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Pharmacokinetics of intravenous and oral prethcamide in horses

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Abstract

The respiratory stimulant prethcamide is a mixture of equal parts of crotethamide and cropropamide. A specific and sensitive gas chromatographic method for the determination of crotethamide and cropropamide in horse plasma and urine is described. Both components of prethcamide were extracted from plasma and urine into dichloromethane. The extracts were analyzed by capillary gas chromatography with thermionic detection in the nitrogen-specific detection mode. The lower limits of quantitation were 4.0 ng ml^{-1} of plasma and 10.0 ng ml^{-1} of urine. Calibration curves were linear from 2.0–100 ng ml^{-1} of plasma for both components. Pharmacokinetic parameters for crotethamide and cropropamide after intravenous and oral dosing were estimated by analysis of plasma concentration versus time data. The total plasma clearance of cropropamide was greater than that of crotethamide and both values were greater than 5 ml min^{-1} kg^{-1}. Renal clearance values of the two drugs were comparable and were much less than estimates of filtration clearance values in horses, indicating extensive re-absorption of both components from the renal tubules. Both compounds were metabolized by N-demethylation of the [(dimethylamino)-carbonyl]-propyl moiety and these metabolites were excreted in urine. The method was demonstrated to be suitable for detecting illicit administration of prethcamide to competition horses.

Keywords: Capillary column GLC; Cropropamide; Crotethamide; Horses; Mass spectrometry; Metabolites; Pharmacokinetics; Prethcamide; Oral bioavailability

1. Introduction

Prethcamide is a mixture of equal parts of crotethamide and cropropamide (Fig. 1) that is used clinically as a respiratory stimulant to produce arousal from drug-induced depression. Prethcamide produces a marked increase in ventilation, primarily as a result of increased tidal volume, with minimal increases in respiratory rate. Prethcamide is marketed for parenteral and oral administration to humans in several European and South American countries but is not approved by the U.S. Food and Drug Administration for use in either human or veterinary medicine in the United States.

Prethcamide is banned by several sports governing bodies, including the International

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Olympic Committee [1], the National Collegiate Athletic Association [2], and the International Amateur Athletic Federation [2], because of its potential to stimulate performance of athletes. Recently there has been concern that prethcamide has been administered to show horses in an attempt to make the horses appear more alert during competition. Although the presence of prethcamide or its metabolites in blood or urine test samples is prohibited by horse show association rules, information concerning the detection and identification of the components of the drug or their metabolites in test samples collected from horses is lacking. Therefore, the objectives of this study were: (1) to develop a gas chromatographic method for detecting crotethamide and cropropamide and their metabolites in extracts of plasma and urine samples collected from horses; (2) to investigate the pharmacokinetics of prethcamide after intravenous and oral dosing to horses; and (3) to isolate and characterize metabolites of prethcamide in urine samples collected from horses.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical-reagent-grade or better. Dichloromethane and ethyl acetate were purchased from Burdick & Jackson Laboratories (Muskegon, MI). Crotethamide and cropropamide were obtained from Ciba-Geigy Limited (Basle, Switzerland), and nikethamide was obtained from Ciba-Geigy Pharmaceutical Company (Summit, NJ).

2.2. Instrumentation for quantitative measurement

The gas chromatograph (model 3700, Varian Associates, Palo Alto, CA) was equipped with a thermionic detector that was operated in the nitrogen-specific detection mode and an on-column capillary injector (J & W Scientific Inc., Folsom, CA). Separations were performed on a 25 m × 0.257 mm i.d. capillary column coated with 14% cyanopropylphenyl siloxane (DB-1701, 0.25 μm film thickness, J & W Scientific Inc.). A 5 m × 0.25 mm i.d. section of deactivated fused silica capillary tubing was used to connect the injector to the analytical column. The column oven was programmed to increase from an initial temperature of 85°C (after a 1.0 min delay following sample injection) at a rate of 15°C min^{-1} to a final temperature of 180°C, which was maintained isothermal for 7 min. For the analysis of urine sample extracts, the oven temperature was then increased from 180°C to 280°C and held for 5 min before returning to the starting temperature. The detector temperature was 330°C. Helium was used as a carrier gas at a flow rate of 2.5 ml min^{-1}. The output was recorded on an electronic integrator (Varian Associates).

2.3. Analytical procedure for quantitative measurements

Stock solutions of crotethamide, cropropamide, and nikethamide were prepared in ethyl acetate at a concentration of 1.00 mg ml^{-1} and stored in the dark at 4°C. Working solutions of crotethamide and cropropamide were prepared by serially diluting both stock solutions to 10.0 μg ml^{-1} and 2.0 μg ml^{-1} with ethyl acetate. The working solution of nikethamide was prepared by diluting the stock solution to 10.0 μg ml^{-1} with ethyl acetate. Working solutions were stored in the dark at 4°C.

