#297

(Author)

Proceedings of the 14th International Conference of Racing Analysts and Veterinarians, Florida, USA

ALBUTEROL BY TORPEX AND APPARENT ELISA 'DETECTION TIMES': A PRELIMINARY REPORT

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ABSTRACT

Torpex is a proprietary inhalation device which allows easy, efficient and effective administration of medications, including albuterol, to horses. Because albuterol administered by Torpex may be used in competitive horses in training, it is important to understand its elimination after administration. We therefore determined the apparent ELISA 'detection times' for albuterol in equine serum, nasal swabs and urine samples after administration by Torpex.

Albuterol was administered by Torpex to 6 Thoroughbred mares at doses of one puff (120 µg/puff), 3 puffs, 6 puffs and then 6 puffs per administration, 4 administrations/day, for 5 days. We considered a significant ELISA response as >50% inhibition of the ELISA response. No significant ELISA responses were observed in any serum sample tested. In urine samples, the mean concentrations of albuterol equivalents exceeded 50% ELISA inhibition for up to 8 h after administration of albuterol at the 1, 3 and 6 puff doses. There was no ELISA evidence of albuterol or its metabolites at 24 h after these single sequence administrations. To be conservative, however, 48 h should be allowed for albuterol to 'clear' the urine after these doses. Following administration of 6 puffs per horse 4 times a day for 5 days, the urine samples showed no ELISA evidence of albuterol or its metabolites at 24 and 48 h post dosing, suggesting a conservative 72 h withdrawal time. On the other hand, swabs from the nasal mucous membranes remained ELISA positive for at least 8 h after the 1, 3 and 6 puff doses, and individual nasal samples have a small probability of inhibiting the ELISA test for up to 8 days after the last administration.

These results are guidelines only, were developed in a small number (n=6) of horses and are based on the ELISA test used and interpreted as described. Modification of this test or interpretations increasing its sensitivity have the potential to increase the probability of a detection-triggering event. Jurisdictional thresholds for albuterol would probably decrease the likelihood of a detection-related event. Factors specific to individual horses may also influence 'detection times'.

INTRODUCTION

Albuterol is a relatively selective ß-2 adrenoreceptor agonist used as a bronchodilator in the treatment of human asthma and chronic obstructive pulmonary disease (Torneke *et al.* 1998, Nava *et al.* 2001). Aerosolised albuterol, at doses of 360 µg and 720 µg, is a safe and effective bronchodilator in horses with recurrent airway obstruction (Derksen *et al.* 1999). Unlike formoterol and salmeterol, which are lipophilic and have long durations of effect, albuterol is hydrophilic and has a rapid onset and short duration of action (Lotvall 2001).

Albuterol has recently been formulated for administration by the 3M Equine Inhaler (Torpex) (3M and Boehringer Ingelheim Vetmedica, Inc., Missouri, USA). Torpex is a new equine inhalation device which effectively and efficiently administers nebulised therapeutic medications in a synchronised manner into the respiratory tract of horses. Because the lung is the target organ for albuterol, administration of this agent by Torpex allows efficient delivery of albuterol to its sites of action in the respiratory tract. The efficiency of Torpex, therefore, serves to reduce the dose of albuterol that must be administered to attain a desired therapeutic response. This reduction in dose may be expected to bring with it the added

benefit of reducing both incidence and intensity of any adverse responses and, in the case of competition horses, may also reduce the probability of inadvertent medication identifications.

As an ARCI class 3 bronchodilator, albuterol may be considered by regulators to have the potential to alter the athletic performance of horses (Bailey *et al.* 1999), particularly if the animal has bronchospasm and bronchoconstriction associated with reversible airway obstruction (Sasse and Hajer 1978; Derksen *et al.* 1999). As such, its detection in post performance samples may lead to significant sanctions against the trainer. In competition horses, the detection of high potency medications such as albuterol is largely dependent on ELISA testing. To evaluate the relationship between administration of albuterol by Torpex and the potential for ELISA detection of such administrations, albuterol was administered by Torpex to 6 Thoroughbred horses and the post-administration detection of albuterol and/or its metabolites by ELISA in serum, urine, and nasal swabs is reported.

