Plasma and urinary concentrations of trimetoquinol by LC-MS-MS following intravenous and intra-tracheal administration to horses with heaves

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Trimetoquinol (TMQ) is a very potent and fast acting bronchodilator in horses with heaves. This study assessed the plasma and urinary concentrations of TMQ in horses with heaves following administration via the intravenous (IV, 0.2 μg/kg) and intra-tracheal (IT, 2 μg/kg) routes. TMQ was administered to six horses affected with heaves (RAO – Recurrent Airway Obstruction, used interchangeably) by the above routes and plasma and urine samples collected and stored at −20 °C until analyzed. Solid Phase Extraction (SPE) of TMQ was followed by highly sensitive ESI(+) - LC-MS-MS (ElectroSpray Ionization, positive mode – Liquid Chromatography – Mass Spectrometry – Mass Spectrometry); with a Limit of Detection (LOD) estimated at 1 pg/mL. Following IV administration, TMQ plasma levels peaked at 1 min at 707 pg/mL, and at 9 min following IT administration. Our results show that TMQ plasma concentrations decline rapidly following IV administration, which is consistent with the fast onset and short duration of TMQ effect that was observed in our previous studies. On the other hand, IT administration showed a very unique plasma concentration pattern. From a regulatory standpoint, the current available TMQ ELISA kit was also used in an attempt to detect TMQ from the plasma and urine samples. We report that the ELISA kit was unable to detect TMQ from any of the samples generated in these studies.

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INTRODUCTION

Trimetoquinol [TMQ, Inolin® Tanabe Seiyaku Co., Ltd. (Chuo-ku, Osaka, Japan)](1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydrossoquinoline, m.w. 345) is a non-selective (Buckner & Abel, 1974; Feller et al., 1975) β-adrenoceptor agonist (Iwasawa and Kiyomoto 1967). We have been investigating its therapeutic efficacy as a bronchodilator in horses and have reported that when administered intravenously, TMQ shows both β1- and β2-adrenoceptor stimulatory activities (Camargo et al., 2006, 2007).

More recently, we reported the bronchodilator effects of TMQ following its intravenous (IV), intra-tracheal (IT), oral (PO) and aerosolized (AER) administrations (Camargo et al., 2006, 2007) to horses with heaves. In summary, our results showed that by the IV and IT routes, TMQ is an extremely potent cardiac stimulant, but a short-lived bronchodilator that produces undesirable side effects that are typical of the responses elicited by epinephrine and related sympathomimetics (Hoffman 2001). From a regulatory standpoint, TMQ produces maximal cardio-respiratory effects after administration of a very small dose (0.2 μg/kg). This in turn will produce very low plasma (or serum) and urinary concentrations that may be difficult to detect using the currently available ELISA screening test. We tested all the urine and plasma samples generated from this work and also from previous pilot experiments using the ELISA test kit to assess if these samples would produce a positive ELISA result. We also now report a new extraction and analytical method to detect and...
quantify the plasma and urinary concentrations of TMQ by LC-MS-MS following administration by the IV and IT routes.

MATERIALS AND METHODS

Horses
Six horses with a history of recurrent airway obstruction (RAO) were used in this present study; these horses included five mares and one gelding of mixed breeds, 17–29 years of age, 483–570 kg. Horses were maintained on pasture and their diet supplemented with pelleted alfalfa feed as necessary. While on pasture, these animals presented no clinical signs of airway obstruction. When brought indoors, housed in stalls, bedded on straw, and fed hay, all the horses developed the characteristic clinical signs of heaves. Animals used in these studies were managed according to the rules and regulations of the All University Committee on Animal Use and Care of Michigan State University, which also approved the experimental protocol.

TMQ and papaverine acquisition
Trimetoquinol was a gift of Tanabe Seiyaku Co. Ltd. Tanabe Seiyaku Co. Ltd. (Chuo-ku, Osaka, Japan), with a certificate of analysis indicating an estimated purity of 100 ± 0.7% (±SD). The purity was confirmed by in-house GC/MS evaluation of a BSTFA + 1% TMCS Pierce Biotechnology, Inc., Rockford, IL, USA derivatized sample. Papaverine, used as an internal standard, was obtained from Sigma (Sigma-Aldrich Co., St Louis, MO, USA).

