JOURNAL OF
Veterinary Pharmacology and Therapeutics

J. vet. Pharmacol. Therap. doi: 10.1111/jvp.12068

The pharmacokinetics of methocarbamol and guaifenesin after single intravenous and multiple-dose oral administration of methocarbamol in the horse

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Rumpler, M. J., Colahan, P., Sams, R. A. The pharmacokinetics of methocarbamol and guaifenesin after single intravenous and multiple-dose oral administration of methocarbamol in the horse. *J. vet. Pharmacol. Therap.* doi: 10.1111/jvp.12068.

A simple LC/MSMS method has been developed and fully validated to determine concentrations and characterize the concentration vs. time course of methocarbamol (MCBL) and guaifenesin (GGE) in plasma after a single intravenous dose and multiple oral dose administrations of MCBL to conditioned Thoroughbred horses. The plasma concentration–time profiles for MCBL after a single intravenous dose of 15 mg/kg of MCBL were best described by a three-compartment model. Mean extrapolated peak (C_0) plasma concentrations were 23.2 (±5.93) μg/mL. Terminal half-life, volume of distribution at steady-state, mean residence time, and systemic clearance were characterized by a median (range) of 2.96 (2.46–4.71) h, 1.05 (0.943–1.21) L/kg, 1.98 (1.45–2.51) h, and 8.99 (6.68–10.8) mL/min/kg, respectively. Oral dose of MCBL was characterized by a median (range) terminal half-life, mean transit time, mean absorption time, and apparent oral clearance of 2.89 (2.21–4.88) h, 2.67 (1.80–2.87) h, 0.410 (0.350–0.770) h, and 16.5 (13.0–20) mL/min/kg. Bioavailability of orally administered MCBL was characterized by a median (range) of 54.4 (43.2–72.8)%. Guaifenesin plasma concentrations were below the limit of detection in all samples collected after the single intravenous dose of MCBL whereas they were detected for up to 24 h after the last dose of the multiple-dose oral regimen. This difference may be attributed to first-pass metabolism of MCBL to GGE after oral administration and may provide a means of differentiating the two routes of administration.

(Paper revised 2 April 2013; accepted for publication 20 June 2013)

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INTRODUCTION

Methocarbamol (MCBL) is indicated for the symptomatic treatment of muscle spasms that may result from various musculoskeletal disorders. It was first synthesized by Murphy (1956), and its pharmacologic effects were investigated by Truitt and Little (1958). It was approved by the US Food and Drug Administration (USFDA) for use in horses more than 40 years ago and continues to be an important therapeutic drug in performance horses. Despite its therapeutic relevance, MCBL has the potential to affect performance, and for this reason, the Association of Racing Commissioners International (ARCI) has designated MCBL a class 4 drug. More than 140 findings for MCBL were reported from U.S. horse tracks between 2005 and 2012 (Racing Medication and Testing Consortium, recent rulings database). Only phenylbutazone, flunixin, and clenbuterol were reported more frequently during this period. Although MCBL is commonly used at racetracks, the relatively large number of findings suggests that the time required for MCBL to decline to undetectable concentrations in blood or urine samples after the last dose may be poorly understood or difficult to predict. Therefore, the purpose of this investigation was to develop and validate a method for determining MCBL in horses and to investigate its disposition after intravenous and oral doses to exercise-conditioned Thoroughbred horses for the purpose of generating

data that could be used to establish a regulatory threshold for use in horse racing.

Methocarbamol is commonly administered orally to horses as 500-mg tablets (Robaxin™-V; Zoetis Inc. (Florham Park, NJ, USA) and various generic manufacturers). Compounding pharmacies advertise that they prepare MCBL for use in horses in various formulations including oral powders, capsules, oral paste, oral suspension in oil, and a transdermal gel. Clinical doses of the injectable formulation range from 15 to 100 mg/kg, while recommended oral doses are often larger due to the drug's incomplete bioavailability after oral dose (Cunningham *et al.*, 1992).

The United States Equestrian Federation (USEF) classifies MCBL as a therapeutic drug and advises a maximum oral dose of 5 g every 12 h. Accordingly, the USEF has stated that the maximum plasma concentration of MCBL allowed in horses participating in USEF events is 4 μg/mL (United States Equestrian Federation, 2011).

The mechanism of action of MCBL remains unknown, but it is thought to act as a CNS depressant (Muir *et al.*, 1984). Methocarbamol is metabolized via dealkylation and hydroxylation followed by conjugation to form both glucuronides and sulfates (Bruce *et al.*, 1971). The disposition of MCBL after intravenous (Muir *et al.*, 1984) and oral administration to horses has been investigated (Cunningham *et al.*, 1992).

Guaifenesin (GGE) is both an expectorant drug that is a common ingredient in human over-the-counter and prescription medications and a centrally acting skeletal muscle relaxant used as a pre-anesthetic agent in veterinary medicine. Therefore, it is a class 4 drug (ARCI) and a prohibited substance under the USEF and ARCI guidelines. Furthermore, it is a metabolite of MCBL (Muir *et al.*, 1984). Its pharmacodynamic effects following a single intravenous pre-anesthetic dose to horses have been studied briefly, and high doses (>100 mg/kg) are well tolerated (Hubbell *et al.*, 1980). However, there are no reports of its disposition following the administration of MCBL to horses, although the metabolism of MCBL to GGE and the excretion of GGE in urine after oral and intravenous administration of MCBL to horses have been known for several decades (Muir *et al.*, 1984; Koupai-Abyazani *et al.*, 1997).

