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Title: Pyrilamine in the horse: II. Detection and pharmacokinetics of pyrilamine and its major urinary metabolite

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SUMMARY

Pyrilamine is an antihistamine used in human and equine medicine. Since antihistamines produce central nervous system effects in horses, pyrilamine has the potential to affect the performance of racing horses. The predominant equine urinary metabolite was observed to be O-desmethylpyrilamine. The presence of O-desmethylpyrilamine-O-glucuronide in post-administration urine samples was confirmed by LC/MS/MS. The pyrilamine ELISA and GC/MS-SIM methods readily detected pyrilamine and O-desmethylpyrilamine in equine blood and urine. After intravenous injection of 100mg pyrilamine the ELISA detected pyrilamine equivalents in serum for up to11hrs and in urine for up to 69hrs. The GC/MS-SIM method was used to quantify pyrilamine and O-desmethylpyrilamine in equine blood and urine. After intravenous administration of pyrilamine (300mg/horse) serum pyrilamine concentrations declined from about 280ng/ml at 5min post-dose to about 2.5ng/ml at 8hrs post-dose. After oral administration of pyrilamine (300mg/horse) serum concentrations peaked at about 33ng/ml at 30min, falling to <2ng/ml at 8hrs post-dose. No pyrilamine was detected in serum at 24hrs post-dosing by either route. The recovery of O-desmethylpyrilamine from dosed horse urine was optimized by incubating the urine samples 12hrs at 37°C with *Helix pomatia* β-glucuronidase. Hydrolysis was followed by solid phase extraction, trimethylsilyl derivatization and GC/MS-SIM analysis to determine the apparent O-desmethylpyrilamine concentrations. After intravenous injection of pyrilamine (300mg/horse) O-desmethylpyrilamine was recovered at a level of about 20µg/ml at 2hr post-dose thereafter declining to about 2ng/ml at 168hrs post-dose. After oral administration, the O-desmethyl-pyrilamine recovery peaked at about 12µg/ml at 8hrs post-dose and declined to <2ng/ml at 168hrs post-dose.

INTRODUCTION

Pyrilamine (N-[(4-methoxyphenyl)methyl]-N',N'-dimethyl-N-2-pyridinyl-1,2-ethanediamine, Figure 1) 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). Pyrilamine is classified by the Association of Racing Commissioners International as a class 3 agent, and its use in racing horses is thereby prohibited because it may alter the performance of horses during competition. Therefore, methods for detection and identification of antihistamines and/or their metabolites in biological samples from horses is needed for effective control of these agents. Detection of pyrilamine and/or its metabolites in post-race blood or urine samples may lead to significant sanctions against trainers.

Detection and identification of pyrilamine in urine samples has been hampered because the drug is extensively metabolized, and only small amounts of the parent drug are excreted in urine (reference). Recently we have reported the isolation, identification and synthesis of the major equine urinary metabolite of pyrilamine, O-desmethylpyrilamine (ODMP) (Sams, *et al*, 2001). The objectives of this study were to use this synthesized metabolite to develop sensitive screening, confirmational and quantitative detection methods for pyrilamine and the major metabolite, and to explore the relationships between dose, route of administration, pharmacological response, and analytical findings after intravenous and oral administrations of pyrilamine to horses. Some of the pharmacokinetics data from one horse has been summarized in a preliminary report (Woods, *et al*, 2000).

MATERIALS AND METHODS

Horses:

Six 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. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). 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 two hours before and eight hours after drug administration. Water was 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:

For the pharmacokinetics study, four horses were administered pyrilamine (Sigma Chemical Co., St Louis, MO) according to one of the following protocols: 100 mg intravenously, 100 mg orally, 300 mg intravenously, and 300 mg orally. From horses receiving the 100 mg doses, urine was collected at 0, 2, 4, 6, 11, 24, 48, and 69 h after dosing, and serum was collected at 0, 0.5, 1, 2, 4, 6, 11,24, 48, and 69 h after dosing. From horses receiving the 300 mg doses, urine was collected at 0, 1, 2, 4, 6, 8, 24, 48, and 72, 96, and 168 h after dosing, and serum was collected at 0, 5, 10, 15, 30 min and 1, 2, 4, 6, 8, 24, and 48 h after dosing.

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. N,O-bis(trimethylsilyl)trifluoroacetimide with 1% trimethylchlorosilane (BSTFA with 1% TMCS) was obtained from Pierce, Rockford, IL. ODMP was synthesized as previously described (Sams, *et al*, 2001).

ELISA Cross-Reactivity:

The reactivity of the pyrilamine enzyme-linked immunosorbent assay (ELISA) was tested with pyrilamine maleate, synthetic ODMP, and a group of compounds similar to pyrilamine (tripelennamine HCl, chlorpheniramine maleate, pheniramine maleate, diphenhydramine HCl, albuterol) Standards for ELISA cross-reactivity studies were prepared in methanol and diluted to appropriate concentrations in assay buffer. The pyrilamine ELISA (Neogen, Lexington, KY) was performed as previously described (Rees *et al.* 1996). 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 1hr, followed by washing and the addition of 150 µl KY-Blue substrate (Neogen, Lexington, KY). The optical density (OD) at a wavelength of 650 nm was measured with an automated microplate reader (Bio-Tek Instruments, Winooski, VT) approximately 30 min after addition of the substrate. All assay reactions were run at room temperature

ELISA quantification of pyrilamine equivalents:

Urine samples from one horse were first screened with a pyrilamine enzyme-linked immunosorbent assay (ELISA, Neogen, Lexington KY) 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, ODMP 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-horseradish peroxidase (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. During the test, the presence of pyrilamine or its metabolites in the sample competitively prevented the binding of

pyrilamine-HRP complex to the antibody. Since the HRP enzyme was responsible for the color-producing reaction in the ELISA, the log of the concentration of pyrilamine and its metabolites in the sample was inversely related to the percent of maximal optical density of the test well, which was determined at a wavelength of 650 nm with an automated microplate reader (Bio-Tek Instruments, Winooski, VT) approximately 30 min after addition of substrate. All assay reactions were run at room temperature (20.5° C).

O-Desmethylpyrilamine-Glucuronide Analysis by MS/MS

Direct infusion injection of urine samples on a Micromass (Beverly, MA) 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 IV. The samples were filtered by centrifugation (90 min at 1200 x g) through a 3000 molecular weight cutoff filter (Amicon, Beverly, MA). 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/hr via a Harvard syringe pump equipped with a 500 ml Hamilton gas-tight syringe. The MS/MS was tuned for positive ion spectra by direct infusion of 10 ng/ml 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).