Plasma calibrators were prepared by adding appropriate volumes of the working solutions of crotethamide and cropropamide to 12 ml screw-cap tubes containing 1.0 ml of normal saline solution. A 6.0 μl aliquot of the working solution of nikethamide was added to each tube for use as internal standard. Then 1.0 ml of drug-free plasma was added to each tube and the contents were mixed well. Plasma calibrators were prepared with crotethamide and cropropamide ranging in concentration from 2.0–100 ng ml^{-1}; the concentration of nikethamide was 60 ng ml^{-1}. A set of plasma calibrators was prepared and analyzed with each set of unknown samples.

Urine calibrators were prepared by adding appropriate volumes of the working solutions of crotethamide and cropropamide to 12 ml screw-cap tubes containing 1.0 ml of normal saline

solution. Drug-free horse urine (1.0 ml) was then added to each of these tubes. The concentrations of crotethamide in urine calibrators ranged from 10.0–100 ng ml^{-1} and those of cropropamide from 4.0–60 ng ml^{-1}.

Unknown plasma samples were prepared for analysis by pipetting 1.0 ml of each into a 12 ml screw-cap test tube containing 1.0 ml of normal saline solution and 6.0 μl of the working solution of nikethamide. If unknown samples were found to contain either crotethamide or cropropamide at a concentration greater than 100 ng ml^{-1} (i.e. the upper limit of the calibration curve) then the sample was reanalyzed using a smaller volume of sample and a sufficient volume of drug-free plasma to produce a total volume of 1.0 ml of plasma.

Each plasma calibrator or unknown sample was mixed with 1.0 ml of carbonate buffer (0.1 M, pH 11.2). The buffered plasma samples were then extracted for 10 min by end-over-end rotation with 5 ml of dichloromethane. The mixtures were centrifuged at 2200*g* for 5 min and the aqueous phases were removed by aspiration. The organic phases were transferred to 8 ml conical centrifuge tubes and evaporated to dryness at 45°C under nitrogen. The residues were then stored overnight in a vacuum desiccator over anhydrous calcium carbonate. The dried residues were dissolved in 50 μl of ethyl acetate and 3 μl of each was injected into the gas chromatograph.

Urine calibrator and unknown samples were extracted as described above for plasma samples except that the organic extracts were washed with 1.0 ml of carbonate buffer (0.1 M, pH 11.2) before evaporation.

Peak heights were determined by electronic integration and peak height ratios of each analyte to that of the internal standard in calibrators and unknown samples were calculated. The regression line of peak height ratios versus calibrator concentration with equal weighting of the data was calculated and the concentrations of each analyte is unknown plasma samples were determined from the regression line. Concentrations of analytes in extracts of urine samples were determined from the regression of peak height versus calibrator concentration.

Accuracy and precision were determined by supplementing drug-free plasma and urine with crotethamide and cropropamide at concentrations of 4.0 ng ml^{-1} of plasma ($n = 12$) and 10 ng ml^{-1} of urine ($n = 10$). Concentrations of each were determined as described above.

2.4. Determination of blood to plasma concentration ratio

The blood to plasma concentration ratio was determined by adding 2.0 ml of fresh, drug-free, heparinized blood to each of 10 screw-top glass tubes containing 400 ng each of crotethamide and cropropamide. The tubes were rotated slowly end-over-end for 30 min at room temperature and then centrifuged at 2200*g* for 20 min. The plasma fractions were separated and concentrations of crotethamide and cropropamide were determined by gas chromatography as described above. The blood to plasma concentration ratios were calculated by dividing the initial drug concentration in blood by the measured drug concentration in plasma.

2.5. Determination of plasma protein binding

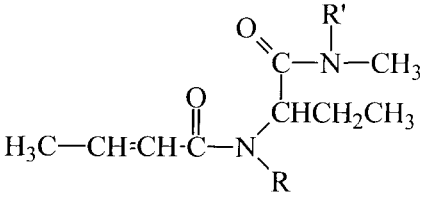
The extent of plasma protein binding was determined by adding 0.5 ml of fresh, drug-free plasma to each of 20 screw-top glass tubes containing either 80 or 400 ng each of crotethamide and cropropamide. The contents of each tube were mixed and transferred to micropartition filtration devices (Centrifree®, Amicon Corporation, Danvers, MA). The devices were centrifuged at 2200*g* for 30 min at 37°C. The concentrations of crotethamide and cropropamide in 0.25 ml aliquots of plasma filtrates were determined as described above except that calibrators were prepared from a filtrate of drug-free plasma. The free fraction was calculated by dividing the drug concentration in the filtrate by the initial plasma drug concentration. The bound fraction was calculated by subtracting the free fraction from unity and the percent protein binding was calculated by multiplying the bound fraction by 100.

2.6. Animal studies

Six female horses aged 2–13 years and weighing 408–546 kg were used for these studies. The horses were judged to be in good health based on clinical examination, clinical chemistry profile, and hemogram. Horses were maintained in a pasture and were provided with hay, trace mineral salt, and water *ad libitum*. On experimental days the horses were transferred to 10 m^2 box stalls approximately 2 h before dose administration, were provided water *ad libitum*, and were fed mixed grass hay 2 h after dose administration.

