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Pyrilamine in the horse: detection and pharmacokinetics of pyrilamine and its major urinary metabolite *O*-desmethylpyrilamine

L. DIRIKOLU*
A. F. LEHNER†
J. D. HARKINS†
W. E. WOODS†
W. KARPIESIUK†
R. S. GATES‡
M. FISHER§ &
T. TOBIN†

**Department of Veterinary Sciences, College of Veterinary Medicine, University of Illinois, Urbana, IL, USA; †Department of Veterinary Science, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY, USA; ‡Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY, USA; §The Kentucky Racing Commission, Ironworks Pike, Lexington, KY, USA*

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Pyrilamine is an antihistamine used in human and veterinary medicine. As antihistamines produce central nervous system effects in horses, pyrilamine has the potential to affect the performance of racehorses. In the present study, *O*-desmethylpyrilamine (*O*-DMP) was observed to be the predominant equine urinary metabolite of pyrilamine. After intravenous (i.v.) administration of pyrilamine (300 mg/horse), serum pyrilamine concentrations declined from about 280 ng/mL at 5 min postdose to about 2.5 ng/mL at 8 h postdose. After oral administration of pyrilamine (300 mg/horse), serum concentrations peaked at about 33 ng/mL at 30 min, falling to <2 ng/mL at 8 h postdose. Pyrilamine was not detected in serum samples at 24 h postdosing by either route. After i.v. injection of pyrilamine (300 mg/horse) *O*-DMP was recovered at a level of about 20 μg/mL at 2 h postdose thereafter declining to about 2 ng/mL at 168 h postdose. After oral administration, the *O*-DMP recovery peaked at about 12 μg/mL at 8 h postdose and declined to <2 ng/mL at 168 h postdose. These results show that pyrilamine is poorly bioavailable orally (18%), and can be detected by sensitive enzyme-linked immunosorbent assay tests in urine for up to 1 week after a single administration. Care should be taken as the data suggest that the withdrawal time for pyrilamine after repeated oral administrations is likely to be at least 1 week or longer.

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Dr Levent Dirikolu, 3830 VMBSB, 2001 South Lincoln Avenue, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, Urbana, IL 61802, USA. E-mail: dirikolu@uiuc.edu

INTRODUCTION

Pyrilamine {*N*-[(4-Methyoxyphenyl)methyl]-*N*′,*N*′-dimethyl-*N*-2-pyridinyl-1,2-ethanediamine} is an antihistamine that is an H_1-receptor antagonist used extensively in human and veterinary medicine for symptomatic relief of allergic reactions. Although the behavioral effects of pyrilamine on the horse are unknown, general side effects of antihistamines include sedation or central nervous system stimulation, depending on the medication, dose, and route of administration (Douglas, 1985).

Medications capable of improving the racing performance of horses are classified by the Association of Racing Commissioners International (ARCI) based on their performance-enhancing potential. Pyrilamine is currently classified as an ARCI class 3 agent (available from the ARCI, 2007. http://www.arci.com/druglisting.pdf). Table 1 gives a synopsis of this classification along with relevant examples. As a class 3 agent, pyrilamine is considered to have significant potential to influence the outcome of a race, and its administration to a horse shortly before post-time would clearly contravene the rules of racing in most jurisdictions. Therefore, pyrilamine is currently a medication whose administration to a horse prior to racing is likely to be considered inappropriate. As such, useful screening and confirmation methods for pyrilamine and its metabolites in equine serum/plasma or urine are required.

In competition horses, the detection of high-potency medications, such as pyrilamine, is largely dependent on enzyme-linked immunosorbent assay (ELISA) testing. To date, there have been no studies that have evaluated the ELISA detection times of pyrilamine and its metabolites in the biological fluids of horses. The primary objective of this study was, therefore,

Table 1. Brief description of Association of Racing Commissioners, International, Inc. Drug Classification Scheme in relation to racehorses (after ARCI, 2007)

ARCI class	Potential to affect performance in racehorses	Accepted therapeutic activities	Examples
1	Very high	None	Stimulant and depressant drugs, opiates, opioids, psychoactive drugs, amphetamine-like drugs, all DEA schedule I drugs
2	High, <class 1	None	Psychotropic drugs, certain nervous system & cardiovascular stimulants, depressants, and neuromuscular blocking agents
3	High, <class 2	Possible	Bronchodilators, other drugs with primary effects on the autonomic nervous system, procaine, antihistamines with sedative properties and high-ceiling diuretics
4	Moderate, <class 3	Yes	Less potent diuretics, anabolic steroids, corticosteroids, antihistamines & certain skeletal muscle relaxants, expectorants & mucolytics, hemostatics, cardiac glycosides & anti-arrhythmics, topical anesthetics, antidiarrheals, mild analgesics
5	Low, <class 4	Yes	Agents with very localized actions only, e.g. dimethylsulfoxide, anti-ulcer drugs, certain anti-allergy drugs, anticoagulant drugs

to evaluate the ELISA detection times of pyrilamine and its metabolites in urine and serum samples of horses by using commercially available ELISA kits. Also, even though pyrilamine is widely used in horses, no pharmacokinetic data are available for pyrilamine at this time. Therefore, we also developed and validated a sensitive and specific gas chromatography/mass spectrometry (GC/MS) quantitative method for pyrilamine and its major equine urinary metabolite, *O*-desmethylpyrilamine (*O*-DMP), in horses (structures in Scheme 1). With the validated method, we determined the drug's absorption, distribution, metabolism and elimination characteristics following its administration to horses. Together, these technologies will have the potential to provide racing industry with the requisite methodologies, ELISA detection/withdrawal times, and pharmacokinetics information for effective control of pyrilamine in competition horses. It is essential for the welfare and integrity of the racing industry that accurate ELISA detection/withdrawal times and pharmacokinetic data exist for pyrilamine in order to control either its inadvertent or intentional misuse. Additionally, portions of the pharmacokinetic data from one horse have previously been communicated in a preliminary report (Woods *et al.*, 2000).

MATERIALS AND METHODS

Horses

Eight healthy mares weighing between 450 and 576 kg were used in this study. The horses ranged in age from 3 to12 years and were either thoroughbreds or standardbreds. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. The horses were placed in box stalls and feed was withheld for 2 h before and 8 h after drug administration. Water and hay were available *ad libitum*. The animals were managed according to the rules and regulations of the Institutional Animal Care Use Committee at the University of Kentucky, which also approved the experimental protocol.

Pyrilamine administration

In the present study, eight mature mares were randomly divided in two equal groups. Pyrilamine was obtained from Sigma Chemical (Sigma Chemical Co., St Louis, MO, USA). Four horses received single oral dose of 300 mg pyrilamine at 0.52–0.66 mg/kg dosage by syringe and the remaining four horses received single intravenous (i.v.) dose of 300 mg of pyrilamine at roughly the same dosage range. The skin over the left jugular vein was washed with Betadine Scrub (Poviderm®; Burns Veterinary Supplies, Westbury, NY, USA) and rinsed with ethanol. An i.v. catheter (Abbocath-T, 14ga × 5½″; Abbott Animal Health, North Chicago, IL, USA) was inserted into the left jugular vein and sutured in place. Pyrilamine was administered with great care as a single i.v. dose into the right jugular vein. Following oral and i.v. administration of pyrilamine, blood samples were collected from the left jugular vein by means of vacutainer tubes (SST Gel & Clot Activator; Becton Dickinson, Franklin Lakes, NJ, USA) for analyses 0, 5, 10, 15, 30 min and 1, 2, 4, 6, 8, 24 and 48 h after dosing. Clots developed after approximately 6–12 h at 4 °C and then the tubes were centrifuged at 4 °C at 2000 ***g*** for 15 min; the serum samples were collected and stored in polypropylene snap-cap tubes and refrigerated (4 °C) in 5-mL aliquots until assayed within 1–2 weeks postdosing. During the first day, complete urine collection was accomplished with a Foley catheter at 0, 1, 2, 4, 6 and 8 h after administration. At 24, 48, 72, 96 and 168 h after administration, a Harris flush tube (24 Fr × 152.4 cm; Seamless, Ocala, FL, USA) was used to collect the urine samples. Urine was divided into appropriate aliquots and stored in 250 mL Nalgene rectangular HDPE bottles (Fisher Scientific, Pittsburg, PA, USA) at −20 °C until assayed.

In a separate experiment, a single horse was used for the ELISA detection of pyrilamine in serum and urine samples. In this experiment, one horse received a single i.v. injection of 300 mg pyrilamine and urine samples were collected at 0, 2, 4, 6, 11, 24, 48 and 69 h postdose and serum samples were collected at 0, 0.5, 1, 2, 4, 6, 11, 24, 48 and 69 h postdose. Samples were handled and stored as indicated above.