Optimization of ODMP hydrolysis and extraction

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

The progress of the *Patella vulgata* β-glucuronidase hydrolysis of ODMP-glucuronide in dosed horse urine was monitored. Urine collected from a horse dosed with 300mg pyrilamine IV 2hr post-dose was diluted 1:100 with blank urine. This diluted urine, along with blank equine urine supplemented with ODMP at a concentration of 40ng/ml, was hydrolyzed with *Patella vulgata* β-glucuronidase (5000 units/ml) at 65°C for 0hr, 1hr, 2hr and 3hr. 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.

The hydrolysis of ODMP-glucuronide by Type H-5 *Helix pomatia* β-glucuronidase preparation was optimized for enzyme concentration and for incubation time. Urine collected from a horse dosed with 300mg pyrilamine IV 2hr post-dose was diluted 1:100 with blank urine. This diluted urine was hydrolyzed with *Helix pomatia* β-glucuronidase (5000 units/ml) at 37°C for 0hr, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 12hr, 16hr, and 22hr. In another experiment the diluted urine was hydrolyzed with *Helix pomatia* β-glucuronidase at 1000 units/ml, 2000 units/ml, 3000 units/ml, 4000 units/ml and 5000 units/ml at 37°C for 16hr. 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 ODMP-glucuronide in dosed horse urine by *Patella vulgata* and *Helix pomatia* β-glucuronidase preparations were compared. Urine collected from a horse dosed with 300mg pyrilamine IV at 0hr, 1hr, 2hr, 4hr, 6hr, and 8hr post-dose was diluted with blank urine (1:100 for *Patella vulgata* preparation; 1:1000 for *Helix pomatia* preparation). The diluted samples were hydrolyzed with *Helix pomatia* β-glucuronidase (5000 units/ml) at 37°C for 16hr or with *Patella vulgata* β-glucuronidase (5000 units/ml) at 65°C for 3hr. 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

Standard solutions of pyrilamine, ODMP, and albuterol were prepared in methanol. Extraction standards were prepared by the addition of a known amount of a pyrilamine and O-desmethylpyrilamine solution to blank urine samples. A known amount of an albuterol standard (20 µl of 10 µg/ml methanol solution) was added to each sample, standard, and blank as an internal standard.

The urine samples, standards, and blanks were placed in culture tubes. To each sample was added 1 ml β-glucuronidase solution (Type H-5, from *Helix 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, the pyrilamine, O-desmethylpyrilamine, and albuterol supplements were added, the samples were sonicated for 90 sec, sodium phosphate buffer (2 ml of 0.1 M, pH6) was added, and the sample pH was adjusted to 6.0 ± 0.5 with 1 M sodium hydroxide or 1 M hydrochloric acid.

Preparation of Serum Samples

The serum samples, standards, and blanks were placed in culture tubes. The pyrilamine, O-desmethylpyrilamine, and albuterol supplements were added, sodium phosphate buffer (2 ml of 0.1 M, pH6) was added, and the sample pH was adjusted to 6.0 ± 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). Clean Screen SPE columns (United Chemicals Technologies-Worldwide Monitoring, Bristol, PA) 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 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 (<40° C). For derivatization each sample was dissolved in 40 µl of BSTFA with 1% TMS, 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). The column was a J&W DB-5MS, 30m x 250µm x 0.25µm (Supelco, Bellefonte, PA). The carrier

gas was helium with a flow of 1 ml/min. A volume of 1 µl 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 MSD temperature was 280° C. Each derivatized sample was transferred to an autosampler vial. A 1 µl aliquot was injected onto the GC/MS, and the MS was run in selective ion mode (SIM) with data collected from 3 to 24.5 min. Ion 121 m/z (pyrilamine) and ion 179 m/z (ODMP-mono-TMS) were utilized along with ion 369 m/z (albuterol-tri-TMS, internal standard)) for quantitation (Table 1).

Validation of quantitative GC/MS method:

The GC/MS method for the quantitation of serum pyrilamine and urine ODMP 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 ODMP), middle (20 ng/ml for serum pyrilamine; 16 ng/ml for urine ODMP), and high (200 ng/ml for serum pyrilamine; 160 ng/ml for urine ODMP) standards.

The between-run precision was determined by comparing the calculated response (in ng/ml backfit of the standard curve) of the low (4 ng/ml for serum pyrilamine; 2 ng/ml for urine ODMP), middle (20 ng/ml for serum pyrilamine; 16 ng/ml for urine ODMP), and high (200 ng/ml for serum pyrilamine; 160 ng/ml for urine ODMP) standards over six consecutive daily runs. Standard curve correlation was measured by the mean coefficient of determination (r^2) for six consecutive daily runs (5). The recovery was determined by comparing the response (in area) of middle (20 ng/ml; 16 ng/ml) 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 6 runs.

RESULTS

Standard curves for the ELISA screening test:

A standard curve for pyrilamine using the pyrilamine ELISA test indicated that an addition of pyrilamine (0.045 ng/ml) to the system produced 50 percent inhibition of color formation (Figure 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 reacts well with ODMP (I_{50}= 0.58 ng/ml), the principle metabolite of pyrilamine, and triplennamine, another antihistamine agent, with 50% inhibition present following addition of 0.33 ng/ml of that compound. There was minimal detectable cross-reactivity with other antihistamine agents chlorpheniramine, pheniramine, and diphenhydramine.

ELISA quantification of "pyrilamine equivalents" in urine and serum:

After an intravenous dose of 100 mg, the concentrations of pyrilamine equivalents in urine and serum reached peaks of 4158 and 36 ng/ml at 2 and 0.5 h after injection, respectively (Figure 2). Pyrilamine equivalents were still detectable in urine at the last sampling time (69 h) and in serum at 11 h after dosing.

Analysis of O-desmethylpyrilamine-O-glucuronide

Urine samples collected from a horse after the administration of 300mg pyrilamine IV were analyzed for ODMP-O-glucuronide by full scan direct infusion positive electrospray ionization tandem mass spectrometry. The presence of ODMP-O-glucuronide was observed by the measurement of the ion of 448 mass units/ charge which is the pseudo ($m+H^+$) molecular ion for this compound. As there was no isolated ODMP-O-glucuronide available, the intensity of the ion of 448 mass units/ charge was used as a gauge of the concentration of the material. The ion 448 intensity peaked at 2hr post-dose and declined to pre-dose levels after 8hr (Figure 3).

Optimization of ODMP hydrolysis

Under standard conditions of hydrolysis with *Patella vulgata* β-glucuronidase (3hr at 65°C) (reference) only about 20% of ODMP supplemented into blank urine was recovered (Figure 4a). Pyrilamine appeared to be stabile under these conditions. When diluted urine (1:100) collected from a horse dosed with pyrilamine was hydrolyzed with the *Patella vulgata* β-glucuronidase preparation, recoverable ODMP rose to a level of about 40ng/ml after 1hr incubation. However, the concentration on recoverable ODMP remained the same at 2h and fell somewhat (to about 35ng/ml) at 3hr incubation (Figure 4b). Recoverable ODMP levels from urine supplemented with ODMP (40ng/ml) decreased with increased incubation time.

