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THE DETECTION, PHARMACOKINETICS AND BEHAVIORAL EFFECTS OF DIISOPROPYLAMINE DICHLOROACETATE (DADA) IN THE HORSE: A PRELIMINARY REPORT

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Abstract—1. Drug administration studies using diisopropylamine dichloroacetate (DADA) and diisopropylamine (DIPA) were conducted in Thoroughbred and Standardbred horses to assess physiological effects and develop detection methods.

2. Four horses received 0.08 mg DADA/kg body wt and showed no changes in heart and respiratory rates or body temperature as measured over a 1-hr period after administration. A transient diuretic effect was found to occur in 2 mares dosed with 0.80 mg DADA/kg body wt.

3. A qualitative detection method using thin-layer chromatography was developed to detect DIPA, the major metabolite of DADA in equine urine. A quantitative detection method (lower limit of detection 0.5 µg/ml urine) for this metabolite was also developed using gas chromatography.

4. Neither DADA or the free base, DIPA, were detectable in equine blood samples using the above-mentioned methodologies.

INTRODUCTION

Diisopropylamide dichloroacetate (DADA) (Fig. 1) was synthesized by Krebs and Krebs in 1952, shortly after these investigators had synthesized pangamic acid ("vitamin B_{15}") and it has been suggested that these two substances are chemically and pharmacologically related (Kruglikova-L'ova et al., 1965). The pharmacology of DADA has been reported in laboratory animals and humans, (Krushaar, et al., 1963) but there are no reports of its pharmacology in horses. More recently, there have been reports of its use in racehorses, and we have therefore studied its detection, actions, and effects in horses.

The principle pharmacological effects of clinical significance reported for DADA in species other than the horse is hypotension. This response was reported to occur after small doses, and was not blocked by atropine. The minimal dose required to produce this effect was 0.25 mg/kg, with up to 1 mg/kg producing this effect in dogs. Clinical studies have reported DADA to be a safe, useful and self-limiting vasodilator in the treatment of cardiac ischemia, valvular coronary sclerosis and myocardial infarction and angina pectoris. Reviewing these results, Stackpole (1969) concluded that DADA is characterized by its low toxicity and high therapeutic index, hypotensive action, a moderate but constant bradycardiac effect, with prolonged increases in stroke and minute volumes and cardiac output, and a slight decrease in body temperature. These actions were associated with little effect on metabolic processes. Beyond this, the drug

was reported to ameliorate the signs and symptoms of many cardiovascular, hepatic, musculoskeletal and alcoholic disorders. In racehorses it is purported to be useful in treating azoturia ("tying up") at a reported dosage of 40 mg/horse (0.08 mg/kg).

Because DADA may be considered an illegal drug when used in racehorses, and because it has been difficult to detect in post-race blood or urine samples, we elected to study the detection, actions and effects of DADA in horses.

DADA is the salt form of diisopropylamine, (DIPA) and dichloroacetic acid (DCA). We studied the activity of DIPA, since this is the portion most likely to be pharmacologically active.

MATERIALS AND METHODS

Horses

Mature Thoroughbred and Standardbred mares and geldings (450–550 kg) were used throughout. All were in good health. These horses were kept at pasture until the day of their experimental session.
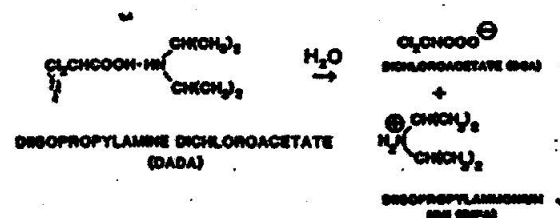

Fig. 1. Chemical structure of diisopropylamine dichloroacetate (DADA) and its dissociation products dichloroacetate (DCA) and diisopropylammonium (DIPA).

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Drugs

DIPA and DCA were obtained from a commercial source (Sigma Chemical Co., St. Louis, MO). For the analytical studies, DADA was prepared by mixing equimolar DIPA and DCA (10 mmol) as by the method of Gelener and Herbert (1982). A solution of DADA at 23% was prepared with distilled water. This solution was steralized by filtration for i.v. injection.

Pharmacokinetic studies

In the pharmacokinetic studies, 4 mares were given DADA at 0.08 mg/kg body wt. Two mares also received DADA at 0.80 mg/kg body wt. In each case, the mares were confined in box stalls (15 square m) before dosing and for the entire experiment. DADA injections were made into the right jugular vein. All pre- and post-dose blood samples were drawn from the left jugular vein into sterile evacuated grey top blood collection tubes (Vacutainer Systems, Rutherford, NJ). Blood samples were drawn before injection and at 5, 10, 15, 20, 30, 45 and 60 min and at 2 and 4 hr after injection. Blood samples were centrifuged and plasma was aliquoted and frozen until analysis. Urine samples for analysis were collected by direct bladder catheterization. For control urine sample collection the bladder was drained completely 30 min before dosing and then again immediately before injection. Post drug administration samples were collected at 30 and 60 min and 2, 4, 6, 8, 12, 24, 36 and 48 hr after dosing in the 0.08 mg/kg studies. In the 0.80 mg/kg studies involving 2 mares, the urine sample protocol was the same except that after dosing, the urine samples were collected at 10 min intervals through the first 60 min and then at 1.5, 2.0, 2.5 and 3.0 hr.

Pharmacodynamic studies

In the pharmacodynamic experiments, 4 horses (mares and geldings) were dosed at 0.08 mg/kg body wt with DIPA. Horses were brought in from pasture at the beginning of each session, allowed 2 hr to acclimate to the experimental setting in a box stall, then confined in stock restraints for the experiment. Heart and respiratory rates and body temperature of the horses were monitored continuously from 30 min before dosing to establish baseline values and then monitored for an additional 60 min after drug administration. Heart rate activity was recorded using a polygraph (Grass Instrument Co, Quincy, MA) with electrodes placed bilaterally at the horses heart girth. Respiration was recorded by monitoring changes in impedence (Impedence Converter, UFI Inc., Morrow Bay, CA) across electrodes placed bilaterally caudal to the diaphragm. Body temperature was recorded using a rectal temperature probe with a digital display monitor (Thermalert TH-6, Bailey Instruments Inc., Saddlebrook, NJ).

Analytical methods

Urine samples were analyzed for DIPA. Two analytical methods were used, a qualitative thin-layer chromatographic (TLC) test suitable for routine screening, and a gas chromatographic (GC) method which allowed quantification of the amount of DIPA in the sample. In addition, mass spectroscopic confirmation was carried out.