MATERIALS AND METHODS

Horses and dosing

Six mature Thoroughbred mares weighing 413–602 kg were used for this study. Horses were kept in a 20-acre field until they were placed in box stalls where they were provided water and hay *ad libitum*. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). A routine clinical examination was performed prior to each experiment. Animals used in these experiments were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care and Use Committee, which also approved the experimental protocol.

All experiments were carried out using the Torpex Equine Inhaler provided by 3M. Six horses were administered single doses of 1, 3 and 6 puffs and a sequence of 6 puffs per dose, 4 times per day, for 5 days which is the maximal recommended duration of aerosol albuterol administration using the 3M Equine Inhaler. A 2-week washout period was allowed between each sequence of administrations.

Sample collection

Nasal swabs were collected with 16-inch (40 cm) cotton nasal swabs (Hardwood Products Company LP, Maine, USA) inserted into the ventral nasal meatus of the horse up to approximately 15 cm. For each horse, a pre-dose nasal swab sample was taken, and then the first swab was taken at a specific time point for each horse, followed by a second swab at the next collection time. Serum samples were collected into serum separator tubes, (Vacutainer, Becton Dickinson, New Jersey, USA) following standard post-race testing practice in Kentucky. During the first day, complete urine collection was accomplished with a Foley catheter and later a Harris flush tube (24 Fr x 152.4 cm; Seamless, Florida, USA) was used to collect urine samples. Urine samples were stored at -20°C until assayed.

General ELISA methods

ELISA tests were performed using the Neogen Bronchodilator Group ELISA kits (Neogen Corporation, Kentucky, USA). ELISA buffer, wash buffer, specific terbutaline-horseradish peroxidase (drug-enzyme) conjugate solution and KY blue substrate were obtained as part of the ELISA kits.

Preparation of standard solutions

Authentic albuterol (salbutamol) (Sigma Chem. Co., Missouri, USA) standard was prepared at 1mg/ml methanol and diluted to appropriate concentrations in assay buffer (0.1 M potassium phosphate-buffered saline pH 7.4 with 0.1% bovine serum albumin).

ELISA procedure

The one-step ELISA tests were performed as described by Stanley *et al.* (1993) and according to the manufacturers' instructions, with albuterol standard curves in assay buffer, blank serum matrix or blank urine matrix. At the time of analysis, 5 ml assay buffer was added to each nasal swab sample in its vial with extraction by vigorous mixing by vortex. The nasal swab samples were assayed using a buffer standard curve. The assay was started by adding 20 µl of standard, test or control samples to the appropriate wells in duplicate, followed by 180 µl of diluted (1:180) drug-enzyme conjugate.

To create the serum or urine matrix (blank serum or urine, pre-dose from each horse) standard curves, the assay was started by adding 20 µl of standard, test or control samples to the appropriate wells in duplicate. For the wells containing the samples, 180 µl of diluted (1:180) drug-enzyme conjugate was added to each well. For the wells containing the standards in buffer, 20 µl blank serum or urine and 160 µl of diluted (1:160) drug-enzyme conjugate was added. The plates were then placed on a microplate shaker

TABLE 1: Mean optical density (OD) of albuterol (~~sse~~) by ELISA from horse urine after administration with 3M Equine Inhalation device

Time (h)	Mean optical density ~~sse~~ (1 puff)	Mean optical density ~~sse~~ (3 puffs)	Mean optical density ~~sse~~ (6 puffs)
0	102.45 ± 3.58	99.98 ± 0.023	103.83 ± 2.17
1	76.09 ± 8.52	76.70 ± 4.61	52.20 ± 9.02
2	55.24 ± 10.31	41.65 ± 8.71	33.23 ± 5.46
4	55.10 ± 8.36	43.56 ± 7.91	35.85 ± 5.06
6	57.96 ± 8.15	47.99 ± 7.22	39.96 ± 5.85
8	63.79 ± 11.50	51.93 ± 7.33	44.24 ± 7.12
24	92.69 ± 10.08	84.15 ± 4.08	109.92 ± 8.42
48	98.16 ± 9.49	94.59 ± 2.75	104.19 ± 4.77

Doses: one puff, 3 puffs and 6 puffs

TABLE 2: Mean urinary concentrations of albuterol equivalents by ELISA from horses dosed with 3M Equine Inhalation device