An alternate hydrolysis method using *Helix pomatia* β-glucuronidase preparation at a lower recommended temperature (37°C) was examined. The *Helix 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 ODMP in urine samples. The highest level added (5000units) resulted in the highest observed ODMP concentration (data not shown). The optimal time of incubation (at 37°C) was observed to be 12hrs with at least 75% of maximum between 6hr and 16hr (Figure 4c). When the hydrolysis of ODMP-O-glucuronide in pyrilamine dosed horse urine samples by the two methods was compared (Figure 4d), there was about a ten-fold increase of recoverable ODMP with the *Helix pomatia* β-glucuronidase preparation over the *Patella vulgata* β-glucuronidase preparation at all investigated collection times.

GC/MS-SIM quantification

The total ion chromatogram of the TMS derivative of chemically synthesized ODMP was generated by GC/MS (Figure 5a). The electron impact (EI)-mass spectrum of the predominate 12.6 min peak seen in the total ion chromatogram indicated a major abundance response at m/z 179 (Figure 5b). 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 (Figure 6). A peak of high abundance was seen at m/z 369. The TMS derivatives of albuterol (retention time 11.0 min) and ODMP (retention time 12.6 min) were well separated by the chromatography of the

GC/MS method (Figure 7a). The GC/MS-SIM qualifier and quantifier ions for albuterol and ODMP TMS derivatives were established (Figure 7b). Table 3 groups the quantifier and qualifier ions (listed in decreasing order) for these two compounds, and for underivatized pyrilamine.

The concentrations of ODMP recovered from urine and the concentrations of pyrilamine in serum of horses following treatment with pyrilamine (300 mg) both intravenously and orally were determined by GC/MS-SIM (Figure 8). The concentrations of ODMP reached peaks in urine (71.0 and 28.4 μg/ml) at 2 and 6 hr after treatment following intravenous and oral administrations, respectively. Peak pyrilamine concentrations in serum (352 and 22.3 ng/ml) were measured at 5 min and 1 hr after treatment following intravenous and oral administrations, respectively.

Validation of GC/MS-SIM method

The within-run precision was determined for the low (2 ng/ml: CV = 21.0% for serum pyrilamine; 2 ng/ml: CV = 8.6% for urine ODMP), middle (20 ng/ml: CV = 14.8% for serum pyrilamine; 16 ng/ml: CV = 8.1% for urine ODMP), and high (200 ng/ml: CV = 2.8% for serum pyrilamine; 160 ng/ml: CV = 4.8% for urine ODMP) concentrations of the pyrilamine and ODMP standard curves. The between-run precision was determined for the low (4 ng/ml: CV = 24.3% for serum pyrilamine; 2 ng/ml: CV = 10.7% for urine ODMP), middle (20 ng/ml: CV = 15.0% for serum pyrilamine; 16 ng/ml: CV = 9.2% for urine ODMP), and high (200 ng/ml: CV = 4.0% for serum pyrilamine; 160 ng/ml: CV=6.0% for urine ODMP) concentrations of the pyrilamine and ODMP standard curves. The mean r^2 for the assays were 0.9982 ± 0.0009 SEM for serum pyrilamine and 0.9988 ± 0.0007 SEM for urine ODMP. The assay recovery for serum pyrilamine was 90.8% and for urine ODMP was 76.2%. The LOD for pyrilamine extracted from serum was 2.25 ng/ml, and the LOD for ODMP extracted from urine was 2.11 ng/ml.

DISCUSSION

The Neogen pyrilamine ELISA kit was easily able to detect pyrilamine equivalents for up to 11 hr in serum and 72 hr in urine following the administration of 100 mg pyrilamine IV. The pyrilamine equivalents are putatively composed of pyrilamine, ODMP, 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. Since 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 of the test well. The ELISA proved to be very sensitive to the parent compound pyrilamine and also sensitive to the synthesized metabolite ODMP 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.

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 ODMP-O-glucuronide. The resultant spectra confirmed ODMP-O-glucuronide in these samples with the pseudo-molecular ion of 448 m/z and reasonable qualifier ions. Without authentic ODMP-O-glucuronide standard, quantitation of the material in the samples was not possible. However, relative quantitation in terms of the intensity of the 448 m/z ion indicated a likely time course of the glucuronide in post-administration urine samples.

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

Pharmacokinetic parameters of pyrilamine administration were calculated using PK Analyst® software (MicroMath, Salt Lake City, UT). Comparison of the Area Under the Curve from 0 to infinity ($AUC_{0-\infty}$) following oral and IV administrations suggested relatively low (12%) bioavailability of pyrilamine in horses.

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The urine concentrations of recoverable ODMP after IV and oral administrations were essentially equivalent at 6hrs post-dosing. This suggests rapid oral absorption and extensive first-pass metabolism followed by enterohepatic circulation of this agent.

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, ODMP, are not uncommon identifications in post-race urine samples. For example, in one racing jurisdiction in United States, when the number of ELISA tests performed on each post-race sample was increased from 3 to about 30, the number of post-race identifications of pyrilamine increased substantially.

Given the high sensitivity of the Neogen ELISA test for pyrilamine and its major urinary metabolite ODMP, 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, ODMP, remains detectable in post-administration samples for up to one week after a single oral or IV dose of this agent. If the dose was larger, or if treatment schedule was prolonged, pyrilamine and or its major urinary metabolites would presumably remain detectable at trace levels for periods significantly longer than one week after the last administration.

Beyond this, it is of interest to note that the oral bioavailability of pyrilamine is relatively poor, in the order of about 12 %, based on these data. Based on this finding, it is even less likely that oral administration of pyrilamine is likely to be associated with significant pharmacological or performance influencing effects in racing horses. 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 for which the horses urine may be at risk of containing detectable traces of pyrilamine or its major urinary metabolite, ODMP.

Similarly, the potential for ODMP 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 presence of pyrilamine or its major urinary metabolite, ODMP in post-race samples.