Extraction and gas chromatography

A standard curve (0.5 μg/ml–10 μg/ml DIPA in blank urine) was set up. Urine samples and standards, in duplicate (2 ml), were pipetted into 125 × 12 mm screw top culture tubes. Diethylamine as internal standard was added to each tube at a concentration of 10 μg/mL. Each tube was adjusted to pH 12 with 1 N sodium hydroxide added dropwise. n-Hexane (4 ml) was added to each tube and the samples were put on a rotary mixer for 10 min. The tubes were centrifuged at 2°C, 1150 g for 30 min. The organic layer was pipetted into a clean tube and 25 μl of 0.5% HCl in methanol was added, followed by 10 min of rotary mixing.

The samples were evaporated to dryness under a stream of nitrogen. The residue quaternary ammonium salts were dissolved in 50 μl isopropanol.

The isopropanol solution (2 μl) was injected onto a Tracor 565 gas chromatograph (Tracor, Austin, TX) equipped with a 28% All-tech 223 + 4% KOH on Gas Chrom R, 80/100 (Alltech-Applied Science, Deerfield, IL) column (6 ft, 2 mm i.d.) and a nitrogen/phosphorous detector. Isothermal oven temperature was 160°C, detector temperature was 280°C and the injector temperature was 180°C. The response was integrated by a SP4270 Integrator (Spectra Physics, Piscataway, NJ).

Thin-layer chromatographic analysis

A TLC method was developed to provide qualitative detection of DIPA. In this procedure 12 ml urine was made basic with 0.8 ml concentrated sodium hydroxide. To this mixture was added 6 ml of a petroleum ether–dichloromethane solvent blend (2:1). The sample was extracted for 5 min with rotary mixing. The mixture was centrifuged, and the organic solvent was transferred to a clean tube for evaporation. 25 μl of 0.5% hydrochloric acid in methanol was added to each tube prior to evaporation to form a nonvolatile DIPA salt.

After solvent evaporation under a stream of nitrogen in a 35°C water bath, the residue in each tube was spotted on a Whatman high performance silica gel thin-layer plate along with an appropriate standard(s). 50 μl methyl acetate was added to the residue for spotting. The thin-layer plate was developed for 5 cm in a ethyl acetate:methanol:acetic acid (7:2:1) system.

Following development the plate was exposed to formalin fumes for 1 min in a sealed tank. The plate was then oversprayed with Dragendorff's (Whatman Reagents, Clifton, NJ) followed by a 5% sodium nitrite spray to yield a brown-gray spot at R_f of 0.32.

RESULTS

Pharmacological effects of DADA

When DIPA was administered to horses at 0.08 mg/kg (40 mg/horse), no overt behavioral responses were observed. Similarly, the heart rates, respiratory rates, and body temperatures of these horses were not affected by this dose of DIPA (Fig. 2). Furthermore, in 2 horses dosed with 10 times this amount of DADA (400 mg/horse), no overt behavioral changes were seen. However, there was some evidence of a transient diuresis in mares given the larger dose of DADA. In two mares treated with this dose of DADA their mean urinary flow rates increased from
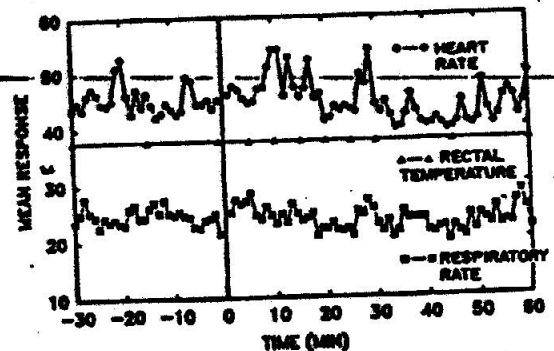

Fig. 2. Physiological effects of DIPA administered to 4 horses at 0.08 mg/kg on heart rate (●) respiratory rate (■) and rectal temperature (▲). All data points represent the mean values of 4 horses.

Table 1. Effect of 0.8 mg DIPA/kg body wt on urine volume output in 2 mares

Time (min)	Mare A	B	X (ml/min)
−60	0.28	11.2	5.70
−30	1.77	10.8	6.30
10	3.40	22.0	12.7
20	2.80	28.5	15.7
30	1.70	13.5	7.60
40	4.50	12.0	8.30
50	2.20	9.60	5.90
60	3.40	7.40	5.40

6 ml/min in control animals to about 15 ml/min at 15 min after dosing (Table 1).

Detection of DADA in urine and plasma

(a) Drug recovery. DIPA is an unusually difficult drug to detect in the blood or urine of horses. This is because it is an exceptionally small molecule, is strongly basic, and is quite volatile. Because it is strongly basic, the pH of the solution used to extract it must be very alkaline. Preliminary work showed that it does not extract well under the pH 9.5 extraction conditions routinely used to extract basic drugs. To successfully extract DIPA it was necessary to increase the pH of the extraction system to about pH 12.0. Beyond this, recovery of this drug after it had been extracted posed further problems. This is because the DIPA molecular is light and volatile and is easily lost during the evaporation and concentration steps used in most drug analyses. We found that a satisfactory way to handle this drug was to render the solution very acidic, thereby quaternizing the drug and reducing its volatility. In this way it was possible to concentrate the sample without losing all of the DIPA by evaporation.

(b) Thin-layer chromatographic analysis. After spiked and post dose urine samples were treated and

DADA
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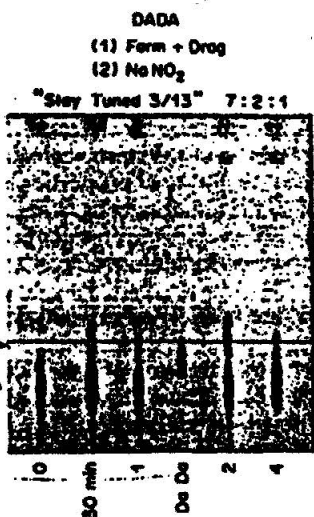(2) NaNO₂
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Fig. 3. Thin-layer chromatogram of DIPA from horse urine. A horse was dosed with 0.8 mg/kg of DADA. Urine samples collected from the horse at the indicated times, extracted and chromatographed as described in methods. The presence of DIPA in the samples is indicated by a brown spot at R_f 0.32 after overspraying with Dragendorfs followed by 5% NaNO₂.
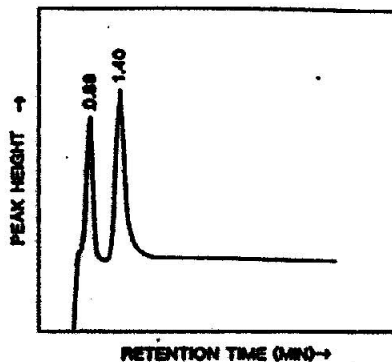

Fig. 4. Typical chromatogram of DIPA (retention time 0.89 min) with isopropylamine (retention time 1.40 min) run as an internal standard under GC conditions outlined in Methods.

developed on the high-performance thin-layer chromatography (HPTLC) plate, a series of brown gray spots with an average R_f of 0.32 were obtained. After administering DADA at 0.08 mg/kg, the residue of DIPA in urine was still readily detectable at 4 hr after dosing (Fig. 3). This method is therefore sufficiently sensitive for routine screening of DADA in post race urine samples.