Several investigators have described the detection of MCBL or GGE in plasma or serum (Hubbell *et al.*, 1980; Weng *et al.*, 1994; Koupai-Abyazani *et al.*, 1997; Zha & Zhu, 2010). Naidong *et al.* (1994) described a LC/UV method for both compounds. Zha and Zhu (2010) reported a rapid and easy protein precipitation method using LC/MS/MS, but their lower limit of quantification (LLOQ) of 150 ng/mL is inadequate for detailed pharmacokinetic analysis of the disposition of MCBL in horses because the compound is rapidly metabolized (Muir *et al.*, 1984). Although previous studies have investigated the pharmacokinetics of MCBL after oral and intravenous administration in the horse (Muir *et al.*, 1984, 1992; Cunningham *et al.*, 1992), the analytical methods applied were unable to provide adequate sensitivity for modern regulatory control of the drug. Recently, more sensitive methods describing the analysis of GGE have been developed (Liao *et al.*, 2007; Wen *et al.*, 2010).

This report describes the disposition of MCBL and GGE in the horse after the administration of clinically relevant doses of MCBL using a validated LC/MS/MS method to quantify MCBL and GGE simultaneously.

MATERIALS AND METHODS

Animals

Twenty, adult, conditioned, Thoroughbred horses (9 mares and 11 geldings) ranging in age from 5 to 10 years and weighing 468–605 kg were used in these studies (Table 1). Horses were housed in grass paddocks at the University of Florida (UF) Veterinary Medical Center (Gainesville, FL, USA), maintained on a diet of commercially available grain mixture and had open access to water and hay at all times. They were regularly exercised (3 days/week) before and throughout the studies.

Horses were conditioned at the UF Equine Performance Laboratory on a high-speed Säto treadmill (Equine Dynamics, Lexington, KY, USA). The standard training regimen used is described in a previous publication (Rumpler *et al.*, 2011).

Drug administration

Twenty horses were dosed intravenously with 15 mg/kg of MCBL (Wedgwood Pharmacy, Swedesboro, NJ, USA) into the right jugular vein to collect plasma concentrations for a withdrawal time study. Fourteen of these horses were subjected to a limited sample collection schedule as described below whereas the remaining six horses were used for an IV and oral pharmacokinetic study with extensive sampling as described

Table 1. Demographics of study subjects. Each number represents a horse

Horse	Gender	Age (years)	Weight (kg)	IV Administration	Oral administration	PK analysis
1	M	5	535	X		
2	M	5	498	X		
3	M	7	563	X		
4	M	7	562	X		
5	M	8	499	X		
6	M	5	542	X		
7	M	7	545	X		
8	M	9	605	X		
9	M	8	468	X		
10	G	9	488	X		
11	G	10	512	X	X	X
12	G	8	560	X	X	X
13	G	10	490	X		
14	G	6	597	X		
15	G	9	548	X		
16	G	10	531	X	X	X
17	G	5	502	X		
18	G	10	530	X	X	X
19	G	7	505	X	X	X
20	G	10	541	X	X	X

M, mare; G, gelding.

below. Six of these horses were dosed once IV and, after an approximate 10-week washout period, they were dosed orally with 5 g of MCBL (Qualitest Pharmaceuticals, Huntsville, AL, USA) five times at 0, 12, 24, 36, and 48 h. For each oral dosage, ten 500 mg tablets were crushed and dispersed in 60 mL of water and delivered to the stomach via nasogastric tube in each dose. Each dose was followed by 60 mL of water to rinse the nasogastric tube.

Sample collections

Whole blood samples were collected from the left jugular vein via needle venipuncture into partially evacuated tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Blood samples were stored on ice until the plasma was separated by centrifugation (776–1318 *g*) at 4 °C for 15 min. Harvesting of plasma took place within 1 h of sample collection, and 2–4 mL aliquots of plasma were immediately frozen at −20 °C and stored within 24 h at −80 °C until analyzed. Collection times for the six horses dosed intravenously in the pharmacokinetic study were before drug administration and at 5, 10, 15, 20, 30, 45, 60, 90 min and 2, 3, 4, 6, 8, 12, 24, 48, and 72 h. Collection times for the remaining 14 horses dosed intravenously in the withdrawal time study were before drug administration and at 24, 48, and 72 h. For the oral dose pharmacokinetic study, whole blood samples were collected at 15, 30, and 45 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 48.25, 48.5, 48.75, 49, 49.5, 50, 51, 52, 54, 56, 60, 64, 72, 96, and 120 h after the first dose. The samples collected at 12, 24, 36, and 48 h were collected immediately before the next dose. Plasma was processed and stored as described in the manner described above. The experimental protocol was approved and facilities were inspected by the UF Institutional Animal Care and Use Committee (IACUC).

Chemicals and reagents

Analytical grade drug standards including MCBL, GGE, MCBL-d_4 (99.9%; 99% D_4), and GGE-d_3 (99.9%; 99% D_4, 0% D_0) were purchased from the United States Pharmacopeial Convention (Rockville, MD, USA), Sigma-Aldrich (St. Louis, MO, USA), Frontier Biopharm (Richmond, KY, USA), and CDN Isotopes (Pointe Claire, Quebec, Canada), respectively. Reagent grade formic acid was purchased from ACROS Organics (Morris Plains, NJ, USA). All solvents including acetonitrile, methanol, and methylene chloride were HPLC grade and were purchased from Thermo Fisher (Pittsburg, PA, USA).

All stock standard solutions were prepared from the solid form and dissolved in methanol. All working standard solutions were diluted to the appropriate concentrations in methanol to prepare nine MCBL calibrators (0.5, 1, 5, 10, 20, 50, 100, 200, 500 ng/mL) in plasma and seven GGE calibrators (5, 10, 20, 50, 100, 200, 500 ng/mL). Calibrators and positive control samples, made from independently prepared stock solutions of MCBL and GGE, were prepared by pipetting sequentially into a tube 0.5 mL of normal saline solution, the appropriate volume of MCBL and GGE working standard solutions, 20 μL of a mixed standard solution containing MCBL-d_4 and GGE-d_3, 1 mL of saturated aqueous borate solution, 1 mL of drug-free control horse plasma, and 5 mL of dichloromethane. The remaining procedural steps are described below. Each deuterated internal standard was prepared in a working standard solution at a concentration of 5 ng/μL. The final deuterated internal standard concentration was 100 ng/mL of plasma for each standard.