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Table 1 Quantifier and qualifier ions (listed in decreasing order of abundance) acquired as part of the pyrilamine SIM routine

Ion type	Albuterol-tris(TMS), I.S.	O-Desmeth yl-pyrilamine	Unmetabolized Pyrilamine
Qualifier-1	86.0 (b.p.)	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	(73.0)
Qualifier-5	179.1	285.2	
Quantifier ion	*369.3*	*179.1 (b.p.)*	*121.1 (b.p.)*

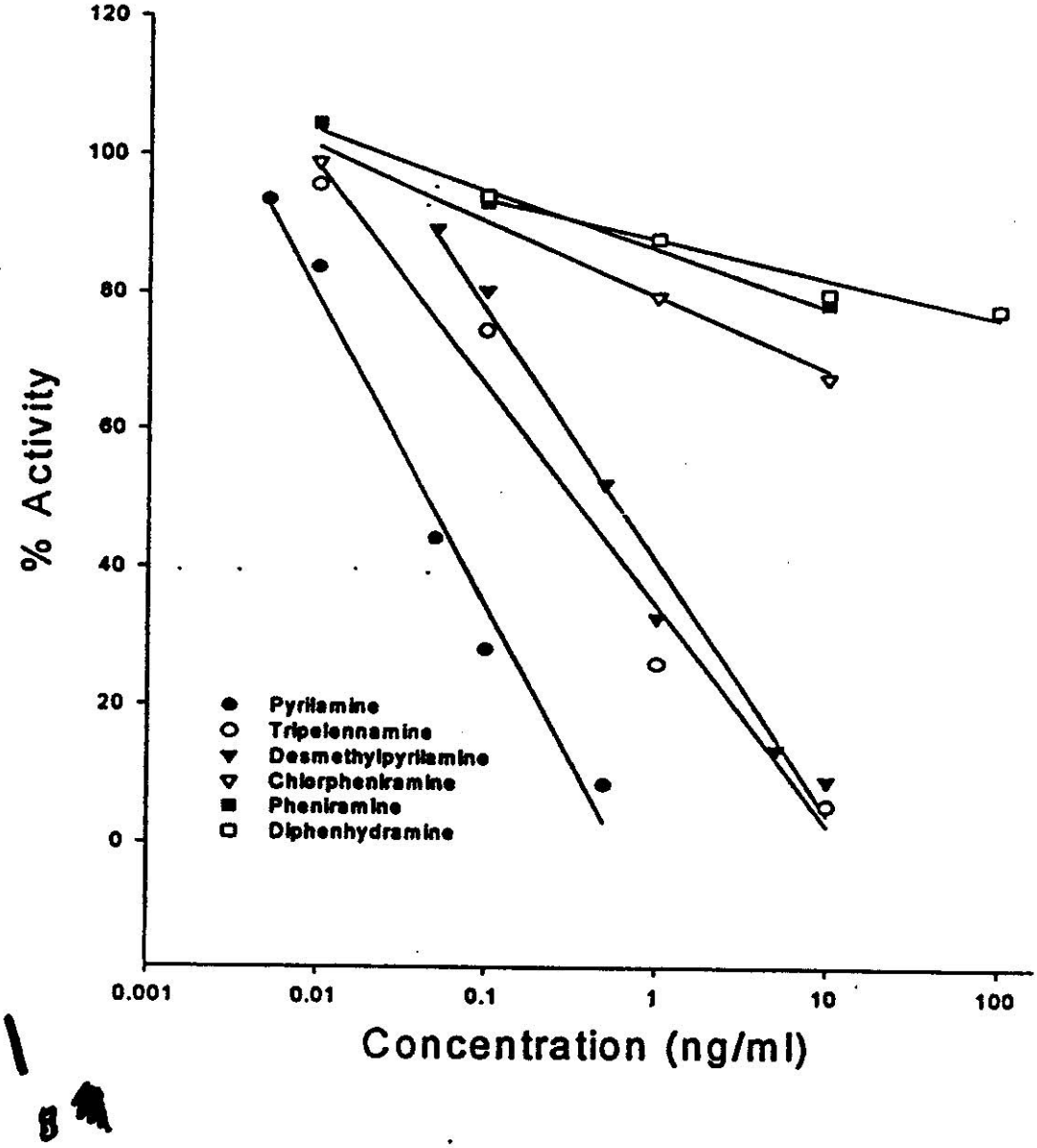


Figure 6 Regression of ELISA standard curves for pyrilamine and its comparison to standard curves for tripelennamine, desmethylpyrilamine, chlorpheniramine, pheniramine, and diphenhydramine showing the cross-reactivity of those agents using the Pyrilamine ELISA test

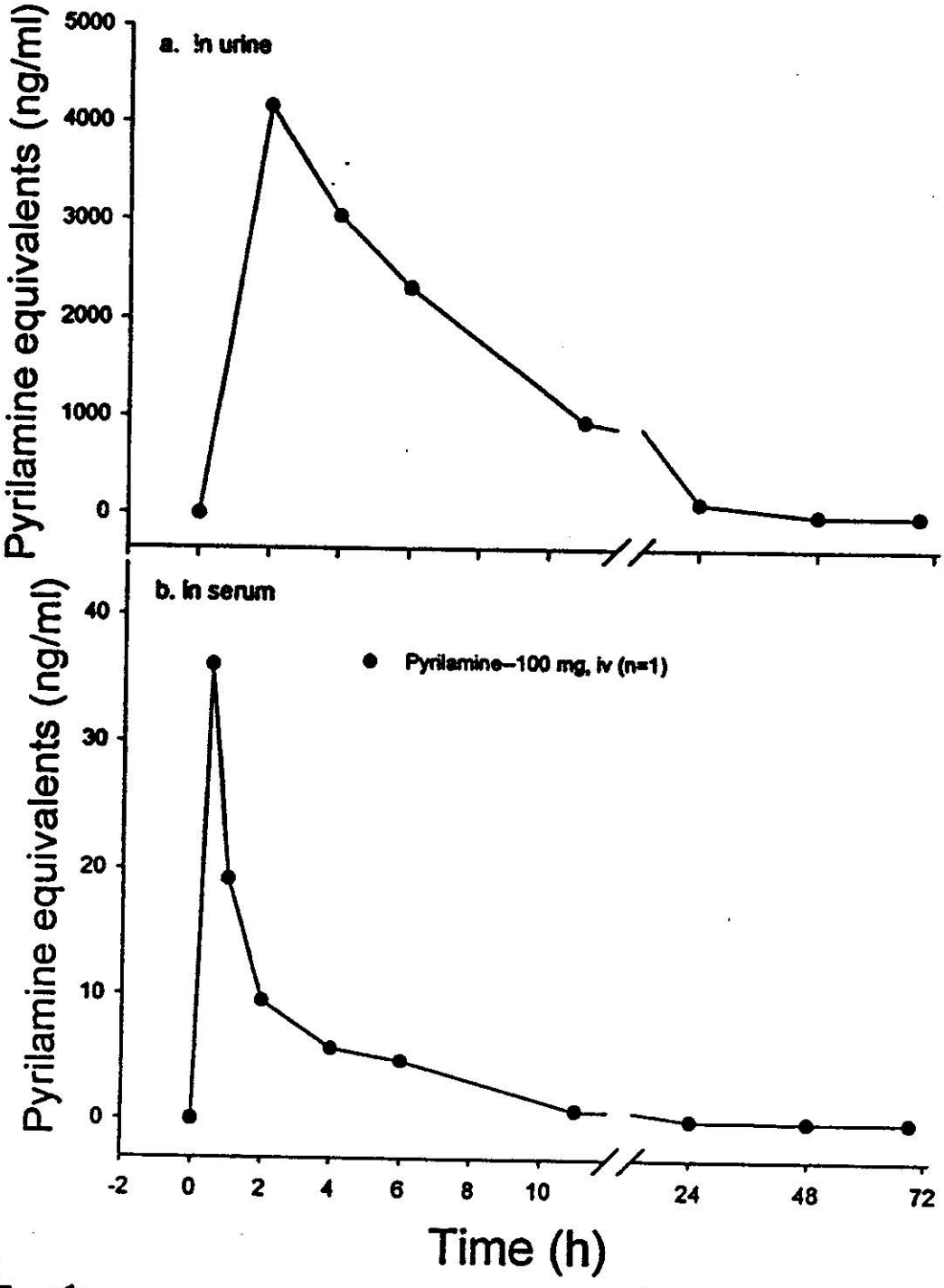


Figure 8 ELISA detection of pyrilamine equivalents in a) urine and b) serum following administration of intravenous pyrilamine (100 mg)