Reagents

β-Glucuronidase types L-II (from *Patella vulgata*) and H-5 (from *Helix pomatia*), pyrilamine maleate, tripelennamine HCl, chlorpheniramine maleate, pheniramine maleate, diphenhydramine HCl and albuterol were obtained from Sigma Chemicals, St Louis, MO, USA. *N,O*-bis(trimethylsilyl)trifluoroacetimide with 1% trimethylchlorosilane (BSTFA with 1% TMCS) was obtained from Pierce, Rockford, IL, USA.

ELISA cross-reactivity

The reactivity of the pyrilamine ELISA was tested with pyrilamine maleate, synthetic *O*-DMP, and a group of compounds similar to pyrilamine (tripelennamine HCl, chlorpheniramine maleate, pheniramine maleate, diphenhydramine HCl). Standards for ELISA cross-reactivity studies were prepared in methanol and diluted to appropriate concentrations in assay buffer. The pyrilamine ELISA (Neogen, Lexington, KY, USA) was performed as follows: Briefly, the assays were started by adding 20 μL of the standard to each antibody coated well, along with 180 μL of a pyrilamine-horseradish peroxidase (pyrilamine-HRP) solution. The wells were allowed to incubate for 1 h, followed by washing and the addition of 150 μL KY-Blue substrate (Neogen). The optical density (OD) at a wavelength of 650 nm was measured with an automated microplate reader (Bio-Tek Instruments, Winooski, VT, USA) approximately 30 min after addition of the substrate. All assay reactions were run in duplicate at room temperature.

ELISA quantification of pyrilamine equivalents

Urine and serum samples from one horse, dosed with a single 300 mg i.v. injection of pyrilamine, were first screened with a pyrilamine ELISA (Neogen) test to determine the concentrations of pyrilamine equivalents (i.e., the concentration of pyrilamine that would produce similar results) in the samples (Woods *et al.*, 2000). The pyrilamine equivalents are putatively composed of pyrilamine, *O*-DMP and other metabolites. The assays were started by adding 20 μL of the standard, test, or control samples to each well, along with 180 μL of the pyrilamine-HRP solution to wells containing the test samples. A quantity of 160 μL of the pyrilamine-HRP solution was added to wells containing standard and control samples along with 20 μL of blank urine/serum to create a matrix comparable to the test samples. Thereafter, the ELISA was performed as above.

O-DMP-glucuronide analysis by MS/MS

Direct infusion of urine samples on a Micromass (Beverly, MA, USA) Quattro II tandem mass spectrometer (MS/MS) was performed as previously described (Korfmacher *et al.*, 1988; Bosken *et al.*, 2000). Full scan positive electrospray ionization (ESI^+) mass spectra were obtained for urine samples collected from a horse administered with 300 mg pyrilamine i.v. The samples were filtered by centrifugation (90 min at 1200 ***g***) through a 3000 molecular weight cutoff filter (Amicon, Beverly, MA, USA). The filtrate was diluted 1:10 with 50% acetonitrile (with 0.05% formic acid) in water. The sample was infused into the electrospray port of the MS/MS at 0.6 mL/h via a Harvard syringe pump equipped with a 500 μL Hamilton gas-tight syringe. The MS/MS was tuned for positive ion spectra by direct infusion of 10 ng/μL pyrilamine in 50% acetonitrile (with 0.05% formic acid) in water. Spectra were analyzed with the aid of Quadtech Mass Spec Calculator™ software (ChemSW Inc, Fairfield, CA, USA).

Optimization of O-DMP-O-glucuronide hydrolysis and extraction

The discovery of a predicted pyrilamine-derived glucuronide (see proposed structure in Scheme 1) necessitated study of the proper enzymatic conditions to digest and release the glucuronic acid portion so as to make the corresponding released pyrilamine metabolite, *O*-DMP, amenable to GC/MS determination. Optimization therefore involved (i) study of the stability of pyrilamine and *O*-DMP to glucuronidase elevated temperatures; (ii) progress of the glucuronidase reaction so as to limit exposure to elevated temperatures; (iii) optimization of glucuronidase concentration; and (iv) comparison of two different glucuronidases, one of which is active at 65 °C, the other at 37 °C.

The stability of pyrilamine and the synthesized *O*-DMP was determined under the *P. vulgata* β-glucuronidase hydrolysis conditions. Blank equine urine was supplemented (spiked) with pyrilamine or *O*-DMP at a concentration of 80 ng/mL. The samples were incubated at 65 °C with added Type L-II *P. vulgata* β-glucuronidase (5000 units/mL) for 0, 1, 3 and 6 h. The samples were extracted, derivatized and analyzed by GC/MS-selected ion mode (SIM) (see GC/MS-SIM section below).

The progress of the *P. vulgata* β-glucuronidase hydrolysis of *O*-DMP-*O*-glucuronide in dosed horse urine was also monitored. Urine collected from a horse dosed with 300 mg pyrilamine i.v. 2 h postdose was diluted 1:100 with blank urine. This diluted urine, along with blank equine urine supplemented with *O*-DMP at a concentration of 40 ng/mL, was hydrolyzed with *P. vulgata* β-glucuronidase (5000 units/mL) at 65 °C for 0, 1, 2 and 3 h. The samples were extracted, derivatized and analyzed by GC/MS-SIM (see GC/MS-SIM section below). The results from the dosed horse urine were not corrected for dilution as we are only interested in relative effects in this experiment.

The hydrolysis of *O*-DMP-*O*-glucuronide by Type H-5 *H. pomatia* β-glucuronidase preparation was also investigated in order to optimize for enzyme concentration and incubation time in the present study. Urine collected from a horse dosed with 300 mg pyrilamine i.v. 2 h postdose was diluted 1:100 with blank urine. This diluted urine was hydrolyzed with *H. pomatia* β-glucuronidase (5000 units/mL) at 37 °C for 0, 1, 2, 3, 4, 5, 6, 12, 16, and 22 h. In another experiment, the diluted urine was hydrolyzed with *H. pomatia* β-glucuronidase at 1000, 2000, 3000, 4000 and 5000 units/mL at 37 °C for 16 h. The samples were extracted, derivatized and analyzed by GC/MS-SIM (see GC/MS-SIM section below). The results were corrected for dilution.

β-Glucuronidase hydrolysis of *O*-DMP-*O*-glucuronide in dosed horse urine by *P. vulgata* and *H. pomatia* *β*-glucuronidase preparations were also compared. Urine collected from a horse dosed with 300 mg pyrilamine i.v. at 0, 1, 2, 4, 6, and 8 h postdose was diluted with blank urine (1:100 for *P. vulgata* preparation; 1:1000 for *H. pomatia* preparation). The diluted samples were hydrolyzed with *H. pomatia* *β*-glucuronidase (5000 units/mL) at 37 °C for 16 h or with *P. vulgata* *β*-glucuronidase (5000 units/mL) at 65 °C for 3 h. The samples were extracted, derivatized and analyzed by GC/MS-SIM (see GC/MS-SIM section below). The results were corrected for dilution.

Quantitative analysis by GC/MS-SIM

Enzymatic hydrolysis of urine

The 5 mL urine samples, standards, and blanks were transferred to 10 × 125 mm screw cap borosilicate glass culture tubes. To each sample was added 1 mL *β*-glucuronidase solution (Type H-5, from *H. pomatia*, 5000 units/mL) and 2 mL of 1 M sodium acetate buffer (pH 5.0). The samples were mixed briefly by vortex and incubated in a water bath at 37 °C overnight (16 h). After cooling, pyrilamine and *O*-DMP were added to standards, and albuterol was added as internal standard to all tubes as follows. Standard solutions of pyrilamine, *O*-DMP, and albuterol were prepared at 1 mg/mL in methanol. Quantitative standards were prepared by the addition of a known amount of pyrilamine (0, 20, 50, 200, 200 and 500 ng/mL) and *O*-DMP (0, 1, 2, 4, 16, 40, 160 and 320 ng/mL) to blank 5 mL (volume before hydrolysis) urine samples. A known amount of albuterol standard (20 *μ*L of 10 *μ*g/mL methanol solution) was added to each sample, standard, and blank as an internal standard. This approach limited any effects of thermally-induced degradation on accurate measurement of pyrilamine and *O*-DMP posthydrolysis, while excluding methanol from the enzymatic digestion.

The samples were then sonicated for 90 sec with a Fisher horn sonicator (Fisher Scientific) while samples were kept on ice. Next, sodium phosphate buffer (2 mL of 0.1 M, pH 6) was added, and the sample pH was adjusted to 6 ± 0.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

Preparation of serum samples

Glucuronidase was not necessary for serum samples. Serum samples, standards, and blanks (1 mL each) were placed in culture tubes. Pyrilamine (0, 1, 2, 4, 10, and 20 ng/mL), *O*-DMP (0, 4, 20, 40, 80 and 200 ng/mL), and albuterol (20 *μ*L of 10 *μ*g/mL methanol solution) supplements were added as required, sodium phosphate buffer (2 mL of 0.1 M, pH 6) was added, and the sample pH was adjusted to 6 ± 0.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

Extraction/derivatization

Solid phase extraction (SPE) was performed on a RapidTrace automated workstation (Zymark Corp., Hopkinton, MA, USA). Clean Screen SPE columns (United Chemicals Technologies-Worldwide Monitoring, Bristol, PA, USA) were conditioned by sequentially adding methanol (3 mL), water (3 mL), and 1 mL of 0.1 M sodium phosphate buffer (pH 6.0). The samples (10 mL total, urine; 3 mL total, serum) were then loaded, and the column was sequentially washed with water (2 mL), 1 M acetic acid (2 mL), and methanol (4 mL). The column was eluted with 3 mL of dichloromethane/isopropanol/ammonium hydroxide (78:20:2). The eluent was evaporated to dryness under a stream of nitrogen gas (38–40 °C). For derivatization each sample was dissolved in 40 *μ*L (final sample volume) of BSTFA with 1% TMCS, vortexed for 15 sec, and incubated at 75 °C for 45 min.