Horses were administered single intravenous and oral doses of prethcamide in a randomized cross-over design with dose administrations separated by 7 days. Intravenous doses were administered rapidly via the left jugular vein at a total dose of 1.0 mg of prethcamide kg^{-1}. Oral doses were administered via capsules (Micoren® capsules, Ciba-Geigy Limited, Basle, Switzerland) at a dose of approximately 1.0 mg of prethcamide kg^{-1} (the dose was administered to the nearest 100 mg).

Blood samples (15 ml) were collected into partially-evacuated glass tubes containing 286 units of heparin via a 14-gauge polyethylene catheter inserted in the right jugular vein. Blood samples were collected immediately before (0 h) and at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min after intravenous drug administration. Blood samples were also collected before (0 h) and at 30, 60, 90, 120, 180, 240, 360, and 480 min after oral administration. The plasma was harvested from centrifuged blood and stored in polyethylene tubes at −20°C until analyzed.



Crotethamide: R = CH_2CH_3; R' = CH_3

Cropropamide: R = $CH_2CH_2CH_3$; R' = CH_3

Norcrotethamide: R = CH_2CH_3; R' = H

Norcropropamide: R = $CH_2CH_2CH_3$; R' = H

Fig. 1. Chemical structures of crotethamide, cropropamide, and their N-demethylated metabolites.

Urine samples were collected via an indwelling balloon-tipped urinary catheter inserted in the bladder. Urine was collected before drug administration and during the following intervals after drug administration: 0–30, 30–60, 60–90, 90–120, 120–180, 180–240, 240–360, and 360–480 min. Urine was removed from the bladder at the end of each collection period and mixed well to insure homogeneity. The total volume of urine collected from each horse during each collection period was recorded and a 5 ml sample was stored in a polyethylene tube at −20°C until analyzed.

2.7. Pharmacokinetic data analysis

Plasma crotethamide and cropropamide concentration versus time data for each animal were analyzed by the RSTRIP™ computer program (MicroMath Software, Salt Lake City, UT) to estimate the parameters of the polyexponential equation best describing the data for each animal, to calculate the areas under the plasma concentration versus time curves (AUC), and to estimate the terminal elimination rate constant (λ_2). All data points were weighted by $1/C_p^2$ where C_p is the plasma concentration. The correlation coefficient, coefficient of determination, and model selection criterion (MSC) for the polyexponential equation describing the data for each component of prethcamide in each horse were calculated by the RSTRIP™ computer program. The number of exponential terms used to describe the data for each horse was selected from the computer fit giving the largest value of MSC.

Estimates of total body clearance (CL_T), volume of distribution at steady-state (Vdss), volume of distribution based on area ($Vd_{(area)}$), and volume of the central compartment (Vc) for each component of prethcamide were obtained from

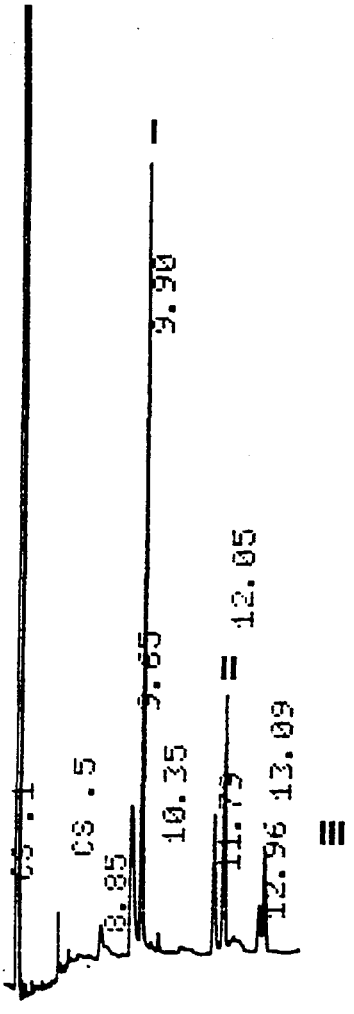


Fig. 2. Chromatogram of an extract of a plasma sample collected from a horse 90 min after intravenous administration of a 1.0 mg prethcamide kg^{-1} dose: peak I = nikethamide; peak II = crotethamide; peak III = cropropamide. The peaks eluting immediately before peaks II and III were identified as norcrotethamide and norcropropamide, respectively.

analysis of the plasma concentration versus time data in horses administered an intravenous dose [3]. The fraction of the dose eliminated unchanged in the urine (fu) was calculated from the cumulative amount of drug recovered unchanged and the dose of drug administered:

$$fu = \sum Cu \times Uv/\text{Dose}$$

where Cu is the concentration of the drug in urine and Uv is the volume of urine collected during each collection interval. The renal clearance (CL_R) was calculated as

$$CL_R = fu \times CL_T$$

The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) after oral administration were determined by examination of the plasma drug concentration versus time data for each horse. The areas under the plasma concentration versus time curves after oral (AUC_{PO}) and intravenous (AUC^*_{IV}) dosing were calculated by the trapezoidal method; areas under the curves were determined from the time of dosing to the last quantifiable concentration. The extent of oral bioavailability (F, %) was then calculated from the areas under the plasma concentration versus time curves and the intravenous and oral doses as follows:

$$F = [AUC_{PO}/AUC^*_{IV}] \times [DOSE_{IV}/DOSE_{PO}]$$

The mean values and standard deviations of C_{max}, T_{max}, λ_2, AUC^*_{IV}, and AUC_{PO} were calculated by standard formulas. The harmonic mean

