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M. Frank
University of Kentucky

T. J. Weckman
University of Kentucky

T. Wood
University of Kentucky

W. E. Woods
University of Kentucky

Chen L. Tai
University of Kentucky

See next page for additional authors

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Hordenine: pharmacology, pharmacokinetics and behavioural effects in the horse

M. FRANK, T. J. WECKMAN, T. WOOD, W. E. WOODS, CHEN L. TAI, SHIH-LING CHANG, A. EWING*, J. W. BLAKE and T. TOBIN†
*Kentucky Equine Drug Research Program and Graduate Center in Toxicology, University of Kentucky, Lexington, Kentucky 40506, USA. *Pharmacology and Toxicology Research Laboratories, 144 Prosperous Place, Lexington, Kentucky 40504, USA.*

Summary

Hordenine is an alkaloid occurring naturally in grains, sprouting barley, and certain grasses. It is occasionally found in post race urine samples, and therefore we investigated its pharmacological actions in the horse. Hordenine (2.0 mg/kg bodyweight [bwt]) was administered by rapid intravenous (iv) injection to 10 horses. Typically, dosed horses showed a flehmen response and defecated within 60 secs. All horses showed substantial respiratory distress. Respiratory rates increased about 250 per cent and heart rates were approximately double that of resting values. All animals broke out in a sweat shortly after iv injection, but basal body temperature was not affected. These effects were transient, and the animals appeared normal within 30 mins of dosing. Treated horses were tested in a variable interval responding apparatus 30 mins after dosing and no residual stimulation or depressant effects of hordenine were apparent. Animals dosed orally with 2.0 mg/kg bwt of hordenine showed no changes in heart rate, respiratory rate, basal body temperature or behaviour. After iv injection of hordenine, (2.0 mg/kg bwt) plasma reached a maximum value of about 1.0 μg/ml, and declined thereafter in a biexponential fashion. Kinetics of plasma concentration satisfied the concept of a two compartment open system, with an α-phase half-life of about 3 mins, and a β-phase half-life of about 35 mins. Total urinary concentrations of hordenine (free and conjugated) peaked at about 400 μg/ml, and then declined exponentially to background levels by 24 h after dosing. Oral administration of hordenine (2.0 mg/kg bwt) showed peak plasma levels of about 0.15 μg/ml 1 h after dosing, followed by a slow multi-exponential decline in blood levels of the drug. Total urinary concentrations of hordenine (free and conjugated) peaked at about 200 μg/ml, remained at this level for about 8 h, and then declined to background levels. Plasma levels of hordenine were reflected by a kinetic model which assumed very slow absorption of hordenine from the gastrointestinal tract and no effect on behaviour, heart rate or respiratory rate were noted after oral administration. Because of the low plasma levels, it would appear to be particularly difficult to obtain a pharmacological effect of hordenine after oral administration.

Introduction

HORDENINE, (p-hydroxy-N,N-dimethylphenethylamine), is an alkaloid found in many plants, particularly sprouting barley (Smith 1977), and is probably present therefore at low levels in most equine diets. It has been reported in post race equine urine in England, Australia, Canada and, more recently, Kentucky.

†Correspondence should be addressed to Dr. Tobin

Whether or not hordenine should be classified as a natural constituent of feedstuffs or an illegal medication is unclear. In England, it is considered part of normal feeding, and disciplinary action has not been taken when identified in equine urine. In Canada, on the other hand, it is a prohibited drug and, therefore, a prohibited medication. In Australia, the Racing Stewards have taken no action on reported positive cases, presumably because of the possibility that the drug arose from horse feed, and not from an attempt to administer an illegal drug. While the current work was in progress, the Canadian authorities have noted the possible source of hordenine in animal feed and not penalised trainers after its identification in post race samples (Beaumier 1984).

Based on its chemistry and pharmacological effects, hordenine has potential as an illegal drug in horse racing. It is structurally related to epinephrine and ephedrine, and is classified as a sympathomimetic stimulant. It stimulates heart function, constricts blood vessels and relaxes bronchioles (Rietschel 1937).