TMQ administration and sample collection
The TMQ dose used for each route of administration was selected based on the pharmacodynamic responses to TMQ in the horse, which have been reported previously (Camargo et al., 2006).

Intravenous administration
A dose of 0.2 µg/kg intravenous (IV) [~ 100 µg/horse] was administered to each of six horses in the right jugular vein. Blood samples were collected using green-top Heparin Vacutainer Becton Dickson, Franklin Lakes, NJ. USA tubes (16 × 100 mm, 10 mL) at times 0 (before drug administration), and at 1, 3, 6, 10, 15, 20, 30, 45 min, and 1, 2, 4 and 6 h after administration from the left jugular vein. After collection, blood samples were centrifuged and plasma transferred to 13 × 100 mm glass tubes, capped, and stored at −20 °C until analyzed. The plasma samples of two horses (one female and the gelding) were excluded from these experiments due to unsuitability for analysis. We are, therefore, reporting results of plasma analyses of four female horses. Urine samples were collected from the four female horses via a Foley catheter at times 0 (before drug administration) and at 1, 2, 4, 6 and 24 h post-administration. Urine samples were stored at −20 °C in 100 mL plastic bottles until analyzed.

Intra-tracheal administration
A dose of 2 µg/kg Intra-tracheal (IT) [~ 1 mg/horse] was administered to each of six horses (five females and one gelding). Plasma samples were collected at times 0 (before drug administration), and at 3, 6, 9, 12, 16, 20, 25, 30, 45 min, and 1, 2, 4 and 6 h post-administration from the left jugular vein. Urine samples were collected from the five female horses at the same time points described above for the IV protocol. Urine samples were not collected from the gelding to avoid complications due to catheterization.

ANALYTICAL PROCEDURES

Sample Preparation and Solid Phase Extraction (SPE):

Calibration curves
Calibration curves were generated by spiking increasing amounts of stock solutions of TMQ into 2 mL of blank urine or plasma, according to the sample matrix tested, with duplicate final concentrations of 0.01, 0.1, 1, 10, 50, 100, 300 and 600 ng/mL. A fixed amount, 20 ng of internal standard, papaverine, was added to each sample, so that the final concentration for the internal standard was 10 ng/mL. These samples were then submitted to the same protocol described below for the test samples. The r² for any given calibration curve was always 0.99 (Fig. 1).

Urine samples
Urine samples were hydrolyzed since TMQ is excreted in urine principally in the form of TMQ-glucuronide (Camargo et al., 2004). For beta-glucuronidase hydrolysis, 2 mL thawed urine samples were treated for 3 h at 65 °C with Patella vulgata beta-glucuronidase (1000 units of Sigma Sigma-Aldrich Co., St Louis, MO, USA Type L-II per ml of urine brought to pH 5 with 1.5 mL 2 M sodium acetate buffer) as previously described (Camargo et al., 2004). The resultant hydrolyzates were brought to pH 6.5 with sodium hydroxide, sonicated, and subjected to SPE.

Plasma
Plasma samples were not hydrolyzed. Two ml of each plasma sample were brought to pH 6.5 with 2 mL 0.25 m ammonium acetate buffer, centrifuged at 3577 g for 15 min and then submitted to SPE.

Solid phase extraction
Solid phase extraction was performed on a battery of 8 Zymark RapidTrace Zymark, Hopkinton, MA, USA SPE Workstation extraction modules.

For SPE of urine, Strata-X-CW Phenomenex, Inc., Torrance, CA, USA cartridges (200 mg, 3 mL) were conditioned by adding sequentially 3 mL of methanol and 3 mL of 2 M sodium acetate buffer (pH 6.5). Samples were slowly loaded, after which the cartridges were washed sequentially three times with 3 mL of a solution of methanol and sodium acetate buffer (50:50, pH 6.5).
Each cartridge was next eluted two times with 4.5 mL of methanol/acetonitrile/formic acid (49:49:2) into glass tubes. Each eluent was evaporated to dryness under a stream of nitrogen in a 55 °C water bath in a Zymark TurboVap LV evaporator Zymark, Hopkinton, MA, USA. Residues were then dissolved directly in 100 μL of mobile phase A, which consisted of deionized water, 5% acetonitrile and 0.03% formic acid, then transferred to a microinjection vial and sealed. For sample analysis 10 μL aliquots were injected into the Varian LC-MS-MS Varian Inc., Palo Alto, CA, USA.