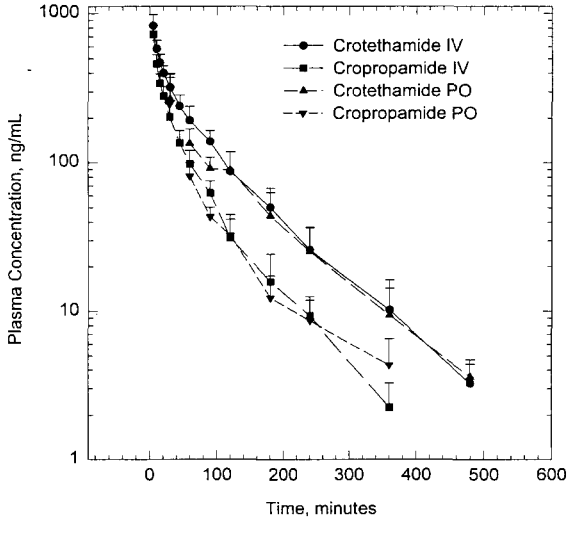


Fig. 3. Mean (+SD) plasma concentration versus time data for crotethamide and cropropamide after intravenous or oral administration of a 1.0 mg prethcamide kg^{-1} dose.

and the pseudo standard deviation of $t_{1/2}$ were calculated by the jack-knife method [4]. Calculated pharmacokinetic parameters are reported as median values and ranges. The terminal elimination rate constants after intravenous and oral administration were compared by the paired t-test with a P value < 0.05 considered significant. The lower limit of quantitation (LOQ) was defined as the lowest concentration of each analyte that could be measured with a precision that did not exceed 20% relative standard deviation [5].

2.8. GC–MS identification of urinary prethcamide metabolites

A 1.0 ml aliquot of a urine sample collected 0–30 min after intravenous administration of prethcamide to horse #12 was extracted as described above. The residue was dissolved in 20 μl of ethyl acetate and 1 μl was injected via a splitless injector into a GC–MS system (model 5970A, Hewlett-Packard, Palo Alto, CA). Analytical separations were performed using a 15 m × 0.242 mm i.d. fused silica capillary column (DB-1, 0.25 μm film thickness, J & W Scientific Inc.). The carrier gas was helium at a linear velocity of 50 cm s^{-1}. The column oven temperature was increased from 80°C 1 min after sample injection at a rate of 10°C min^{-1} to a final temperature of 150°C. Mass spectra were obtained under electron impact ionization conditions at an ionizing voltage of 70 eV.

A 1 μl aliquot of the residue was also analyzed by injection via an on-column injector (J & W Scientific Inc.) into a gas GC–MS system (model 4500, Finnigan Corporation, San Jose, CA) operated in the chemical ionization mode with methane as the reagent gas (source pressure 0.3 Torr). A 5 m × 0.25 mm i.d. section of deactivated fused silica capillary tubing was used to connect the injector to the analytical column. Separations were carried out on a 30 m × 0.242 mm i.d. fused silica capillary column coated with DB-1 (0.25 μm film thickness, J & W Scientific Inc.). The column oven temperature was increased from 80°C 1 min after sample injection at a rate of 10°C min^{-1} to a final temperature of 280°C. The mass spectrometer was operated at a source temperature of 260°C. The carrier gas was helium at a linear velocity of 50 cm s^{-1}.

3. Results

3.1. Quantitative measurement of prethcamide in extracts of plasma and urine samples

Crotethamide and cropropamide were determined in extracts of plasma and urine samples by a capillary GC–NPD method using nikethamide as the internal standard. Chromatographic peaks for crotethamide, cropropamide, and nikethamide extracted from plasma were well resolved from each other and from those of other substances. Plots of peak height ratio versus concentration of plasma calibrators were linear ($r > 0.999$) over the concentration range 2.0–100 ng ml^{-1} of plasma and passed through the origin. The LOQ for crotethamide and cropropamide was approximately 4.0 ng ml^{-1} of plasma. Determinations of crotethamide and cropropamide in plasma at a concentration of 4.0 ng ml^{-1} were characterized by precision estimates with percent relative standard deviations (%RSD) of 14.1% and 14.9% respectively and accuracies of 104 and 101% respectively. A typical chromatogram of an extract of a plasma collected from a horse 90 min after intravenous administration of a 1.0 mg prethcamide kg^{-1} dose is shown in Fig. 2.

Chromatographic peaks for crotethamide and cropropamide but not nikethamide extracted from urine were resolved from each other and from endogenous substances. Peaks for metabolites eluted just before the drug peaks but were resolved. Interference with the nikethamide peak precluded its use as internal standard and therefore peak heights were used. Plots of peak height versus concentration of urine calibrators were linear ($r > 0.995$) over the concentration ranges 4.0–100 ng ml^{-1} of urine for crotethamide and 2.0–60 ng ml^{-1} for cropropamide and passed through the origin. The LOQ for both crotethamide and cropropamide was approximately 10.0 ng ml^{-1} of urine. Determinations of crotethamide and cropropamide in urine at a concentration of 10.0 ng ml^{-1} were characterized by precision estimates

with %RSD of 12.6% and 7.90% respectively and accuracies of 104 and 105% respectively.