Time (h)	Mean urinary concentration ~~sse~~ (one puff) (ng/ml)	Mean urinary concentration ~~sse~~ (3 puffs) (ng/ml)	Mean urinary concentration ~~sse~~ (6 puffs) (ng/ml)
0	0	0	0
1	0.21 ± 0.0948	0.34 ± 0.195	3.81 ± 1.46
2	1.02 ± 0.285	4.89 ± 1.459	10.15 ± 3.86
4	0.96 ± 0.358	4.28 ± 1.679	7.14 ± 1.597
6	0.88 ± 0.469	3.12 ± 1.218	7.30 ± 1.45
8	0.80 ± 0.492	2.68 ± 1.245	6.22 ± 1.68
24	0.084 ± 0.0497	0.18 ± 0.136	0.006 ± 0.0049
48	0.056 ± 0.0511	0.035 ± 0.03	0.042 ± 0.038

Doses: 1 puff, 3 puffs and 6 puffs. There was a good correlation of the mean peak urinary concentrations and doses of albuterol (r^2: 0.999)

briefly, covered and incubated at room temperature for 45 min.

After the incubation was completed, the wells were inverted and any remaining liquid was removed by tapping the plate on a lint-free towel. Then each well was washed with 300 ~~ml~~ of diluted wash buffer 3 times. The plates were inverted and tapped dry between each washing and 150 µl of KY blue substrate was added to each well, and the plates were mixed by shaking them gently. The plates were placed on a microplate shaker for 30 minutes. The optical density (OD) of each well was read at a wavelength of 650 nm with an automated microplate reader (EL 310, Microplate Autoreader, Bio-Tech. Inc., Vermont, USA). Samples with OD less than that of the highest standard were diluted appropriately with assay buffer and re-assayed.

Estimated albuterol equivalents concentration were calculated for the serum samples using the serum matrix standard curve, for the urine samples using the urine matrix standard curve, and for the nasal swab samples using the buffer standard curve. The data were plotted as % maximum activity (OD at 650 nm) versus time post-dose, or as albuterol equivalent concentration (ng/ml) versus time post-dose.

Results

The Neogen Bronchodilator Group ELISA kit readily detects various ß-2 adrenoreceptor agonists and antagonist with varying sensitivities depending on the sample matrix and the compound being tested. In the current study, tests for albuterol and/or or its metabolites were performed using the Neogen Bronchodilator Group ELISA on equine serum, urine and nasal swab samples following administrations of albuterol by Torpex. Because ELISA testing does not specifically identify albuterol, its metabolites or structurally related substances, all estimated ELISA quantifications, are identified as albuterol equivalents.

Analysis of the post-administration serum samples showed relatively little inhibition of ELISA response in any of the samples tested. For all time points, the % maximum activity post-administration averaged less than 25% inhibition, and was thus not considered substantially different from the 'background' value for the pre-dose sample (data not shown). These results suggest that it is very unlikely that screening of serum samples by ELISA testing as described herein will lead to chemical

TABLE 3: Mean urinary optical density (OD) of albuterol by ELISA from horses (n=6) dosed with 3M equine inhalation device

Time during administration (days)	Mean optical density ± se (6 puffs/dose, 4 times/day)
0	108.24 ± 9.22
1	24.89 ± 1.402
2	27.69 ± 5.044
3	23.12 ± 4.033
4	23.99 ± 2.213
5	23.40 ± 4.084
Time post-last dose administration (h)	
1	22.52 ± 4.13
2	19.27 ± 2.71
4	21.43 ± 3.46
8	57.68 ± 19.92
24	110.98 ± 14.34
48	139.02 ± 9.66
72	118.09 ± 16.3062
96	121.15 ± 24.12
120	105.75 ± 10.96
144	132.22 ± 17.12
192	101.83 ± 11.75

Dose: 6 puffs/dose 4 times a day for 5 days. The post administration 'washout' of albuterol starts at Day 5

TABLE 4: Mean urinary concentrations of albuterol equivalents (± se) from horses (n=6) dosed with 3M Equine Inhalation device

Time during administration (days)	Mean urinary concentrations ± se (6 puffs/dose, 4 times/day) (ng/ml)
0	0
1	12.21 ± 5.79
2	17.43 ± 6.87
3	14.60 ± 3.50
4	15.25 ± 4.73
5	17.13 ± 5.33
Time post-last dose administration (h)	
1	29.47 ± 10.89
2	35.63 ± 10.47
4	29.42 ± 11.06
8	10.95 ± 5.17
24	0
48	0
72	0
96	0
120	0
144	0
192	0

Dose: 6 puffs/dose 4 times a day for 5 days. The post administration 'washout' of albuterol starts at Day 5

identifications of albuterol in equine serum samples following administration by Torpex at the reported doses.