Instrumentation

The instrument used for GC analysis was a Hewlett-Packard Model 6890 GC equipped with a Model 5972A mass selective (MS) detector (Hewlett-Packard, Palo Alto, CA, USA). The column was a J&W DB-5MS, 30 m × 250 *μ*m × 0.25 *μ*m (Supelco, Bellefonte, PA, USA). The carrier gas was helium with a flow rate of 1 mL/min. Each derivatized sample was transferred to an autosampler vial. A volume of 1 *μ*L was injected in splitless mode at an injector temperature of 250 °C. Initial oven temperature was 70 °C (held 2 min), ramping at 20 °C/min to 280 °C (held 12 min). Total run time was 24.5 min, and MS detector temperature was 280 °C. The MS was run in SIM with data collected from 3 to 24.5 min. Ion 121 *m/z* (underivatized pyrilamine) and ion 179 *m/z* [*O*-DMP-mono-trimethylsilyl (TMS)] were utilized along with ion 369 *m/z* (albuterol-tri-TMS, internal standard) for quantitation (Table 2).

Standard curve generation

Standard curves were constructed by plotting standard pyrilamine and *O*-DMP concentrations vs. the ratio of pyrilamine:internal standard and *O*-DMP:internal standard peak areas, respectively. Each standard was run in duplicate and the response ratios for each were averaged before plotting. Standard curves were generated with Sigma Plot for Windows (Aspire Software International, Leesburg, VA, USA). The areas of peaks

Table 2. Mass spectrometric quantifier and qualifier ions (listed in decreasing order of abundance) acquired as part of the pyrilamine SIM routine

Ion type	Albuterol-tris-trimethylsilyl (TMS), Internal standard, *m/z*	*O*-desmethylpyrilamine-mono-(TMS), *m/z*	Underivatized pyrilamine, *m/z*
Qualifier-1	86.0 (base peak)	58.0	58.0
Qualifier-2	73.0	73.0	215.2
Qualifier-3	294.3	272.2	285.2
Qualifier-4	163.1	163.1	73
Qualifier-5	179.1	285.2	
Quantifier ion	369.3	179.1 (base peak)	121.1 (base peak)

corresponding to pyrilamine, *O*-DMP and the internal standard were recorded, and the internal standard values were thereby used to normalize the pyrilamine and *O*-DMP areas. Integrated peak values were entered into QuattroPro for Windows (Borland Software Corporation, Scotts Valley, CA, USA) for statistical analysis of standards and for calculation of unknown amounts of pyrilamine and *O*-DMP in test animals. Calculations of pyrilamine and *O*-DMP concentrations in unknown samples were obtained by graphical interpolation of each unknown:internal standard area ratio obtained from each sample on the corresponding pyrilamine or *O*-DMP standard curve. Standard curves were linear in the range 0.4–500 ng/mL for pyrilamine, and 0.4–320 ng/mL for *O*-DMP, with typical R^2 values >0.99. Samples out of range were diluted and re-run as necessary.

Validation of quantitative GC/MS method

The GC/MS method for the quantitation of serum pyrilamine and urine *O*-DMP was validated by examining the measurement of consistency of results (within-run and between-run), correlation (coefficient of determination of the standard curve), and recovery of the assay. The within-run precision was calculated from responses from six repeats of three standards in one run. The within-run precision was determined by comparing the calculated response (in ng/mL backfit of the standard curve) of the low (2 ng/mL for serum pyrilamine; 2 ng/mL for urine *O*-DMP), medium (20 ng/mL for serum pyrilamine; 16 ng/mL for urine *O*-DMP), and high (200 ng/mL for serum pyrilamine; 160 ng/mL for urine *O*-DMP) standards.

The between-run precision was determined by comparing the calculated response (in ng/mL backfit of the standard curve) of the low (2 ng/mL for serum pyrilamine; 2 ng/mL for urine *O*-DMP), medium (20 ng/mL for serum pyrilamine; 16 ng/mL for urine *O*-DMP), and high (200 ng/mL for serum pyrilamine; 160 ng/mL for urine *O*-DMP) standards over six consecutive daily runs. Standard curve correlation was measured by the mean coefficient of determination (r^2) for six consecutive daily runs. The recovery was determined by comparing the response (in area) of medium (20 ng/mL for serum pyrilamine; 16 ng/mL for urine *O*-DMP) methanol standards to the equivalent extracted standards. The limit of detection (LOD) was calculated as the mean of the assay background plus two times the standard deviation of the mean for six runs. The limit of quantitation (LOQ) was calculated as the mean of the assay background plus five times the standard deviation of the mean for six runs.

Pharmacokinetic analysis

Pharmacokinetic analyses was performed, using a nonlinear regression program (WinNonlin, version 5.01, Pharsight Corporation, Cary, NC, USA). The goodness of fit was evaluated by the Akaike Information Criterion (AIC), residual plots and visual inspection. The data was weighted as $1/(y_{\text{pred}})^2$, where y_{pred} is the model-predicted concentration at the actual time. Area under the curve (*AUC*) following i.v. administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (β) of the log serum drug concentrations vs. time curve was determined by the method of least-squares regression (Gibaldi & Perrier, 1982).

The compartmental model used for the determination of the pharmacokinetic parameters of pyrilamine is represented by the general equation *a* where Cp is plasma concentration of compound at any time (t), A and B are the Y intercepts associated with the distribution and elimination phases, respectively, and α and β represent the rate constant of the distribution and terminal elimination phases, respectively (Riviere, 1997). The rate constant of distribution (α), and distribution half-life ($t_{1/2}\ \alpha$) were determined by using the method of residuals (Gibaldi & Perrier, 1975). The terminal half-life ($t_{1/2}\ \beta$) (Martinez, 1998a,b) was calculated according to Equation 1.

$$\text{Cp} = \text{A} \times \text{e}^{-\alpha t} + \text{B} \times \text{e}^{-\beta t} \qquad (a)$$

$$t_{1/2}\beta = \ln 2/\beta \qquad (1)$$

Total body clearance (Cl_s) was calculated by use of Equation 2 (Martinez, 1998a,b).

$$\text{Cl}_s = \text{i.v.Dose}/AUC_{0-\text{inf}}(\text{i.v.}). \qquad (2)$$

The volume of distribution in central compartment (Vd_c), volume of distribution in terminal elimination phase (Vd_β) and volume of distribution at steady-state (Vd_{ss}) were calculated according to Equations 3, 4 and 5, respectively (Benet & Zia-Amirhosseini, 1995).

$$\text{Vd}_c = \text{Dose(i.v.)}/\text{A} + \text{B} \qquad (3)$$

$$\text{Vd}_\beta = \text{i.v.Dose}/AUC_{0-\text{inf}} \times \beta \qquad (4)$$

$$\text{Vd}_{ss} = \text{i.v.Dose} \times AUMC_{0-\text{inf}}/(AUC_{0-\text{inf}})^2 \qquad (5)$$

AUMC is the area under the first moment curve and was calculated by the trapezoidal method and extrapolated to infinity (Gibaldi & Perrier, 1975). K_{10} is first order elimination rate constant, which describes elimination of drug from the central compartment. K_{12} and K_{21} are the distribution rate constants from the central to the peripheral and from the peripheral to the central compartments, respectively. K_{10}, K_{12}, and K_{21} were calculated according to Equations 6, 7, 8, respectively.

$$\text{K}_{10} = \alpha \times \beta/\text{K}_{21} \qquad (6)$$

$$\text{K}_{12} = \alpha + \beta - \text{K}_{21} - \text{K}_{10} \qquad (7)$$

$$\text{K}_{21} = \text{B} \times \alpha + \text{A} \times \beta/(\text{A} + \text{B}) \qquad (8)$$

Noncompartmental analysis was performed for the determination of the kinetic parameters of pyrilamine following oral administration. The terminal elimination rate constant (K) was

estimated from the log-linear portion of the plasma concentration–time courses. The *AUC* of plasma concentration–time was estimated from the plasma concentrations at different time points using the linear trapezoidal rule with extrapolation to infinity. The area under the moment curve (*AUMC*, on a plot of concentration times time vs. time) was also calculated by the trapezoidal rule. From the *AUC* and *AUMC* values, the mean residence time (*MRT*) was calculated (*MRT* = *AUMC/AUC*) (Martinez, 1998a,b).