3.2. Plasma concentrations and pharmacokinetics after intravenous and oral doses

Mean (+SD) plasma concentration versus time curves for both crotethamide and cropropamide after intravenous administration are shown in Fig. 3. Plasma concentrations of both drugs declined biexponentially in all horses after intravenous administration. Mean (±SD) plasma concentrations of crotethamide and cropropamide were approximately equal in plasma samples obtained 5 min after dose administration (830 ± 157 and 725 ± 111 ng ml^{-1} respectively). However, the mean plasma crotethamide concentration (10.2 ± 6.08 ng ml^{-1}) 6 h after dose administration was approximately five times greater than the corresponding mean plasma cropropamide concentration (2.25 ± 1.03 ng ml^{-1}). Plasma cropropamide concentrations were below the LOQ in all horses 6 h after dose administration whereas the corresponding plasma crotethamide concentrations were above the LOQ in all horses.

Pharmacokinetic parameter estimates for crotethamide and cropropamide after intravenous administration of prethcamide are reported in Table 1. The median values (ranges) for total body clearance (CL_T) of crotethamide and cropropamide were 5.87 (5.19–7.43) and 10.3 (9.35–11.2) ml min^{-1} kg^{-1} respectively. The median values (ranges) of renal clearance (CL_R) were 0.0133 (0.00811–0.0209) and 0.0106 (0.00934–0.0233) respectively. The harmonic means (± pseudo SD) of the terminal elimination half-life ($t_{1/2}$) were 65.6 (±11.1) and 40.2 (±13.2) min respectively.

Mean (+SD) plasma concentrations of crotethamide and cropropamide after oral administration of prethcamide are shown in Fig. 3. Mean values of C_{max}, T_{max}, AUC, and λ_2 after oral administration are reported in Table 2. The mean (±SD) values of C_{max} for crotethamide and cropropamide were 268 ± 126 ng ml^{-1} and 248 ± 125 ng ml^{-1} respectively after oral administration and were observed at the earliest sampling time. The median values (ranges) of the extents of oral bioavailability of crotethamide and cropropamide were 65.6 (41.0–74.8)% and 51.1 (36.5–94.7)% of the values obtained after intravenous administration.

3.3. Blood to plasma concentration ratio

The blood to plasma concentration ratios for crotethamide and cropropamide were 1.01 and 0.99 respectively at a concentration of 200 ng of each per milliliter of blood.

3.4. Plasma protein binding

The mean (±SD) values of plasma protein binding of crotethamide and cropropamide were 25.0 (±7.64)% and 28.2 (±4.98)% respectively at a concentration of 80 ng ml^{-1}. The extent of plasma protein binding for crotethamide and cropropamide decreased to 10.2 (±9.73)% and 18.9 (±7.81)% respectively at 400 ng ml^{-1}.

3.5. Urine concentrations after intravenous and oral doses

Mean concentrations of crotethamide and cropropamide in urine samples collected after intravenous and oral doses of prethcamide are shown in Fig. 4. The lower LOQ of crotethamide and cropropamide in urine was 10 ng ml^{-1}. Cumulative urinary recoveries of unchanged crotethamide and cropropamide accounted for only 0.225% and 0.120% of the dose respectively after intravenous administration and 0.0946% and 0.0360% of the dose respectively after oral administration (Fig. 5).

3.6. Identification of urinary metabolites of prethcamide

Electron impact ionization and methane chemical ionization mass spectra of metabolites isolated from urine are reported in Figs. 6 and 7. Metabolites of crotethamide and cropropamide were identified as norcrotethamide and norcropropamide (Fig. 1) by comparison of their mass spectra with those previously reported from extracts of human urine samples [6].

Table 1
Summary of pharmacokinetic parameter values for crotethamide and cropropamide in horses after intravenous administration of a 1.0 mg prethcamide kg^{-1} dose

Parameter	Crotethamide		Cropropamide	
	Median	Range	Median	Range
$C_1(\mu g\ ml^{-1})$	0.736	0.415–1.47	0.802	0.344–3.12
$\lambda_1(min^{-1})$	0.0837	0.0249–0.152	0.0866	0.0581–0.411
$C_2(\mu g\ ml^{-1})$	0.312	0.237–0.434	0.239	0.124–0.441
$\lambda_2(min^{-1})$	0.0103	0.0080–0.0143	0.0148	0.011–0.0290
$t_{1/2}$(min)	65.6 [a]	11.1 [b]	40.2 [a]	13.2 [b]
AUC($\mu g\ min^{-1}\ ml^{-1}$)	40.0	33.7–48.2	23.6	20.4–26.7
$CL_T(ml\ min^{-1}\ kg^{-1})$	5.87	5.19–7.43	10.3	9.35–11.2
AUMC ($\mu g\ min^{-2}\ ml^{-1}$)	3.24×10^3	$1.76–4.52 \times 10^3$	1.21×10^3	$0.543–1.35 \times 10^3$
Vdss(1 kg^{-1})	0.955	0.764–1.136	0.958	0.522–1.27
$Vd_{(area)}$(1 kg^{-1})	1.21	0.977–1.50	1.33	0.756–2.08
Vc(1 kg^{-1})	0.465	0.275–0.755	0.485	0.140–0.779
fu(%)	0.225	0.141–0.367	0.120	0.0837–0.250
$CL_R(ml\ min^{-1}\ kg^{-1})$	0.0133	0.00811–0.0209	0.0106	0.00934–0.0233
MRT(min)	80.8	51.9–95.0	50.9	23.8–53.6