(c) Gas chromatographic analysis. The critical step in the GC analysis of DIPA was identifying an optimal solvent to redissolve the DIPA. We found isopropanol to give the best results. When this sequence of extraction-recovery steps was followed we found about 50% recovery of DIPA added to urine samples. The method is therefore sufficiently accurate and sensitive to allow quantification of DIPA in horse urine samples. A typical chromatogram of DIPA is shown in Fig. 4.

The threshold for detection of DIPA is about 0.5 µg/ml. A typical standard curve for DIPA in horse urine is nonlinear but well fitted by a quadratic function (Fig. 5). Using this method urinary concentrations of DIPA in horse urines after administration of different doses of DADA was followed.

After administration of 0.08 mg/kg of DADA to horses no blood amounts of DADA or DIPA were detected using the techniques outlined above. Urinary concentrations of DIPA were detected at 30 min after dosing, peaked at about 2.0 µg/ml at 1 hr after dosing
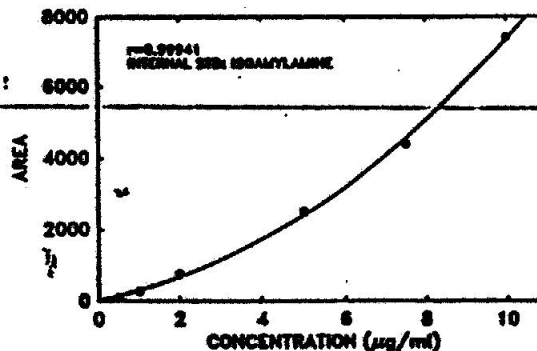

Fig. 5. Standard curve of the concentration of DIPA recovered from urine against peak area by GC analysis. The points were well fitted by a curve following the quadratic function $y = ax^2 + b$.
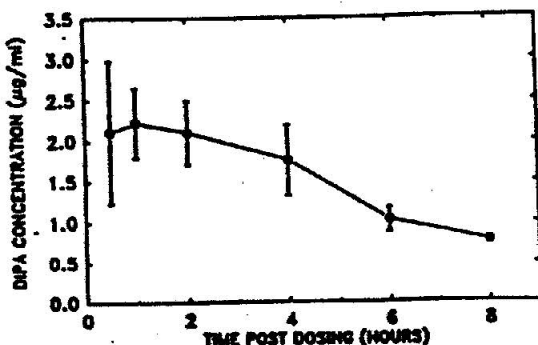
Fig. 6. DIPA concentration in urine after administration of 0.08 mg/kg DADA (n = 4). Plot shows the concentration recovered over time.

Fig. 8. Mass spectrum of a propionyl derivative of diiso-propylamine. A molecular ion is apparent at 157 M/E with a base peak shown at 86. Inset shows the structure of the derivative.

and then declined in an apparently zero order fashion to become undetectable at about 8 hr after dosing (Fig. 6). These data are in good agreement with the HPTLC data presented in Fig. 3, and suggest that the period for which DADA is likely to be detectable in urine is relatively short compared to most other agents.

Because no pharmacological effects of DIPA were seen after administration of the 0.08 mg/kg dose, we administered 10 times this dose to 2 horses during our preliminary evaluations of this drug. After administration of 0.80 mg DADA/kg body wt, the concentrations of DIPA found in horse urine peaked at about 80 μg/ml at 30 min after dosing, and declined rapidly at first, then more slowly, to remain detectable in urine at 3 hr after administration of the drug (Fig. 7A). Since DADA has an apparent diuretic affect in horse urine these concentrations do not exactly parallel the rate of excretion of the drug. Thus a plot of the actual amounts of the drug excreted against time (Fig. 7B) shows that the great bulk of the drug excretion occurs in the first 30 min after dosing.

and that excretion at 90 min after dosing was greatly reduced. The total amount of DADA excreted in urine as DIPA was about 11% of total drug.

(d) Mass spectroscopic confirmation. The DIPA fragment of this compound was difficult to confirm by mass spectrometry (MS) because of its volatility and short retention time on our GC columns. Therefore we derivatized DIPA with propionic anhydride. When derivatized a good mass spectrum of the propionyl derivative of DIPA was obtained with a molecular ion at 157 M/E units and a base peak at 86 (Fig. 8).

DISCUSSION

Based on the structure of DADA a number of characteristics of this drug may be expected. Since it is composed of two relatively small molecules which are paired to form a salt, in vivo it may be expected that these molecules will dissociate to yield the individual ions, DIPA and DCA. As a secondary amine, DIPA is likely to be highly charged at physiological pH and as such will not enter cells readily. It will therefore tend not to be metabolized in the horse, but to be excreted by glomerular filtration. As such the duration of action of its pharmacological effects will be short, a relatively large proportion of a dose will be excreted in the urine unchanged and, if the administered dose is large enough, an osmotic diuretic effect may be expected. In addition, because of the small size of the DIPA molecule it may be expected that loss of this molecule by volatilization in extraction and recovery could be substantial, and interfere with the recovery of this agent from blood and urine samples. Finally, because of the small size and very simple structure of this molecule it seems unlikely that specific pharmacological effects will be found after injection.

In general, the results reported here support these expectations. After i.v. injection of DADA the pharmacological effects observed are small, even when the dose of drug is 10 times the reported clinical dose. This dose, however, was large enough to produce what appears to be an osmotic diuresis, suggesting that the drug is being excreted in relatively large amounts in the urine. This observation is
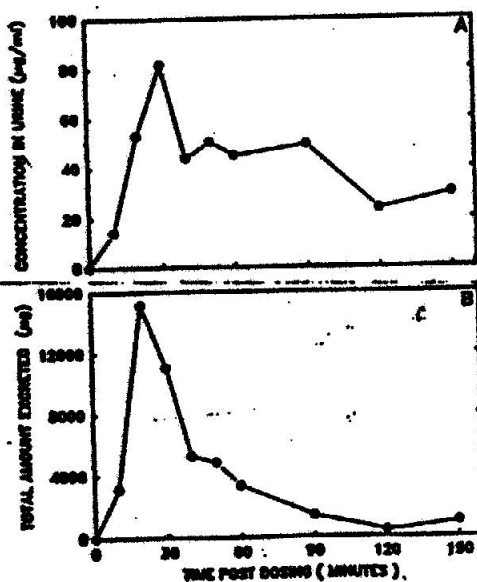