The terminal elimination half-life ($t_{1/2}$ K) was calculated according to equation 9.

$$t_{1/2}\mathrm{K} = \ln 2/\mathrm{K} \quad (9)$$

Total oral clearance (Cl_o) was calculated by use of Equation 10

$$Cl_o = \mathrm{Dose(Oral)}/AUC_{0-\inf} \quad (10)$$

The maximum drug concentration after oral administration (C_{max}) and the time at which C_{max} is achieved (T_{max}) were determined directly from the experimental data.

The absolute bioavailability (*F*) was calculated from the AUC_{0-inf} ratio obtained following oral and i.v. administration according to Equation 11 (Benet & Zia-Amirhosseini, 1995).

$$F = AUC_{0-\inf}(\mathrm{Oral})/AUC_{0-\inf}(\mathrm{i.v.}) \times \mathrm{i.v.Dose/OralDose} \quad (11)$$

RESULTS

ELISA cross-reactivity and ELISA quantification of 'pyrilamine equivalents' in urine and serum

A standard curve for pyrilamine using the pyrilamine ELISA test indicated that an addition of 0.065 ng/mL of pyrilamine to the system produced 50% inhibition of color formation (I_{50}) (Fig. 1). Higher concentrations of pyrilamine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 0.1 ng/mL of pyrilamine. This ELISA test also reacted well with *O*-DMP (I_{50} = 0.54 ng/mL), the principal metabolite of pyrilamine, and tripelennamine, another antihistamine agent (I_{50} = 0.35 ng/mL). There was minimal detectable cross-reactivity with other antihistaminic agents chlorpheniramine (I_{50} = 35 ng/mL), pheniramine (I_{50} = 75 ng/mL), and diphenhydramine (I_{50} = 1000 ng/mL).

After an i.v. dose of 300 mg, the concentration of pyrilamine equivalents in urine reached a peak of 4160 ng/mL at 2 h after injection (Fig. 2, top). The concentration of pyrilamine equivalents in serum samples at 0.5 h after injection was 36 ng/mL (Fig. 2, bottom). Pyrilamine equivalents were still detectable in urine at the last sampling time (69 h, 3.3 ng/mL) and in serum at 11 h (0.8 ng/mL) after injection.

Analysis of O-DMP-O-glucuronide by ESI/MS/MS

Urine samples collected from a horse after the administration of 300 mg pyrilamine i.v. were analyzed for *O*-DMP-*O*-glucuronide

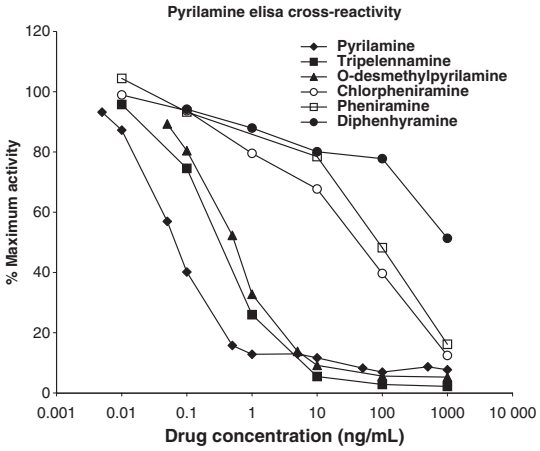


Fig. 1. Enzyme-linked immunosorbent assay (ELISA) cross-reactivity on the Neogen pyrilamine test. Assay buffer solutions of pyrilamine, its metabolite *O*-desmethylpyrilamine and other structurally related compounds were analyzed by the Neogen pyrilamine ELISA. Reactivity of the substance with the ELISA antibody is expressed as the concentration at 50% maximum activity (I_{50}).

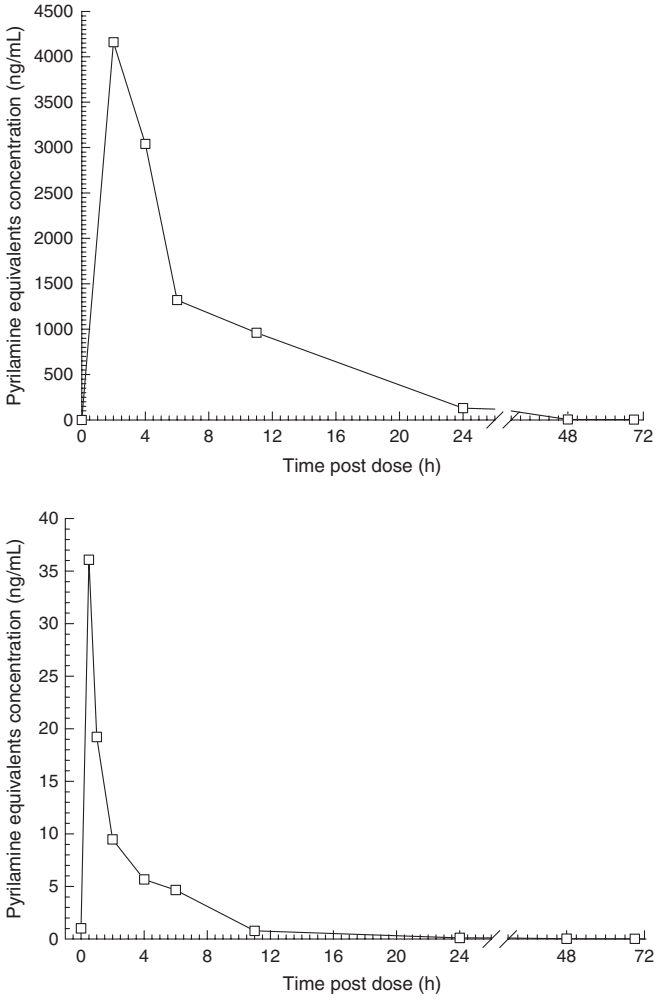


Fig. 2. Enzyme-linked immunosorbent assay detection of pyrilamine equivalents in urine (Top), and serum samples (Bottom) following single intravenous administration of 300 mg pyrilamine (n = 1).

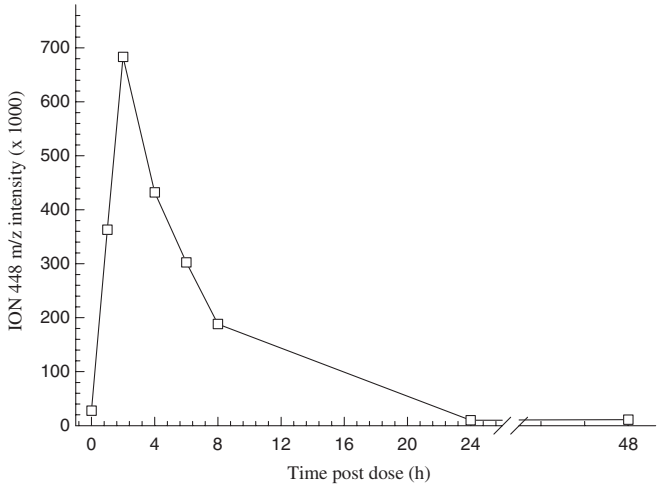


Fig. 3. Time course of *O*-DMP-*O*-glucuronide following single 300 mg i.v. injection of pyrilamine (n = 1). *O*-DMP-*O*-glucuronide was measured by full scan direct infusion positive electrospray ionization tandem mass spectrometry. The presence of *O*-DMP-*O*-glucuronide was confirmed by measurement of the *m/z* 448 ion which is the [M + H^+] pseudomolecular ion for this compound.

by full scan direct infusion positive electrospray ionization tandem quadrupole mass spectrometry. The presence of *O*-DMP-*O*-glucuronide was confirmed by measurement of the *m/z* 448 ion which is the [M + H^+] pseudomolecular ion for this compound. Reference standards for glucuronide metabolites are generally difficult to synthesize or obtain, and no attempt was made to synthesize the *O*-DMP-*O*-glucuronide; nevertheless, the intensity of the *m/z* 448 ion could be used as a gauge of the relative concentration of the material. *m/z* 448 ion intensity peaked at 2 h postdose and declined to predose levels after 8 h (Fig. 3). Figure 4a shows an ESI-MS spectrum of the *O*-DMP-*O*-glucuronide from a 2 h postdose urine sample in comparison to such a spectrum from a standard of *O*-DMP. Cleavages responsible for significant ions are indicated.

Optimization of O-DMP-O-glucuronide hydrolysis

Under standard conditions of hydrolysis with *P. vulgata* β-glucuronidase (3 h at 65 °C) only about 20% of *O*-DMP supplemented into blank urine was recovered (Fig. 5a). Pyrilamine appeared to be stable under these conditions.