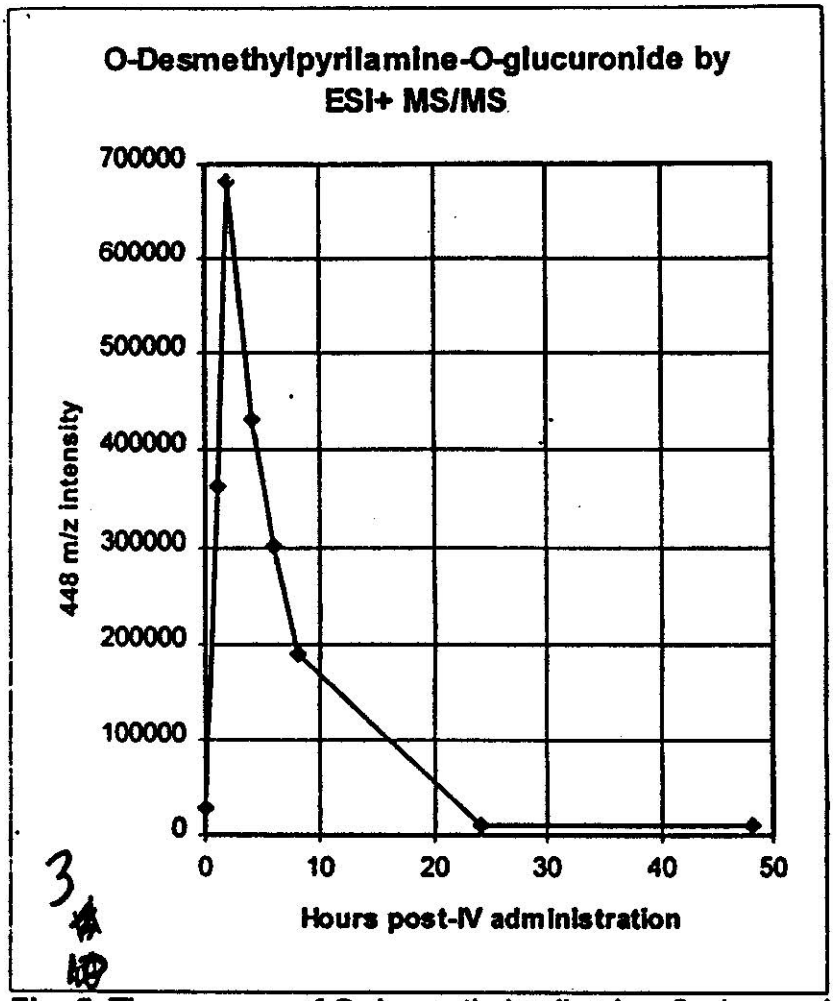


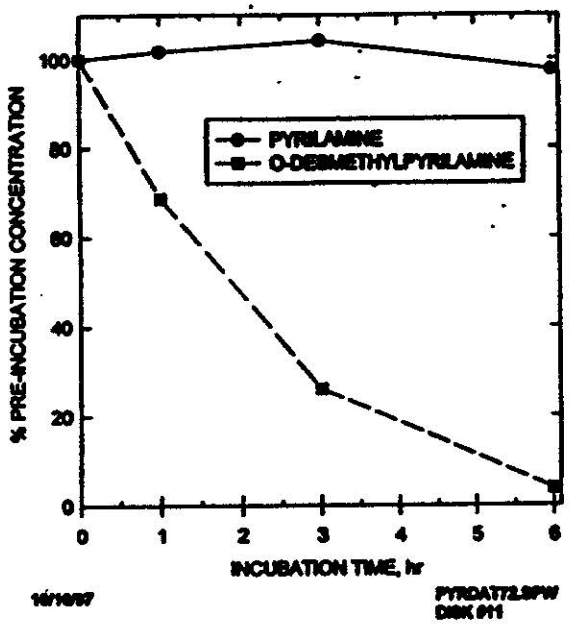
Fig. β. Time course of O-desmethylpyrilamine-O-glucuronide post-IV administration. The glucuronide was measured by full scan ESI$^+$ MS/MS as the 448 m/z monoprotonated cation; the mass spectrum underwent background subtraction and peak smoothing prior to plotting the intensity of this ion relative to the number of hours post-administration.

electrospray Ionization
direct Infusion

RRF 17

A

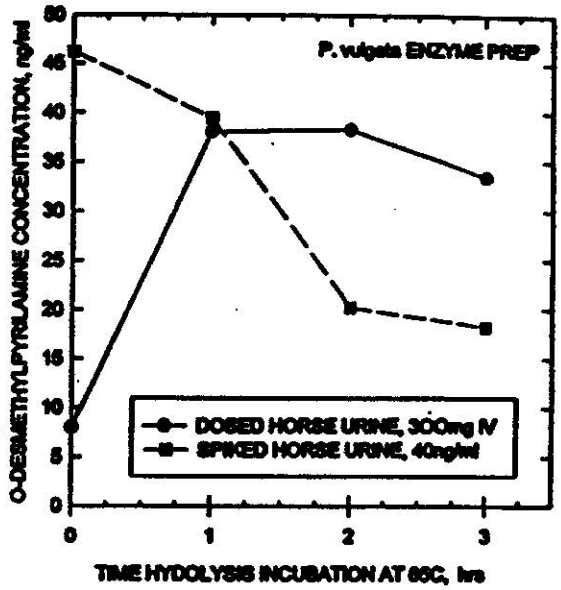
PYRILAMINE & O-DESMETHYLPYRILAMINE
BY GC/MS-SIM
STABILITY IN HORSE URINE AT 65C



10/10/97 PYRDAT72.SPW DISK #11

B

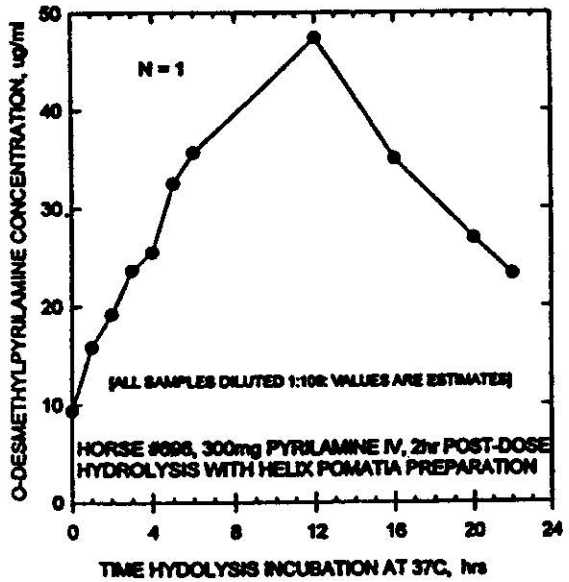
O-DESMETHYLPYRILAMINE BY GC/MS
FROM SPIKED AND DOSED HORSE URINE
WITH INCREASED INCUBATION TIME