For SPE of plasma samples, Strata-X-CW Phenomenex, Inc., Torrance, CA, USA cartridges (200 mg, 3 mL) were conditioned by adding sequentially 3 mL of methanol, a second time 3 mL of methanol and then 3 mL of ammonium acetate buffer (0.25 M, pH 6.5). Samples were slowly loaded, and then the cartridges were washed with 3 mL of water and subsequently with 3 mL of a solution of methanol and ammonium acetate buffer (70:30, pH 6.5). The samples were then submitted to the same elution, evaporation and reconstitution protocol as described above for urine samples.

**MS-MS tuning and HPLC parameters**

The mass spectrometer parameters were optimized and set as follows, while directly infusing 100 μg/mL of TMQ dissolved into ACN:H2O (50:50) and 0.03% formic acid. The mass spectrometer was operated in positive ion mode with the ESI (Electrospray Ionization) chamber installed. Nitrogen was used as the drying and nebulizing gas. The drying gas temperature was set at 300 °C at 20 psi, and the nebulizing gas at 50 psi. The needle voltage was set at 5000 V, and the shield at 600 V. The capillary voltage was set at +30 V. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the collision cell by optimizing settings as needed for the second quadrupole. Collision gas was set at 2 mTorr. The electron multiplier was set at 1700 V. The collision energy was set at −18 V after 5 min (time allowed for elution of TMQ) and −18 V after 5 min (elution of papaverine) (Fig. 1).

The Varian LC-MS-MS Varian Inc., Palo Alto, CA, USA was equipped with a Phenomenex 4 mm × 2 mm phenylpropyl guard column and Phenomenex Luna phenyl-hexyl column (30 mm × 1 mm with 3 μ particle size). Gradient chromatography with the LC-ESI(+)−MS-MS was performed as in Table 1.

**Table 1.** LC-MS-MS gradient. Flow was set at 0.15 mL/min throughout

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<td>7:00</td>
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A: Water: 5% ACN: 0.03% Formic Acid.
B: ACN: 0.03% Formic Acid.

**TMQ detection by LC-MS-MS**

In Mobile Phase A, TMQ readily produced an m/z 346 [M+H]+ protonated pseudomolecular ion. This species produced one prominent daughter ion at m/z 164 on collision-dissociation in the tandem quadrupole instrument (Camargo et al., 2004). Production of the m/z 164 daughter ion allowed ready detection of TMQ using MRM (multiple reaction monitoring) detection methodology.

The limit of detection (LOD) of this method, based on the signal-to-noise ratio with a minimum value of 3, is estimated, by instrumental analysis of non-extracted standards of decreasing number of pg injected, at least 10-fold below the lowest calibrator (10 pg/mL matrix, 2 pg on column), so the estimated LOD is in the area of 1 pg/mL of matrix. The lowest calibrator was taken as the LOQ, and values calculated below this value were assigned a value of zero.

**TMQ detection by ELISA**

Testing Components Corp. TMQ ELISA kits TCC, Collinsville, IL were utilized to detect and quantify TMQ equivalents in the urine and plasma samples, as a means to determine if these available ELISA test kits would be able to detect TMQ adequately. The ELISA kit consisted of ELISA standards (10–10 000 ng/mL TMQ), pre-coated microtiter plates, antibody # 1, antibody # 2, assay buffer (PBS buffer, phosphate buffered saline), wash solution (20X concentrate), and substrate. The assay was performed as specified according to the manufacturer’s instructions which are summarized as follows: urine samples were diluted 1:5 in PBS buffer. Fifty (50) μL of each standard or sample solution were added into the appropriate wells, in duplicate. Antibody # 1 (100 μL) was added to each well, followed by gentle mixing and incubation for 30 min at 37 °C. The plate was then washed three times with diluted wash solution and patted dry. Freshly diluted antibody # 2 (150 μL) was added to each well with gentle mixing. The plate was incubated for 30 min at 37 °C. The plate was again washed three times and patted dry. The substrate (150 μL) was carefully added to each well to prevent the formation of bubbles, and the plate was incubated at room temperature for 15 min. The optical density was read at 650 nm with a Benchmark Plus ELISA microplate reader Bio-Rad Laboratories, Hercules California.