Fig. 7. DIPA excretion patterns in urine after administration of 0.8 mg/kg DADA. Panel A shows recovery pattern of DIPA when plotted as concentration. Panel B shows total amount of drug recovered from urine over 2.5 hr.
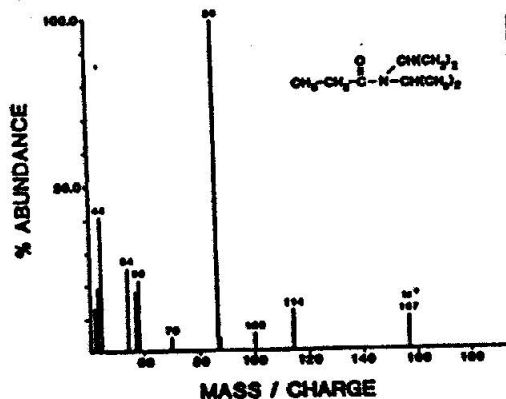
supported by the data of Fig. 7, which shows that a large proportion of this drug is excreted unchanged in the urine, consistent with the drug being an agent which enters cells poorly, is excreted by glomerular filtration and is found in the urine in relatively high concentrations.

If DIPA is rapidly excreted, its detection in blood is likely to be difficult since after i.v. injection plasma levels of the drug can be expected to decline rapidly. In agreement with this we were unable to detect DIPA in plasma after administration of doses as high as 400 mg/horse. Similarly, we also had trouble initially in detecting this drug in urine, due in part to the difficulty in recovery and analysis for this drug and also in part to the relatively rapid excretion of DADA in urine.

Our initial problem in detecting DADA was apparently due to the very basic nature of this drug. While the pK_a of DADA is not known, it is apparently more basic than the average basic drug recovered in equine drug testing. Most medications found in equine urine extract well at a pH of about 9.5, while to extract DIPA, a pH of about 12 was required. The simplest explanation for this fact is that the pK_a of DADA is much higher than that of most basic drugs, and for this reason the pH of the extracting medium has to be higher. When the pH of the extraction medium was increased to 12.0, DIPA extracted well and recovery of this drug from urine was greatly improved.

As well as being difficult to extract from urine DADA turned out to be difficult to handle in the concentration process. This is because as a small molecule it tends to be volatile and is readily lost during the evaporation process. To overcome this problem the concentrating solution was made acidic, thereby forming a molecular salt and rendering it nonvolatile. When this precaution was taken recovery of the drug was greatly improved, and quantitative recovery of the drug from urine became possible.

Considerable difficulty was encountered in identifying a solvent suitable for dissolving the extracted material for GC analysis. Methanol appeared to react with DIPA to yield a material with different chromatographic characteristics from authentic DIPA. Isopropanol was selected as the material into which DIPA could be dissolved and chromatographed satisfactorily.

Dosing horses with the clinically suggested dose of DADA (0.08 mg/kg or 40 mg/horse) produced no apparent overt effects. In the physiology studies after doses of 0.08 mg/kg DIPA, no changes in respiratory rate suggesting central-nervous stimulation were observed, and neither were there changes in basal body temperature. More significantly, however, in view of the reported pharmacological actions of DADA as a hypotensive agent there were no apparent effects of this dose of DIPA on the cardiac rates of these horses. In general, one might expect that if this drug was able to reduce blood pressure in a horse significantly at this dose it would also be likely to increase the heart rate of these horses. No such effect was found, suggesting that the clinically recommended doses of this agent are not likely to produce a pharmacologically effective hypotension in horses.

In addition to not producing pharmacological effects, these doses of DADA did not give rise to detectable levels of DIPA in the bloodstream of horses after either the 40 or 400 mg/horse doses. The reason for our inability to detect the drug in blood is unclear, but is likely related to the relatively low sensitivity of our detection method and the rapid clearance of this drug from blood. The likelihood of detecting this drug in blood after clinical dose (40 mg/horse) appears small since we were unable to detect the drug in blood after the larger 400 mg/horse dose.

The detection methods reported here were more than sufficient to detect the drug in urine, however. After the 40 mg/horse dose the drug was detected in urine for up to 8 hr. Although this is a relatively short period by the standards of other agents, it is likely sufficient to allow control of the use of this agent in racehorses. This is because if the duration of action (if any) of this drug is short it will have to be given shortly before post time to be effective, and the methods reported here allow easy detection of this drug for up to 4 hr or more after its administration.

Beyond simple detection of the drug, it must also be confirmed by MS analysis to enable its regulatory control. Since underivatized DIPA is quite volatile and therefore difficult to chromatograph, we derivatized DIPA with propionic anhydride to increase the mass of the molecule and reduce its volatility. When treated in this way the propionyl derivative of DIPA yielded a molecular ion at 157 M/E and a base peak at 86. This derivative forms readily, chromatographs well and enables the development of good MS evidence of the presence of DIPA in a sample.

The regulatory control of DADA is also complicated by the fact that this substance is marketed to horsemen as the supposed "vitamin B_{15}". Since most rules of racing allow the use of vitamins and no restrictions are placed on their administration to horses, or on their inclusion in the horses diet, it can be difficult to regulate the use of this agent on forensic grounds. This is despite the fact that "vitamin B_{15}" has no recognized chemical structure, and is not recognized by the U.S. Food and Drug Administration as either a discrete chemical structure or as a vitamin.

In conclusion, therefore, we have studied the detection, disposition and behavioral effects of DADA in horses. When administered i.v. at a dose of 40 mg/horse no effects of DIPA on the behavior, heart or respiratory rates or basal body temperature of horses was observed. Using an extraction and recovery method reported here, DIPA could be readily recovered from urine but not from blood of these treated horses. Using HPTLC, DIPA was detectable in horse urine for at least 4 hr after dosing. Similarly, it was detectable by a GC detection method for at least 8 hr after dosing. MS confirmation of the presence of the drug in the urine samples was possible using a propionic anhydride derivative of DIPA. Together these experimental techniques should be sufficient to allow effective screening and MS confirmation of this drug in post race urine samples and allow for effective regulatory control of this agent in horse racing.

SUMMARY

Diisopropylamine dichloroacetate (DADA) is a medication marketed for use in horses in Australia.

In this communication we report on the detection, actions and effects in horses of diisopropylamine, the pharmacologically active portion of this agent.

To assess the physiological effects of DIPA 4 horses were dosed with DIPA at 0.08 mg/kg body wt and monitored for changes in physiological parameters. No apparent changes in heart and respiratory rates or body temperature were recorded in horses dosed with 0.08 mg/kg DIPA. However, a transient diuretic effect was found in 2 mares dosed with DADA at 0.80 mg/kg body wt.