6/23/98 PYRDAT14.SPW DISK #39

C

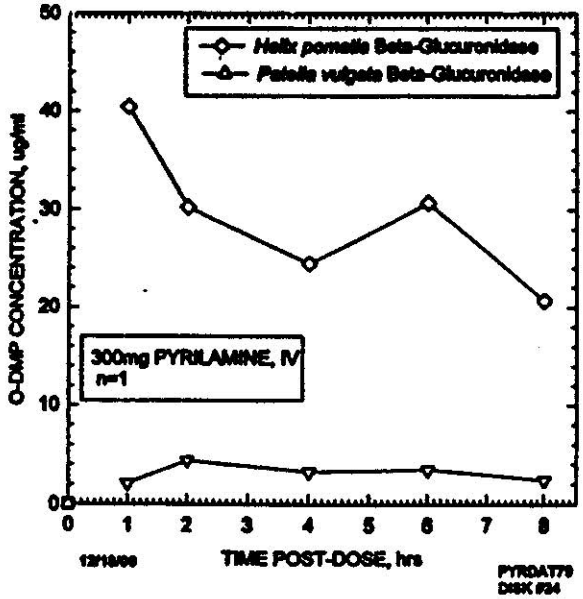
O-DESMETHYLPYRILAMINE BY GC/MS
FROM DOSED HORSE URINE
WITH INCREASED HYDROLYSIS TIME



09/16/98 PYRDAT2.SPW DISK #16

D

O-DMP BY GC/MS: DOSED HORSE URINE
COMPARISON OF ENZYME PREPARATIONS



12/10/98 PYRDAT79 DISK #34

Figure # 2 4

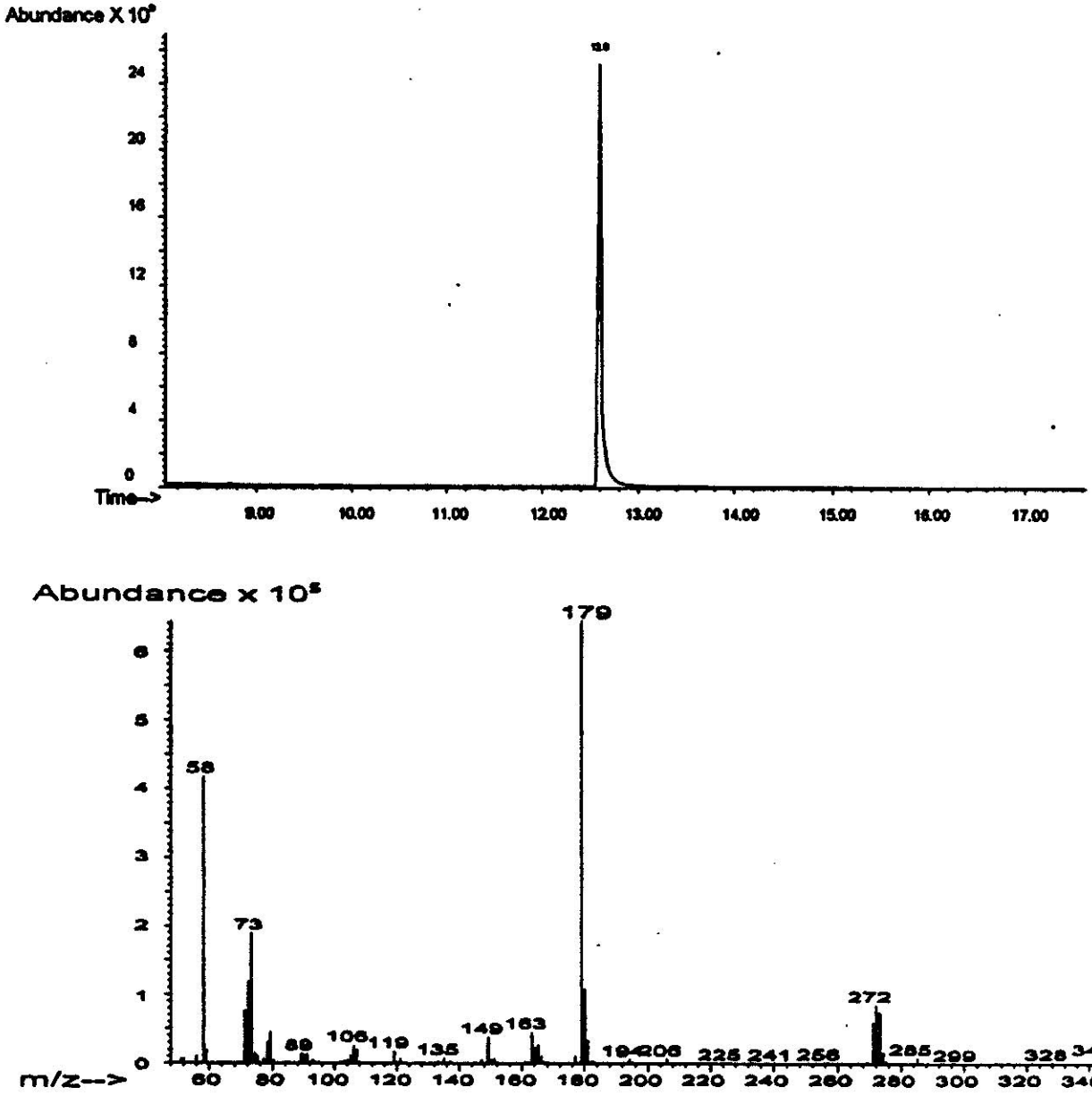


Figure 4 a) Total ion chromatogram of the synthesized O-desmethylpyrilamine compound; b) EI-mass spectrum of the 12.6 min peak seen in Figure 4a.

