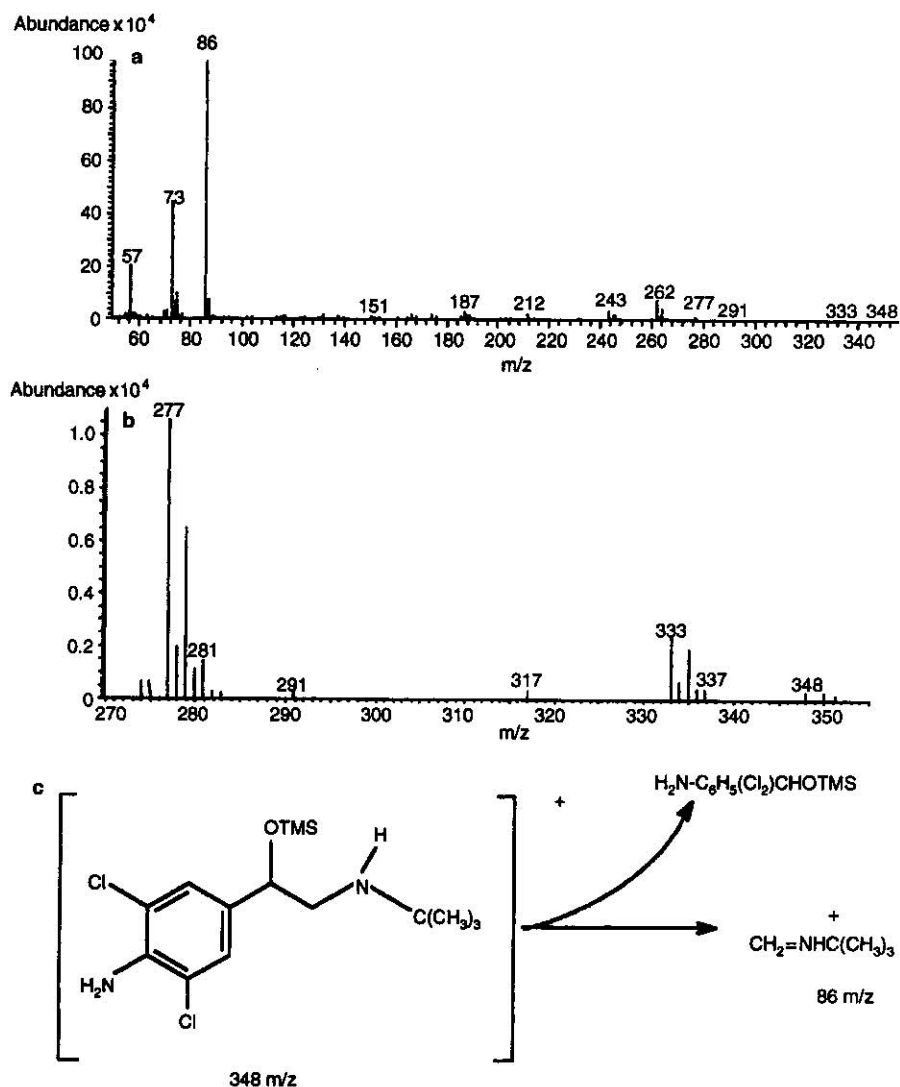


Fig. 4. Full scan mass spectrum of peak at 10.5 min seen in Fig. 3a, (b) mass spectrum of the higher range (270–355 m/z) to better illustrate the ions in that range; and (c) the origin of the clenbuterol–TMS 86 m/z base peak by intramolecular α -cleavage with loss of the aminodichlorobenzyl–OTMS fragment (loss of 262 amu as a neutral radical).

postadministration, which was unexpected. By day 11 after dosing, the concentrations of apparent clenbuterol had dropped sharply to again become essentially undetectable. Also note in Fig. 2c that the recurrence of apparent clenbuterol was evident not just on day 10 but was cyclic, reappearing on days 17 and 22 as well.