To develop detection methods in equine urine and blood, DADA was prepared and horses were injected with 0.08 mg/kg ($n = 4$) or 0.80 mg/kg ($n = 2$) of DADA intravenously. The major metabolite of DADA in horse urine was found to be DIPA. A qualitative detection method for DIPA in equine urine using thin-layer chromatography (TLC) was developed. Additionally a quantitative detection method for DIPA in equine urine samples was developed using gas chromatography (GC).

Extraction and alkalinization of samples allowed for detection of DIPA in urine. DIPA was readily detectable using TLC screening after 0.80 mg/kg for at least 4 hr after administration. GC analysis allowed for detection of DIPA down to about 0.5 μg/ml for about 8 hr after administration of a clinical dose of DADA at 0.08 mg/kg. However, neither DADA or DIPA were detectable in blood by either of these methodologies.

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4. Neither DADA or the free base, DIPA, were detectable in equine blood samples using the above-mentioned methodologies.

INTRODUCTION

Diisopropylamide dichloroacetate (DADA) (Fig. 1) was synthesized by Krebs and Krebs in 1952, shortly after these investigators had synthesized pangamic acid ("vitamin B_{15}") and it has been suggested that these two substances are chemically and pharmacologically related (Kruglikova-L'ova *et al.*, 1965). The pharmacology of DADA has been reported in laboratory animals and humans, (Krushaar, *et al.*, 1963) but there are no reports of its pharmacology in horses. More recently, there have been reports of its use in racehorses, and we have therefore studied its detection, actions, and effects in horses.

The principle pharmacological effects of clinical significance reported for DADA in species other than the horse is hypotension. This response was reported to occur after small doses, and was not blocked by atropine. The minimal dose required to produce this effect was 0.25 mg/kg, with up to 1 mg/kg producing this effect in dogs. Clinical studies have reported DADA to be a safe, useful and self-limiting vasodilator in the treatment of cardiac ischemia, valvular coronary sclerosis and myocardial infarction and angina pectoris. Reviewing these results, Stackpole (1969) concluded that DADA is characterized by its low toxicity and high therapeutic index, hypotensive action, a moderate but constant bradycardiac effect, with prolonged increases in stroke and minute volumes and cardiac output, and a slight decrease in body temperature. These actions were associated with little effect on metabolic processes. Beyond this, the drug

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DADA is the salt form of diisopropylamine, (DIPA) and dichloroacetic acid (DCA). We studied the activity of DIPA, since this is the portion most likely to be pharmacologically active.

MATERIALS AND METHODS

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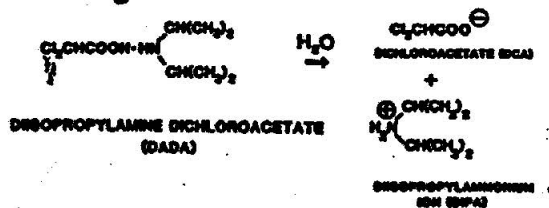

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DIPA and DCA were obtained from a commercial source (Sigma Chemical Co., St. Louis, MO). For the analytical studies, DADA was prepared by mixing equimolar DIPA and DCA (10 mmol) as by the method of Geleter and Herbert (1982). A solution of DADA at 23% was prepared with distilled water. This solution was steralized by filtration for i.v. injection.

Pharmacokinetic studies

In the pharmacokinetic studies, 4 mares were given DADA at 0.08 mg/kg body wt. Two mares also received DADA at 0.80 mg/kg body wt. In each case, the mares were confined in box stalls (15 square m) before dosing and for the entire experiment. DADA injections were made into the right jugular vein. All pre- and post-dose blood samples were drawn from the left jugular vein into sterile evacuated grey top blood collection tubes (Vacutainer Systems, Rutherford, NJ). Blood samples were drawn before injection and at 5, 10, 15, 20, 30, 45 and 60 min and at 2 and 4 hr after injection. Blood samples were centrifuged and plasma was aliquoted and frozen until analysis. Urine samples for analysis were collected by direct bladder catheterization. For control urine sample collection the bladder was drained completely 30 min before dosing and then again immediately before injection. Post drug administration samples were collected at 30 and 60 min and 2, 4, 6, 8, 12, 24, 36 and 48 hr after dosing in the 0.08 mg/kg studies. In the 0.80 mg/kg studies involving 2 mares, the urine sample protocol was the same except that after dosing, the urine samples were collected at 10 min intervals through the first 60 min and then at 1.5, 2.0, 2.5 and 3.0 hr.

Pharmacodynamic studies

In the pharmacodynamic experiments, 4 horses (mares and geldings) were dosed at 0.08 mg/kg body wt with DIPA. Horses were brought in from pasture at the beginning of each session, allowed 2 hr to acclimate to the experimental setting in a box stall, then confined in stock restraints for the experiment. Heart and respiratory rates and body temperature of the horses were monitored continuously from 30 min before dosing to establish baseline values and then monitored for an additional 60 min after drug administration. Heart rate activity was recorded using a polygraph (Grass Instrument Co, Quincy, MA) with electrodes placed bilaterally at the horses heart girth. Respiration was recorded by monitoring changes in impedence (Impedence Converter, UFI Inc., Morrow Bay, CA) across electrodes placed bilaterally caudal to the diaphragm. Body temperature was recorded using a rectal temperature probe with a digital display monitor (Thermalert TH-6, Bailey Instruments Inc., Saddlebrook, NJ).

Analytical methods

Urine samples were analyzed for DIPA. Two analytical methods were used, a qualitative thin-layer chromatographic (TLC) test suitable for routine screening, and a gas chromatographic (GC) method which allowed quantification of the amount of DIPA in the sample. In addition, mass spectroscopic confirmation was carried out.

Extraction and gas chromatography

A standard curve (0.5 µg/ml–10 µg/ml DIPA in blank urine) was set up. Urine samples and standards, in duplicate (2 ml), were pipetted into 125 × 12 mm screw top culture tubes. Diethylamine as internal standard was added to each tube at a concentration of 10 µg/ml. Each tube was adjusted to pH 12 with 1 N sodium hydroxide added dropwise. n-Hexane (4 ml) was added to each tube and the samples were put on a rotary mixer for 10 min. The tubes were centrifuged at 2°C, 1150 g for 30 min. The organic layer was pipetted into a clean tube and 25 µl of 0.5% HCl in methanol was added, followed by 10 min of rotary mixing.

The samples were evaporated to dryness under a stream of nitrogen. The residue quaternary ammonium salts were dissolved in 50 µl isopropanol.