Standard curve for ELISA test was generated by spiking known amounts of TMQ in duplicate and averaging the results. This process was repeated five different times, and five curves were generated.

All post-TMQ administration urine samples collected after the following administrations were analyzed by ELISA: 4 mg IV, 100 mg PO, 0.2 μg/kg IV, 2 μg/kg IT, 60 μg/kg PO and 1 mg aerosolized TMQ (Camargo et al., 2004, 2006, 2007).

**Experimental design**

At the time of TMQ administrations, at which time the pharmacodynamics were assessed, the drug administrations were designed...
as a randomized crossover experiment with washout period of at least 2 weeks between treatments (Camargo et al., 2006).

Statistical analysis

Results for TMQ plasma and urine concentrations are reported as median and range.

RESULTS

Plasma and urinary TMQ concentrations

Intravenous administration

Plasma concentrations Following IV administration, TMQ plasma concentrations had the highest concentration at 1 min at 707 pg/mL (446–880) and declined thereafter. At 6 h post-administration, the median TMQ concentration in the samples was 10 pg/mL (0–26). Detailed results for plasma concentrations are shown in Table 2. Individual results of plasma TMQ concentrations for each horse and the median result after IV administration are presented in Fig. 2.

Urinary concentrations Following IV TMQ administration, all horses showed peak urinary TMQ concentration at the 1 h post-administration collection. The median urinary concentration of TMQ at 1 h post-administration was 51.76 ng/mL (20.93–100.49), at 2 h the concentration was 15.73 ng/mL (5.23–29.62). By 6 h post-administration all horses had TMQ urine concentrations below 2 ng/mL. Detailed results of urinary TMQ concentrations following IV administration are presented on Table 3 and Fig. 3.

Intra-tracheal administration

Plasma concentrations Following IT administration, the peak median TMQ plasma concentration was observed at 9 min post-administration, with median concentration of 305.5 pg/mL (40–549), and declining thereafter. At 6 h post-administration, the median plasma TMQ concentration was 10 pg/mL (0–48). Detailed results for plasma concentrations are shown in Table 2. Individual horse results and median results for TMQ plasma concentration after IT administration are presented in Fig. 4.

Urinary concentrations Following IT administration of TMQ, the peak urinary TMQ concentration was observed at 2 h, with median concentration of 112.44 ng/mL (76.24–696.42). One horse had peak urinary concentrations at 1 h (119.66 ng/mL). At 6 h post-administration, TMQ urinary concentration was 12.56 ng/mL (8.46–27.17). The urinary concentrations of TMQ observed following IT administration are depicted in Table 3 and Fig. 3.

Post-administration detection of TMQ in urine by ELISA

The commercially available ELISA test for TMQ had an I-50, or detection sensitivity, of around 1000 ng/mL or 1 000 000 pg/mL, as presented in Fig. 5. We ELISA screened
50 different post-TMQ administration urine samples from horses administered doses of TMQ ranging from 100 µg to 100 mg per horse, a 1000-fold dose range. Only the samples from the horses administered 100 mg PO tested positive by this ELISA test, and all the other samples tested negative by the ELISA test kit (Fig. 5).

**DISCUSSION**

In previous work we demonstrated that Trimetoquinol is an exceptionally potent and rapidly acting cardiac stimulant and bronchodilator, although its actions on these systems are short-lived (Camargo et al., 2006). We now report the plasma and urinary concentrations of TMQ following IV and IT administrations and the new analytical method and results reported here are of importance for the regulatory control of TMQ.