In 1985, hordenine began to appear at higher than usual concentrations in post race urine samples in Kentucky, and a systematic investigation of its pharmacology, pharmacokinetics and behavioural effects in the horse was therefore undertaken.

Materials and methods

Three separate experiments were conducted:

1) horses were dosed intravenously (iv) and orally with 2 mg/kg bodyweight [bwt] hordenine (hordenine hemisulfate, Sigma Chemical Co., St. Louis, MO). Plasma and urine samples were collected for gas chromatography (GC) analysis.
2) horses were dosed iv (2 mg/kg bwt) and tested in a variable interval responding regime.
3) horses were dosed iv and orally and monitored for cardiac, respiratory and basal temperature changes.

Horses

Mature Thoroughbred and Standardbred mares of about 450 kg bwt were used. These animals were in good health and kept at pasture until the morning of the experiment. All iv injections were into the right jugular vein and all blood samples were drawn into Vaccutainer (Becton-Dickinson & Co., Rutherford, NJ) grey top (potassium oxalate-sodium fluorate) tubes from the left jugular vein and stored at 4°C until analysed. Urine samples were collected by direct bladder catheterisation and stored at -20°C until analysed. Oral dosings were administered by stomach tube.

Overt behavioural effects

With each experimental session of hordenine dosing an observation form was used to record behavioural changes. In the iv dosing sequences several gross behavioural changes occurred consistently.

These were recorded as flehmen response, postural and attention/awareness changes, defecation, changes in respiration and flush or sweating reactions.

Physiological parameters

Horses dosed iv and orally were evaluated for changes in heart rate, respiratory rate, body temperature and behaviour. The horses were placed in an isolated laboratory equipped with equine stock restraints and pre-gelled disposable electrodes (Red Dot 3-M Co., St Paul, MN) were placed bilaterally at the heart girth and the diaphragm to record heart rate and respiration respectively. Heart impulses were recorded on Grass Model 7 polygraph (Grass Instrument Co., Quincy, MA) and respiratory changes through an impedence converter (UFI, Inc., Morrow Bay, CA) and recorded on the same polygraph. Basal body temperature was measured with a deep rectal probe and digitally displayed (Therm-Alert TH-6, Bailey Instruments Inc., Saddlebrook, NJ).

Each animal was monitored for 30 mins after entering the stocks to establish its baseline activities. Continuous recordings were made of heart rate and respiration in the iv dosed subjects, from 30 mins pre-dose to 60 mins post dose. Horses receiving an oral dose were monitored for 30 mins pre-dose, removed to an open stall to be intubated, and then returned to the stock restraints. Within 5 mins of oral dosing, the recorders were replaced and the horses monitored for an additional 60 mins. In both groups, rectal temperature was recorded at three points pre-dose and then every 5 mins post dose for 60 mins.

Five horses were schooled to respond to a conditioning apparatus as described previously (Shults, Combie, Dougherty and Tobin 1979; 1982). This apparatus trains the horse to obtain a reward of food by cutting a light beam by head movement. The pattern of head movement in an individual is variable within very narrow limits. The horses were given 2 mg/kg bwt hordenine iv on a Monday morning and 30 mins later placed in a stall equipped with a variable interval responding apparatus for 30 mins. The number of responses per animal, within each 30 min period was recorded daily for the remainder of the week. These data were compared with those of control periods which consisted of one week of daily 30 min sessions and a similar week following a Monday morning iv injection of saline.

Analytical methods

Urine: A sample of urine (1 ml) was added to a screw-top glass culture tube. The sample was hydrolysed with β-glucoronidase from *Patella vulgata* (Sigma Chemical Co., St. Louis, MO) (50 µl containing 5000 units) and 0.1 M sodium acetate buffer (2 ml) of pH 5.0 then incubated for 3 h at 63°C. Concentrated ammonium hydroxide (4 drops) was then added to each tube to adjust the pH to 9.5. Following the addition of 4 ml dichloromethane (DCM), the tubes were rotoracked for 4 mins and centrifuged for 30 mins, 6°C at 1150 g.