[a] Harmonic mean.
[b] Pseudo standard deviation.

Table 2
Summary of pharmacokinetic parameter values for crotethamide and cropropamide in horses after oral administration of a ≈ 1.0 mg prethcamide kg^{-1} dose

Parameter	Crotethamide			Cropropamide		
	Mean	SD		Mean	SD	
C_{max}(ng ml^{-1})	268	126		248	125	
T_{max}(min)	30	NA [a]		30	NA [a]	
$t_{1/2}$(min)	60.1 [b]	9.68 [c]		40.4 [b]	12.9 [c]	
	Median	Range		Median	Range	
fu(%)	0.0946	0.0642–0.167		0.0360	0.0292–0.0584	
AUC_{PO}(μg min^{-1} ml^{-1})	24.5	18.9–31.9		12.1	9.81–21.6	
AUC^{*}_{IV} (μg min^{-1} ml^{-1})	41.0	35.6–49.6		25.6	21.2–28.0	
F(%)	65.6	41.0–74.8		51.1	36.5–94.7	

[a] NA = not applicable.
[b] Harmonic mean.
[c] Pseudo standard deviation.

Metabolites were detected during gas chromatographic analysis of extracts of horse urine samples. Although the heights of the peaks attributed to these metabolites were greater than those of crotethamide and cropropamide in samples collected after both intravenous and oral administration, quantitation was not possible because synthetic reference standards were not available.

4. Discussion

Plasma concentrations of crotethamide and cropropamide in samples collected from horses after intravenous and oral administration of single doses of 1.0 mg of prethcamide kg^{-1} were readily determined by capillary GC–NPD. Pharmacokinetic analysis of plasma crotethamide and cropropamide concentrations indicated rapid elimination of both drugs from plasma, metabolism by N-demethylation of the [(dimethylamino)-carbonyl]-propyl moiety with very little unchanged drug excreted in the urine, and extensive distribution as indicated by relatively large volumes of distribution. Furthermore, cropropamide was eliminated more rapidly than crotethamide.

Total body clearances of crotethamide and cropropamide were similar to those of several other rapidly cleared drugs such as diazepam (7.48 ml min^{-1} kg^{-1}) [7], propoxyphene (25.6 ml min^{-1} kg^{-1}) [8], and doxapram (10.9 ml min^{-1} kg^{-1}) [9]. The hepatic extraction ratios for crotethamide and cropropamide were estimated by assuming that hepatic metabolism accounted for the total body clearance since renal clearance was negligible, equating total plasma clearances to total blood clearances since the blood to plasma concentration ratios were unity, and then dividing the total blood clearances by estimates of the total hepatic blood flow in the horse.

The total plasma flow to the liver in the horse was approximated from the plasma clear-

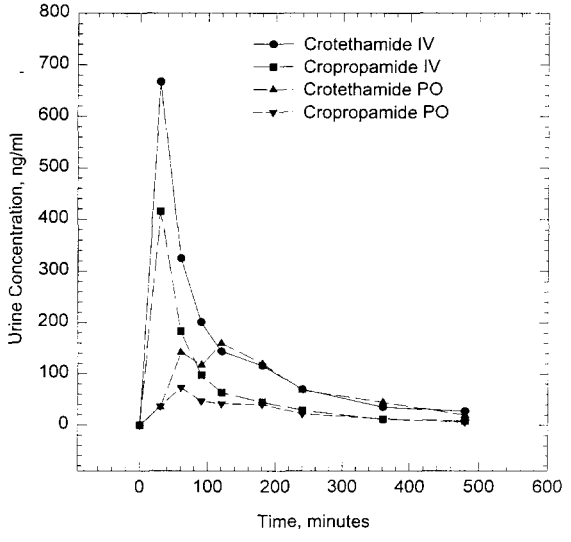


Fig. 4. Mean urine concentration versus time data for crotethamide and cropropamide after intravenous or oral administration of a 1.0 mg prethcamide kg^{-1} dose.

ance value for sulfobromophthalein (BSP) of 10.5 ml min^{-1} kg^{-1} [10], assuming that BSP is completely extracted by the liver. This value for total plasma flow was converted to total blood flow by assuming that BSP does not enter red blood cells and that the hematocrit is approximately 40%. The estimate for total hepatic blood flow based on BSP clearance was therefore 17.5 ml min^{-1} kg^{-1}.