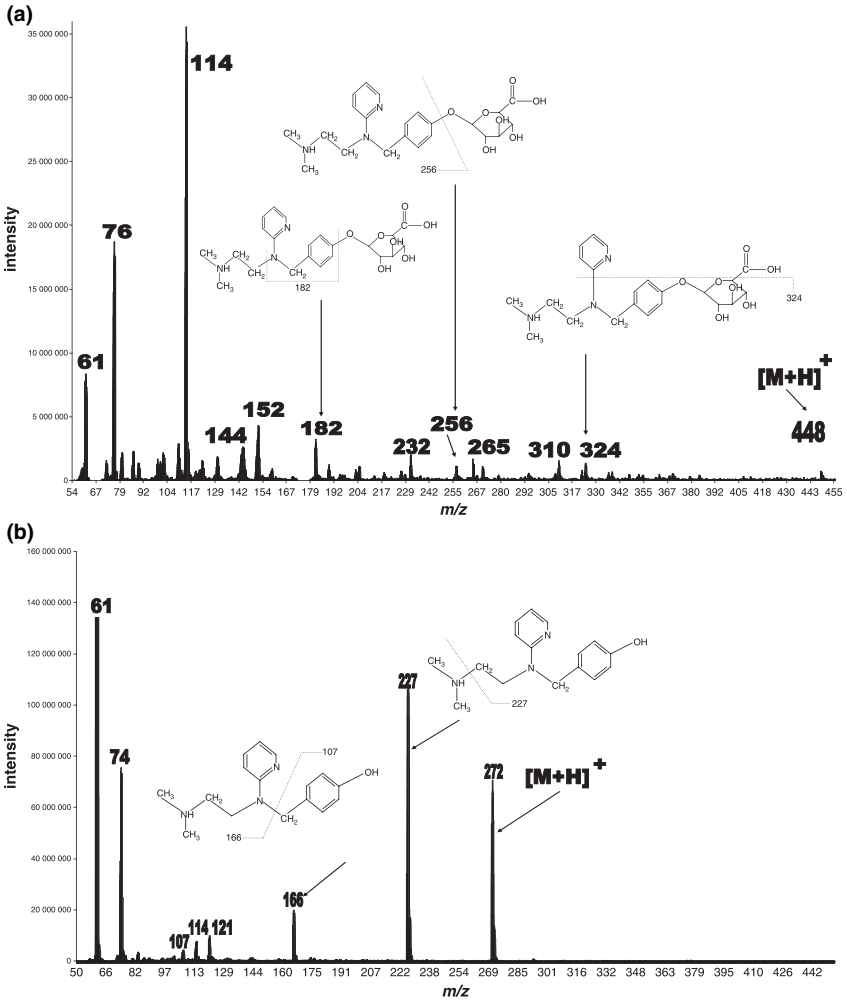
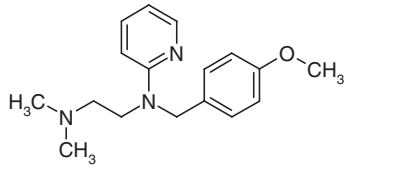
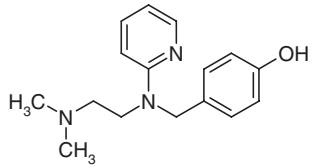


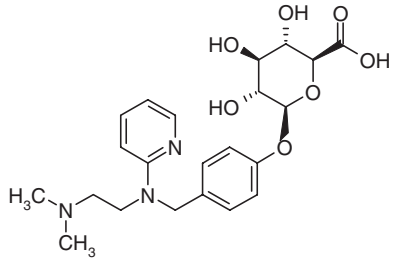
Fig. 4. ESI-[+]-mass spectra of the putative *O*-DMP-*O*-glucuronide (a) in comparison to the standard of *O*-DMP (b) run under the same conditions. Urine from the 2 h postintravenous dose of 300 mg extracted as in Materials and methods was infused at 0.6 mL/h with a cone voltage setting of 30 V to provide the glucuronide spectrum in a, whereas 10 ng/uL *O*-DMP was infused to provide the spectrum in b. Fragmentation schemes for significant ions are provided.



Pyrilamine, 285 m.w.



O-Desmethylpyrilamine, 271 m.w.



O-Desmethylpyrilamine-O-glucuronide, 447 m.w.

Scheme 1. Structure of pyrilamine, *O*-desmethylpyrilamine, (O-DMP) and O-glucuronide of O-DMP

When diluted urine (1:100) collected from a horse dosed with pyrilamine (300 mg i.v.) was hydrolyzed with the *P. vulgata* β-glucuronidase preparation, recoverable *O*-DMP rose to a level of about 40 ng/mL after 1 h incubation (Fig. 5b). However, the concentration of recoverable *O*-DMP remained the same at 2 h and fell somewhat (to about 35 ng/mL) at 3 h incubation (Fig. 5b). Recoverable *O*-DMP levels from urine supplemented with *O*-DMP (40 ng/mL) decreased with increased incubation time to around 17 ng/mL following a 3 h incubation period.

An alternate hydrolysis method using *H. pomatia* β-glucuronidase preparation at a lower recommended temperature (37 °C) was examined. The *H. pomatia* β-glucuronidase hydrolysis method was optimized for enzyme concentration and incubation time. Increasing the amount of β-glucuronidase in the incubation mixture increased the detectable *O*-DMP in urine samples. The highest level added (5000 units) resulted in the highest observed *O*-DMP concentration (data not shown). The optimal time of incubation (at 37 °C) was observed to be 12 h with at least 75% of maximum recovery between 6 and 16 h (Fig. 5c). When the hydrolysis of *O*-DMP-*O*-glucuronide in pyrilamine dosed horse urine samples by the two methods was compared (Fig. 5d), there was about a tenfold increase of recoverable *O*-DMP with the *H. pomatia* β-glucuronidase preparation over the *P. vulgata* β-glucuronidase preparation at all investigated collection times.

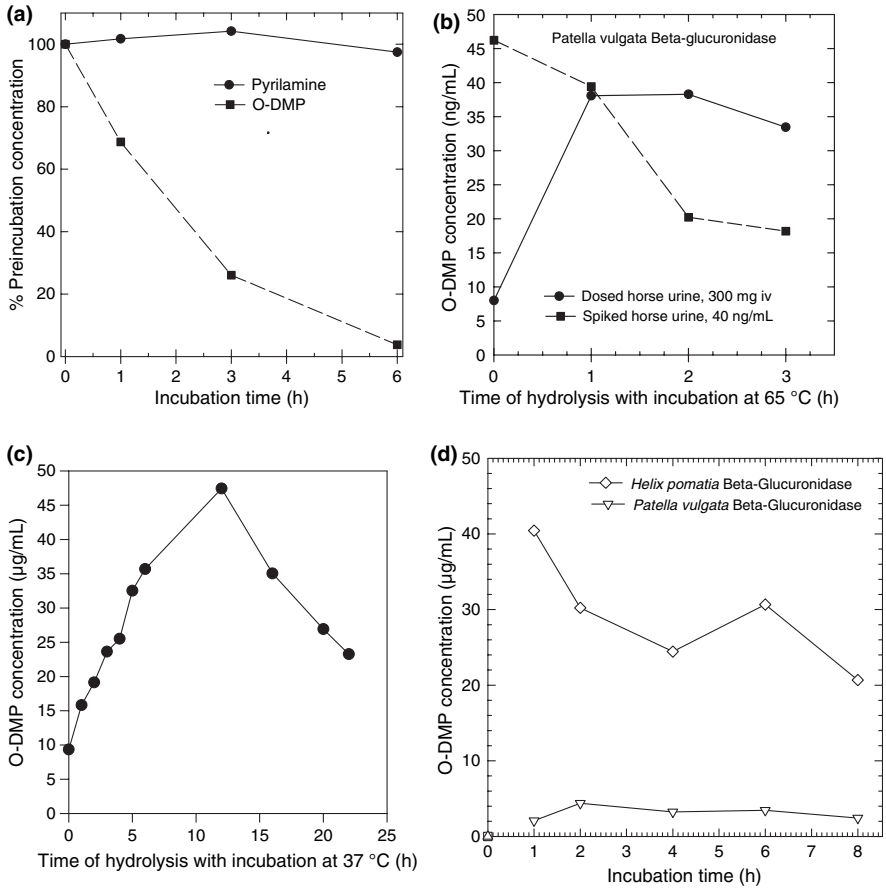


Fig. 5. Time course of the stability of pyrilamine and *O*-desmethylpyrilamine (*O*-DMP) at 65 °C for 6 h (a). Concentration of *O*-DMP from spiked and dosed horse urine with increased incubation time following hydrolysis with the *Patella vulgata* β-glucuronidase preparation (b). Concentration of *O*-DMP from dosed horse urine [300 mg intravenous (i.v.)] with increased incubation time following hydrolysis with the *Helix pomatia* β-glucuronidase preparation (c). Concentration of *O*-DMP from dosed horse urine (300 mg i.v.) at various time points following hydrolysis with the *P. vulgata* β-glucuronidase and the *H. pomatia* β-glucuronidase preparations (d). In these experiments, concentrations of pyrilamine and *O*-DMP were determined using validated gas chromatography/mass spectrometry-selected ion mode method.