Consistent with ELISA, the mass spectral data using the TMS method showed that clenbuterol was again undetectable by day 5 after administration. However, by day 9, the mean clenbuterol concentration had increased to ~0.8 ng/mL and then reached a peak on day 10 of ~1 ng/mL (Fig. 7b). The consistency of this pattern is again illustrated with the individual urine concentrations of the five horses (Fig. 7a).

Possible contributing factors for the increase in apparent clenbuterol were examined in retrospect to help explain the phenomenon. The experiments were performed in October, and the ambient temperature dropped about 6 °C from day 9 to day 10. However, there was no profound temperature change on days 8 and 9, days when the clenbuterol concentration in urine had clearly begun to increase. Another possible factor

could have been the reproductive cycle. Although the experiment was performed in mares, any effects of the oestrous cycle should not have been a factor as testing occurred late in the year (October), after most mares would have stopped cycling. A remote possibility could be differential prolonged excretion of one enantiomer of clenbuterol, as the commercial preparation is a mixture of (+) and (–) enantiomers (Gausepohl & Blaschke, 1998). These enantiomers are indistinguishable in our system.

Because the recurring peak of urine clenbuterol and/or its metabolite(s) was unexpected, analyses of urine samples from days 3 and 8–11 were repeated using the MBA derivatization method. Derivatization with MBA offered significant advantages for the mass spectral detection of clenbuterol including specificity and sensitivity, primarily because of a high molecular weight base peak (243 m/z), which was ideal for quantitative purposes. However, to maintain the derivative, the MBA procedure required injection of a relatively large amount of the derivatizing reagent which caused excessive damage to the column and the detector (C. Natrass, N.Heater, S. Stanley,

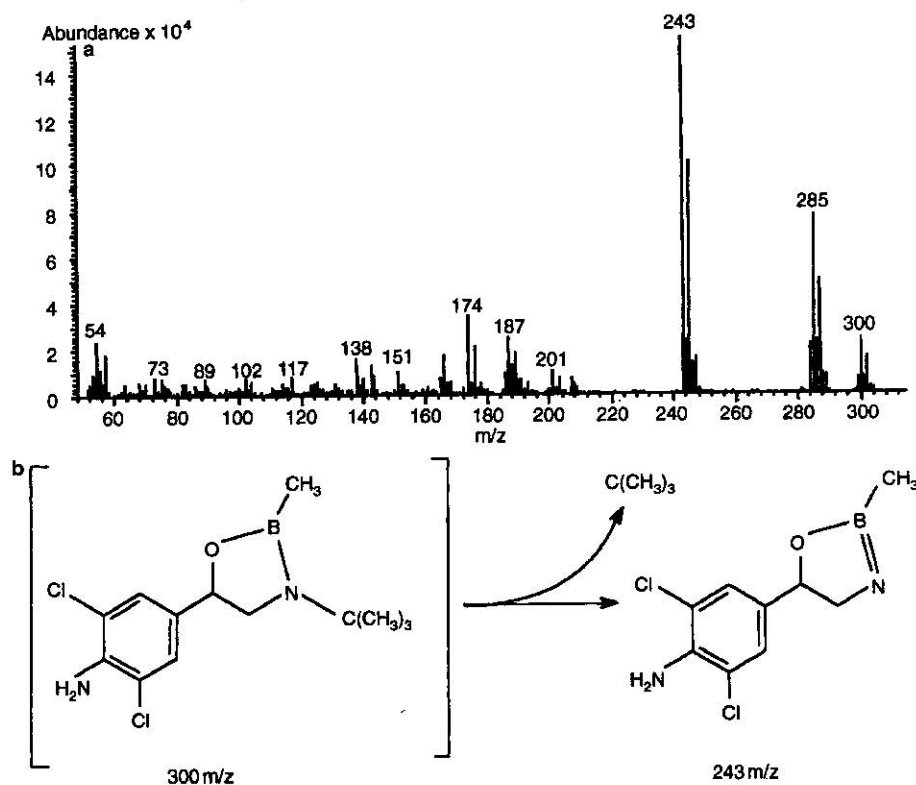


Fig. 5. (a) Full scan mass spectrum of peak at 12.1 min seen in Fig. 3b, and (b) the origin of the clenbuterol-MBA 243 m/z base peak by α -cleavage of the tert-butyl group (loss of 57 amu as a neutral radical).

personal communication). There was a good quantitative relationship between the concentrations of clenbuterol identified using the TMS and MBA methods. At concentrations above 1 ng/mL, the correlation was very good, although it was less satisfactory at lower concentrations.