One very interesting finding was that the highest plasma concentrations of TMQ observed were those following IV administration, despite the fact that the dose administered intravenously was 10-fold lower than the intra-tracheal dose. On
the other hand, by comparing the IV and IT administrations, it is clear that the elimination phase by the IT route was considerably slower than that following administration by the IV route, which shows that by the IT route, TMQ is more slowly absorbed, distributed and eliminated.

An important pharmacodynamic characteristic of TMQ is that it is an exceptionally potent agent; as such, the administered doses were very small, in the order of 100 micrograms per horse. In a pilot study (unpublished data), six healthy horses (not affected with RAO) received TMQ IV (2, 0.2, and 0.02 l g⁄kg), epinephrine IV (20, 2 and 0.2 l g⁄kg), and norepinephrine IV (20, 2 and 0.2 l g⁄kg), and heart rate was recorded. When the data were analyzed, we found that the response elicited by intravenous TMQ was stronger (higher HR) and longer lasting than that following administration by the IV route, which shows that by the IT route, TMQ is more slowly absorbed, distributed and eliminated.

As such, the very small doses of TMQ administered may be expected to yield very low blood levels of TMQ. Consequently, development of a highly sensitive analytical method with the ability to detect the extremely low (part per trillion) resultant blood levels of TMQ was both an absolute requirement for carrying out the study on the drug and also a considerable analytical challenge.

The analytical method reported here is one of the most sensitive analytical methods currently available. The recovery step, Solid Phase Extraction (SPE) method, utilizes cation exchange on a polymeric sorbent, and an acidic elution solvent. In this recovery method, TMQ attaches to the polymeric sorbent, which is then neutralized by the acidic elution (pH below 2) solvent, thereby releasing the adsorbed TMQ. This method

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Fig. 4. Plasma TMQ concentration following IT administration of 2 l g⁄kg. (a) Results for individual horses, linear vertical axis; (b) median results, vertical axis in log scale. Also note that TMQ concentration is reported in picogram⁄mL.

Fig. 5. TMQ ELISA standard curve (a). Note that the IC-50 (Inhibition Concentration 50%), or sensitivity of this ELISA kit was around 1000 ng⁄mL. Results presented are the mean for five standard curves and the standard deviation. ELISA detection and quantitation of TMQ (b). The TMQ ELISA test did not detect TMQ from the urine samples collected for our experimental design, i.e. IV, IT, PO and aerosolized administration. Therefore we used the urine of two horses which had been administered 4 mg IV and 100 mg PO in a pilot experiment previous to our now reported experiments. As can be noted, the ELISA test was able to detect TMQ from the horse administered 100 mg PO yet was unable to detect any TMQ from the horse administered 4 mg IV. The dose of 4 mg IV is about 40 times higher than the final dose administered IV in our reported experiments, which was about 100 µg⁄horse, dose which conferred maximum pharmacological effects.
provided recoveries between 70 and 100%, which are very satisfactory at the exceptionally low analyte concentrations involved in these TMQ studies. When compared to other reported methods of extraction and quantitation (Suzuki et al., 1982; Brode et al., 1986), this present method is more sensitive and more efficient, as follows. Both these groups reported sensitive methods of detection of TMQ, with calibrators ranging from 2–50 ng/mL and 0.05–5 ng/mL, and calculated LODs of 100 pg/mL, and 40 pg/mL, respectively. While both methods described are sensitive, they were utilized in studies involving considerably higher doses of TMQ, doses of 6.7 mg/kg and 10 mg/kg respectively, which are up to 100 000-fold higher than the doses utilized in our experiments. The dosage administered plays a very important role in method development, and this is because it correlates with the plasma concentration of the drug. Although extraction methods enable us to concentrate the analyte and thus improve detection, if one already starts with an extremely low administration the number of drug molecules distributed in the plasma will evidently be also low, and thus more challenging for extraction and detection. Also, both groups reported methods of extraction that used 5 mL of plasma, while we extracted 2 mL of plasma. And finally, one group reported a 40% extraction efficiency, the other group reported 70–75%, while we now report a 70–100% extraction efficiency at substantially lower analyte concentrations.