The isopropanol solution (2 µl) was injected onto a Tracor 565 gas chromatograph (Tracor, Austin, TX) equipped with a 28% Ali-tech 223 + 4% KOH on Gas Chrom R, 80/100 (Alltech-Applied Science, Deerfield, IL) column (6 ft, 2 mm i.d.) and a nitrogen/phosphorous detector. Isothermal oven temperature was 160°C, detector temperature was 280°C and the injector temperature was 180°C. The response was integrated by a SP4270 Integrator (Spectra Physics, Piscataway, NJ).

Thin-layer chromatographic analysis

A TLC method was developed to provide qualitative detection of DIPA. In this procedure 12 ml urine was made basic with 0.8 ml concentrated sodium hydroxide. To this mixture was added 6 ml of a petroleum ether–dichloromethane solvent blend (2:1). The sample was extracted for 5 min with rotary mixing. The mixture was centrifuged, and the organic solvent was transferred to a clean tube for evaporation. 25 µl of 0.5% hydrochloric acid in methanol was added to each tube prior to evaporation to form a nonvolatile DIPA salt.

After solvent evaporation under a stream of nitrogen in a 35°C water bath, the residue in each tube was spotted on a Whatman high performance silica gel thin-layer plate along with an appropriate standard(s). 50 µl methyl acetate was added to the residue for spotting. The thin-layer plate was developed for 5 cm in a ethyl acetate:methanol:acetic acid (7:2:1) system.

Following development the plate was exposed to formalin fumes for 1 min in a sealed tank. The plate was then oversprayed with Dragendorff's (Whatman Reagents, Clifton, NJ) followed by a 5% sodium nitrite spray to yield a brown-gray spot at R_f of 0.32.

RESULTS

Pharmacological effects of DADA

When DIPA was administered to horses at 0.08 mg/kg (40 mg/horse), no overt behavioral responses were observed. Similarly, the heart rates, respiratory rates, and body temperatures of these horses were not affected by this dose of DIPA (Fig. 2). Furthermore, in 2 horses dosed with 10 times this amount of DADA (400 mg/horse), no overt behavioral changes were seen. However, there was some evidence of a transient diuresis in mares given the larger dose of DADA. In two mares treated with this dose of DADA their mean urinary flow rates increased from
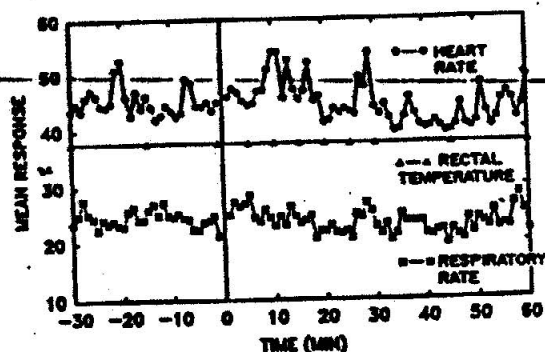

Fig. 2. Physiological effects of DIPA administered to 4 horses at 0.08 mg/kg on heart rate (●) respiratory rate (■) and rectal temperature (▲). All data points represent the mean values of 4 horses.

Table 1. Effect of 0.8 mg DIPA/kg body wt on urine volume output in 2 mares

Time (min)	Mare A	B	X (ml/min)
−60	0.28	11.2	5.70
−30	1.77	10.8	6.30
10	3.40	22.0	12.7
20	2.80	28.5	15.7
30	1.70	13.5	7.60
40	4.50	12.0	8.30
50	2.20	9.60	5.90
60	3.40	7.40	5.40

6 ml/min in control animals to about 15 ml/min at 15 min after dosing (Table 1).

Detection of DADA in urine and plasma

(a) Drug recovery. DIPA is an unusually difficult drug to detect in the blood or urine of horses. This is because it is an exceptionally small molecule, is strongly basic, and is quite volatile. Because it is strongly basic, the pH of the solution used to extract it must be very alkaline. Preliminary work showed that it does not extract well under the pH 9.5 extraction conditions routinely used to extract basic drugs. To successfully extract DIPA it was necessary to increase the pH of the extraction system to about pH 12.0. Beyond this, recovery of this drug after it had been extracted posed further problems. This is because the DIPA molecular is light and volatile and is easily lost during the evaporation and concentration steps used in most drug analyses. We found that a satisfactory way to handle this drug was to render the solution very acidic, thereby quaternizing the drug and reducing its volatility. In this way it was possible to concentrate the sample without losing all of the DIPA by evaporation.

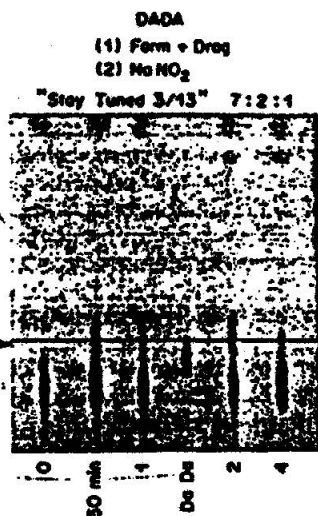(b) Thin-layer chromatographic analysis. After spiked and post dose urine samples were treated and

Fig. 3. Thin-layer chromatogram of DIPA from horse urine. A horse was dosed with 0.8 mg/kg of DADA. Urine samples collected from the horse at the indicated times, extracted and chromatographed as described in methods. The presence of DIPA in the samples is indicated by a brown spot at R_f 0.32 after overspraying with Dragendorfs followed by 5% NaNO$_2$.
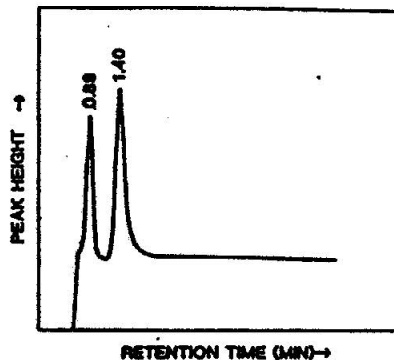
Fig. 4. Typical chromatogram of DIPA (retention time 0.89 min) with isopropylamine (retention time 1.40 min) run as an internal standard under GC conditions outlined in Methods.

developed on the high-performance thin-layer chromatography (HPTLC) plate, a series of brown gray spots with an average R_f of 0.32 were obtained. After administering DADA at 0.08 mg/kg, the residue of DIPA in urine was still readily detectable at 4 hr after dosing (Fig. 3). This method is therefore sufficiently sensitive for routine screening of DADA in post race urine samples.

(c) Gas chromatographic analysis. The critical step in the GC analysis of DIPA was identifying an optimal solvent to redissolve the DIPA. We found isopropanol to give the best results. When this sequence of extraction-recovery steps was followed we found about 50% recovery of DIPA added to urine samples. The method is therefore sufficiently accurate and sensitive to allow quantification of DIPA in horse urine samples. A typical chromatogram of DIPA is shown in Fig. 4.