The supernatant was removed by aspiration and the organic phase was poured into a clean tube. A 0.2 N solution of sulphuric acid (2 ml) was added to each sample and rotoracked for 4 mins. The aqueous layer was removed by pipette into a clean tube to which DCM (4 ml) and 0.6 N ammonium hydroxide (2 ml, pH 9.3) were added and the tubes rotoracked for 4 mins and centrifuged. The aqueous layer was again aspirated off and the organic phase was poured into a clean tube. The tubes were placed in a water bath (40°C) and evaporated to near dryness (20 µl) under a stream of nitrogen. Ethyl acetate (25 µl) and N,0-bis(trimethylsilyl)-trifluoroacetamide) (Pierce Chemical, Rockford, IL) (25 µl) were added and the tubes incubated at room temperature for 15 mins. Following evaporation to near dryness under a stream of nitrogen, the samples were reconstituted with 50 µl of hexane and vortex-mixed (10s). A portion of this mixture (2 µl) was then injected into the chromatograph. Where necessary, the urine samples were diluted with control urine of each respective horse to prevent off-scale readings on the integrator.

Plasma: Each plasma sample was added to a glass screw-top culture tube. The sample was made basic (pH 9.5) by the addition of 4 drops of ammonium hydroxide. Following the addition of DCM (4 ml), the samples were rotoracked and centrifuged as described above. The supernatant was removed by aspiration and the organic phase was poured into a clean tube. From this point on the samples were subjected to the same procedure of derivatisation and reconstitution as the urine samples.

Internal standard

Ephedrine was used as an internal standard to allow accurate quantification of hordenine. The hordenine peak areas were normalised to the ephedrine peak areas which minimised the effect of volumetric errors. All samples were analysed in duplicate.

Gas chromatography

A gas chromatograph (Tracor 565, Tracor Inc., Austin, TX) equipped with a nitrogen-phosphorus detector, was used to measure hordenine in samples from horses given the drug by iv and oral routes (Reilly 1981).

The chromatograph contained a 1.83 x 2 mm id glass column with 3 per cent OV-101 mesh packing (Tracor Inc., Austrin, TX). A column temperature programme was used with an initial temperature of 130°C held for 1 min. The oven temperature then increased 16°C/min until it reached 250°C where it held for 2 mins. The injection port and interface (manifold) temperatures were set at 250°C and 300°C respectively. The carrier gas flow (nitrogen) was 30 ml/min, the detector air flow was 100 ml/min, and the detector hydrogen flow was 30 ml/min. A computing integrator (Spectra-Physics, San Jose, CA) was used to integrate peak areas. The integrator chart speed was set at 10 mm/min and the attenuation at 1.0.

Statistical and pharmacokinetic analysis

Pharmacokinetic analysis was model independent by means of PCNONLIN model (Anon 1986). Statistical analysis was by means of an analysis of variance (ANOVA) in which the variance among subjects, sessions, treatments as well as treatment by variance session is calculated. Linearity over treatments was determined by partitioning variance among treatments into linear and non-linear components and using regression analysis. Comparisons between saline and drug treatments were made using a Duncan's multiple range test. All calculations were performed on an IBM 3083 computer using a statistical analysis system program.

Results

Overt behavioural effects

A typical pattern of behavioural and physiological response to hordenine emerged after administration to 10 horses over 22 experimental sessions. Administration of 0.77 mg/kg bwt iv produced no observable changes in the behaviour of four horses. Increasing the dose to 2.0 mg/kg bwt, however, produced marked behavioural and physiological responses.

The initial reaction at this higher level was usually a flehmen response and an alert attitude with the head and ears erect. The majority of horses also defecated within 60 secs of dosing. The accentuated alertness was followed by a marked increase in heart and respiratory rates within 2 mins after rapid drug administration. The horses often made a dry swallowing sound from the glottal area and extended their neck and head to facilitate air intake. Some subjects were observed to walk forward with their forelimbs while keeping their hindlimbs fixed thus achieving hyperextension of their thorax in addition to the head and neck extension. This posture is unique in our experience of drug administrations.