Table 1 shows the inhibition of ELISA activity by urine samples following 1, 3 and 6 puffs of Torpex. Review of these data shows that at least 24 h should be allowed after intranasal administration of 6 or fewer puffs for the urine to 'clear' albuterol. Conservatively, however, it would be prudent to allow at least an extra 24 h for a total of 48 h for albuterol and or its metabolites to clear after such administrations.

In these post-administration urine samples the apparent peak concentrations of albuterol equivalents were dose-related (r^2=0.999). The mean peak urinary concentrations of albuterol equivalents occurred within 2–4 h post-administration, and were 1.02, 4.89 and 10.15 ng/ml at the 1, 3 and 6 puff doses, respectively (Table 2). The mean peak urinary concentrations of albuterol equivalents were 0.8, 2.68 and 6.22 ng/ml at 8 h post-dose following administrations at the 1, 3 and 6 puffs doses, respectively, and the urine samples did not contain ELISA detectable levels of albuterol equivalents at 48 h post-administration (Table 2). The examination of the percent inhibition of the ELISA test data in nasal swab samples following increasing doses of albuterol by Torpex suggests that nasal swabs collected up to at least 6 h post-dose can give a 'positive' ELISA response after 3 and 6 puffs (data not shown).

Table 3 shows the urinary inhibition of the ELISA tests following administration of 6 puffs/dose 4 times a day for 5 days. Table 4 shows mean urinary concentrations of albuterol equivalents following 6 puffs/dose 4 times a day for 5 days. The mean peak urinary concentrations of albuterol equivalents ranged from 15–17.5 ng/ml during these 5 days and rapidly declined after the last administration with concentrations of albuterol equivalents being 10.95 ng/ml at 8 h post-dose (Table 4). During the dosing and for at least 8 h after the last dose, all urine samples gave strong positive indications of the presence of albuterol. None of the urine samples had detectable concentrations of albuterol equivalents at 24 h after the last dose. Conservatively, however, it would be prudent to allow at least an extra 48 h for a total of 72 h for albuterol and or its metabolites to clear after such a schedule of administration.

Table 5 shows the ELISA inhibition produced by nasal swabs following 6 puffs/dose 4 times a day for 5 days of albuterol with Torpex. The data suggest that nasal swabs collected up to at least 24 h after the last dose may give rise to detectable levels of albuterol equivalents by ELISA. The apparent peak concentrations of albuterol equivalents in these nasal swab samples ranged

TABLE 5: Mean optical density (OD) of albuterol by ELISA from nasal swab samples of horses (n=5) dosed with 3M Equine inhalation device

Time post-last dose administration (h)	Mean optical density ± SE (6 puffs/dose, 4 times/day)
0	9.73 ± 1.32
1	12.15
2	11.76
4	17.28 ± 9.43
8	18.70
24	50.34 ± 7.44
48	66.33 ± 9.97
72	59.84 ± 7.63
96	59.63 ± 5.38
120	59.00 ± 2.93
144	68.49 ± 7.35
192	71.59 ± 13.67

Dose: 6 puffs/dose 4 times a day for 5 days. Only single horse nasal swab sample was analysed where SEM is not being reported in the table. The post administration 'washout' of albuterol starts at 0 h following last dose administration

from 11–90 ng/ml. The mean estimated concentrations of albuterol equivalents from these nasal swab samples were 40 and 0.13 ng/ml at Day 5 treatment and 120 h post initial-dose, respectively (data not shown).

Discussion

Albuterol, an ARCI class 3 agent, is marketed for application by the 3M Equine Inhaler (Torpex) (3M and Boehringer Ingelheim Vetmedica, Inc., Missouri, USA). Each actuation (puff) of the Torpex device delivers 120 µg of albuterol sulphate. As a bronchodilator, albuterol has the potential to alter the athletic performance of horses (Bailey *et al.* 1999), particularly if the animal experiences bronchospasm and bronchoconstriction associated with reversible airway (Derksen *et al.* 1999, Sasse and Hajer 1978). Because albuterol is an ARCI class 3 agent, its detection in post-performance samples may lead to sanction against the trainer or other connections of the horse.