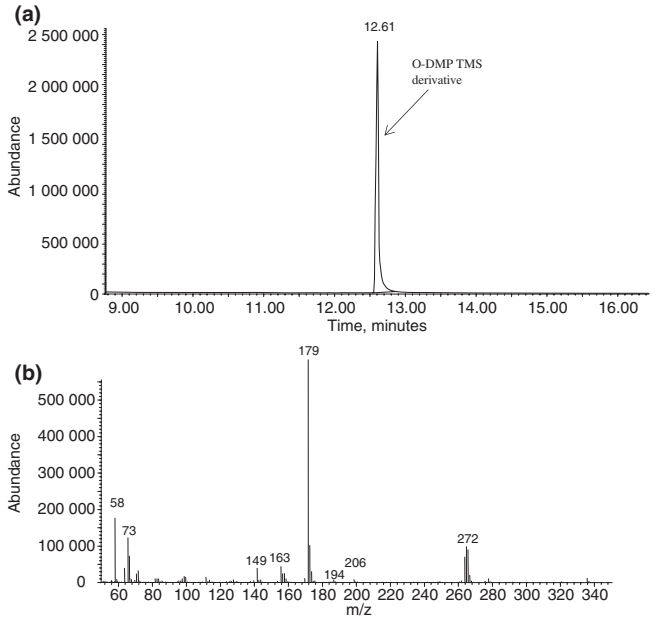


Fig. 6. Total ion chromatogram of the trimethylsilyl-derivatized synthetic *O*-desmethylpyrilamine compound (a), and electron impact-mass spectrum of the 12.6 min peak seen above (b).

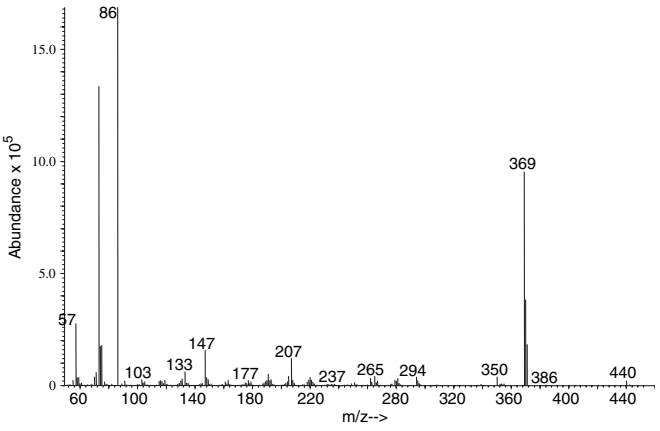


Fig. 7. Electron impact-mass spectrum of the tris-trimethylsilyl derivative of albuterol.

GC/MS-SIM quantification

The total ion chromatogram (TIC) of the TMS derivative of chemically synthesized *O*-DMP was generated by GC/MS (Figure 6a). The electron impact (EI)-mass spectrum of the predominant 12.6 min peak seen in Fig. 6a displayed a base peak at *m/*z 179 and molecular ion at *m/*z 343 (Figure 6b). *m/*z 179 represents the product of C–N bond cleavage to form a para-trimethylsilyloxytoluyl fragment. The EI-mass spectrum of the tris-TMS derivative of albuterol, the internal standard for the GC/MS-SIM quantitative pyrilamine assay, was also determined (Fig. 7). A significant peak at *m/*z 369 was likely the product of C–C bond cleavage with loss of a $CH_2NHC(CH_3)_3$ fragment. The

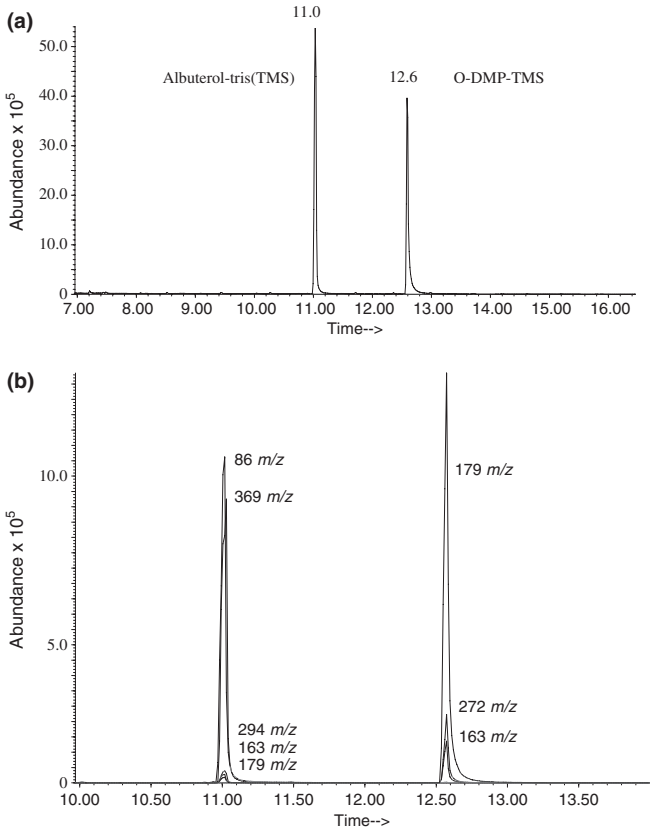


Fig. 8. (a) The chromatographic separation of the trimethylsilyl derivatives of albulterol (11.0 min) and *O*-desmethylpyrilamine (*O*-DMP) (12.6 min). (b) The overlap of qualifier and quantifier ions for albuterol and *O*-DMP, and Table 2 summarizes the quantifier and qualifier ions (listed in decreasing order) for these two compounds along with underivatized pyrilamine.

TMS derivatives of albuterol (retention time 11.0 min) and *O*-DMP (retention time 12.6 min) were well separated by the chromatography of the GC/MS method (Fig. 8a). The GC/MS-SIM qualifier and quantifier ions for albuterol-tris-TMS and *O*-DMP-mono-TMS derivatives were each demonstrated to be superimposable (Fig. 8b). The TIC of pyrilamine was also generated by GC/MS (Fig. 9a). Pyrilamine did not react with the derivatization agent used in this experimental protocol, but revealed an equimolar amount of 2-butanedioic acid-TMS (Fig. 9b). The EI-mass spectrum of the predominant 12.3 min peak seen in the TIC showed a base peak for pyrilamine at *m/*z 121 (Fig. 9c). Table 3 summarizes fragments from pyrilamine in comparison with those in *O*-DMP-mono-TMS, indicating fragment origin.

Pharmacokinetic results

The concentrations of *O*-DMP recovered from urine and the concentrations of pyrilamine in serum of horses following treatment with pyrilamine (300 mg) both i.v. and orally were determined by GC/MS-SIM. The concentrations of *O*-DMP