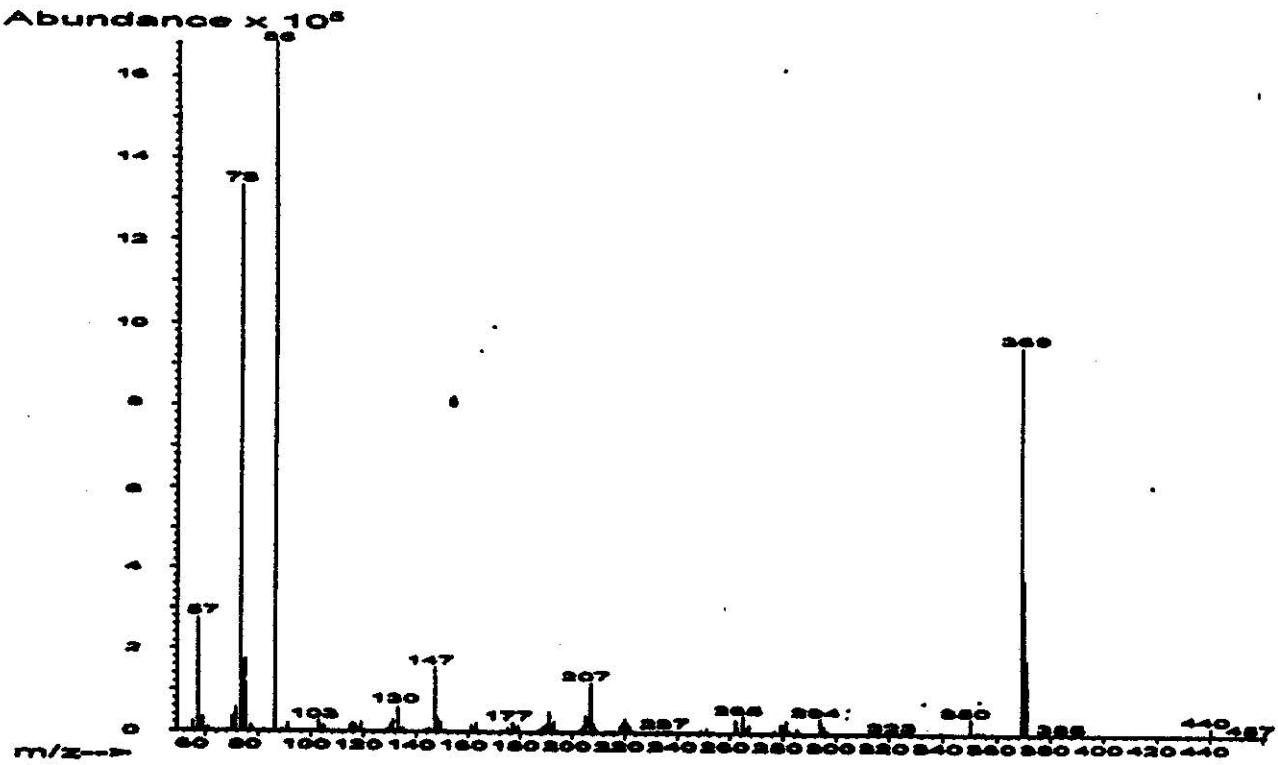
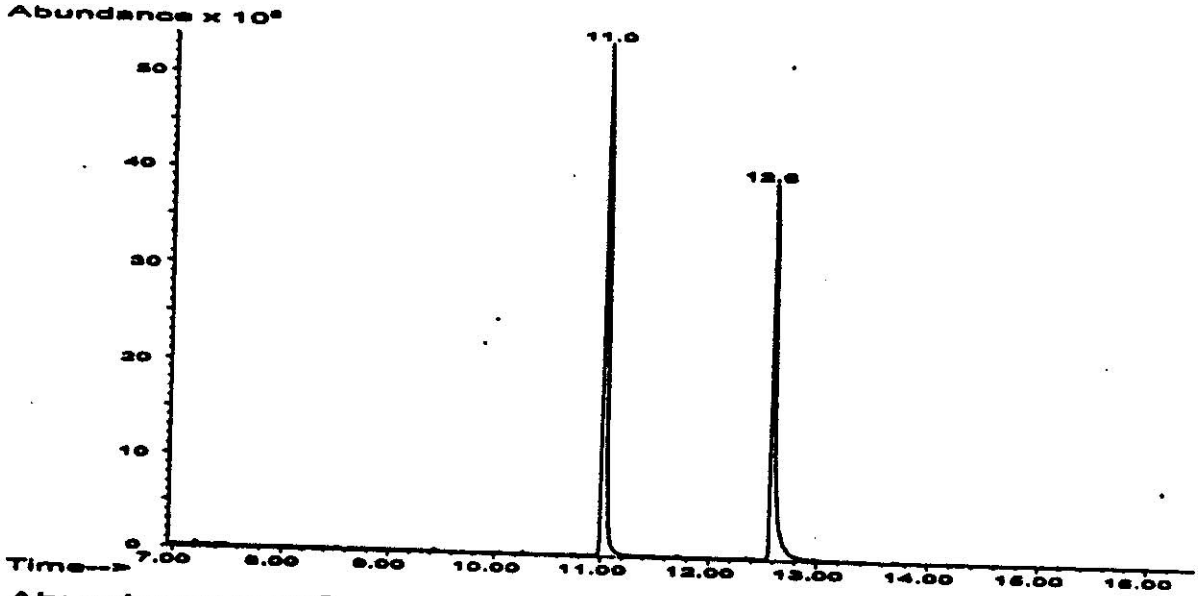