Sample preparation

In duplicate, a 1-mL aliquot of each plasma sample was pipetted into 0.5 mL of 0.9% sodium chloride solution and 20 μL of 5 ng/μL mixed internal standard (MCBL-d_4 and GGE-d_3) working solution in 15-mL disposable, screw-cap centrifuge tubes. If sample dilution was required, an aliquot of the plasma sample was appropriately diluted with 0.9% sodium chloride solution. For the liquid–liquid extraction, 1 mL of saturated aqueous borate solution was added to each tube followed by 5 mL of dichloromethane. The contents of each tube were vortex-mixed after the addition of each component. All tubes were mixed end-over-end for 10–15 min and then centrifuged at 1508 *g* for 15 min to separate the phases. The upper (i.e., aqueous) layer was aspirated from each tube, and the organic layer was transferred to a clean 5 mL conical centrifuge tube. The organic contents were evaporated to dryness under nitrogen using a TurboVap® LV evaporator (Zymark, Hopkington, MA, USA). Finally, sample extracts were dissolved in 100 μL of methanol:water (10:90) containing 0.1% formic acid and transferred to glass autosampler vials.

Instrumentation

LC/MS/MS analysis was performed on a triple-stage quadrupole Quantum Ultra mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a heated electrospray ionization (HESI) source and interfaced with a HTC PAL autosampler (Leap Technologies, Carrboro, NC, USA) and Accela LC pump (ThermoFisher). XCALIBER™ software (ThermoFisher), version 2.0.7 and LCQUAN (ThermoFisher), version 2.5.6 were used for data acquisition and analysis.

Chromatographic separations were achieved with an Aqcuity C18 HSS T3 (2.1 mm × 50 mm × 1.7 μm) column (Agilent, Santa Clara, CA, USA). Gradient elution was begun with a mobile phase of 0.1% (v/v) formic acid in water (80%; Solvent A) and 0.1% (v/v) formic acid in methanol (20%; Solvent B). The initial mixture, maintained at a constant flow rate of 250 μL/min, was held isocratically for 0.75 min, then Solvent A was decreased linearly to 5% and Solvent B increased by 95%, over 2.75 min and held for 0.75 min. The mobile phase was then returned to the initial conditions for the remaining 0.5 min for a total run time of 4.0 min. The flow into the mass spectrometer was diverted into the waste stream from 0–0.75 min to 2.5–3.5 min. The column temperature was isothermal at 35 °C, and 20 μL of extractant dissolved in 100 μL

of methanol:water (10:90) containing 0.1% formic acid was injected. Mass spectral data were acquired in positive ion mode using the HESI technique with the following MS parameters: ESI spray voltage; 4100, vaporizer temperature; 240 °C, sheath gas pressure; 60 (arbitrary units), ion sweep gas; 0 (arbitrary units), auxiliary gas pressure; 6 (arbitrary units), capillary temperature; 300 °C, tube lens offset; 89 V, and skimmer offset; −10 V.

Identification and quantification of the analytes were based on selected reaction monitoring. Compound specific optimization (tuning) of MS/MS parameters was performed before analyses via direct infusion of 10 ng/μL of each analyte and internal standard dissolved in mobile phase. Tuning for MCBL yielded collision energies of 8, 13, and 11 V for transitions m/z 242→199, m/z 242→163, and m/z 242→118, respectively. The most abundant ion transition for MCBL was m/z 242→199 and was used for quantification. The second and third most abundant transitions were used as qualifier transitions. Tuning for MCBL-d4 yielded a collision energy of 8 and tube lens offset of 96 for transition m/z 246→203. Tuning for GGE yielded collision energies of 9, 11, and 24 V for transitions m/z 199→163, m/z 199→151, and m/z 199→109, respectively. The most abundant ion transition for GGE was m/z 199→163 and was used for quantification. The second and third most abundant transitions were used as qualifier transitions. Tuning for GGE-d3 yielded a collision energy of 8 and tube lens offset of 85 for transition m/z 202→165.

Data analysis

All standards, controls, calibrators, and samples were prepared in duplicate and peak ion area ratios of the analyte and internal standard were calculated for each. Individual values of the duplicate concentrations were averaged. Quantification was performed using a simple least squares linear regression analysis of the calibrator data with a $1/C_p$ weighting factor, where C_p was the nominal plasma concentration. Quality control and sample acceptance criteria have been specified according to the following guidelines and standard operating procedures of the UF Racing Laboratory, Research Section. The requirement is that the %CV for all calibrators, positive controls, and samples must not exceed 10% (15% at the LLOQ). In addition, for calibrators and controls, the difference between the back-calculated concentration and the nominal concentration must not exceed 10% (15% at the LLOQ). All study samples that did not meet the %CV criteria were reanalyzed. Calibrators that did not meet the criteria set forth were excluded from the calibration curve, and each batch was required to include at least six calibrators.

Compound identification was based on a requirement to detect the quantifier and qualifier ions with a signal to noise >10:1 for concentrations above the LLOQ and >3:1 for concentrations between the limit of detection (LOD) and the LLOQ. Also, the relative retention time of these ions for the analyte in each test was required to vary less than ±50% of the half height peak width or 3 sec, whichever was greater. Lastly, ion area ratios for quantifier and qualifier ions for the analytes in test samples were compared with the average ion area ratios calculated for these ions from the calibrators and required to differ <20% (relative).

Method validation

The method was validated in accordance with the U.S. Food and Drug Administration recommended guidelines (U.S. Department of health & Human Services, Food & Drug Administration, 2001) for specificity, sensitivity, linearity, carryover, accuracy, precision, extraction efficiency, matrix effect, process efficiency, dilution integrity, ruggedness, and stability. Each validation and study sample run contained nine (MCBL) and seven (GGE) calibration standards prepared in drug-free horse plasma, three nonfortified (analyte) control samples, and five positive control samples spanning the calibration range, all prepared in duplicate. Run acceptability was determined by the accuracy and precision of the calibration standards and positive control samples, an examination of a plot of weighted residuals, the coefficient of determination of the calibration curves, and the absence of MCBL or GGE in the negative control samples.