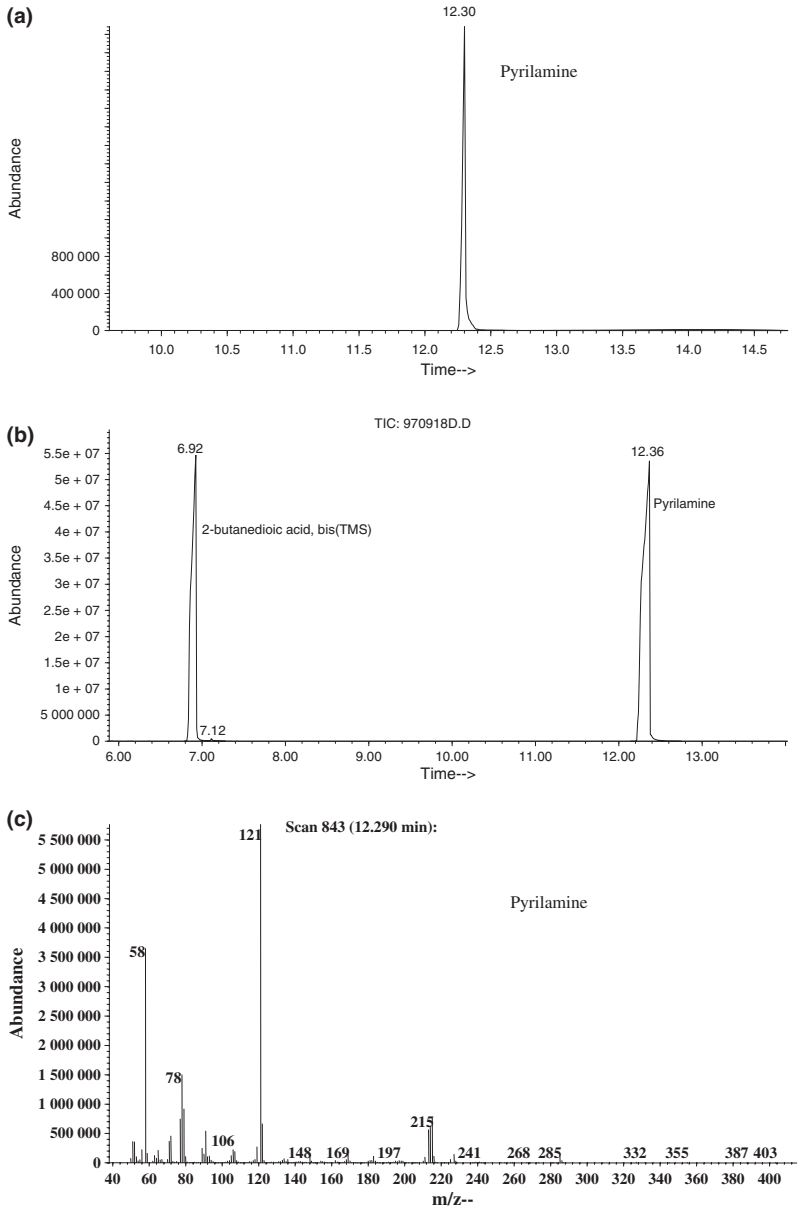


Fig. 9. Total ion chromatogram (TIC) of pyrilamine in methanol (a); TIC of pyrilamine following BSTFA+1% TMCS reaction indicating no reaction with pyrilamine, but revealing an equimolar amount of 2-butanedioic acid (b); electron impact-mass spectrum of the 12.3 min peak of pyrilamine (c).

reached peaks in urine [20 398 ± 3668 (SEM) ng/mL and 12 000 ± 4331 (SEM) ng/mL] at 2 and 8 h after treatment following i.v. and oral administrations, respectively (Fig. 10). Analysis of the serum samples showed rapid absorption of pyrilamine following oral administration in horses included in our study with the mean serum concentration of pyrilamine being 27.6 ng/mL at 10 min following oral administration (Fig. 11). Peak pyrilamine concentrations in serum [282 ± 28 ng/mL (SEM) and 36.6 ± 3.7 (SEM) ng/mL] were measured at 5 min and 0.5 h following i.v. and oral administrations, respectively (Fig. 11). Following i.v. administration serum level of pyrilamine was 2.58 ± 1.18 ng/mL (SEM) at 8 h postadministration time with the apparent elimination half-life of 1.7 h. Observed C_{max} of pyrilamine was obtained within 0.44 h (T_{max}) after oral administration, and the observed C_{max} values were in reasonably close agreement (Table 5). The serum concentration of pyrilamine following oral administration was 2.66 ± 0.37 ng/mL (SEM) at 4 h postadministration time with the apparent elimination half-life of 0.85 h. The apparent pharmacokinetic parameters of pyrilamine following i.v. and oral administration are shown in Table 4 and 5, respectively. As this study did not follow a cross-over design, for the determination of the oral bioavailability of pyrilamine, AUC_{0-inf} for each horse following oral administration was compared to the mean AUC_{0-inf} following i.v. administration of pyrilamine. The oral bioavailability of pyrilamine ranged from 9.3% to 26.7% with the mean oral bioavailability of 18.4%.

Validation of GC/MS-SIM method

The mean r^2 for the assays were 0.9982 ± 0.0009 (SEM) for serum pyrilamine and 0.9988 ± 0.0007 (SEM) for urine *O*-DMP.

Table 3. Comparison of fragmentations in pyrilamine with those in *O*-desmethylpyrilamine (*O*-DMP)-mono-trimethylsilyl (TMS), indicating losses due to fragmentation or the resultant identified fragment

Pyrilamine	*O*-DMP-TMS	Origin of each fragment
285	343	M^+
214 cluster	272 cluster	M^+ minus $CH_2{=}CHN(CH_3)_2$
182		M^+ minus $CH_2{=}CHN(CH_3)_2$ and OCH_3
171		M^+ minus $CH_2{=}CHN(CH_3)_2$ and $C{=}OCH_3$
148	206	M^+ minus C_5NH_4 and $CH_2N(CH_3)_2$
121, b.p.	179, b.p.	M^+ minus C_5NH_4-*N*-$CH_2CH_2N(CH_3)_2$
	163	CH_2-C_6H_4-p-OTMS minus CH_3
	149	*m/z* 163 minus CH_2
135 cluster	135 cluster	$C_5NH_4(CH_2)CH_2CH_2$
119	119	C_5NH_4-$CH{=}CH_2$
106	106	*O*-$C_6H_4CH_2$
	89	OTMS
78 cluster	78 cluster	C_5NH_4
	73	TMS
58	58	$CH_2N(CH_3)_2$

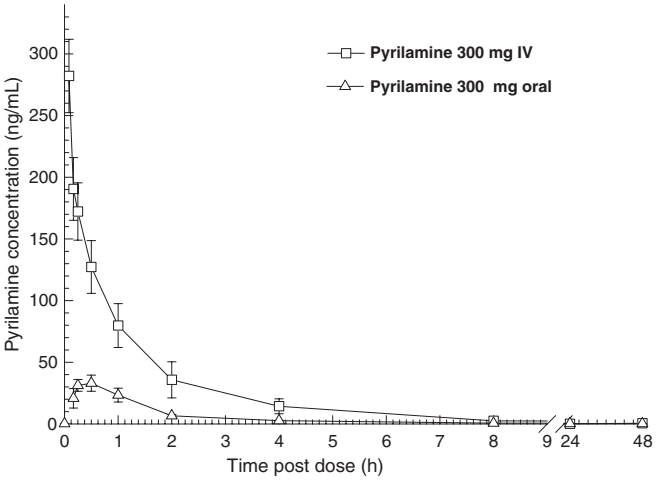


Fig. 11. Mean serum concentrations of pyrilamine (± SEM) following single 300 mg intravenous and oral administration of pyrilamine by gas chromatography/mass spectrometry-selected ion mode.

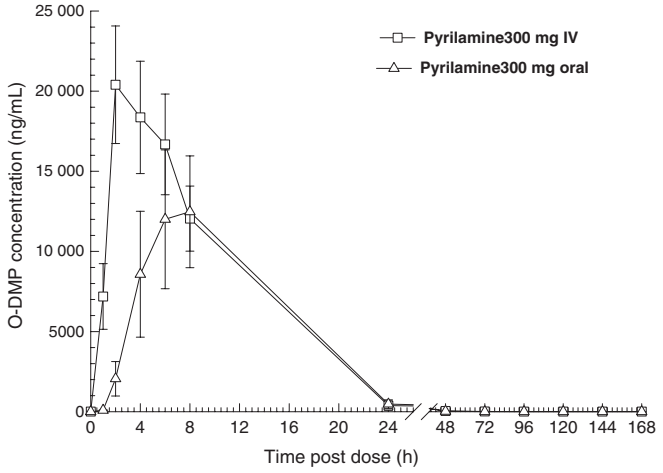


Fig. 10. Mean urinary concentrations of *O*-desmethylpyrilamine (± SEM) following single 300 mg intravenous and oral administration of pyrilamine by gas chromatography/mass spectrometry-selected ion mode (n = 4).

The assay recovery for serum pyrilamine was 90.8% and for urine *O*-DMP was 76.2%. The LOD and LOQ for pyrilamine extracted from serum were 2.25 and 6.4 ng/mL, respectively. The LOD and LOQ for *O*-DMP extracted from urine were 2.11 and 5.3 ng/mL, respectively. The validation parameters are summarized in Table 6.

DISCUSSION

The Neogen pyrilamine ELISA is exceptionally sensitive, with an I_{50} for parent pyrilamine of <100 pg/mL as such, this test readily detected pyrilamine equivalents for up to 11 h in serum and 69 h in urine following the administration of 300 mg pyrilamine i.v. The pyrilamine equivalents are putatively composed of pyrilamine, *O*-DMP-*O*-glucuronide, 5′-hydroxypyrilamine, *N*,*O*-desmethylpyrilamine and possibly other metabolites. During the analysis of the samples, the presence of pyrilamine or its metabolites in the sample competitively prevented the binding of pyrilamine-HRP complex to the antibody in the well. As the HRP enzyme was responsible for the color-producing reaction in the ELISA, the log of the concentration of pyrilamine and its metabolites (pyrilamine equivalents) in the sample was inversely related to the percent of maximal OD in the test well. The ELISA proved to be very sensitive to the parent compound pyrilamine and also sensitive to the synthesized metabolite *O*-DMP and to the analog tripelennamine. As the Neogen ELISA Racing Kit Manual reports that this assay does not require urine sample dilution to avoid background interaction, the test should be adequately sensitive for regulatory purposes.

Note that the sensitivity of the pyrilamine ELISA can be expressed as that concentration at which 50% of maximum color change was observed, denoted I_{50}. This was calculated as 0.075 ± 0.06 (SD) ng/mL for pyrilamine, agreeing well with Neogen Corporation's calculation of 0.07, and as 0.74 ± 0.24 ng/mL for *O*-DMP. Generally, ELISA estimates of individual compound concentrations will be fairly accurate in the vicinity of the I_{50}, whereas severe deviation from the I_{50} will result in significant under- or overestimates of analyte concentrations. This is compounded by the presence of multiple metabolites in most dosed animal plasma or urine samples, each with its own I_{50}, resulting in report of 'pyrilamine equivalents' as in Fig. 2 (top and bottom).