The subjects, although alert, did not appear excited and little if any locomotor stimulation was observed. With the increase in heart

and respiratory rates the horses also quickly broke out in a sweat, usually starting along the neck and becoming more generalised within 5 to 8 mins. This effect lasted only about 20 mins, after which the animals began to cool down. The tachypnoeic and tachycardia phase occurred within 120 mins post injection in all subjects receiving the 2 mg/kg bwt iv dose. These physiological changes were of relatively short duration, with most of the grossly laboured breathing and rapid heart rate passing in 8 to 10 mins and tapering back to pre-treatment levels within 20 mins.

These dramatic behavioural changes were not observed when the same dose was administered orally by stomach tube. Four horses dosed orally with 2 mg/kg bwt hordenine and allowed to move at will in loose box stalls exhibited no observable behavioural changes.

Physiological effects

After rapid iv administration of 2.0 mg/kg bwt, mean heart rate increased about 100 per cent over baseline in all subjects within the first 5 mins post injection, was sustained for about 15 mins, and returned to control levels by 25 mins (Fig 1). Similarly, respiration rate had more than doubled within the first 5 mins post injection. Respiratory minute volume was not measured, but the subjects displayed a dramatic increase in respiratory effort and a significant 'blowing' effect was seen. Respiratory rates returned to control levels by about 20 mins post dose (Fig 1). Despite these substantial changes in heart and respiratory rate, the acute behavioural changes and the marked sweating displayed by these animals, no change in rectal temperature was recorded during these experiments (Fig 2).

Four horses were similarly evaluated after receiving 2 mg/kg bwt orally via a stomach tube. Heart rate, respiratory rate, basal body temperature and behaviour did not differ significantly from the pre-treatment control period, when monitored for 60 mins post administration (Table 1).

Variable interval responding

The results of administration of 2 mg/kg bwt hordenine iv to five horses assayed in the variable interval apparatus are presented in Table 2. Statistical analysis of the sessions post hordenine and control week response rates showed no significant difference ($P>0.05$). No horses showed visually apparent changes in their behaviour patterns during these experiments.

Pharmacokinetic data

Hordenine was readily detectable by nitrogen-phosphorous detectors in plasma and urine samples as shown in Figure 3. Hordenine chromatographed with a retention time of about 3.4 mins and was well separated from the ephedrine internal standard (Fig 3). Enzyme hydrolysis of urine samples increased recovery of hordenine to about 300 times above that of unhydrolysed samples. Recovery of hordenine from hydrolysed urine samples average about 75 per cent, being significantly higher for single extraction plasma recovery than the urine back extraction method. Using this method, standard curves for recovery of hordenine from plasma and urine were constructed and typical curves are presented in Figures 4a and b. The method yielded close to linear standard curves, and was sensitive down to about 0.1 µg/ml in plasma and 0.5 µg/ml in urine.

The plasma levels found after rapid iv injection and oral administration are shown in Figure 5. After iv injection, plasma levels peaked at about 1 µg/ml 5 mins after dosing, and then declined with an α-phase half-life of about 3.5 mins, followed by a β-phase half-life of about 32 mins. Plasma concentrations were followed for 120 mins at which time they were about 0.25 µg/ml (Table 3).

Plasma concentration increased slowly, to peak at about 1 h after oral administration. Thereafter they declined slowly, with an apparent half-life much slower than that after iv administration. These data were reflected by a model which assumed very slow absorption from the gastrointestinal tract, with an apparent mean half-life for absorption of the drug of about 150 mins. The bioavailability of hordenine after oral administration was approximately 100 per cent (Table 3).

Total urinary concentrations of hordenine (free and conjugated) peaked at about 350 µg/ml 1 h after iv administration (Fig 6). Thereafter, concentrations declined exponentially with a half-life of about 2.5 h to reach a concentration of 1 µg/ml about 24 h after dosing, which was still somewhat above background concentrations of the drug, which rarely exceed 0.5 µg/ml in post race urines, or in control urines from horses on our experimental farm.