In summary, therefore, we have developed a highly sensitive analytical method, which allowed quantitation of TMQ down to our lowest calibrator concentration of 10 pg/mL. The LOD of this method was estimated in the order of 1 pg/mL. When administered intravenously at a dose of 0.2 µg/kg (approximately 100 µg/horse), plasma levels of TMQ peaked at around 700 pg/mL (range: 446–880 pg/mL), but rapidly declined to on the order of 15 pg/mL by 30 min post-administration. This decline was then followed by a secondary increase in plasma concentration to about 30 pg/mL at about 1 h post-administration. Thereafter, TMQ plasma concentrations declined slowly, but remained detectable at about 10 pg/mL until the sixth hour post-administration (Fig. 2 and Table 2). Likewise, urinary concentrations of TMQ peaked at 1 hr post-administration, with 100 ng/mL being the highest individual urinary concentration observed (range: 23–100 ng/mL), and declined thereafter. At 24 h post-administration, TMQ was still readily detectable in urine at concentrations of about 50 pg/mL (Fig. 3 and Table 3).

When administered by the IT route at a dose of 2 µg/kg (approximately 1 mg/horse), plasma concentrations of TMQ
peaked at about 250 pg/mL (range: 50–1500 pg/mL) and declined thereafter, reaching about 10 pg/mL by 6 h post-administration. As can be seen in Fig. 4, the decline in TMQ plasma concentrations was more gradual post-IT administration, in contrast with the sharp decline post-IV administration (c.f. Fig. 2). It can also be noted in Fig. 4 and Table 2 that TMQ plasma levels peaked within the first 20 min post-administration, then declined, but thereafter rose again in some horses. One suggested interpretation is that these observations could reflect different amounts of mucus accumulation (horses with heaves vary in the amount of tracheal mucus accumulation) in each individual horse’s trachea and upper respiratory system, to which the administered drug would attach to and be slowly released to yield a secondary absorption peak. The mucus may be a barrier between the administered drug and the tracheal mucosa, which may impede the drug from effectively having direct contact with the mucosa in a steady fashion.

Although the IT administration dose, at about 1 mg per horse, was 10-fold higher than the IV dose, the highest plasma concentration after IT administration was about 1500 pg/mL, or only about twice the 880 pg/mL observed after IV administration (Table 2). One interpretation for this observation is that TMQ is rapidly metabolized and biotransformed after it is absorbed through the tracheal mucosa, thereby reducing the peak plasma concentration observed after IT administration. In that regard, review of the urinary TMQ concentration data confirmed such observations, with individual horses’ peak urinary concentrations observed on the order of 100 ng/mL post-IV administration and around 700 ng/mL post-IT administration, a 7-fold difference (Table 3 and Fig. 3), which more closely corresponds to the 10-fold increase in the dose.

In reviewing the IV and IT administrations’ pharmacological effects (Camargo et al., 2006) with the goal of relating pharmacological effects to plasma TMQ concentration, the IV administration of TMQ was associated with an extremely rapid increase in HR, peaking at 160 bpm at 1 min post-administration (Fig. 7, Camargo et al., 2006). This HR response declined to baseline by 2 h post-administration. In the respiratory system, this dose of TMQ produced an essentially immediate and highly significant bronchodilation (measured as a significant decrease in ΔPplmax, or maximal change in pleural pressure), which had returned to pretreatment values by 30 min post-administration. However, an unexpected secondary phase of bronchodilation was seen, commencing at later than 1 h post-administration, and remaining statistically significant for the remainder of the six-hour experimental period (Camargo et al., 2006).

The IT administration route was associated with relatively delayed cardiac and bronchodilatory responses, with peak responses in both systems occurring at 9 min post-administration, as compared with 1 and 3 min post-IV administration (Fig. 8). The HR increase had returned to baseline levels by 4 h post-administration and the respiratory response returned to pretreatment values by 45 min post-administration. However, there was a secondary bronchodilation post-IT treatment commencing at later than 2 h post-administration, and remaining statistically significant for the remainder of the 6-h experimental period (Camargo et al., 2006).