Specificity of the method was assessed by supplementing negative control horse plasma with various licit and potentially interfering substances. The purpose of this study was to determine whether such compounds altered the response of the analyte or internal standard or both. Three replicates each of five concentrations (1, 25, 75, 250, and 400 ng/mL) of positive control samples were evaluated in the presence of high concentrations of phenylbutazone and furosemide, substances that are frequently present in race horse plasma specimens.

Sensitivity was determined by establishing the LOD and LLOQ for both analytes. LOD was defined as the lowest concentration of analyte that could be detected with acceptable chromatography, the presence of quantifier and qualifier ions each with a signal-to-noise ratio of at least 3, and a retention time within ±0.2 min of the average retention time. LLOQ was the lowest concentration that met the LOD criteria but with a signal-to-noise ratio of 10 and acceptable accuracy and precision as defined below. The upper limit of quantification (ULOQ) corresponded to the concentration of the highest calibration standard.

Linearity of the calibration line was assessed using a simple least squares regression analysis with a $1/C_p$ weighting factor, where C_p was the plasma concentration. Evidence of linearity was provided when the coefficient of determination (R^2) was at least 0.998, and the back-calculated concentrations for the calibrators were within 15% and 10% of the nominal concentration at the LLOQ, and all other concentrations, respectively. However, as these methods alone are inadequate measures for demonstrating linearity (Araujo, 2009), the peak area response factors and peak area ratio (i.e., analyte area/internal standard area) response factors were plotted against the nominal concentrations. Acceptable linearity was indicated by a straight line with a slope at or close to zero; the slopes of these plots were compared with 0 for a statistical difference.

Carryover was evaluated by measuring the ion intensities of the characteristic ions of MCBL and GGE in an internal standard fortified negative plasma sample analyzed immediately after each of the four highest calibrators. Concentrations of MCBL and GGE in the negative plasma samples were calculated, and carryover was determined to occur if the analyte concentrations exceeded the LOD.

Accuracy, precision, matrix effect, and extraction efficiency were investigated at five (MCBL) and four (GGE) positive control concentrations that spanned the calibration range. Intra- and interbatch accuracy and precision were assessed with five replicates at each concentration over 1 ($n = 5$) and 4 days ($n = 20$), respectively. An estimate of precision, expressed as percentage relative standard deviation (%RSD), was obtained using a one-way analysis of variance (ANOVA), using Microsoft Excel (DeSilva *et al.*, 2003). Precision was required to be within ±15% for all, but the lowest concentration control material which was required to be within ±20%. Accuracy was determined by comparing the mean ($n = 20$) measured concentration of the analyte with the nominal value and expressing it as a percent of the nominal concentration with an acceptance criterion being 80–120% for the lowest concentration control and 85–115% for all other controls.

Matrix effects were evaluated according to a simplified method described by Matuszewski *et al.* (2003). For matrix effect, analyte peak areas of positive control samples supplemented after extraction were compared with peak areas of samples at the same nominal concentration prepared in mobile phase (neat). Matrix suppression or enhancement, expressed as a percentage, was calculated as follows: (mean peak area of the postextraction supplement/mean peak area of neat) × 100. Extraction efficiency (recovery) was evaluated by comparing results for positive control samples supplemented before extraction to those from samples supplemented after extraction. Extraction efficiency, expressed as a percentage, was calculated by dividing the mean peak area of the pre-extracted supplemented positive control samples by the mean peak area of the postextracted supplemented positive control samples and multiplying by 100. In addition, to evaluate the influence of different sources of matrices on analyte quantification, five different lots of negative control plasma were supplemented with five positive control concentrations and compared with a single lot supplemented with the same nominal positive control concentrations as described in Matuszewski (2006).

Concentrations of MCBL in plasma samples collected immediately after intravenous drug administration exceeded the upper limit of the calibration range. Therefore, sample dilutions were required for a portion of the samples. Dilution integrity was assessed by supplementing negative control plasma with MCBL at three concentrations (0.25, 3.75, and 7.5 μg/mL), and diluting the samples over the range of dilution factors used for the study samples. Dilution factors evaluated were 1:2, 1:50, and 1:100. Dilutional integrity was considered acceptable if replicate ($n = 5$) values were within ±10% of the nominal concentrations.

Method ruggedness was investigated to determine whether small variations in sample preparation affected analyte quantification. Positive control samples at five concentrations (1, 25, 75, 250, and 400 ng/mL) were evaluated under specific test conditions, such as altering the volume of diluent, buffer, and organic solvent content, and the results were compared with those of positive control samples prepared under the usual conditions.

Stabilities of MCBL and GGE were evaluated over short-term intervals at −20 °C (14 days) and −80 °C (90 days) storage with three replicates at each of three concentrations (1, 20, and 400 ng/mL). Freeze–thaw stability was evaluated after three freeze/thaw cycles. Extracted analyte stability was evaluated at 24, 48, and 72 h at 20 °C in autosampler conditions.

Pharmacokinetic analysis

Methocarbamol plasma concentration vs. time data for each horse in the PK study group after intravenous and oral administration were evaluated using compartmental and noncompartmental analysis with the software program PHOENIX WINNONLIN® 6.1 (Pharsight, St. Louis, MO, USA) based on the Gauss–Newton (Levenberg and Hartley) method. Methocarbamol and metabolite were not detected in the pre-administration samples collected before intravenous or oral dosing. Log plasma concentrations were plotted vs. time to assess goodness of fit. The best-fit model and appropriate weighting factor were selected based on a combination of coefficient of variation, Akaike's Information Criterion (Yamaoka *et al.*, 1978), and Schwartz's Bayesian Criterion as well as visual assessment of the graphical output, including model fits (actual vs. predicted concentration) and residual plots. Secondary parameters calculated include area under the curve (AUC), terminal half-life, mean residence time (MRT), apparent volumes of distribution, systemic clearance, and microdistribution rate constants. MCBL systemic bioavailability was calculated by dividing the AUC_{0-12} (oral) by the AUC_{0-12} (intravenous). MCBL mean absorption time (MAT) was calculated by subtracting the MRT obtained after intravenous administration from the mean transit time (MTT) after oral administration (MTT_{oral}-MRT_{iv}; Cheng, 1992). All calculations for pharmacokinetic parameters were based on methods described by Gibaldi and Perrier (1982). All pharmacokinetic parameter estimates are expressed as median (range). Plasma MCBL and GGE concentrations from six horses from the PK study are expressed as mean ± SD.