The primary reason the TMS method was less satisfactory at concentrations <1 ng/mL is the low molecular weight of the quantifying ion (86 m/z). In the clenbuterol-TMS based assay, the peak area calculation of clenbuterol standards below 1 ng/mL is compromised by a fluctuating baseline resulting from a high apparent background. This baseline interference prevented the establishment of a rigorous limit of detection (LOD) for this method, and all clenbuterol values <1 ng/mL carry a considerable level of quantification uncertainty. In addition, specificity was hampered by degrading qualifier ion ratios for clenbuterol-TMS in this range, requiring invocation of higher cutoffs for acceptable identification of clenbuterol.

Furthermore, the signal to noise ratio of clenbuterol-TMS for values below 1 ng/mL was calculated at mass spectral SIM peaks and compared with average baseline noise; such values were quite close to an artificial cutoff criterion of $s/n = 2$, whereas qualifier ions m/z 243 and 262 were below this cutoff. The qualifiers surpassed this criterion at 8 and 4 ng/mL, respectively, suggesting that the TMS method be used principally for pharmacokinetic studies and not for forensic confirmation of drug presence, except above 10 ng/mL. These shortcomings of the TMS method contrast with the MBA method, which exceeded the $s/n = 2$ criterium for quantifier and qualifier ions down to 0.4 ng/mL.

On the other hand, preliminary work with the clenbuterol-MBA method yielded a much lower background for the quantitative ion (243 m/z), with an estimated LOD of <0.1 ng/mL. However, this method was not pursued extensively because of the corrosive effects of MBA on instruments, columns, and detectors, as evidenced by rapid deterioration of MS tune quality, visible column damage, and decreased column performance. On this basis, it is reasonable to suggest that samples containing more than 1 ng/mL can be reliably quantitated using the clenbuterol-TMS method with less damage to instruments, columns and detectors, while the clenbuterol-MBA method should be reserved for those samples containing concentrations of <1 ng/mL.

The prolonged, intermittent detection of clenbuterol in urine reported here is not unique. In another recent work, clenbuterol was administered for 4 weeks at a dose of 3.2 g/kg and was determined as its MBA derivative using a LOD of 250 pg/mL. In individual horses, clenbuterol concentrations in urine persisted above 1 ng/mL for 5–11 days after the last administration. For up to 28 days after administration, urine clenbuterol ranged from undetectable to 370 pg/mL. During the period when urine clenbuterol was <1 ng/mL (11–28 days), its detection was sporadic, being undetectable in some urine samples for a few days then reappearing again (Sams, 1999). Furthermore, this phenomenon has been observed with other drugs. In a study of procaine concentrations in plasma and urine of four horses, urine procaine decreased to the point of being almost undetectable on day 9, at which time it began to increase again, reaching a peak on day 11 and then declining again to day 14