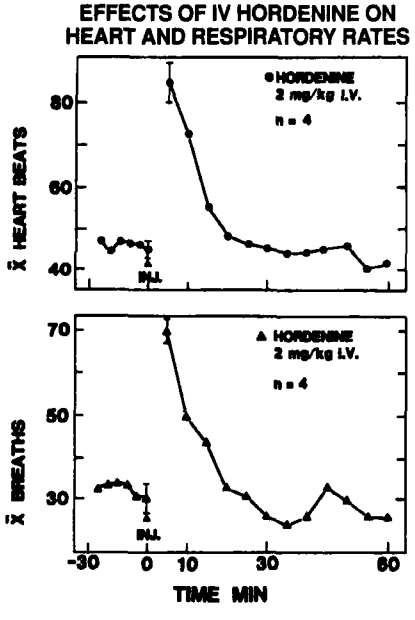

Fig 1: Illustrating the effect of rapid intravenous injection of hordenine (2.0 mg/kg bwt) on heart (● – ●) and respiratory (▲ – ▲) rates. All data points are the means of determinations in four horses. The vertical bars indicate the standard errors of the mean

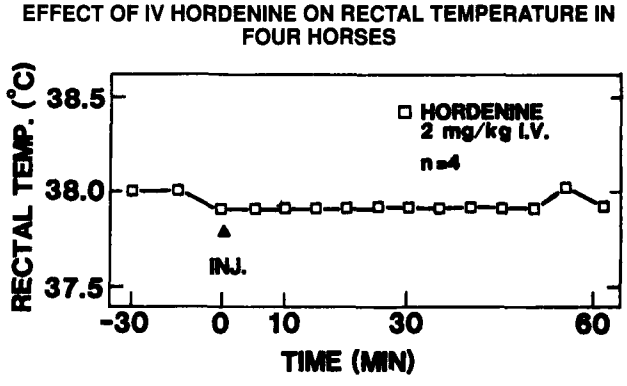

Fig 2: Mean rectal temperature (□ – □) in four horses for 30 mins prior to, and 60 mins after, rapid iv injection of 2.0 mg/kg bwt of hordenine

TABLE 1: Comparison of mean pre-treatment vs mean post treatment values for heart rate (HR), respiratory rate (RR) and rectal temperature (RT) after 2.0 mg/kg bwt hordenine orally (n=4)

	Pre-treatment			Post treatment		
Time (mins)	HR (/min)	RR (/min)	RT (°C)	HR (/min)	RR (/min)	RT (°C)
5	43.75	21.00	37.8	43.25	19.00	-
10	42.00	20.00	-	43.75	19.25	-
15	42.75	21.00	37.8	41.50	19.25	37.8
20	44.25	21.25	-	40.25	19.00	-
25	44.50	19.00	-	43.00	17.75	-
30	42.25	22.25	37.8	39.00	16.50	37.8
35	-	-	-	39.50	17.50	-
40	-	-	-	41.75	21.75	-
45	-	-	-	43.25	23.00	37.8
50	-	-	-	40.75	17.25	-
55	-	-	-	40.75	21.75	-
60	-	-	-	40.25	18.00	37.8

TABLE 2: Effect of pre-treatment with hordenine on variable interval responding

Horse	Control	Saline	Hordenine
1	718 ± 66	727 ± 172	629 ± 49
2	468 ± 38	471 ± 75	363 ± 46
3	420 ± 43	413 ± 43	395 ± 19
4	450 ± 47	430 ± 15	424 ± 22
5	213 ± 22	214 ± 36	204 ± 14

Horses were treated with hordenine iv at 2.0 mg/kg bwt by rapid bolus injection. They were then held for 20 mins and placed in a variable interval responding stall. Each data point represents the average number of responses per 30 min session and the standard error of the mean. Horses were tested once daily for each treatment week. Comparison of these weekly means using the ANOVA test showed no significant differences at P<0.05