The threshold for detection of DIPA is about 0.5 μg/ml. A typical standard curve for DIPA in horse urine is nonlinear but well fitted by a quadratic function (Fig. 5). Using this method urinary concentrations of DIPA in horse urines after administration of different doses of DADA was followed.

After administration of 0.08 mg/kg of DADA to horses no blood amounts of DADA or DIPA were detected using the techniques outlined above. Urinary concentrations of DIPA were detected at 30 min after dosing, peaked at about 2.0 μg/ml at 1 hr after dosing
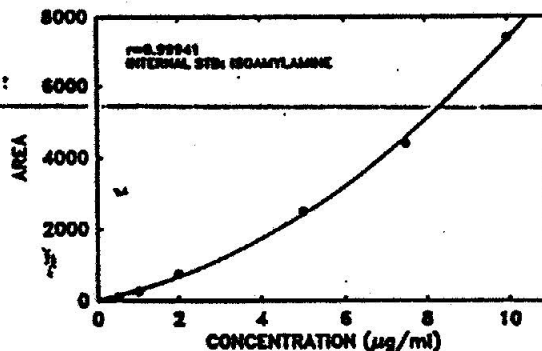
Fig. 5. Standard curve of the concentration of DIPA recovered from urine against peak area by GC analysis. The points were well fitted by a curve following the quadratic function $y = ax^2 + b$.
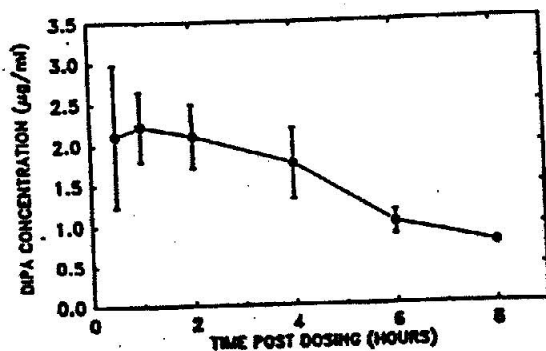
Fig. 6. DIPA concentration in urine after administration of 0.08 mg/kg DADA ($n = 4$). Plot shows the concentration recovered over time.

Fig. 8. Mass spectrum of a propionyl derivative of diisopropylamine. A molecular ion is apparent at 157 M/E with a base peak shown at 86. Inset shows the structure of the derivative.

and then declined in an apparently zero order fashion to become undetectable at about 8 hr after dosing (Fig. 6). These data are in good agreement with the HPTLC data presented in Fig. 3, and suggest that the period for which DADA is likely to be detectable in urine is relatively short compared to most other agents.

Because no pharmacological effects of DIPA were seen after administration of the 0.08 mg/kg dose, we administered 10 times this dose to 2 horses during our preliminary evaluations of this drug. After administration of 0.80 mg DADA/kg body wt, the concentrations of DIPA found in horse urine peaked at about 80 μg/ml at 30 min after dosing, and declined rapidly at first, then more slowly, to remain detectable in urine at 3 hr after administration of the drug (Fig. 7A). Since DADA has an apparent diuretic affect in horse urine these concentrations do not exactly parallel the rate of excretion of the drug. Thus a plot of the actual amounts of the drug excreted against time (Fig. 7B) shows that the great bulk of the drug excretion occurs in the first 30 min after dosing.
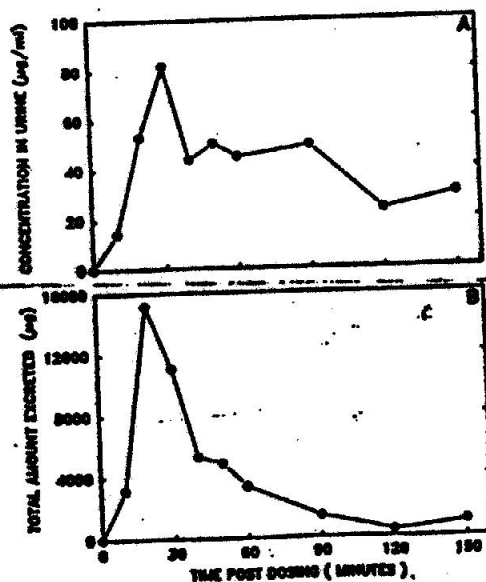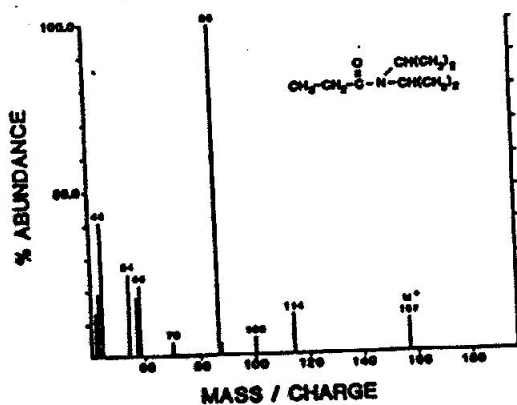

Fig. 7. DIPA excretion patterns in urine after administration of 0.8 mg/kg DADA. Panel A shows recovery pattern of DIPA when plotted as concentration. Panel B shows total amount of drug recovered from urine over 2.5 hr.

and that excretion at 90 min after dosing was greatly reduced. The total amount of DADA excreted in urine as DIPA was about 11% of total drug.

(d) *Mass spectroscopic confirmation.* The DIPA fragment of this compound was difficult to confirm by mass spectrometry (MS) because of its volatility and short retention time on our GC columns. Therefore we derivatized DIPA with propionic anhydride. When derivatized a good mass spectrum of the propionyl derivative of DIPA was obtained with a molecular ion at 157 M/E units and a base peak at 86 (Fig. 8).

DISCUSSION

Based on the structure of DADA a number of characteristics of this drug may be expected. Since it is composed of two relatively small molecules which are paired to form a salt, *in vivo* it may be expected that these molecules will dissociate to yield the individual ions, DIPA and DCA. As a secondary amine, DIPA is likely to be highly charged at physiological pH and as such will not enter cells readily. It will therefore tend not to be metabolized in the horse, but to be excreted by glomerular filtration. As such the duration of action of its pharmacological effects will be short, a relatively large proportion of a dose will be excreted in the urine unchanged and, if the administered dose is large enough, an osmotic diuretic effect may be expected. In addition, because of the small size of the DIPA molecule it may be expected that loss of this molecule by volatilization in extraction and recovery could be substantial, and interfere with the recovery of this agent from blood and urine samples. Finally, because of the small size and very simple structure of this molecule it seems unlikely that specific pharmacological effects will be found after injection.