In six horses administered 15 mg/kg of MCBL intravenously for pharmacokinetic analysis, the observed plasma concentration–time profile was best characterized by a three-compartment open model. An equation describing MCBL plasma concentration as a function of time after intravenous bolus administration for this model is:

$$C_t = A\exp^{-\alpha \cdot t} + B\exp^{-\beta \cdot t} + C\exp^{-\gamma \cdot t}$$

where C_t is the plasma concentration at time (t), A is the zero-time intercept for the initial phase, B is the zero-time intercept

for the rapid elimination phase, and C is the zero-time intercept for the slow elimination phase. Further, α, β, and γ are the exponential terms for each phase, and exp is the base of the natural logarithm. The weighting factor used for fitting this model to the data was $1/(C_p)$, where C_p was the observed plasma concentration.

A noncompartmental model was fitted to plasma concentration vs. time data of each horse after oral administration of MCBL. Noncompartmental pharmacokinetic parameter estimates were determined using the linear trapezoidal model with linear interpolation.

Statistical analysis

All *P*-values used to detect a difference between two values were determined using a two sample Student's *t*-test and were computed using Microsoft Excel 2010. A *P*-value of <0.05 was considered statistically significant. Goodness of fit evaluations (linearity) were performed using GRAPHPAD PRISM, version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Upper limits of the 95/95 tolerance intervals for plasma MCBL concentrations at different collection times were computed to contain at least 95% of the population with 95% confidence and were calculated using the software program JMP 9.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Method validation

Method specificity was demonstrated by adding therapeutically relevant but potentially interfering concentrations of the regulated substances phenylbutazone (5000 ng/mL) and furosemide (100 ng/mL) to positive control samples. No interferences with the determination of MCBL, GGE, or the deuterated internal standards were detected in the analysis of plasma samples containing phenylbutazone or furosemide. Specificity was assessed by the presence and retention time of all three product ions, which varied ≤0.05 min for each analyte, quantifier-to-qualifier ion ratios, and the accuracy at all five concentrations (91–101%) of the control samples for MCBL and all four concentrations (95–106%) of the control samples for GGE. Moreover, a major transition for MCBL is attributed to loss of CHNO (m/z 242 → 199) from the carbamate moiety. This transition is uncommon and sufficiently unique that it is unlikely to resemble the product ions of other commonly encountered substances (Smyth, 2005).

Method linearity was observed over a range of 1–500 and 5–500 ng/mL, with a coefficient of determination (R^2) of >0.998 (n = 5) and >0.996 (n = 5) for MCBL and GGE, respectively. The corresponding LOD, LLOQ, and ULOQ were 0.5, 1.0, and 500 ng/mL for MCBL and 5, 25, and 500 ng/mL for GGE. The back-calculated concentrations of both compounds in calibrators were within 15% and 10% of the target concentration for the LLOQ and all other concentrations, respectively. No carryover was observed in a mobile phase blank for MCBL or GGE after injection of the four highest calibrators.

Precision, accuracy, matrix effects, and extraction efficiency of the method were evaluated over the linear range at five concentrations (1, 25, 75, 250, and 400 ng/mL) for MCBL and at four concentrations (25, 75, 250, and 400 ng/mL) for GGE. The intra- (n = 5) and interbatch (n = 20) imprecision values were <10% (expressed as %RSD) for both compounds. Inaccuracy ranged from 0.5–4.7% to −2.4–1.5 for MCBL and GGE, respectively. Calculations required for accuracy and precision are those described by DeSilva *et al.* (2003). Absolute matrix effects for MCBL and GGE were observed with a range of 95–107% and 95–102%, respectively. Relative matrix effect was evaluated using five different lots of horse plasma. MCBL and GGE concentrations in positive control samples prepared in each of the five different lots of matrix differed from those of positive control samples prepared in a single lot used for the calibrators by <15%.

Dilutional Integrity was evaluated at three dilution factors (2, 50, 100), with five replicates at each factor, to encompass the range of dilutions that were required for sample analysis. All dilutions were prepared using 0.9% sodium chloride solution. The average concentrations for five replicates at each dilution factor did not differ from the nominal value (*P*-values > 0.05). Mean accuracy at all three concentrations ranged from 99.2% to 102.3% for MCBL and 99.8–103.9% for GGE.

Method ruggedness was evaluated to determine whether small variations in the proposed method affected the quantification of MCBL, GGE, or both. Changes in volume of the aqueous sodium chloride and borate solutions had minimal effects on MCBL. However, doubling the volume of dichloromethane to 10 mL resulted in unacceptable values for the mean recovery (range: 99–190%). Moreover, when the organic solvent volume was decreased to 2.5 mL, the mean recovery ranged from 99% to 106% and quantification was unaffected for MCBL. Mean recovery results indicated that increasing the volume of the organic solvent was a critical variable when analyzing low (ng/mL) concentrations of MCBL. Ruggedness evaluations for GGE revealed that volume changes in aqueous sodium chloride solution, aqueous borate solution, and dichloromethane had minimal effects on the analyte response (P > 0.05).

The stabilities of MCBL and GGE from extracted quality control samples over the range of the calibration curve were evaluated at 20 °C autosampler conditions for up to 72 h. After storage for 48 h, the mean concentrations of MCBL and GGE extracts differed <10% compared with those in freshly prepared samples whereas those determined after storage for 72 h differed more than 10% from freshly prepared samples. Additionally, the stability of MCBL through three freeze–thaw cycles at −80 °C was demonstrated as no appreciable degradation was found compared with freshly prepared samples. Short-term stability of MCBL and GGE at three concentrations in plasma after storage at −20 and −80 °C for 14 and 90 days, respectively, was evaluated.