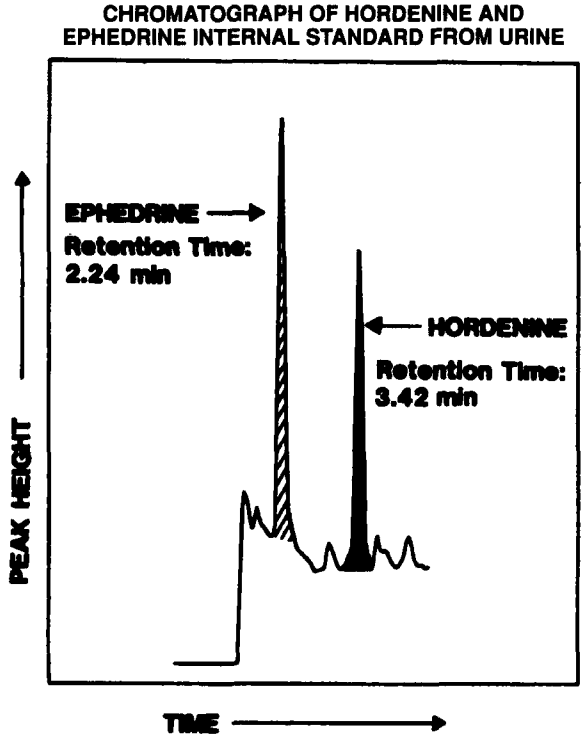

Fig 3: Detection of hordenine on a gas chromatograph equipped with a nitrogen/phosphorous detector. Typical chromatograph from spiked urine analysed for hordenine as described in methods. The solid peak represents hordenine (retention time 3.42 mins) and the hatched peak ephedrine (retention time 2.24 mins) run as an internal standard

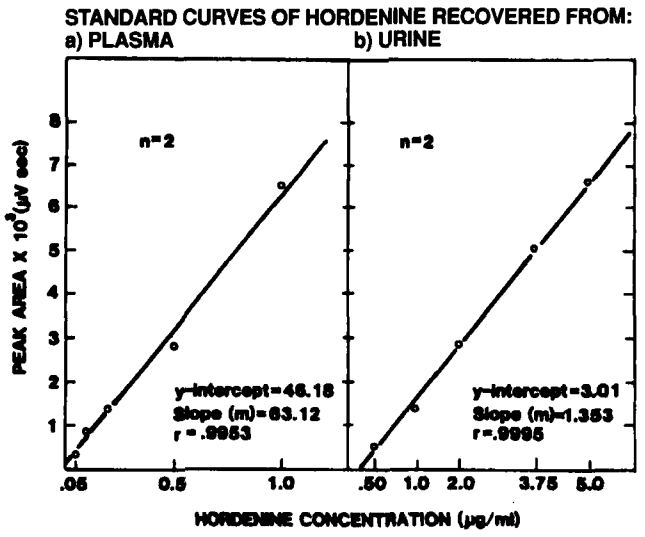

Fig 4: Hordenine standard curves using Tracor 565 gas chromatograph. The points represent the means of standard curves from a) plasma and b) urine analysis as described in materials and methods. Peak height (measured in μV. sec) on the recorder was plotted against μg of hordenine injected on column from a) spiked plasma and b) spiked urine. Correlation coefficients are given from the line representing the linear regression on the data points from the greatest to the least amounts of hordenine detected