The full scan ESI^+ MS/MS analysis of directly infused urine samples resulted in mass spectra that were examined by spectral analysis for the presence of *O*-DMP-*O*-glucuronide. The resultant spectra confirmed *O*-DMP-*O*-glucuronide in these samples with the pseudo-molecular ion of *m/z* 448 and reasonable qualifier ions. Without authentic *O*-DMP-*O*-glucuronide standard, quantitation of the material in the samples was not possible. However, relative quantitation in terms of the intensity of the *m/z* 448 ion indicated a likely time course of the glucuronide in postadministration urine samples, peaking at 2-h after i.v. administration.

Table 4. Pharmacokinetic parameters of pyrilamine after a single intravenous administration (300 mg)

Parameter	Horse 1	Horse 2	Horse 3	Horse 4	Mean (± SD)
$t_{1/2}$ α (h)	0.318	0.383	0.237	0.295	0.308 (± 0.06)
$t_{1/2}$ β (h)	1.608	1.698	1.607	1.75	1.67 (± 0.07)
$t_{1/2}$ K_{10} (h)	0.704	0.950	0.815	0.448	0.729 (± 0.213)
A (ng/mL)	190	191	141	228	187.4 (± 35.5)
B (ng/mL)	80.9	145.3	102.9	26.7	88.97 (± 49.4)
$AUC_{o-inf.}$ (ng·mL/h)	274	462	287	165	297 (± 123)
Cl_s (L/h)	1092	650	1046	1824	1153 (± 489)
Vd_c (L)	1107	890	1230	1181	1102 (± 150)
Vd_β (L)	2532	1592	2419	4607	2788 (± 1283)
Vd_{ss} (L)	1884	1310	2076	2335	1901 (± 435)
AUMC (ng·h^2/mL)	475	930	570	211.92	547 (± 297)
MRT (h)	1.73	2.02	1.99	1.29	1.76 (± 0.34)
R^2	0.980	0.997	0.996	0.979	0.988 (± 0.009)

Table 5. Pharmacokinetic parameters of pyrilamine after a single oral administration (300 mg)

Parameter	Horse 1	Horse 2	Horse 3	Horse 4	Mean (± SD)
F (%)	23.8	13.7	9.3	26.7	18.4 (± 8.2)
$t_{1/2}$ K_{10} (h)	0.95	0.41	0.85	1.17	0.85 (± 0.32)
$AUC_{o-inf.}$ (ng·mL/h)	70.7	40.8	27.5	79.4	54.6 (± 24.5)
Oral clearance (L/h)	4243	7353	10 909	3778	6571 (± 3299)
T_{max} (h)	0.5	0.5	0.25	0.5	0.44 (± 0.13)
C_{max} (ng/mL)	41.7	31.1	29.5	44.2	36.6 (± 7.4)
AUMC (ng.h^2/mL)	90.3	78.6	32.4	118.9	80 (± 36)
MRT (h)	1.28	1.95	1.18	1.49	1.48 (± 0.34)
R^2	0.97	0.64	0.99	0.90	0.88 (± 0.16)

Table 6. Within-run and between-run precisions of gas chromatography/mass spectrometry assay used to quantify pyrilamine in serum samples, and *O*-desmethylpyrilamine in urine samples

Theoretical concentration (ng/mL) of pyrilamine in serum (n = 6)	Measured concentration (ng/mL; mean ± SD) (n = 6)	Coefficient of variation (%)
Within-run		
2	3.14 ± 0.66	21
20	22.28 ± 3.29	14.8
200	187.08 ± 5.23	2.8
Mean		12.9
Between-run		
2	2.08 ± 0.13	6.3
20	19.71 ± 2.95	15
200	194.31 ± 7.74	4
Mean		8.4
Theoretical concentration (ng/mL) of *O*-DMP in urine (n = 6)	**Measured concentration (ng/mL; mean ± SD) (n = 6)**	**Coefficient of variation (%)**
Within-run		
2	3.01 ± 0.26	8.6
16	15.95 ± 1.29	8.1
160	163.55 ± 7.91	4.8
Mean		7.2
Between-run		
2	1.96 ± 0.21	10.7
16	15.19 ± 1.40	9.2
160	156.06 ± 9.37	6
Mean		8.6

β-glucuronidase hydrolysis of metabolite-glucuronide complexes in postadministration equine urine samples have usually been performed in our laboratory using a *P. vulgata* (type L-II) enzyme preparation with incubation at 65 °C for 3 h (Harkins *et al.*, 1998). However, the synthesized *O*-DMP was found to be relatively unstable under these conditions. After incubation for 3 h at 65 °C only 20% *O*-DMP remained. Joujou-Sisic *et al.* (1996) have described β-glucuronidase hydrolysis of isoxsuprine-glucuronide using an enzyme preparation isolated from *H. pomatia* and incubation at 37 °C overnight. Hydrolysis under these milder conditions, and with the *H. pomatia* preparation, achieved a much higher concentration of recoverable isoxsuprine from urine samples. Therefore, a similar hydrolysis procedure was investigated in order to optimize the recovery of *O*-DMP from urine samples. Our optimization studies with this enzyme preparation indicated the maximum recoverable *O*-DMP with an incubation time of between 6 and 16 h, and with at least 5000 units enzyme/sample.

Pharmacokinetic parameters of pyrilamine administration were also calculated for both i.v. and oral administrations (Tables 4, 5). Comparison of the AUC_{0-inf} following oral and i.v. administrations suggested relatively low (18.4%) bioavailability of pyrilamine in horses. As this study did not follow a cross-over design, for the determination of the oral bioavailability of pyrilamine, AUC_{0-inf} for each horse following oral administration was compared to the mean AUC_{0-inf} following i.v. administration of pyrilamine. It is known that the best way to determine the bioavailability of drugs is to use a Latin Square design (cross-over study) by comparing each animal to itself as a control following appropriate washout period. On the other hand, the primary

objective of this study was not to determine the exact magnitude of the bioavailability, but to have an idea about the rate and extent of absorption of pyrilamine following oral administration in horses. It should be remembered that one of the disadvantages of Latin square design is the washout period, and in this model we assumed that systemic clearance does not change in each study subject during the waiting period. Therefore, we strongly believe that we would not see drastically different results if the same horses were crossed over. Pyrilamine is also rapidly cleared from blood samples of horses with the apparent elimination half-life of 0.85 and 1.67 h following oral and i.v. administration of pyrilamine, respectively.

Pyrilamine is widely used in equine medicine in the United States, and oral preparations of pyrilamine in combination with other agents are widely marketed to horsemen. Consistent with this widespread use of pyrilamine, pyrilamine or its major urinary metabolite, *O*-DMP, are not uncommon identifications in postrace urine samples. For example, in one racing jurisdiction in United States, when the number of ELISA tests performed on each postrace sample was increased from 3 to about 30, the number of postrace identifications of pyrilamine increased substantially.

Given the high sensitivity of the Neogen ELISA test for pyrilamine and its major urinary metabolite *O*-DMP, it seems likely that many, if not most, of these identifications could represent detection of sub-therapeutic or trace residues of these agents at relatively long periods after the last administration of pyrilamine. Consistent with this belief, the data presented here show that pyrilamine, as its major urinary metabolite, *O*-DMP, remains detectable in postadministration samples for at least 2-days after a single oral or i.v. dose. If the dose was larger, or if the treatment schedule was prolonged, pyrilamine and/or its major urinary metabolites would presumably remain detectable at trace levels for periods significantly longer after the last administration.

It should be also noted that the oral bioavailability of pyrilamine in horses is relatively poor, on the order of about 18.4% based on data in the present study. This relatively poor oral bioavailability of pyrilamine suggests less likely induction of significant pharmacological or performance influencing effects of pyrilamine in racehorses following oral administrations. These factors should be borne in mind by horsemen and veterinarians when pyrilamine is recommended for oral administration to horses, where the modest likelihood of a significant pharmacological or therapeutic effect must be balanced against a relatively long period during which the horse's urine may contain detectable traces of pyrilamine or its major urinary metabolite, *O*-DMP. Additionally, the ELISA test for pyrilamine is exceptionally sensitive, and there is a considerable potential for pyrilamine and *O*-DMP to be retained in horse urine at trace levels for relatively long periods after the last administration of this agent, especially if the administration is oral. Finally, the potential for pyrilamine and *O*-DMP to be retained in horse urine at trace levels for relatively long periods after the last administration of this agent should be kept in mind by regulatory or veterinary authorities reviewing trace level evidence for the presence of pyrilamine and/or *O*-DMP in postrace or postcompetitive event samples.

ACKNOWLEDGEMENTS

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