One of the objectives of analyzing plasma concentrations of TMQ was to determine whether the secondary phase of bronchodilation correlated with parallel secondary increases in TMQ plasma concentrations. Review of TMQ plasma concentration data revealed there was no obvious correlation between the increased plasma drug concentrations observed and the onset and time course of the observed secondary phase of bronchodilation.

Overall, the number of horses that presented a secondary bronchodilation was 8 (IV and IT combined results, see Table 4). Those secondary bronchodilations only occurred at times 2, 4 and 6 h measurements. The number of horses that presented a secondary increase in TMQ plasma concentrations for those time periods was 4. There were other occasions where secondary increase in plasma concentrations were observed, but those happened at 45 min and 1 h, time points which did not parallel with the secondary bronchodilations. As can be noted in Table 4, out of the secondary bronchodilation observations, there were only two times in that horses had a secondary...
Table 4. This table presents the observations of secondary bronchodilation and secondary increase of TMQ plasma concentrations following IV and IT administrations. Horses only showed secondary bronchodilation at time points 2, 4 and 6 h. This table details which horses had a secondary bronchodilation and whether or not they also had a secondary increase in TMQ plasma concentrations. Note that only in two occasions (darker grey) the same horse had both the bronchodilation and the secondary increase in plasma concentrations.

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<td>Observed secondary increase in plasma concentration</td>
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bronchodilation at a time point that it also presented a secondary increase in plasma concentration.

One possible explanation for this discrepancy could be that the secondary bronchodilation was caused by an undetected metabolite. The detector of the Varian LC-MS-MS was set to detect only transition compounds with the m/z 346 → 164 for TMQ, as described above; thus, such metabolite, having a different m/z [M+H]+, would not be detected by our system. In that regard, as described by Camargo et al. (2004), one possible metabolite could be 7-O-methylated-TMQ (m/z 360 → 178), which, described by Sato et al. (1971), produced bronchodilation in cats. What is interesting about this metabolite is that, because the methyl group confers more lipophilicity than non-methylated TMQ, 7-O-methylated TMQ is more slowly distributed and eliminated than TMQ, with urinary concentrations peaking at 2 h and slowly decreasing thereafter (Camargo et al., 2004). This could explain a secondary bronchodilator, and why it happens at 2, 4 and 6 h post administration depending on the horse and route of administration.

The analytical method reported in this study would appear to be the most sensitive analytical method for TMQ currently available, with an estimated LOD in the order of 1 pg/mL in plasma or urine. However, in sharp contrast to the high sensitivity of our analytical method of detection, the currently available ELISA screening test is much less sensitive. In our hands, this test did not detect TMQ from the samples developed in this study, which should be of regulatory concern, because, ideally, screening tests should be at least of sufficient sensitivity to match pharmacology. Given the extreme potency of this drug, in which as little as 5 µg/horse IV is sufficient to double the basal heart rate, there is a need for a highly sensitive screening methodology.

The need for a more sensitive screening test is particularly strong in light of reports in racing circles which suggest the use of TMQ as a ‘pre-race adjustment’ to some racing horses. In support of the basic hypothesis of the use of bronchodilators as performance enhancing substances in racing horses, the Association of Racing Commissioners International (ARCI) have reported 40 identifications of terbutaline, a bronchodilator not recognized by the American Association of Equine Practitioners (AAEP) as an equine therapeutic agent, between the years of 1977–2006. Moreover, between August 2004 and August 2005, a total of 58 horses tested positive for bronchodilators, which included horses that were positive for drugs not recognized as therapeutic bronchodilators by the AAEP, such as ipratropium bromide, pirbuterol, terbutaline and theophylline. It is anticipated that, were a highly sensitive TMQ screening test available, those numbers would have been much higher.

In conclusion, TMQ is a highly potent cardiac stimulant and bronchodilator in the horse. These responses commence within minutes following its IV administration, but decline rapidly, and are essentially over within 1 h. These dramatic findings are in good agreement with the plasma and urinary concentration data, which show that following IV administration TMQ is extremely rapidly distributed and eliminated from the body.

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REFERENCES