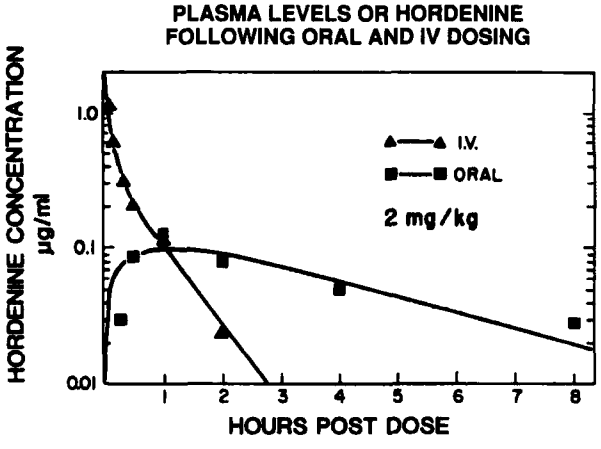

Fig 5: Plasma kinetics of hordenine. Plasma levels of hordenine after administration of 2.0 mg/kg bwt iv to four horses (▲ – ▲) and orally to three horses (■ – ■). The lines indicate the best fit to these data obtained with a two compartment open model and the pharmacokinetic parameters presented in Table 3

We investigated the pharmacokinetics of hordenine after oral administration because one of the ways in which it may enter the horse's system is as a food additive or constituent. Four horses were given 2 mg/kg bwt hordenine by stomach tube, and plasma and urinary concentrations of the drug determined.

As described previously, plasma levels of hordenine after oral administration were very low, peaking at about 0.17 μg/ml 1 h after dosing, and declining thereafter (Fig 5). Urinary concentrations after oral administration averaged about 200 μg/ml after 1 h and remained relatively high for 8 h (Fig 7). They then declined rapidly, and by 24 h post dosing were about 1.5 μg/ml. Urine levels reached a steady state final level of about 0.5 μg/ml at 72 h after dosing.

Discussion

Hordenine is an alkaloid found in many plants and therefore may occasionally be ingested by animals. The present study was instigated by the finding of a number of unusually high concentrations of hordenine in post race urine samples of Kentucky harness horses.

When administered iv, the dose of hordenine necessary to produce a behavioural effect is approximately 2 mg/kg bwt. Initial experiments at a dose level of 0.77 mg/kg bwt produced no apparent behavioural change in four horses. However when the dose was increased to 2.0 mg/kg bwt behavioural changes and effects on the respiratory and cardiovascular system were seen within minutes, peaking within 10 mins of administration. Thereafter these effects declined rapidly, with a half-life of about 3 mins. The very short half-life of the respiratory and cardiovascular effects suggest that they are associated with a bolus effect in the CNS, rather than in the blood.

The rapid disappearance of behavioural effects related to iv administration was supported by the lack of effects on variable

TABLE 3: Pharmacokinetic parameters of hordenine in the horse

	Chelsie	Silk purse	Point Finale	T-11	Mean
iv administration					
A ug/ml	1.828	0.64	8.79	1.65	1.93
B ug/ml	0.40	0.082	0.58	0.35	0.39
t1/2 (min)	2.60	8.80	1.75	6.80	3.54
t1/2 (min)	15.95	62.56	30.95	52.43	32.37
Oral administration					
A µg/ml	1.774	-	9.07	1.68	1.97
B µg/ml	0.305	-	0.598	0.247	0.407
t1/2 (min)	2.930	-	1.717	7.51	3.45
t1/2 (min)	21.18	-	29.88	28.23	31.05
Ka (min)	252.47	-	86.86	109.70	152.8
Fa	3.038	-	0.409	0.77	1.13

The values represent the pharmacokinetic values obtained after best fit of the individual and mean data points of Fig 5 were calculated. The model assumed was a two compartment open model. Ka refers to the half-life of absorption after oral administration of the drug; Fa refers to the fraction of drug absorbed