Figure 3 EI-mass spectrum of the tris(TMS) derivative of albuterol

36



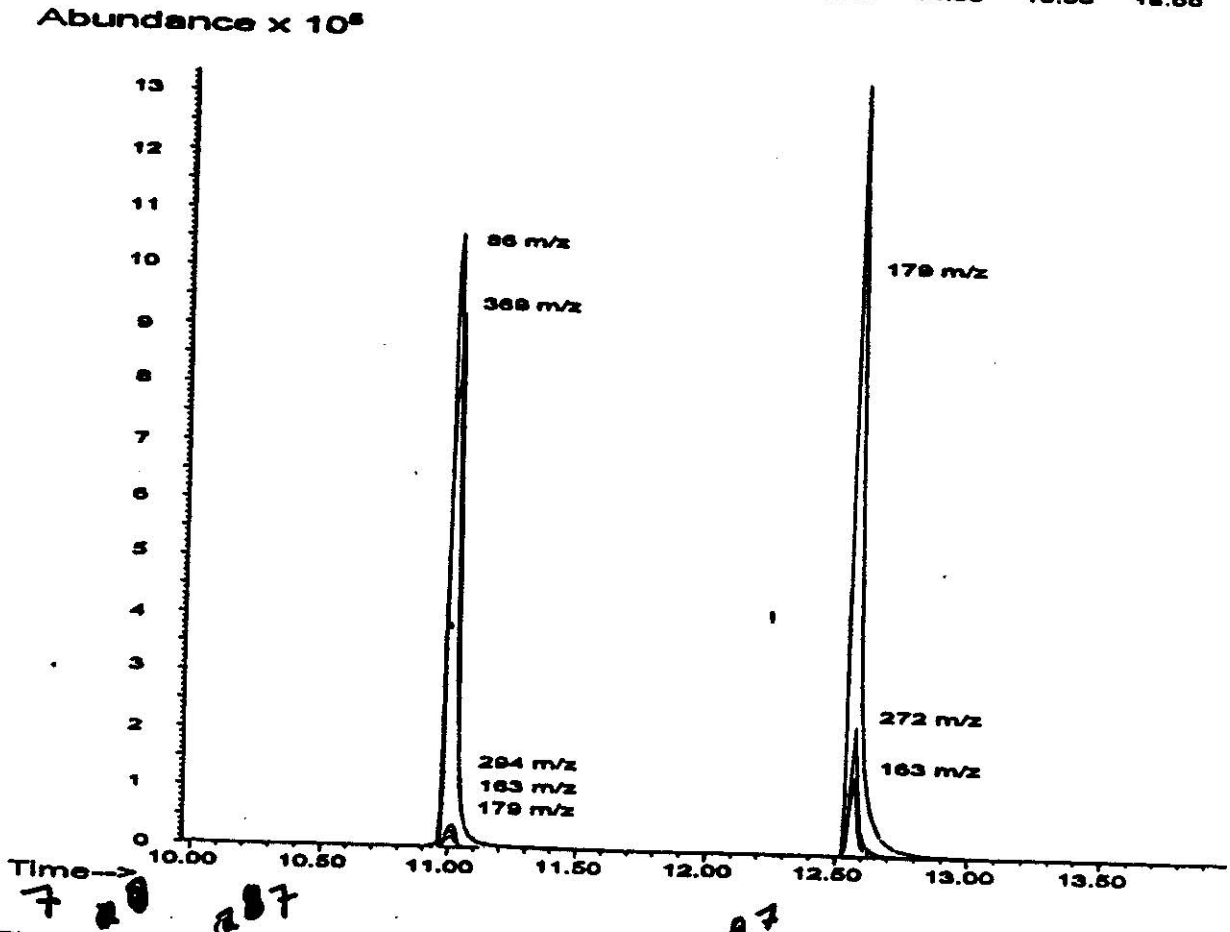


Figure 5 Figure 5a shows the chromatographic separation of the TMS derivatives of albuterol (11.0 min) and O-desmethylpyrilamine (12.6 min). Figure 5b shows the overlap of qualifier and quantifier ions for albuterol and O-desmethylpyrilamine, and Table 3 groups the quantifier and qualifier ions (listed in decreasing order) for the two compounds.

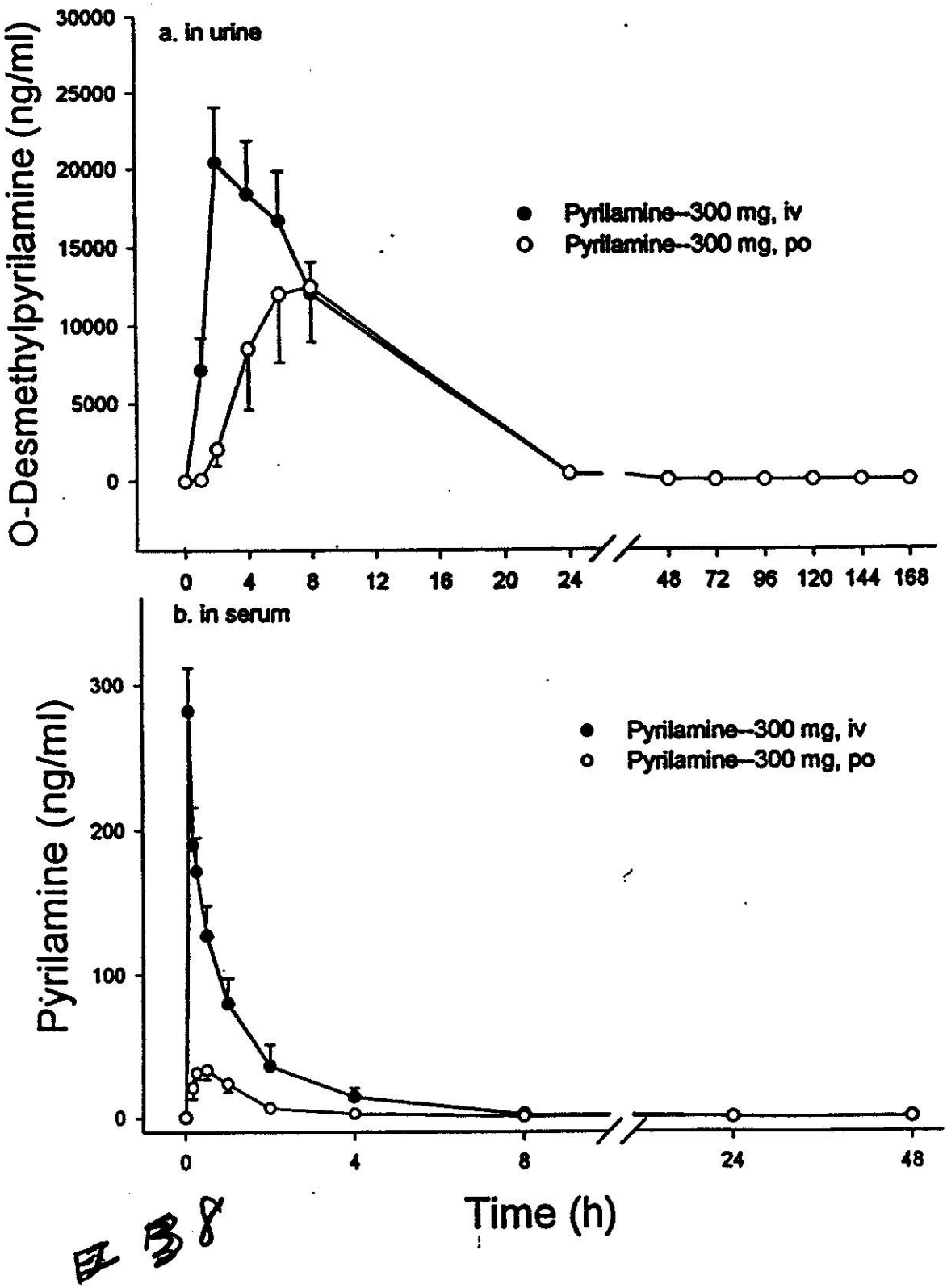


Figure 8 Concentration of a) O-desmethylpyrilamine in urine and b) pyrilamine in serum following oral (300 mg) and intravenous (300 mg) administration of pyrilamine