Intravenous administration

After rapid bolus intravenous dose of MCBL at a dose of 15 mg/kg, plasma MCBL was determined by the validated method through the 24 h collection time point in all 20 horses. Plasma MCBL concentrations at 24 h were characterized by a median (range) of 3.10 (1.0–13.4) ng/mL. The upper limit of the 95/95 tolerance interval for the MCBL plasma concentration at 24 h after intravenous dose to 20 horses was 17.2 ng/mL. MCBL was not detected in any samples collected more than 24 h (i.e., 48 and 72 h) after intravenous dose.

Plots of plasma MCBL concentration vs. time through 24 h for six horses are depicted in Fig. 1. The mean peak plasma concentration (5 min) was 23.2 (±5.93) μg/mL. Data characterizing the disposition of MCBL are reported in Table 2. The terminal half-life, volume of distribution at steady-state, and systemic clearance were characterized by a median (range) of 2.96 (2.46–4.71) h, 1.05 (0.943–1.21) L/kg, and 8.99 (6.68–10.8) mL/min/kg, respectively. After intravenous dosing of MCBL at 15 mg/kg, the metabolite, GGE, was not detected in any sample collected from 5 min to 72 h (LOD of 5 ng/mL).

Oral administration

Multiple oral dose administration of MCBL was characterized by a short terminal half-life of 2.89 (2.21–4.88) h and MTT of 2.67 (1.80–2.87) h. The extent of bioavailability of MCBL was characterized by a median (range) of 54.4 (43.2–72.8)%, and an oral clearance of 16.5 (13.0–20) mL/min/kg was observed (Table 2). Plots of plasma MCBL concentration vs. time after intravenous and oral administration are depicted in Fig. 2. Plasma concentrations of MCBL after oral administration increased rapidly as evidenced by the short median (range) MAT of 24.6 (20.9–46.0) min. The upper limit of the 95/95 tolerance interval for the MCBL plasma concentration at 24 h after the final oral dose to six horses was 7.56 ng/mL. By 48 h after the last oral dose MCBL concentrations were below the LLOQ of the analytical method in all horses studied following multiple oral administration of MCBL, GGE was quantified up to 8 h and detected up to 16 h after the

Table 2. Pharmacokinetic parameter estimates for methocarbamol after a single intravenous (15 mg/kg) and multiple oral (5000 mg) dose of methocarbamol in six horses

Parameter	Median	Range
Intravenous administration		
$t_{1/2}$ (h)	2.96	2.46–4.71
Vd_{ss} (L/kg)	1.05	0.943–1.21
CL (mL/min/kg)	8.99	6.68–10.8
$AUC_{0-\infty}$ (h·μg/mL)	28.0	23.4–37.4
$AUMC_{0-\infty}$ (h^2·μg/mL)	54.8	34.7–92.0
MRT (h)	1.98	1.45–2.51
Oral administration		
$t_{1/2}$ (h)	2.89	2.21–4.88
F	0.544	0.432–0.728
CL/F (mL/min/kg)	16.5	13.0–20.0
k_e (h^{-1})	0.245	0.142–0.313
k_a (h^{-1})	2.47	1.31–2.87
MAT (h)	0.410	0.350–0.770
MTT (h)	2.67	1.80–2.87
$AUC_{first\ dose}$ (h·μg/mL)	10.7	8.4–12.4
$AUC_{last\ dose}$ (h·μg/mL)	13.3	12.6–24.3
$AUC_{last\ dose}/AUC_{first\ dose}$	1.33	1.07–2.77
$AUMC_{last}$ (h^2·μg/mL)	24.1	20.0–38.8

$t_{1/2}$, terminal half-life; Vd_{ss}, volume of distribution at steady-state; CL, total systemic clearance; $AUC_{0-\infty}$, area under the plasma concentration–time curve to infinity; $AUMC_{0-\infty}$, area under the moment curve to infinity; MRT, mean residence time; F, oral bioavailability; CL/F, fractional oral systemic clearance; k_e, elimination rate constant, k_a, absorption rate constant; MAT, mean absorption time; MTT, mean transit time.

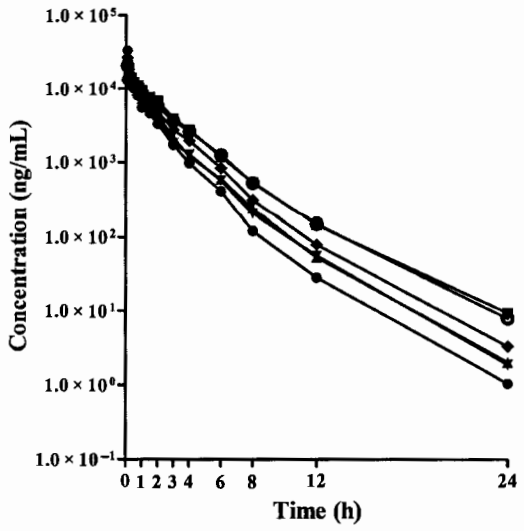

Fig. 1. Plasma concentration vs. time profile of methocarbamol (MCBL) after a single 15 mg/kg intravenous dose to six conditioned Thoroughbreds.

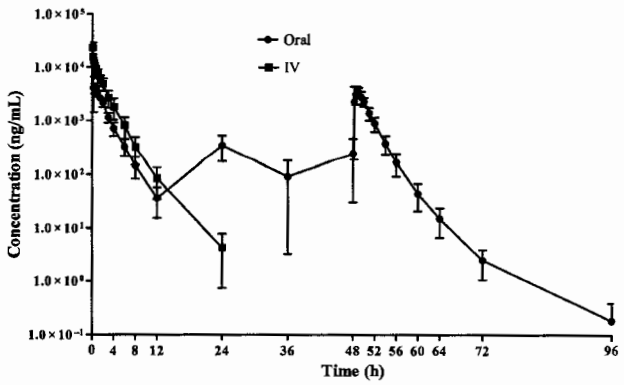

Fig. 2. Mean (±SD) plasma concentrations of methocarbamol (MCBL) after five (0, 12, 24, 36, and 48 h) oral doses of 5000 mg each or a single 15 mg/kg intravenous dose of MCBL in six conditioned Thoroughbreds. Plasma MCBL concentrations were below the lower limit of quantification (LLOQ) by 24 h after the IV dose and the last oral dose in all horses.