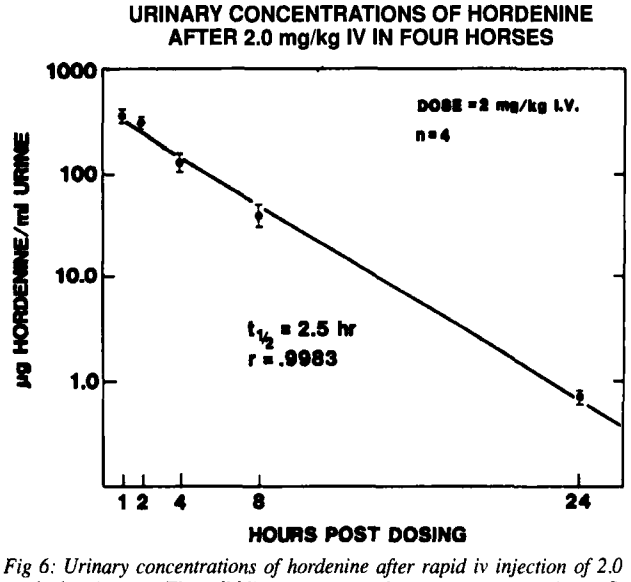

Fig 6: Urinary concentrations of hordenine after rapid iv injection of 2.0 mg/kg bwt (● – ●). The solid line represents a least squares regression to fit the experimental data

interval responding 30 mins after administration. As shown in Table 2, no significant behavioural effects were seen when these animals were tested in the behavioural responding apparatus at 30 mins post administration. This apparatus is one of the more sensitive ways of measuring behavioural activity in the horse and therefore these experiments support suggestions that behavioural and other pharmacological effects of hordenine require relatively high concentrations of the drug at its site of action.

When the drug was administered orally, peak blood levels averaged about 0.1 µg/ml and were not sufficient to produce a pharmacological response. Nevertheless, urinary levels of hordenine found after such oral doses were relatively high, averaging about 200 µg/ml. Based on this information, it appears that high urinary concentrations of hordenine do not necessarily indicate pharmacological effect.

The low plasma concentrations of hordenine after oral administration appear to be related to slow absorption of the drug rather than poor bioavailability which was estimated to be in the order of 100 per cent. It is difficult, however to estimate the true bioavailability of hordenine because it is a constituent of the natural diet of a horse, and its plasma and urinary levels may vary throughout the day depending on the diet of a horse and its feeding

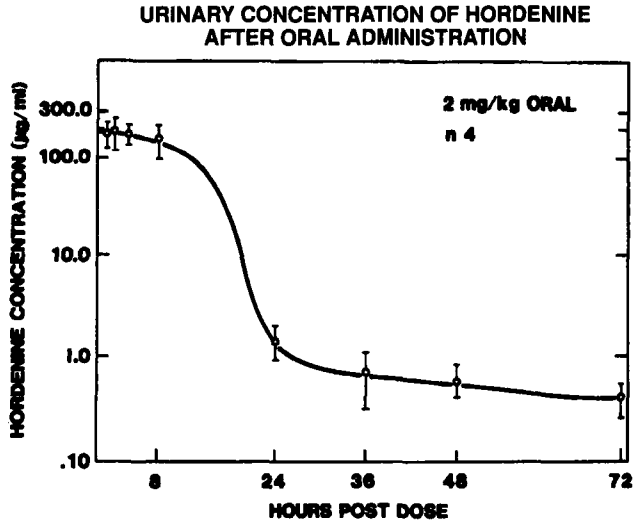

Fig 7: Urinary levels of hordenine after oral administration of 2.0 mg/kg bwt (○ – ○). The curve was fitted by eye

schedule. For this reason therefore, determination of the true bioavailability of hordenine would require more elaborate experiments than those reported here.

The plasma concentrations of hordenine, however, reflected a model in which the absorption of hordenine from the gastrointestinal tract was slow, with an apparent half-life of about 150 mins. The plasma kinetics of hordenine, after oral administration, therefore follow a flip-flop model, where slow oral absorption determines apparent half-life of the drug in urine. Because of this slow absorption, a pharmacological effect would be achieved only with difficulty after oral administration. In summary, hordenine requires very high blood concentrations to produce pharmacological effects in horses, found only after rapid iv injection of 2.0 mg/kg bwt.

When administered orally at the same dose, no behavioural changes were observed and plasma concentrations were substantially below those associated with behavioural changes. However, urinary concentrations of the conjugated metabolite of hordenine appeared in the urine at levels comparable to those found after iv administration. Hordenine is absorbed from the gastrointestinal tract completely but also, apparently, very slowly which adds to the difficulty of producing a pharmacological effect after oral administration.

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