Total hepatic blood flow was also estimated from studies of tissue mass and residual blood volume [11]. These studies suggest a total blood flow to the liver (arterial flow plus portal flow from the intestines) of approximately 17.1 ml min^{-1} kg^{-1} [11], in excellent agreement with the estimate of 17.5 ml min^{-1} kg^{-1} based on BSP clearance. Thus, the hepatic extraction ratios (E_H) were calculated from:

$$E_H = CL_T/Q_H$$

where CL_T was an estimate of hepatic blood clearance and Q_H was an estimate of the hepatic blood flow rate based on BSP. The estimates of E_H for crotethamide and cropropamide were 0.335 (5.87/17.5) and 0.589 (10.3/17.5) respectively.

The incomplete bioavailability of the drugs after oral administration suggested either incomplete absorption or losses resulting from first-pass metabolism. The time to C_{max} was short (30 min or less) and the terminal elimination rate constant after oral administration did not differ from that after intravenous administration, indicating that absorption was rapid and did not affect the rate of elimination. Therefore, incomplete bioavailability was probably not due to slow absorption but to first-pass metabolism. The estimated values for E_H were then used to calculate the fraction of the dose escaping first-pass metabolism (F^*):

$$F^* = 1 - E_H$$

The estimated values of the oral bioavailabilities of crotethamide and cropropamide were 66.5% and 41.1%, in good agreement with measured values (65.6% and 51.1% respectively), suggesting that incomplete bioavailability was due to first-pass metabolism during absorption. However, the AUC after oral dosing may have been underestimated due to an incomplete characterization of

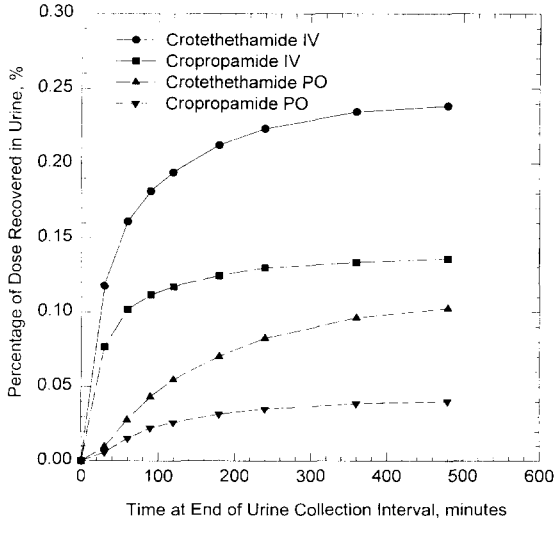


Fig. 5. Cumulative urinary recoveries of unchanged crotethamide and cropropamide after intravenous or oral administration of a 1.0 mg prethcamide kg^{-1} dose.

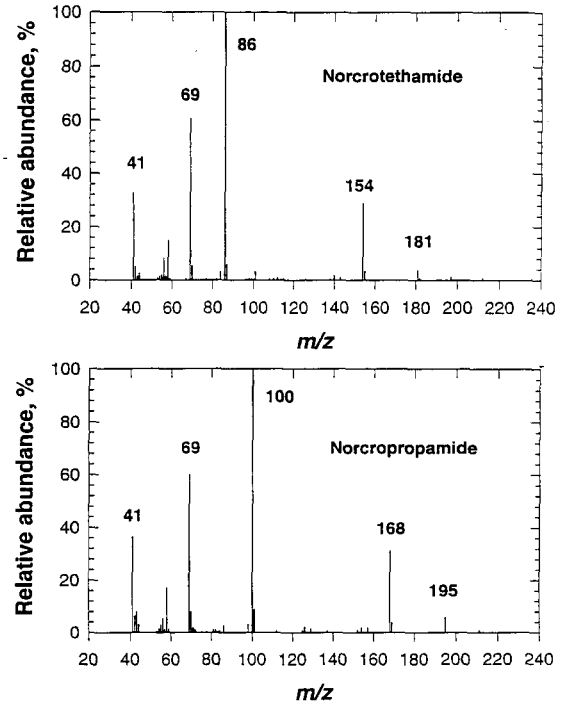


Fig. 6. Electron impact ionization mass spectra of (A) norcrotethamide and (B) norcropropamide isolated from a urine sample collected after intravenous administration of a 1.0 mg prethcamide kg^{-1} dose.

plasma concentrations during the absorptive phase since T_{max} was the first sample collected after oral dosing and, therefore, the extent of bioavailability may have been underestimated. However, the fraction of the dose excreted unchanged in the urine after oral dosing was less than after intravenous dosing for both crotethamide and cropropamide, suggesting that oral bioavailability was incomplete.

The median values of the renal clearances of crotethamide and cropropamide after intravenous administration were only 0.0133 and 0.0106 ml min^{-1} kg^{-1} respectively. These low values suggested extensive reabsorption of the drugs from the renal tubules, since neither drug was extensively bound to plasma proteins and the glomerular filtration rate estimated from the creatinine clearance in adult horses is approximately 1.92 ml min^{-1} kg^{-1} [12].