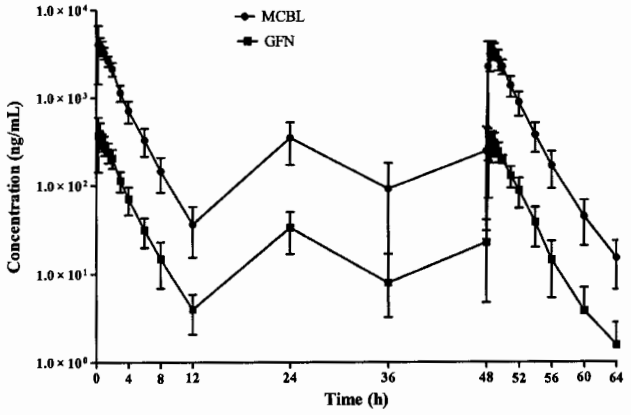

Fig. 3. Mean (±SD) plasma concentrations of methocarbamol (MCBL) and guaifenesin (GGE) after five (0, 12, 24, 36, and 48 h) oral doses of 5000 mg of MCBL to each of six conditioned Thoroughbreds. Plasma GGE concentrations are below the lower limit of quantification (LLOQ) by 8 h after the first and last dose in all horses.

Table 3. Pharmacokinetic parameter estimates of GGE after multiple-dose oral (5000 mg per dose for each of five doses) administration of MCBL to six horses

Parameter	Median	Range
$t_{1/2}$ (h)	1.78	0.941–2.72
MTT (h)	1.64	1.07–2.48
$AUC_{first\ dose}$ (h·μg/mL)	0.902	0.631–1.36
$AUC_{last\ dose}$ (h·μg/mL)	1.57	0.941–2.19
AUC_{last}/AUC_{first}	1.39	1.20–2.40
$AUMC_{last}$ (h^2·μg/mL)	2.45	1.63–4.07

$t_{1/2}$, half-life; $AUC_{0-\infty}$, area under the plasma concentration–time curve; $AUMC_{0-\infty}$, area under the moment curve; MTT, mean transit time; GGE, guaifenesin; MCBL, methocarbamol.

last dose. MCBL and GGE plasma concentration vs. time profiles are displayed in Fig. 3. The median (range) MTT of GGE was 1.64 (1.07–2.48) and was similar to the MTT of MCBL after oral administration (Table 3).

DISCUSSION

The stable isotope dilution method described in this report is fully validated according to USFDA guidance requirements and standard industry practices (DeSilva *et al.*, 2003; FDA Guidance document; Araujo, 2009). The method is characterized by LLOQs for MCBL and GGE of 1 and 25 ng/mL, respectively and is accurate, precise, rugged, and simple to perform.

Plasma MCBL and GGE concentrations after oral administration of MCBL were below the method's LLOQ at 48 and 8 h, respectively, after the last dose in all horses. Therefore, to reduce the bias associated with replacing these values with zero, replacing them with the LLOQ or a fraction of the LLOQ, or omitting the values from the calculations (Beal, 2001; Duval & Karlsson, 2002; Ahn *et al.*, 2008), calculated values that fell between the LOD and LLOQ were used for pharmacokinetic analysis (Jusko, 2012).

In earlier studies of the disposition of MCBL in horses, Muir *et al.* (1984) reported dose-dependent systemic clearance of MCBL over the intravenous dose range of 4.4–17.6 mg/kg to six horses, and Cunningham *et al.* (1992) reported the systemic clearance after an 11.2 (±0.979) mg/kg intravenous dose to six horses. In the previous studies, the systemic clearance after a dose of 17.6 mg/kg was 8.55 (±1.52) mL/min/kg (Muir *et al.*, 1984) and that after a dose of 11.2 mg/kg was 7.58 (±2.30) mL/min/kg (Cunningham *et al.*, 1992). These values compare well with the current study mean (SD) of 8.90 (±1.77) mL/min/kg. Similarly, the volume of distribution at steady-state was 0.724 (±0.094) L/kg (Muir *et al.*, 1984) and 0.812 (±0.235) L/kg (Cunningham *et al.*, 1992). These values compare favorably with the current study values of 1.05 (±0.09) L/kg. Thus, systemic clearance and volume of distribution values for MCBL in the horse from the three studies are in good agreement despite the differences in methodology and the fact that the horses used in the current study were exercise-conditioned Thoroughbred horses compared with unconditioned horses of various breeds in the other studies.

Median (range) terminal half-life of MCBL for the intravenous [2.96 (2.46–4.71) h] and oral [2.89 (2.21–4.88) h] routes were similar indicating that the rate of absorption was sufficiently rapid that flip-flop kinetics were not occurring and therefore had minimal effect on the terminal half-life. The MAT was calculated as $MTT_{oral}-MRT_{iv}$ and was short with a median (range) of 0.410 (0.35–0.77) h. This finding is consistent with the rapid rise in MCBL concentrations after oral dosing. We found MCBL accumulation (AUC_{48-0}/AUC_{0-12}) to be 2.7-fold in one horse, yet accumulation was limited to no more than 10–30% over this time period in the other horses in the study. These findings are consistent with the short terminal half-life of MCBL (2–3 h) relative to the much longer dosing interval of 12 h.