The advantage of Torpex is that the system directly delivers active compound deep into the lung resulting in rapid onset of therapeutic action (5–15 min). Administration by this route also bypasses the gastrointestinal tract and liver metabolism, either of which can significantly reduce and/or alter the bioavailability of an orally administered drug. Administration of albuterol by Torpex, which yields direct, synchronised delivery of albuterol into the respiratory tract, significantly reduces the dose required for therapeutic efficacy of this compound. These characteristics of Torpex provide higher therapeutic efficacy and a presumably lower incidence of side effects. A further advantage is that, in the case of competition horses, administration by Torpex can also serve to reduce the probability of an inadvertent medication identification.

The goal of this study was to generate an ELISA database to provide guidelines for veterinarians and regulators regarding the period after administration of albuterol by Torpex in which residues of albuterol may be expected to be detected in biological fluids of the horse. Regulatory control of albuterol generally involves ELISA screening of post-race urine samples, followed by mass spectral confirmation of its presence. In this study, albuterol ELISA tests were performed on serum, urine and nasal swab samples following administration of albuterol by Torpex.

With regard to the detection of albuterol in serum, the ELISA test showed very little response in any of the samples tested. In the absence of unusual extraction procedures, this ELISA test is unlikely to detect albuterol in serum samples after administration of these doses by Torpex. On the other hand, albuterol equivalents were readily detected in urine samples with the mean peak urinary concentrations of albuterol equivalents occurring within 2–4 h post-administration, and were 1.02, 4.89 and 10.15 ng/ml at the 1, 3 and 6 puff doses, respectively. The majority of the urine samples did not contain ELISA detectable concentrations of albuterol equivalents at 24 h post-administration. To be conservative, however, it would be prudent to allow at least an extra 24 h for a total of 48 h for albuterol and or its metabolites to clear after such administrations.

ELISA analysis of nasal swab material showed that peak concentrations of albuterol equivalents were obtained within 1–2 h post-administration and were 6.76, 34 and 210 ng/ml following 1, 3 and 6 puff administrations. The data suggest that nasal swabs collected up to 6 h after the 3 and 6 puff administrations may yield a 'positive' ELISA response.

The maximal recommended duration of Torpex albuterol administration is 6 puffs per dose, 4 times per day for 5 days. Following this dose the mean peak urinary concentrations of albuterol equivalents ranged from 15–17.5 ng/ml. Once dosing ceased, the mean urinary concentrations of albuterol declined rapidly, and none of the urine samples had a detectable level of albuterol equivalents at 24 h after the last dose. However, in view of the larger dose and longer duration of administration, it would be prudent to allow an extra 48 h, for a total of 72 h, for albuterol and or its metabolites to clear after such administrations.

The mean concentrations of albuterol equivalents from the nasal swab samples were 40 and 0.13 ng/ml at Day 5 of treatment and 120 h post-initial-dose. The data suggest that any nasal swab collected up to 24 h or longer after the last dose administered may have the potential to give a detectable level of albuterol equivalents by ELISA.

It must be kept in mind that these results provide guidelines only. They are based on the sensitivity of the ELISA test used and modification of this test or its interpretation to increase its sensitivity, may increase the probability of a detection triggering event. Beyond this, factors specific to individual horses may also influence these 'detection times'. As always, prior to administration of any medication to a horse about to enter a medication or drug tested event, it is advisable to consult the veterinarian(s) and/or other experts concerning any unique regulatory or testing characteristics related to the horse or the event in question.

Acknowledgements

Publication No. 297 from the Equine Pharmacology and Experimental Therapeutics Program at The Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky.

Published as Kentucky Agricultural Experiment Station Article No. with approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station. Supported by Boehringer Ingelheim Vetmedica Inc.

This research was supported by grants from 3M and Boehringer Ingelheim Vetmedica, Inc., Missouri and also by National, Florida, Kentucky, Nebraska, Arkansas, Pennsylvania, Ohio and Canadian Horsemen's Benevolent and Protective Associations.

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