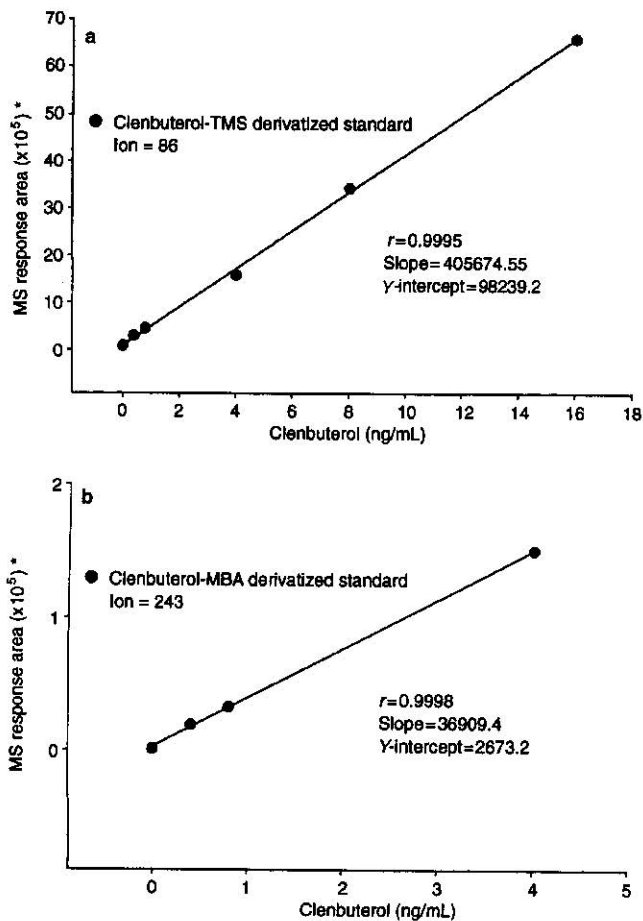


Fig. 6. Standard curves for (a) derivatized clenbuterol-TMS extracted from urine and generated by least squares fit of integrated GC/MS areas for ion 86 and (b) derivatized clenbuterol-MBA extracted from urine and generated by least squares fit of integrated GC/MS areas for ion 243. *MS response area is determined relative to the internal standard.

(Tobin & Blake, 1977). Tolmetin is an arthritis treatment that demonstrated biphasic elimination from plasma (Grindel *et al.*, 1979). Etoprozine is a psychotropic drug that showed irregular plasma concentration time profiles, resulting in secondary peaks in some patients (deVries *et al.*, 1991). Vincristine showed a biphasic disappearance from blood in rats and dogs (Castle *et al.*, 1976).

It is unlikely that accidental re-administration of the horses occurred because the consistent, cyclic reappearance of clenbuterol/metabolite (Fig. 2c; days 10, 17, and 22) means that all horses would have been re-administered on at least three different occasions, days after all planned dosing had ceased. Recent work has demonstrated that horses often contaminate themselves from urine-soaked bedding and contamination of feed areas when administered orally, causing identification of the drug/metabolite in subsequent urine samples (Wennerlund *et al.* 2000). That is unlikely in this study because the horses were at pasture at all times postadministration, except during urine collection. Nevertheless, the cyclic observations of clenbuterol in urine should be

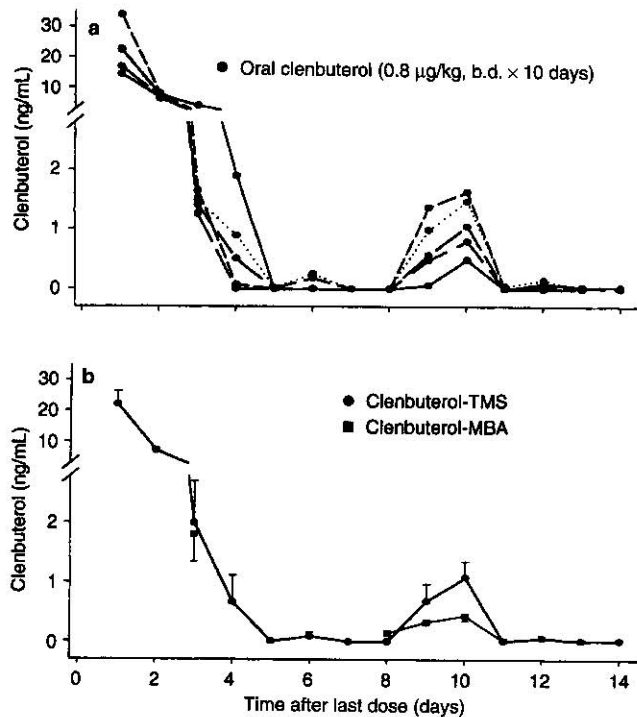


Fig. 7. (a) Individual urine concentrations of clenbuterol as measured by the TMS-derivatization protocol for GC/MS following oral administration of clenbuterol (0.8 µg/kg, b.i.d. for 10 days), and (b) mean comparison of methods for the analysis of clenbuterol-TMS and clenbuterol-MBA derivatives in horse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

considered preliminary until such time that others have the opportunity to repeat this work.

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