Methocarbamol is rapidly absorbed and extensively metabolized after oral administration to horses as indicated by a short absorption time and modest systemic bioavailability [median (range) of 54.4 (43.2–72.8)%]. Our estimate of total systemic clearance 8.99 (6.68–10.8) mL/min/kg after intravenous dose is nearly half of estimates of hepatic blood flow in the horse (Dyke *et al.*, 1998), leading us to predict that 40–50% of orally administered MCBL is eliminated through first-pass metabolism. This estimate was based on a comparison of systemic clearance to hepatic blood flow and was similar to the measured extent of bioavailability indicating that the modest bioavailability of MCBL is likely the result of first-pass metabolism and is not due to incomplete absorption. Cunningham *et al.* (1992) also investigated oral pharmacokinetics of MCBL in horses after a 5-g dose. Bioavailability was lower and less variable than reported in the current study with a mean (SD) of 36.3 (4.7)%. The lower bioavailability reported by Cunningham *et al.* (1992) may be due in part to the manner in which the MCBL tablets were administered in the current study. The tablets were crushed, mixed with water to form a slurry, adminis-

tered by a nasogastric tube, and the tube was then rinsed with water to assure maximal delivery of the oral dose.

The fact that the measured extent of systemic bioavailability agreed reasonably well with the estimate based on the ratio of systemic clearance to hepatic blood flow, suggests that the systemic clearance of MCBL is largely due to hepatic clearance and that renal clearance is not a substantial contributor to the total systemic clearance. Renal clearance was not determined in this study, and no reports of renal clearance in horses were found. Methocarbamol is detected in urine samples collected after intravenous and oral administration indicating that renal clearance occurs but to an unknown extent (R. A. Sams, personal observation).

Guaifenesin was not detected in plasma samples collected after intravenous dose of MCBL, and these findings are similar to those reported previously (Muir *et al.*, 1984) despite a lower detection limit in the current study (5 ng/mL). On the other hand, GGE was quantified in plasma for 8 h after oral dose of 5 g of MCBL and plasma GGE concentrations paralleled those of MCBL (Fig. 3). When expressed as molar concentrations, GGE concentrations were approximately 11% of the corresponding MCBL concentrations, and the $AUC_{metabolite}/AUC_{parent}$ ranged from approximately 9–14% after oral administration.

Formation of the metabolite GGE from MCBL was rate limiting as evidenced by the parallelism between the concentrations (Rowland & Tozer, 1995) of GGE and those of MCBL (Fig. 3) during the terminal phase after oral administration (Houston & Taylor, 1984). Furthermore, the metabolite also accumulated from 11% to 240% between the first and last dose, similar to what was observed for MCBL. Finally, the terminal half-life reported for MCBL after intravenous administration has been reported to be 1.32 h (Hubbell *et al.*, 1980) and 1.77 h (Matthews *et al.*, 1997). Both of these estimates are less than the measured terminal half-life reported after oral administration in this study (2.89 h), indicating that the rate of formation of GGE by metabolism from MCBL is the rate determining step in its elimination.

The MTT of GGE after oral administration of MCBL ranged from 1.1 to 2.5 h whereas that for MCBL ranged from 1.8 to 2.9 h of after oral administration. Chan and Gibaldi (1990) pointed out that the difference between MTT for a metabolite and the MTT for the parent drug that undergoes first-pass metabolism after oral administration (i.e., ΔMRT) can be expressed as follows:

$$\Delta MRT = (F-1)MRT_{p,p(IV)} + MRT_{m,m(iv)}$$

Where ΔMRT is the difference between the MTT of the metabolite and the MTT of the parent drug after oral administration, F is the fraction of the absorbed dose of parent drug that escapes first-pass metabolism, $MRT_{p,p(IV)}$, is the mean residence time of the parent drug after IV administration, and $MRT_{m,m(iv)}$ is the mean residence time of the metabolite after IV administration of the metabolite. An estimate for F was obtained from the extent of bioavailability for MCBL after oral administration assuming that all of the dose was absorbed, and $MRT_{p,p(IV)}$ was determined for MCBL after IV administration. Therefore, subtracting the quantity $(F-1)MRT_{p,p(IV)}$ from ΔMRT provides an estimate for $MRT_{m,m(iv)}$. $MRT_{m,m(iv)}$ was not estimated from this study but was determined in a study of the pharmacokinetics of GGE in horses after IV administration (Matthews *et al.*, 1997). Values of 2.12 h, 3.07 h, and 2.63 h were reported for the three horses investigated. Assuming a mean value of 2.61 h for the MRT for GGE after IV administration to horses, a mean value of 1.98 h for the MRT of MCBL after IV administration, and a mean value of 0.544 for F, we estimated that ΔMRT would be 1.7 h. The calculated median (range) ΔMRT is 2.29 (1.66–3.56) h. The agreement between the values for ΔMRT seems reasonable given the assumptions used to estimate it using the MRT for GGE after IV administration to horses from Matthews *et al.* (1997).

Guaifenesin was not detected in plasma after intravenous dose of 15 mg/kg of MCBL but was quantifiable after oral MCBL dose. These findings are similar to those reported for norcocaine in rats after IV and oral administration of cocaine (Sun & Lau, 2001). That study revealed that cocaine is metabolized to norcocaine by first-pass metabolism after oral administration but not after parenteral administration to rats (Sun & Lau, 2001). As GGE was not detected in plasma after intravenous administration but was quantifiable after oral administration, it may be possible to infer the route of administration of MCBL by analysis of a plasma sample for both MCBL and GGE.

In conclusion, we report a fully validated stable isotope dilution method suitable for the identification and quantification of MCBL and GGE in postrace plasma samples with limits of quantification of 1 and 25 ng/μL, respectively. Further, we have characterized the disposition of MCBL after single intravenous dose administration and that of MCBL and GGE after multiple-dose oral administration. The results of this research support the development of thresholds and detection time or withdrawal time guidelines for regulating the use of MCBL in the horseracing industry.

ACKNOWLEDGMENTS

The authors wish to thank the Racing Medication and Testing Consortium, Inc. (Lexington, KY) and the Florida Division of Pari-Mutuel Wagering (Tallahassee, FL) for financial support for this work. We also thank the veterinary and research personnel at the University of Florida Equine Performance Laboratory for performing the treadmill exercise and sample collections.

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