Metabolites of crotethamide and cropropamide were excreted in the urine and were tentatively identified as norcrotethamide and norcropropamide (Fig. 1) based on comparison of their mass spectra (Figs. 6 and 7) with those previously reported for cropropamide and crotethamide and for metabolites isolated from human urine [6]. Mass spectral analysis of crotethamide and its urinary metabolite under electron impact ionization conditions (70 eV) indicated low intensity molecular ion peaks (m/z 226 and 212 respectively) and identical fragment peak ions at m/z 181, both of which provided valuable diagnostic evidence for the proposed structure of norcrotethamide. Firstly, the decreased mass (14 amu) of the molecular ion of the metabolite relative to that of crotethamide was consistent with the loss of a methyl group. Secondly, in an analysis of the fragmentation pattern of crotethamide,

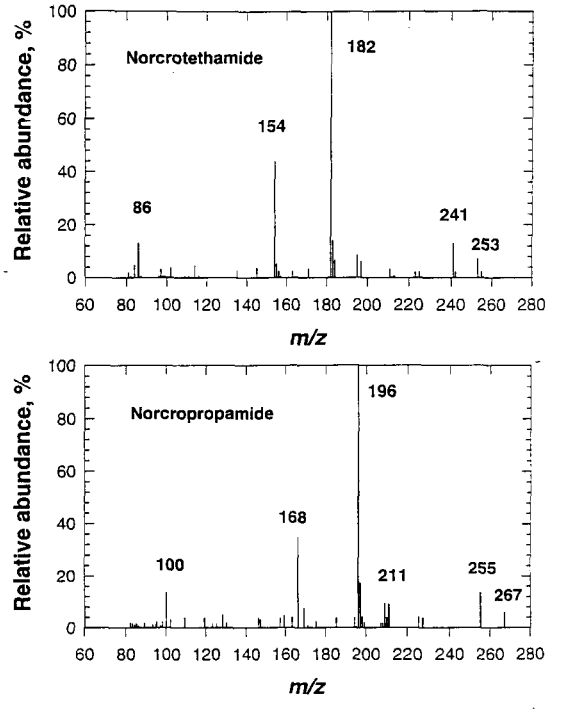


Fig. 7. Methane chemical ionization mass spectra of (A) norcrotethamide and (B) norcropropamide isolated from a urine sample collected after intravenous administration of a 1.0 mg prethcamide kg^{-1} dose.

Delbecke et al. [6] proposed that the fragment ion peak at m/z 181 resulted from intramolecular hydrogen transfer from the ethyl substituent to the nitrogen atom with subsequent displacement of the dimethylamino group. The current authors have attributed the presence of this prominent fragment ion peak at m/z 181 in the mass spectrum of the metabolite to an analogous mechanism with displacement of the methylamino group. Therefore, metabolic loss of a methyl group from this site must have occurred since loss from any other site would not result in the formation of this fragment ion but one at 14 amu less (i.e. m/z 167). However, the mass spectrum did not produce any evidence for this ion. Therefore, it was concluded that the metabolite of crotethamide resulted from loss of a methyl group from the dimethylamino moiety. A similar analysis of the mass spectral data for cropropamide and its metabolite led to the conclusion that this metabolite also resulted from loss of a methyl group from the dimethylamino moiety.

The prethcamide dose investigated in this study, 1.0 mg kg^{-1}, was substantially less than that required to produce respiratory or central nervous system stimulation in other species. Prethcamide doses producing respiratory or central nervous system stimulation range from 13 mg kg^{-1} in humans [13] to 200 mg kg^{-1} in narcotized cats [14]. Prethcamide increases pulse rate and blood pressure but does not affect tidal volume or respiratory rate at an intravenous dose of 13 mg kg^{-1} in humans [13]. Central sympathetic effects of prethcamide in narcotized (chloralose–urethane) cats increase with dose from 35–40 mg kg^{-1} to 165–200 mg kg^{-1} [14]. Smaller doses of 10–20 mg kg^{-1} increase efferent vagal activity in narcotized cats [15]. Furthermore, prethcamide increases locomotor activity in rats after intraperitoneal doses of 60–107 mg kg^{-1} [16]. Respiratory stimulant doses of prethcamide determined in another study were 11.6 mg kg^{-1} in the cat, 21.4 mg kg^{-1} in the rabbit, 34.7 mg kg^{-1} in the rat, and 47.2 mg kg^{-1} in the mouse [17]. Thus, the dose of prethcamide administered to the horses in this study was substantially less than those investigated in other species. Since both components of prethcamide were readily detected at this comparatively low dose, it is assumed that doses likely to produce a stimulant effect in horses would be detectable by analysis of plasma or urine test samples by the gas chromatographic method described in this study.

5. Conclusions

A sensitive and specific GC–NPD method has been developed for identification and quantitation of prethcamide administered to horses. Crotetha-mide and cropropaide were identified in extracts of plasma and urine samples collected up to 8 h after intravenous and oral administration of 1.0 mg of prethcamide kg^{-1}. Metabolites resulting from N-demethylation were also identified from urine extracts. The method was therefore suitable for determination of prethcamide after administration to horses.

Acknowledgments

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