In general, the results reported here support these expectations. After i.v. injection of DADA the pharmacological effects observed are small, even when the dose of drug is 10 times the reported clinical dose. This dose, however, was large enough to produce what appears to be an osmotic diuresis, suggesting that the drug is being excreted in relatively large amounts in the urine. This observation is

supported by the data of Fig. 7, which shows that a large proportion of this drug is excreted unchanged in the urine, consistent with the drug being an agent which enters cells poorly, is excreted by glomerular filtration and is found in the urine in relatively high concentrations.

If DIPA is rapidly excreted, its detection in blood is likely to be difficult since after i.v. injection plasma levels of the drug can be expected to decline rapidly. In agreement with this we were unable to detect DIPA in plasma after administration of doses as high as 400 mg/horse. Similarly, we also had trouble initially in detecting this drug in urine, due in part to the difficulty in recovery and analysis for this drug and also in part to the relatively rapid excretion of DADA in urine.

Our initial problem in detecting DADA was apparently due to the very basic nature of this drug. While the pK_a of DADA is not known, it is apparently more basic than the average basic drug recovered in equine drug testing. Most medications found in equine urine extract well at a pH of about 9.5, while to extract DIPA, a pH of about 12 was required. The simplest explanation for this fact is that the pK_a of DADA is much higher than that of most basic drugs, and for this reason the pH of the extracting medium has to be higher. When the pH of the extraction medium was increased to 12.0, DIPA extracted well and recovery of this drug from urine was greatly improved.

As well as being difficult to extract from urine DADA turned out to be difficult to handle in the concentration process. This is because as a small molecule it tends to be volatile and is readily lost during the evaporation process. To overcome this problem the concentrating solution was made acidic, thereby forming a molecular salt and rendering it nonvolatile. When this precaution was taken recovery of the drug was greatly improved, and quantitative recovery of the drug from urine became possible.

Considerable difficulty was encountered in identifying a solvent suitable for dissolving the extracted material for GC analysis. Methanol appeared to react with DIPA to yield a material with different chromatographic characteristics from authentic DIPA. Isopropanol was selected as the material into which DIPA could be dissolved and chromatographed satisfactorily.

Dosing horses with the clinically suggested dose of DADA (0.08 mg/kg or 40 mg/horse) produced no apparent overt effects. In the physiology studies after doses of 0.08 mg/kg DIPA, no changes in respiratory rate suggesting central-nervous stimulation were observed, and neither were there changes in basal body temperature. More significantly, however, in view of the reported pharmacological actions of DADA as a hypotenisve agent there were no apparent effects of this dose of DIPA on the cardiac rates of these horses. In general, one might expect that if this drug was able to reduce blood pressure in a horse significantly at this dose it would also be likely to increase the heart rate of these horses. No such effect was found, suggesting that the clinically recommended doses of this agent are not likely to produce a pharmacologically effective hypotension in horses.

In addition to not producing pharmacological effects, these doses of DADA did not give rise to detectable levels of DIPA in the bloodstream of horses after either the 40 or 400 mg/horse doses. The reason for our inability to detect the drug in blood is unclear, but is likely related to the relatively low sensitivity of our detection method and the rapid clearance of this drug from blood. The likelihood of detecting this drug in blood after clinical dose (40 mg/horse) appears small since we were unable to detect the drug in blood after the larger 400 mg/horse dose.

The detection methods reported here were more than sufficient to detect the drug in urine, however. After the 40 mg/horse dose the drug was detected in urine for up to 8 hr. Although this is a relatively short period by the standards of other agents, it is likely sufficient to allow control of the use of this agent in racehorses. This is because if the duration of action (if any) of this drug is short it will have to be given shortly before post time to be effective, and the methods reported here allow easy detection of this drug for up to 4 hr or more after its administration.

Beyond simple detection of the drug, it must also be confirmed by MS analysis to enable its regulatory control. Since underivatized DIPA is quite volatile and therefore difficult to chromatograph, we derivatized DIPA with propionic anhydride to increase the mass of the molecule and reduce its volatility. When treated in this way the propionyl derivative of DIPA yielded a molecular ion at 157 M/E and a base peak at 86. This derivative forms readily, chromatographs well and enables the development of good MS evidence of the presence of DIPA in a sample.

The regulatory control of DADA is also complicated by the fact that this substance is marketed to horsemen as the supposed "vitamin B_{15}". Since most rules of racing allow the use of vitamins and no restrictions are placed on their administration to horses, or on their inclusion in the horses diet, it can be difficult to regulate the use of this agent on forensic grounds. This is despite the fact that "vitamin B_{15}" has no recognized chemical structure, and is not recognized by the U.S. Food and Drug Administration as either a discrete chemical structure or as a vitamin.

In conclusion, therefore, we have studied the detection, disposition and behavioral effects of DADA in horses. When administered i.v. at a dose of 40 mg/horse no effects of DIPA on the behavior, heart or respiratory rates or basal body temperature of horses was observed. Using an extraction and recovery method reported here, DIPA could be readily recovered from urine but not from blood of these treated horses. Using HPTLC, DIPA was detectable in horse urine for at least 4 hr after dosing. Similarly, it was detectable by a GC detection method for at least 8 hr after dosing. MS confirmation of the presence of the drug in the urine samples was possible using a propionic anhydride derivative of DIPA. Together these experimental techniques should be sufficient to allow effective screening and MS confirmation of this drug in post race urine samples and allow for effective regulatory control of this agent in horse racing.

SUMMARY

Diisopropylamine dichloroacetate (DADA) is a medication marketed for use in horses in Australia.

In this communication we report on the detection, actions and effects in horses of diisopropylamine, the pharamacologically active portion of this agent.

To assess the physiological effects of DIPA 4 horses were dosed with DIPA at 0.08 mg/kg body wt and monitored for changes in physiological parameters. No apparent changes in heart and respiratory rates or body temperature were recorded in horses dosed with 0.08 mg/kg DIPA. However, a transient diuretic effect was found in 2 mares dosed with DADA at 0.80 mg/kg body wt.

To develop detection methods in equine urine and blood, DADA was prepared and horses were injected with 0.08 mg/kg ($n = 4$) or 0.80 mg/kg ($n = 2$) of DADA intravenously. The major metabolite of DADA in horse urine was found to be DIPA. A qualitative detection method for DIPA in equine urine using thin-layer chromatography (TLC) was developed. Additionally a quantitative detection method for DIPA in equine urine samples was developed using gas chromatography (GC).

Extraction and alkalinization of samples allowed for detection of DIPA in urine. DIPA was readily detectable using TLC screening after 0.80 mg/kg for at least 4 hr after administration. GC analysis allowed for detection of DIPA down to about 0.5 μg/ml for about 8 hr after administration of a clinical dose of DADA at 0.08 mg/kg. However, neither DADA or DIPA were detectable in blood by either of these methodologies.

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